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MUTATIONS IN ERGOSTEROL 11 GENE OF FLUCONAZOL RESISTANT *Candida albicans* ISOLATED FROM DIFFERENT CLINICAL SAMPLES

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Abstract

Fluconazole was used to test the susceptibility of *Candida albicans* isolated from different clinical samples**,** and to detect mutations in *ERG11* gene, and their relationship to fluconazole resistance. Forty-eight isolates of *Candida albicans* were tested for susceptibility using the disc diffusion method (M-44). *ERG11* genes of six isolates were amplified (four resistant, two susceptible) and sequenced. The sequenced genes were analyzed to detect the mutations. Out of 48 isolates of *Candida albicans*, 4 (8%) were resistant to fluconazole. Sixteen-point mutations were detected included 13 silent mutations, and three missense mutations. The mutations of A945C (E266D) and G1609A (V488I) were found only in susceptible *Candida albicans* isolates, while the mutation of G1456A (V437I) was detected only in resistant *Candida albicans* isolates. *Candida albicans* had a high susceptibility against fluconazole. The amino acid substitutions of E266D and V488I have no role in fluconazole resistance, while the substitution of V437I may have a role in developing resistance against fluconazole. Multiple point mutations in *ERG11* gene may develop resistance to fluconazole.

INTRODUCTION

Candida albicans is the most common human opportunistic fungal pathogen among other *Candida* species [1]. *Candida* spp. infects different anatomical sites of the human body. It resides on the mucosa of the gastrointestinal tract, vagina, mouth, and esophagus. [2][3] Candidiasis is classified as superficial in cutaneous and mucosal infections of healthy individuals, and invasive or systemic in deep and widespread infections of immunocompromised individuals. [4] *Candida albicans* is the most prevalent species developing candidiasis. About 50% of world wild candidiasis infections are caused by *C. albicans*.

[5] Immunosuppression and azole long-term therapy are the main factors for developing azole resistance. Because of its high oral bioavailability, fluconazole is prescribed to treat AIDS patients with oropharyngeal candidiasis. Most of HIV-patients have fluconazole-resistant *C. albicans* in their oral cavities. [6] While azole-resistant *C. albicans* is less common in patients with vaginal candidiasis and Candidemia. These patients have azole resistance incidence about 0-5% because they receive a shorter course of azole therapy [7]. The mechanism of Azole resistance can be either qualitative or quantitative changes in the target 14 α-demethylase enzyme.

Qualitative changes include reducing the affinity between the antifungal and the target enzyme. Quantitative changes include the accumulation of the enzyme inside the cell. Modifications in ergosterol of Candida cell wall lead to impaired uptake of the drug, or decreased accumulation of drug inside the cell [8] [9]. Different molecular mechanisms contribute to cell wall modifications. These mechanisms include alteration in *ERG11* gene encoding of the 14 α-demethylase enzyme, and overexpression of genes encoding for membrane transport proteins [10].

ERG11 gene (previously *ERG16* and *CYP51A1*) encodes the enzyme lanosterol demethylase in ergosterol biosynthesis pathway. This gene contains 1851 bp of nucleotides. The transcription start codon is at 148-150 bp and the stop codon is at 1732-1734 bp. *ERG11* reference sequence in gene bank is deposited under the no. of X13296 [11]. Molecular alteration in *ERG11* gene is either point mutations in the encoding region, which lead to decreased

affinity between the enzyme and azoles, or overexpression of *ERG11* gene that increases the production of the enzyme, making it difficult for the drug to inhibit the enzyme [10]. *ERG11* point mutations lead to native amino acid replacement in the product protein (ERG11P). By analyzing the sequence of *C. albicans* resistant isolates, point mutations in *ERG11* were identified. These mutations not distributed randomly. They were clustered in a hot spot region of the protein's structure, therefore, reducing the affinity of the ERG11P protein to the drug [12].

MATERIALS AND METHODS

Samples Collection

Samples collected from forty-eight (48) patients of candidiasis. They were isolated from sputum, vagina, mouth, and urine and according to ethical considerations.

Fluconazole Susceptibility Test

Susceptibility of *C. albicans* isolates to fluconazole was measured using M-44 disc diffusion method, which is a global guideline recommended by CLSI for disk diffusion testing of *Candida* sp. Mueller Hinton agar (2% glucose + 0.5 methylene blue dye), and Fluconazole 25 µg disc were used to perform the test.

Reading of Results of Disc Diffusion

After 24 hours of incubation the diameter of the inhibition zone was measured in millimeters using a metric ruler. According to the measured diameters, the results translated as sensitive (S), susceptible dose-dependent (S-DD), and resistance (R), and they were compared with the standard inhibition zone in (Table 1).

Table 1: Zone diameter and corresponding minimal inhibitory concentrations (MIC) breakpoints for *Candida* spp. (NCCLS, 2004)

Antifungal agent	Disk content	Zone diameter (mm)			Equivalent MIC breakpoints (µg/mL)		
		R	S-DD	S	R	S-DD	S
Fluconazole	25 µg	≤ 4	15-18	≥ 19	≥ 64	16-32	≤ 8

DNA Extraction

DNA extraction was performed using Quick-DNATM Fungal/Bacterial Miniprep Kit from zymoresearch. Quick-DNATM Fungal/Bacterial Miniprep Kit is designated for simple, rapid isolation of DNA from tough to lyse fungi including *Aspergillus fumigatus*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and from mycelium and Gram (+) and (–) bacteria. The procedure is easy and can be completed in 15 minutes. The fungal and bacterial samples were added to a ZR Bashing BeadsTM Lysis tube (0.1mm & 0.5 mm) and rapidly and efficiently lysed by bead beating without using organic denatures or proteinases. The DNA was isolated and purified using Zymo-spinTM technology and ideal for downstream molecular-based applications.

Amplifying and Sequencing of *ERG11* Gene

ERG11 gene of six isolates (four resistant, two susceptible) was amplified and sequenced. Three pairs of primers were designated by referring to Xu *et al.* (2008) (Table2). Polymerase chain reaction (PCR) was carried out in 25 µl reaction containing 12.5 µl of green master mix, 1µl of 10 pmol/µl of each primer, and 2µl of the template DNA. The volume was topped up to 25 µl by adding 8.5 µl of nuclease-free water. PCR condition was as follows: 95 ºC for 5 min, 95 ºC for 30 sec, 56 ºC for 30 sec, 72 ºC for 30 sec, for 33 cycles, and 72 ºC for 7 min. The PCR products were sequenced using ABI3730XL automated DNA sequencer.

RESULTS AND DISCUSSION

Fluconazole susceptibility

Out of 48 *C. albicans* isolates only 8% was resistant to fluconazole. The four isolates (89, 90, 82, and 20) were isolated from vaginal swab, and sputum. About 10.42% was susceptible to dose-dependent, and 81.25% was susceptible to fluconazole (Table3).

Amplifying and mutations of *ERG11* gene

The gene amplified was classified into three sections with a length of 488, 454, and 462 bps, respectively. The three sections were analyzed by comparing them with the referenced *ERG11* sequence in the gene bank under the accession number of X123296 using BioEdit multiple alignment. The amplified product was extended from 322 to 1664 bps of the gene. Based on the results of gene sequence analysis, thirteen silent mutations, and three missense mutations were detected in the *ERG11* gene (Table 4). The amplified sequences are under the accession number of MH468713-MH468727 in NCBI gene bank.

Table2: Primers and expected PCR products for *ERG11* amplification

Primer	Strand	Sequence	Expected PCR product
*ERG11*sec1	Forward	5̀-TTAGTGTTTTATTGGATTCCTTGGTT-3̀	259-777 bp
	Reverse	5̀-TCTCATTTCATCACCAAATAAAGATC-3̀	
*ERG11*sec2	Forward	5̀-ACCAGAAATTACTATTTTCACTGCTTCA-3̀	723-1204 bp
	Reverse	5̀-AAGTCAAATCATTCAAATCACCACCT-3̀	
*ERG11*sec3	Forward	5̀-AGGTGGTGATTTGAATGATTTGACTT-3̀	1179-1667 bp
	Reverse	5̀-GAACTATAATCAGGGTCAGGCACTTT-3̀	

Table 3: Distribution of *Candida albicans* in samples and their fluconazole susceptibility

Site of isolation (%)					Fluconazole susceptibility (%)		
Fungus	**Vaginal swab**	**Sputum**	**Oral swab**	**Urine**	**S**	**S-DD**	**R**
Candida albicans	18 (37.50)	17 (35.42)	8 (16.67)	5 (10.42)	39 (81.25)	5 (10.42)	4 (8.33)

*S: sensitive, R: resistant, S-DD: susceptible dose-dependent

Amplifying and Mutations of *ERG11* Gene

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Disc diffusion is a qualitative test, but CLSI provided a correlation between MIC values in broth dilution methods and zone inhibition measurements of disc diffusion. It is suitable for the susceptibility testing of water-soluble antifungals such as fluconazole. [13] *Candida albicans* in the area of the study showed a high susceptibility to fluconazole. The isolates were from patients with no immunosuppression or patients with HIV, the latest usually show a lower susceptibility to fluconazole, due to long-term antifungal therapy. [11]

The amplified product in our study was extended from 332 to 1664 bp of the gene, since the hot spot regions of ERG11P located at 105-165, 266-287, and 405-488 [14]. The isolates (3, 10) were isolated from different patients, but sharing the same mutations with nine silent (T462C, C558T, C805T, T1143C, A1173G, C1257T, T1350C, C1443T, and T1449G) including two missense mutations (A945C, and G1609A), and four silent mutations (A945C, G1609A, C1443T, and T1449C) detected only in these susceptible isolates (Table 5). These data can be an evidence that these two isolates are belonging to the same strain. The point mutations are allelic variation between different strains, not necessarily associated with resistance [15].

The missense mutations (A945C) and (G1609A) led to the substitution of Glutamic acid by Aspartic acid in position of 266 (E266D), and Valine by Isoleucine in position of 488 (V488I), respectively found together in two fluconazole susceptible isolates (48, 70) with no other missense mutations in both of them. The same mutations in *C. albicans* isolates presented by Maebashi *et al* **.(2003) .**The substitution of E266D located in G-helix of an enzyme product, which covers a part of the active site, but probably does not contribute to fluconazole resistance, because it detected in both resistant and susceptible *C. albicans* isolates [11][15]. Golabek *et al*. (2014) reported that D266E decreasing gene expression. The amino acid encoded by the mutation of G1609A with the substitution of V488I is at a position far from the active site [16]. Maebashi *et al.* (2003) had detected this mutation in resistant and susceptible isolates, so it may not confirm fluconazole resistance. The substitution of V437I was found in three resistant isolates (89, 90, and 82). The substitution of V437I located in the third portion of the hot spot region indicating V437I may have a role in raising resistance to fluconazole. V437I also reported in resistant *C. albicans* by Wang *et al*. (2015).

The silent mutations of T462C, C558T, C805T, A1173G, C1257T, and T1350C were found in resistant and susceptible isolates. While C1443T and T1449C were found only in susceptible isolates. These two mutations were also reported by Taraskina *et al*. (2016), but in resistant isolates. These mutations have no role in fluconazole-resistant but may have if they combine with specific mutations or other mechanisms [12]. Four silent mutations T696C, T1404C,

Table 4: Mutations detected in *Candida albicans* isolates showing the nucleotide changes and resulting amino acid substitutions in the protein.

Mutations (Nucleotide changes)	Mutations in codons	Type of mutations	ERG11p changes/positions (amino acid substitution)
T462C	TTT→TTC	Silent mutation	F105
C558T	TCC→TCT	Silent mutation	S137
T696C	CAT→CAC	Silent mutation	H183
C805T	CTA→TTA	Silent mutation	L220
A945C	GAA→GAC	Missense mutation	E266D
T1143C	GTT→GTC	Silent mutation	V332
A1173G	AAA→AAG	Silent mutation	L340
C1257T	CTC→CTT	Silent mutation	K342
T1350C	TAT→TAC	Silent mutation	Y401
T1404C	CCT→CCC	Silent mutation	P419
C1443T	GCC→GCT	Silent mutation	A432
T1449C	GCT→GCC	Silent mutation	A434
G1456A	GTT→ATT	Missense mutation	V437I
A1587G	TTA→TTG	Silent mutation	L480
G1609A	GTT→ATT	Missense mutation	V488I
T1617C	AAT→AAC	Silent mutation	N490

Table 5: Distribution of nucleotide changes and the resulted amino acid substitutions of each isolate.

	Samples					
ERG11p	**48 (S)**	**70 (S)**	**89 (R)**	**90 (R)**	**82 (R)**	**20 (R)**
F105	T462C	T462C	T462C	T462C	T462C	T462C
S137	C558T	C558T	C558T	C558T	C558T	C558T
H183	---	---	T696C	T696C	---	T696C
L220	C805T	C805T	C805T	C805T	C805T	C805T
E266D	A945C	A945C	---	---	---	---
V332	T1143C	T1143C	T1143C	T1143C	T1143C	---
L340	A1173G	A1173G	---	---	---	A1173G
K342	C1257T	C1257T	C1257T	C1257T	C1257T	C1257T
Y401	T1350C	T1350C	T1350C	---	---	---
P419	---	---	T1404C	---	---	T1404C
A432	C1443T	C1443T	---	---	---	---
A434	T1449C	T1449C	---	---	---	---
V437I	---	---	G1456A	G1456A	G1456A	---
L480	---	---	A1587G	A1587G	A1587G	A1587G
V488I	G1609A	G1609A	---	---	---	---
N490	---	---	T1617C	T1617C	T1617C	T1617C

*S= sensitive, R= resistant.

C1587G, and T1617C were detected only in resistant isolates. T696C was reported by Taraskina *et al.* (2016) in resistant *C. albicans*, while T1404C, C1587G, and T1617C were detected by Strzelczyk *et al.* (2013) in both sensitive and susceptible isolates. Isolate no. 20, which was FLC resistant, contained nine-point mutations, and no amino acid substitution. These silent mutations that present only in resistant isolates may be associated with FLC resistance by effecting gene expression or by combining with other mutations. Multiple mutations in *ERG11* can result in decreasing azole susceptibility, since one mutation is not enough to raise the resistance. Some mutations do not have an effect until they combine with one or more mutation(s). [17][12].

Resistance to fluconazole evolves when the target enzyme changed either by point mutations in *ERG11* gene or increasing gene expression. *ERG11* genes characterized by a genetic polymorphism, more than 140 missense mutations have been reported. The presence of single point mutation in the gene is not enough to change the affinity between fluconazole and the target enzyme, whereas the presence of multiple mutations could raise the resistance of *C. albicans* [12]. Other mechanisms like upregulation of active efflux transporter gene, alterations in other genes that responsible for ergosterol biosynthesis pathway polymorphism, and biofilm formation also contribute to fluconazole resistance [18].

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CONFLICT OF INTEREST

The article including the tables is original. It was written by the stated authors and was not published elsewhere. This manuscript was also not submitted to, nor under review at another journal or other publishing venue. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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