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A Point Mutation in the 14α-Sterol Demethylase Gene *cyp51A* Contributes to Itraconazole Resistance in *Aspergillus fumigatus*

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The genes encoding 14α-sterol demethylases (*cyp51A* and *cyp51B*) were analyzed in 12 itraconazole (ITC)-resistant and three ITC-susceptible clinical isolates of *Aspergillus fumigatus*. Six ITC-resistant strains exhibited a substitution of another amino acid for glycine at position 54, which is located at a very conserved region of the Cyp51A protein. The *cyp51A* gene from the *A. fumigatus* wild-type strain (CM-237) was replaced with the mutated *cyp51A* gene copy of an ITC-resistant strain (AF-72). Two transformants exhibited resistance to ITC, both of which had incorporated the mutated copy of the *cyp51A* gene.

Aspergillus infections are an important cause of mortality and morbidity in the immunocompromised host, and *Aspergillus fumigatus* is one of the most prevalent airborne fungal pathogens causing infection worldwide (13). *A. fumigatus* is intrinsically resistant to fluconazole (FCZ), in contrast to itraconazole (ITC), which has been shown to have good in vitro and in vivo activity against this species (3). However, a number of *A. fumigatus* isolates with in vitro ITC resistance have been described (1, 6, 9). In some instances, the resistance detected in vitro has been confirmed in vivo with animal models (5, 10).

The azole-derived antifungal agents inhibit the ergosterol biosynthesis pathway via the inhibition of 14α-demethylase, the enzyme that removes the methyl group at position C-14 of precursor sterols. The emergence of resistance to azoles in yeast species has accelerated studies of the mechanisms implicated in this resistance, and several alterations of the gene encoding 14α-demethylase (*ERG11*/*cyp51*) have been reported for FCZ-resistant clinical isolates of *Candida albicans* (12, 14, 16, 21, 24). The mechanisms of resistance to azoles have been studied in phytopathogenic fungi to some extent, but the information about human pathogenic filamentous fungi is very meager. The occurrence of a phenylalanine rather than a tyrosine residue at position 136 of the *cyp51* product sequence has been reported for field isolates of *Uncinula necator* (8) and *Erysiphe graminis* (7). In these fungi, the Y136F mutation is related to resistance to demethylation inhibitors. To date, two molecular mechanisms of resistance to ITC have been proposed for *A. fumigatus*. One is reduced intracellular accumulation of ITC due to an efflux pump (9, 25); a reduced penetration of the drug has also been suggested (15). The other mechanism is related to a possible modification of the target enzyme, 14α-sterol demethylase, or its overexpression (9, 20).

Recently, we have described the existence of two genes coding for two different 14α-demethylases in *A. fumigatus*, *cyp51A* and *cyp51B*. We have also shown that both genes are expressed (17). The *cyp51A* sequence exactly matched one previously described sequence for *A. fumigatus* (11). The objective of this work was to compare the sequences of these two genes in ITC-susceptible and ITC-resistant isolates of *A. fumigatus*, and to look for specific residues that could account, at least in part, for the resistance of *A. fumigatus* to ITC.

A total of 15 clinical strains of *A. fumigatus* were selected on the basis of their susceptibilities to ITC. The MICs of ITC for the resistant isolates, AF-72, AF-90, AF-91, AF-1422, F/6929, F/7075, Br130, Br181, AF-1237, CM-796, CM1910, and SO/3829, were >8.0 μg/ml (4, 9, 18). The MICs of ITC for the susceptible isolates, AF-1119, CM-1369, and CM-237, were 0.5 μg/ml. AF-1119 was isolated 4 months before AF-1237 was isolated and before ITC treatment (4). Isolates CM-1369 and CM-796 were obtained 11 months apart from two bronchoalveolar lavage fluid samples from one human immunodeficiency virus-positive patient. Strain CM-1910 was obtained from the sputum of a patient. *A. fumigatus* CM-237 was used as a control strain. This isolate was utilized for describing the sequences of the *cyp51A* and *cyp51B* genes (17). *Paecilomyces variotii* ATCC 22319 and *A. fumigatus* ATCC 9197 were used as quality control strains for susceptibility testing.

Antifungal susceptibility testing. Despite the fact that most of the strains had been previously checked for ITC susceptibility, antifungal susceptibility testing was repeated. A broth microdilution test was performed by following the NCCLS reference method (19) with minor modifications (3). ITC (Janssen Pharmaceutica, Madrid, Spain), voriconazole (VCZ) (Pfizer S.A., Madrid, Spain), FCZ (Pfizer S.A.), and ketoconazole (KTZ) (Janssen Pharmaceutica) were obtained as standard powders from their respective manufacturers. Drugs were dissolved in dimethyl sulfoxide (Sigma, Madrid, Spain) to obtain stock solutions of 1,600 μg/ml that were conserved at −70°C. For ITC, VCZ, and KTZ, the final concentration range assayed was 8.0 to 0.015 μg/ml. FCZ was assayed using concentration ranges from 6,400 to 0.03 μg/ml. Visual readings were performed with a microtiter reading mirror. MICs were defined as the lowest concentration of the antifungal agent that completely inhibited fungal growth after 48 h of incubation at 35°C. Susceptibility tests were performed at least twice with each strain on different days.

The ITC MICs for three isolates, CM-1369, AF-1119, and

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TABLE 1. Nucleotide and amino acid substitutions in the *cyp51A* and *cyp51B* genes from *A. fumigatus* clinical isolates

Isolate	Base change							MIC (μg/ml)[b]			
	cyp51A		*cyp51B*								
	Codon 54	Codon 236	Codon 35	Codon 42	Codon 187	Codon 387	Codon 394	ITC	VRZ	FCZ	KTZ
CM-237	GGG	ATG	TCT	CAG	TTC	GAT	CCT	0.25	0.5	640	8.0
AF-1119	GGG	ATG	TCT	CAG	TTC	GAT	CCT	0.5	0.5	640	8.0
CM-1369	GGG	ATG	TCT	CAG	TTC	GAT	CCT	0.25	0.5	640	8.0
AF-72	^{161}G**A**G[b]	ATG	TCT	^{125}**C**TG[i]	^{664}TT**T**[j]	GAT	CCT	>8.0	0.25	160	1.0
AF-1237	^{160}**A**GG[f]	ATG	TCT	CAG	TTC	GAT	CCT	>8.0	0.5	160	2.0
CM-796	^{161}G**T**G[d]	ATG	^{105}TC**C**[h]	CAG	TTC	GAT	CCT	>8.0	1.0	640	8.0
CM-1910	GGG	ATG	^{105}TC**C**[h]	CAG	TTC	GAT	CCT	>8.0	4.0	3,200	>8.0
AF-91	GGG	^{729}**G**TG[c]	^{105}TC**C**[h]	CAG	TTC	GAT	CCT	>8.0	1.0	3,200	>8.0
AF-90	GGG	^{729}**G**TC[a]	^{105}TC**C**[h]	CAG	TTC	GAT	CCT	>8.0	1.0	3,200	>8.0
AF1422	GGG	^{729}**G**TG[c]	^{105}TC**C**[h]	CAG	TTC	GAT	^{1285}CC**G**[l]	>8.0	1.0	3,200	>8.0
F/6919	GGG	^{730}**A**AG[e]	TCT	CAG	TTC	GAT	CCT	>8.0	2.0	3,200	8.0
F/7075	^{161}G**A**G[b]	ATG	TCT	CAG	TTC	GAT	^{1285}CC**G**[l]	>8.0	0.5	320	2.0
Br130	^{161}G**A**G[b]	ATG	TCT	^{125}**C**TG[i]	^{664}TT**T**[j]	GAT	^{1285}CC**G**[l]	>8.0	0.5	160	1.0
Br181	^{161}G**T**G[d]	ATG	TCT	CAG	TTC	GAT	CCT	>8.0	0.25	160	2.0
SO/3829	GGG	^{730}**A**CG[g]	TCT	CAG	TTC	^{1284}G**A**A[k]	^{1285}CC**A**[l]	>8.0	1.0	1,600	8.0
T-21	^{161}G**A**G[b]	ATG	TCT	CAG	TTC	GAT	CCT	>8.0	0.5	160	1.0
T-23	^{161}G**A**G[b]	ATG	TCT	CAG	TTC	GAT	CCT	>8.0	0.25	80	1.0

[a] Nucleotides are numbered from the translation start codon ATG of *cyp51A* and *cyp51B*. The numbers indicate the position at which a base change occurs (in bold).
[b] Amino acid substitution, **G54E.**
[c] Amino acid substitution, **M**236**V.**
[d] Amino acid substitution, **G**54**V.**
[e] Amino acid substitution, **M**236**K.**
[f] Amino acid substitution, **G**54**R.**
[g] Amino acid substitution, **M**236**T.**
[h] Amino acid substitution, S35S.
[i] Amino acid substitution, **Q**42**L.**
[j] Amino acid substitution, F187F.
[k] Amino acid substitution,**D**387**E.**
[l] Amino acid substitution, P394P.

CM-237, were ≤0.5 μg/ml. The rest of the isolates were confirmed to be resistant in vitro to ITC, with the MICs for them being >8.0 μg/ml (Table 1). Although there are no defined breakpoints for ITC, those isolates for which the ITC MICs were ≤0.5 μg/ml were considered susceptible and the strains for which the ITC MICs were >8.0 μg/ml were considered resistant. These putative breakpoints were chosen on the basis of previous works that correlated an ITC MIC of >8 μg/ml with the lack of a clinical response to treatment with ITC and the lack of response in animal models (5, 10). The MICs for other azole drugs are shown in Table 1.

PCR amplification and sequencing of *A. fumigatus cyp51A* and *cyp51B* genes. Conidia from each strain were inoculated into 3 ml of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and grown overnight at 37°C. Mycelial mats were recovered and subjected to a DNA extraction protocol described previously (26). The full coding sequences of *cyp51A* and *cyp51B* were amplified by using specific primer sets: P450-A1 (5′-ATGGTGCCGATGCTATGG-3′) and P450-A2 (5′-CTGT C-TCACTTGGATGTG-3′) for the *cyp51A* gene and P450-B1 (5′-ATGGGTCTCATCGCGTTC-3′) and P450-B2 (5′-TCAGG CTTTGGTAGCGG-3′) for the *cyp51B* gene. The amplifications were performed in a 50-μl volume as previously described (17). Negative controls including all PCR constituents but without DNA were included in each amplification run. The PCR products were analyzed by electrophoresis on agarose gels and stained with ethidium bromide. Sequence analysis of the *cyp51A* and *cyp51B* genes showed some point mutations. To verify that these point mutations were not due to errors in the PCR amplification of the genes, the *cyp51A* and *cyp51B* genes from all isolates were newly amplified and sequenced a second time. Exactly the same point mutations were found again, indicating that they were not artificially introduced during the PCR.

The target of the azole antifungals is 14α-demethylase, and alterations in the sequence of the gene encoding this enzyme (*ERG11*/*cyp51*) have been demonstrated for azole-resistant strains of *C. albicans* (12, 14, 16, 21, 24) and for some phytopathogenic fungi that are resistant to demethylation inhibitors (7, 8). Thus, in this work, the alteration of the *cyp51* genes of *A. fumigatus* is proposed as one of several potential mechanisms for resistance to ITC. The detection of amino acid substitutions in the Cyp51A and/or Cyp51B proteins of those isolates with phenotypic resistance to ITC that were absent in the same proteins of ITC-susceptible strains would allow us to associate this specific alteration with resistance to ITC. PCR amplification and sequence analysis of the *cyp51A* genes showed some point mutations. Six ITC-resistant strains (CM-796, AF-72, AF-1237, F/7075, Br130, and Br181) showed a single base change (Table 1). These single base mutations led to changes at the triplet encoding the amino acid at position 54 (glycine). This residue is located at a very conserved region of the Cyp51 protein. These mutations were the G-to-T change at position 161 (G161T) in the CM-796 and Br181 strains, G161A in the AF-72, F/7075, and Br130 strains, and G160A in the AF-1237 strain. In addition, five other strains (AF-90, AF-1, AF-1422, F/6929, and SO/3829) showed a different mutation which yielded an amino acid change from methionine (M) to

either valine (V), lysine (K), or threonine (T) at codon 236 (Table 1). Methionine 236 is not a conserved amino acid between the Cyp51 proteins of different yeast species and molds. Regarding the *cyp51B* gene, five additional point mutations were observed in the *cyp51B* sequence of some of the ITC-resistant strains (Table 1). Only two of these changes led to amino acid substitutions. In the AF-72 and Br130 strains, the A125T change led to an amino acid change from glutamine (Q) to leucine (L) at codon 42 (these two strains harbored another mutation, C664T, which is not responsible for an amino acid substitution). One strain (SO/3829) had a T1264A nucleotide change at codon 387 that would be responsible for an aspartic acid (D)-to-glutamic acid (E) change. Three ITC-resistant isolates, Br-181, F/6919, and AF-1237, did not show any change in their *cyp51B* gene sequences with respect to the reference strain CM-237. Strains CM-796, AF-90, AF-91, CM-1910, and AF-1422 showed a base change at position 105 (T105C) in the *cyp51B* sequences when compared to that of the reference strain (CM-237), but this change was not associated with an amino acid alteration. In total, four different point mutations were detected among the 12 ITC-resistant isolates that yielded amino acid substitutions. Strain AF-1237, which harbored the amino acid change G54R in Cyp51A, was matched with the initially ITC-susceptible strain AF-1119. Both were obtained from the same patient and were shown to have identical DNA patterns by different molecular typing methods (4). These two strains differed only in the amino acid at position 54 of the Cyp51A sequence. These findings demonstrate that the mutation at glycine 54 is responsible, at least in part, for the ITC resistance of strain AF-1237. The fact that another five ITC-resistant strains harbored a mutation at the same position in the Cyp51A sequence suggests that there is a high correlation of this specific change with resistance to ITC. In addition, fragments containing the area with the mutation (position 160 or 161) were PCR amplified (using primers A7 and A5, see below) and sequenced from 22 *A. fumigatus* ITC-susceptible strains from our collection of clinical mold strains. All *A. fumigatus* ITC-susceptible strains have the triplet GGG at positions 160 to 162 (encoding glycine 54).

Regarding the other two mutations resulting in amino acid substitutions, previous work has suggested that an altered membrane transporter is the mechanism of resistance operating in strain AF-72, as a reduced intracellular concentration of azoles was found in this strain (9). Strain AF-72 harbored one mutation in each of the two 14α-sterol demethylases, G54E in Cyp51A and Q42L in Cyp51B. The Q42L mutation detected in this strain was present only in another resistant strain. This fact, together with a G54E mutation from AF-72 resulting in ITC resistance in the wild-type CM-237 recipient strain (which does not bear the Q42L mutation), suggests that the Q42L amino acid change is not directly involved in the resistance of *A. fumigatus* to ITC. On the other hand, the resistance of strains AF-90 and AF-91 to ITC was previously reported to be mediated by either increased expression of the 14α-sterol demethylase or an altered enzyme affinity for azoles (9). Both strains were from the same patient and are resistant to ITC. We found the A729T mutation leading to a M236V change in a variable region of the Cyp51A protein in strains AF-91, AF-90, AF1422, F/6919, and SO/3829. Moreover, the M236V and Q42L changes are both situated in protein areas which are not conserved in other Cyp51 enzymes. Therefore, it seems unlikely that neither the M236V nor the Q42L change would be related to *A. fumigatus* ITC resistance. Nevertheless, the appearance of the change at methionine 236 in five strains with similar azole drug resistance patterns deserves consideration. Sequencing of the *cyp51A* and *cyp51B* genes of more ITC-resistant strains is needed before further conclusions can be drawn.

Replacement of the CM-237 wild-type *cyp51A* gene with the mutated strain AF-72 *cyp51A* gene copy by DNA-mediated transformation with electroporation. The *cyp51A* gene from strain AF-72 was PCR amplified as previously described and introduced into the wild-type strain CM-237. Transformations of *A. fumigatus* CM-237 were achieved by electroporation with a protocol previously described for *Aspergillus nidulans* (23) that was adapted for *A. fumigatus* (27). The last step of the protocol was slightly modified, as follows. After electroporation and incubation at 30°C for 90 min on a rotary shaker, transformation mixtures were cultured on 20-ml minimal medium (2) agar plates containing 1% glucose and 5 mM ammonium tartrate and the plates were incubated overnight at room temperature. The following day, 10 ml of melted minimal medium (0.6% agarose) containing 200 μg of ITC was pour plated over the medium to make a final concentration of 8 μg/ml. The plates were incubated at 37°C for 1 week. ITC-resistant transformants appeared at times ranging from 2 to 7 days. Mutants were named with a letter (T) followed by a number. DNA from ITC-resistant transformants was digested with two different restriction enzymes (*Sal*I and *Eco*RI) (Amersham-Pharmacia Biotech, Madrid, Spain), fractionated by electrophoresis through 0.8% agarose gels in Tris-acetate-EDTA buffer, and subjected to Southern blot analysis with a fragment of the *cyp51A* gene as a probe (17, 22).

The *cyp51A* gene from strain AF-72 was introduced into the CM-237 wild-type strain by electroporation. After 48 h of incubation, two transformant strains (T-21 and T-23) resistant to ITC were obtained. Both transformants had only one copy of the *cyp51A* gene (as shown by the fact that only a single hybridization band appeared after digestions with two different restriction enzymes) (results not shown). In order to ensure that no errors occurred during gene replacement (such as DNA deletions or insertions that could have affected *cyp51A* gene transcription), PCR amplification of the 5′ and 3′ *cyp51A* ends from both ITC-resistant transformants was used. Two primer sets (A7 plus A5 and A9 plus A6), previously described (17), were used to PCR amplify DNA fragments of 600 and 500 bp, respectively. Those fragments included the noncoding sequences surrounding the 5′ and 3′ *cyp51A* ends. The ITC resistance (MIC > 8.0 μg/ml) of T-21 and T-23 was confirmed by the microdilution method (3, 19). The MICs of the rest of the azole drugs showed a similar pattern to that of the six strains carrying the change at G54 (Table 1). PCR amplification of the *cyp51A* and *cyp51B* genes from these two transformants and full sequencing of the genes confirmed that both of them had incorporated the mutant copy of the *cyp51A* gene (G161A nucleotide change at codon 54 leading to a glycine-to-glutamic acid change). None of the mutants had any other base change present in either the rest of the *cyp51A* gene or in the *cyp51B* gene.

The replacement of the *cyp51A* gene of the *A. fumigatus*

wild-type strain (CM-237) with the mutated *cyp51A* gene of AF-72 changed the susceptibility pattern of CM-237 to one of ITC resistance. Because we have used direct selection for ITC resistance for identifying the gene replacement event incorporating the mutation, we cannot rule out the possibility of a spontaneous mutation by drug-selective pressure. However, two facts should be considered: (i) no ITC-resistant transformants were obtained when the wild-type spores were electroporated with distilled water without DNA, ruling out in part the possibility of a spontaneous mutation, and (ii) the six strains with changes at G54 harbored three different base changes (GAG, GTG, or AGG) so that the existence of two independent transformants harboring exactly the same mutation at codon 54 (GAG) will support the gene replacement event incorporating the *cyp51A* mutated copy of strain AF-72. In conclusion, results strongly suggest that a point mutation leading to an amino acid change at G54 is related to *A. fumigatus* ITC resistance.

It is noteworthy that the MICs of FCZ and KTZ for all strains (besides CM-796) carrying the amino acid change at G54 were fourfold lower than those without this substitution while the MICs of VCZ for the same strains were not (Table 1). Also, mutants T-21 and T-23 had similar susceptibility patterns. In contrast, the MICs of FCZ for the other six strains were >1,600 μg/ml (at least a 2.5-fold increase) and those of VCZ and KTZ were moderately elevated (Table 1). The 20-fold fluctuation in the MICs of FCZ between ITC-resistant strains with or without the amino acid change at G54 seems remarkable. Whatever the nature of the change due to the substitution at G54, it seems to produce a better molecular environment for access by and/or interaction with FCZ and KCZ. Strain CM-796 exhibited no variation in its susceptibility to the three antifungal agents tested. However, the coexistence of a different mechanism of resistance cannot currently be discarded. At least two different azole susceptibility patterns have already been described for some of these strains (18). The results presented here may match one of these patterns with the amino acid change at G54.

The surveillance of ITC-resistant strains of *A. fumigatus* and the study of the resistance mechanisms operating in them could provide help to understand the mechanisms of drug resistance and to develop new and more active molecules. Further straightforward investigations of the functional analysis of the Cyp51A and Cyp51B proteins of *A. fumigatus* are needed.

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AUTHOR'S CORRECTION

A Point Mutation in the 14α-Sterol Demethylase Gene *cyp51A* Contributes to Itraconazole Resistance in *Aspergillus fumigatus*

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Volume 47, no. 3, p. 1120–1124, 2003. Page 1121, Table 1: "Codon 236" should read "Codon 220," "**M**236**V**" should read "**M**220**V**" in footnote *c*, "**M**236**K**" should read "**M**220**K**" in footnote *e*, and "**M**236**T**" should read "**M**220**T**" in footnote *g*.

Page 1122, column 1, line 1: "codon 236" should read "codon 220."

Page 1122, column 1, line 2: "Methionine 236" should read "Methionine 220."

Page 1122, column 1, lines 57 and 59, and column 2, line 2: "M236V" should read "M220V."

Page 1122, column 2, line 4: "methionine 236" should read "methionine 220."