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Original article

Presence of HSP90 Gene in Amphotericin B Resistant *Aspergillus* Species Isolated from Iranian Immunocompromised Patients

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Abstract

Background: Aspergillosis is an opportunistic infection caused by *Aspergillus* spp in immunocompromised patients. The role of HSP90 in *Aspergillus* drug resistance is still unknown. The aim of this study was to evaluate the correlation between the presence of HSP90 gene and polyene resistance in *Aspergillus spp* using PCR.
Materials and Methods: In this study, 32 *Aspergillus* strains were used, which were isolated from patients susceptible to aspergillosis through Bronchoalveolar lavage (BAL) and identified by conventional methods. The isolates were cultured on Sabouraud dextrose agar (SDA). Susceptibility testing against amphotericin B was conducted according CLSI standards (M38-A). Also, the presence of HSP90 gene was evaluated using PCR.
Results: Of 32 *Aspergillus* strains used in this study, 16 (50%) isolates were identified as *A. Flavus*, 12 (37.5%) isolates as *A. fumigatus*, and 4 (12.5%) isolates as *A. terreus*. Among these species, 19 (59.37%) isolates were sensitive to amphotericin B whereas 13 (40.62%) were resistant. Moreover, there was a significant difference between the presence of HSP90 gene and resistance to amphotericin B in *Aspergillus* species.
Conclusions: The presence of HSP90 gene provides evidence that shows this gene may play important role in resistance to amphotericin B in *Aspergillus* isolates. Although numerous regulatory genes are involved in resistance mechanisms, they remain to be more clarified.

Keywords: Aspergillus spp, Amphotericin B, HSP90 gene, PCR

1. Background

Aspergillosis is an opportunistic infection caused by *Aspergillus* species including: *A. fumigatus*, *A. flavus*, *A. nidulans*, and *A. terreus*. The majority of cases occur in patients with underlying diseases such as tuberculosis, haematological malignancies, organ transplantation, and immunodeficiency or chemotherapy (1-3).

A. fumigatus and *A. flavus* are the two most prevalent species involved in human aspergillosis. Although common antifungal agents are used for patients' treatment, the rate of invasive aspergillosis (IA) and mortality has remained excessively high in immunocompromised individuals (4-5) Despite the excessive antifungal agents, the choice of suitable drug for IA treatment is limited because of the high prevalence of Azole resistance in *Aspergillus* spp (6-9). Voriconazole is used as the first-line therapy against IA; however, caspofungin seem to be suitable therapeutic alternative due to elevated voriconazole resistance in *A. fumigatus* (10). Amphotericin B (AMB) and caspofungin are licensed as intravenous drugs for IA treatment, but they are used for chronic and allergic aspergillosis (5). Fungi-static activity and loss of efficacy resulted from using higher concentrations of caspofungin against *A. fumigatus* may cause clinical side effects (11-14). Resistance to antifungal drugs is not as worrying as resistance to anti-bacterial agents; however, increasing frequency has been observed in the number of both types of primary and secondary resistance among the agents causing human mycosis (15-16).

It is well-known that the primary mechanism of resistance in clinical isolates of *A. fumigatus* is mutation of lanosterol 14 α-demethylase, contributing to ergosterol biosynthesis and leading to the alteration of the enzyme structure (13).

HSP90 (Heat Shock Protein) belongs to molecular chaperone family having multifunctional roles in eukaryote cells, such as signaling transduction, tumor suppression, and protein remodeling. Moreover, more recent studies have demonstrated that HSP90 may play an important role in the emergence of resistance to antifungal drugs in *Candida albicans* and *A. fumigates* (10). A broad range of diseases such as fungal and parasitic pathogens can be eliminated using HSP90 inhibitors both in human and animal models. Few studies have demonstrated HSP90 role in filamentous fungi such as *A. fumigatus* in which resistance mechanisms may substantially differ. Some studies have shown that definite recombinant monoclonal antibody against fungal molecular chaperone HSP90 increases susceptibility to AMB (17-19).

Herein, in vitro role of HSP90 gene was investigated in AMB resistance in clinical isolates of *Aspergillus* species.

2. Objectives

The purpose of current study was to investigate the correlation between HSP90 gene presence and Amphotericin B resistance in clinical isolates of *Aspergillus spp*. from BAL samples.

3. Materials and Methods

3.1. Patient's population

In this study, 32 *Aspergillus* strains were used, isolated from 400 BAL specimens of hospitalized patients with predisposing factors to aspergillosis, including corticosteroid, cancer (leukemia), haematological malignancy, and organ transplantation (lung and

bone marrow), who referred to the bronchoscopy unit of Masih Daneshvari hospital from 2015 to 2016.

3.2. Culture of isolates

The isolates were sub cultured on Sabouraud dextrose agar slants (Merck, Germany) and incubated at 26 and 35˚C. *Aspergillus* species were identified with colony morphology, microscopic evaluation, and slide culture.

3.3. DNA extraction

For this purpose, the isolates were cultured on Czapek broth (Merck, Germany) medium and incubated at 30˚C for 4 days. Genomic DNA was extracted as briefly described. The mycelium was harvested from the medium surface and washed twice by phosphate Buffer Saline (PBS). Then it was disrupted by adding liquid nitrogen and lysis buffer containing EDTA (20mM), Tris-HCL (100 mM), Nacl (1.4 mM), and CTAB 2% PH (8) (Merck, Germany). Next, it was incubated at 60ºC, Phenol- isoamyl alcohol was added and centrifuged in 10000 rpm for 10 min. Then 3 m sodium acetate and cooled isopropanol were added to supernatant. The mixture was kept at -20ºC and subsequently centrifuged. Ethanol 75% was added and centrifuged several times. Finally, the distilled water was added to precipitation containing DNA.

3.4. HSP90 gene amplification using Polymerase Chain Reaction (PCR)

PCR was performed to amplify HSP90 gene. The sequences of HSP90 primers were 5'-TCTTCGGAAACCTTTGAATTCC-3' and 5'-TCGTCAGGAACCTCCTTCTCAG-3'. For PCR reaction, 2.5 µL of 10x PCR buffer, 1.5 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.4 µM primers, 1.25 units of Taq polymerase (Cinaclon, Iran), 1 µL of template DNA, and up to 25 µL molecular grade dH_2O were added to the tube. Thirty-three amplification cycles were done in the thermo cycler (Bio Rad) at 95ºC for 5 min with an annealing step at 59ºC for 30 s, an extension step at 72ºC for 1 min, and a final extension at 72ºC for 5 min, following the last cycle. Electrophoresis was done through 1.8% agarose gel and then visualized by ethidium bromide staining. Negative control was used in all PCR reactions. PCR with ITS1 - ITS4 primers was performed to verify the efficiency of DNA extraction.

3.5. Antifungal susceptibility testing

The minimal inhibitory concentrations (MICs) of amphotericin B was assessed by Clinical and Laboratory Standards Institute (CLSI) (M38-A) document. Briefly, *Aspergillus* species were cultured on Czapek agar medium at 30ºC for seven days, spore suspensions were prepared in sterile saline 0.05% and adjusted to a concentration of 10^6 spores/mL and diluted in RPMI 1640 broth (Sigma-Aldrich, USA). As the standard assay powders, amphotericin B (Sigma) stock solutions were prepared in dimethyl sulfoxide (Merck, Germany), ranging from 0.06 to 32 µg/mL according to the manufacturers instruction. Then 100 µL of RPMI 1640 broth containing spore suspensions and 100µL of final concentration of amphotericin B dilution were added to each well. After inoculation of the trays (96 U-bottom shaped, Germany), all micro-dilution trays were incubated at 35ºC for 48 h, and MICs were determined by visual examination at the first well where 100% growth inhibition was present. As two CLSI quality control strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were tested in the same manner (20).

3.6. Statistical analysis

The MICs obtained from at least three different experiments are presented as mean ± SD. Data were analysed using SPSS software version 20 (SPSS, Chicago, IL, USA).

4. Results

The distribution of *Aspergillus* species was as follows: 16 (50%) *A. flavus*, 12 (37.5 %) *A. fumigatus*, and 4 (12.5%) *A. terreus* (Figure1). Moreover, these positive isolates had been separated from 400 BAL specimens of patients with predisposing factors in previous study (data unpublished). (Figure1). The *Aspergillus* positive patients aged from 40 to 83 years (mean = 59 and median = 60 years). Fifteen (46.87%) patients were male, and 17(53.12%) were female.

The MIC results revealed that from among 32 *Aspergillus* strains, 19 (59.37%) strains were sensitive to amphotericin B, and 13 (40.62%) were resistant. Of 16 *A. flavus* isolates, 6 (37.5%) isolates were sensitive to amphotericin B, and 10 (62.5%) were resistant. Also, 7 (58.3%) out of 12 *A. fumigates* isolates were shown to be sensitive while 5 (41.66%) were resistant. In *A. terreus* strains, the frequency in sensitivity and resistance to amphotericin B was the same (50%). MIC values of amphotericin B for resistant *Aspergillus* species were >1µg/mL while in sensitive *Aspergillus* species, MIC values were ≤1 µg/ mL. (Table1)

The result of PCR analysis showed that HSP90 gene was successfully amplified in resistant isolates (the length of HSP9 gene in resistant isolates was 700 bp) while this gene was not detectable in sensitive isolates (Figure 2).

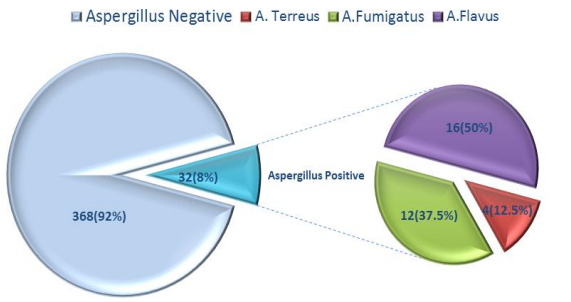

Figure 1. Distribution of *Aspergillus* isolates in patients with underlying diseases.

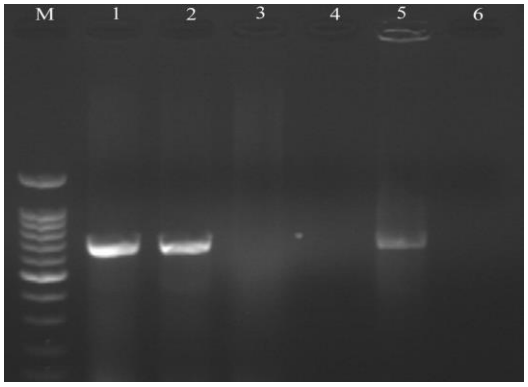

Figure 2. DNA of *Aspergillus* spp was extracted, and polymerase chain reaction was performed using specific primers of HSP90 gene. M: Marker molecular weight 100bP; Line 1, 2, 5: HSP9 gene in resistant isolates (700 bp); Line 3, 4: absent of HSP90 gene in susceptible isolates; Line 6: Negative control.

Table 1. Correlation between HSP90 expression and susceptibility to amphotericin B in isolates.

Species	Number of isolates	HSP90 expression in sensitive isolates (S)		HSP90 expression in resistant isolates (R)	
		Negative	Positive	Negative	Positive
A.Fumigatus	12	7	0	0	5
A.Flavus	16	6	0	0	10
A. Terreus	4	2	0	0	2
Total	32	19	0	0	13

5. Discussion

Invasive Aspergillosis (IA) with new emerging resistant isolates is increasing in immunocompromised patients. Various drugs are commonly used in the treatment of IA, including voriconazole and amphotericin B. Since resistance to antifungal drugs is developing, susceptibility testing for these drugs can help in the selection of appropriate drug to avoid mortality and morbidity in patients. However, susceptibility of *Aspergillus* spp to different antifungal agents is still controversial.

In previous studies, susceptibility of *Aspergillus* spp to amphotericin B has been reported. Rath (1998) revealed that MICs of amphotericin B in *A. fumigatus* and *A. niger* strains ranged from 0.125–0.5 mg/L in contrast with the strains of *A. flavus* and *A. nidulans* in which MICs ranged from 2–16 mg /L (21).

In our study, the concentration of amphotericin B tested for *A. fumigatus*, *A.flavus*, and *A.terreus* was reported as 0.06-32µg/mL; additionally, the presence of HSP90 gene was evaluated in sensitive and resistant species. The MIC results indicated that from among 32 *Aspergillus* isolates, 19(59.37%) isolates were sensitive to amphotericin B whereas 13 (40.62%) were resistant. Consistent with our study, Alborzi et al. (2012) evaluated the susceptibility of *Aspergillus* species isolated from clinical samples to amphotericin B and other antifungal drugs using Etest and CLSI methods. Their result showed that 63.9 and 36.1% of *Aspergillus* isolates were sensitive and resistant to amphotericin B, respectively (22).

The results obtained in the present study were compatible with the presence of HSP90 gene in PCR because HSP90 gene was successfully amplified in the amphotericin B resistant isolates whereas it was not detectable in susceptible isolates. This correlation reveals that HSP90 gene may be responsible for amphotericin B resistance in *Aspergillus* isolates; as Cowen et al. (2005) declared that HSP90 plays a major role in antifungal drug resistance (23). Nevertheless, other important genes such as Mdr1, Mdr3, and Mdr4 are also involved in *Aspergillus* spp drug resistance (24). In another study, Blum et al. (2013) showed that using HSP90 gene inhibitor (blocking) reduced amphotericin B resistance in *Aspergillus* spp clinical samples in vitro, but this inhibition did not show any beneficial effect on Balb/c mice in vivo (25). Previous studies have confirmed that emergence of intrinsically resistant fungal species and clinical resistance should be surveyed to be able to handle invasive aspergillosis in susceptible patients using suitable antifungal drug (26-27).

The growing incidence of invasive fungal infections with a high rate of mortality in immunosuppressed patients shows that resistance to antifungal agents is nowadays greater than ever. Most isolates of *A. terreus* in vivo and in vitro are resistant (MIC >2 mg/L) to amphotericin B; however, the precise mechanisms of sensitivity and resistance to AMB are not thoroughly obvious (28).

Collectively, the critical role of HSP90 gene in other fungi is well-recognized; HSP90 was recently showed to contribute in Azole and Echinocandin resistance in *C. albicans* via the PKC cell wall integrity pathway. Moreover, HSP90 depletion may lead to the destabilization of the terminal mitogen-activated protein kinase (MAPK) Mkc1, consequently the second HSP90 client protein eventually decreases in drug resistance (29). Taken together, our findings implicate the role of HSP90 in AMB resistance; thus, using HSP90 inhibitors can be supported as a therapeutic strategy to manage fungal infections.

6. Conclusions

In conclusion, it is suggested that antifungal susceptibility testing be carried out especially for patients with invasive aspergillosis in order to be able to plan successful treatment approaches. Regarding the obtained results in this study, it can be said that HSP90 plays an important role in AMB resistance. Therefore, it seems that inhibition of HSP90 gene in *Aspergillus* spp by specific mediators diminishes antifungal resistance. Also, by testing more resistant and susceptible isolates, more information would be provided.

Conflict of interest

No potential conflict of interest was reported by the authors.

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Authors' Contribution

All of the authors contributed to this study.

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