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 Amphotericin B resistant Aspergillus species is not well known. The purpose 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%) 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 a more important role in resistance to amphotericin B in Aspergillus isolates. Although many regulatory genes 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). Fungal 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 antibacterial 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 14α-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. fumigatus (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).

Here, 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 60°C. Phenol- isoamyl alcohol was added and centrifuged in 10000 rpm for 10 min. Then 3 m sodium acetate and cooled isopropanol was added to supernatant. The mixture was kept at -20°C 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'-TCTCGGAAACCTTTGAATTC-3' and 5'-TCGTACGGAACCTCTTCTCAG-3'. For PCR reaction, 2.5 µL of 10x PCR buffer, 1.5 mM MgCl2, 0.5 µL of 10 mM dNTPs, 0.4 µM primers, 1.25 units of Taq polymerase (Cmaclon, Iran), 1 µL of template DNA, and up to 25 µL molecular grade dH2O were added to the tube. Thirty-three amplification cycles were done in the thermo cycler (Bio Rad) at 95°C for 5 min with an annealing step at 59°C for 30 s, an extension step at 72°C for 1 min, and a final extension at 72°C 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 30°C for seven days, spore suspensions were prepared in sterile saline 0.05% and adjusted to a concentration of 10⁶ 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 35°C 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 (Figure 1). Moreover, these positive isolates had been separated from 400 BAL specimens of patients with predisposing factors in previous study (data unpublished). (Figure 1). The Aspergillus positive patients aged from 40 to 83 years (mean = 59 and median = 60 years). Fifteen (46.87%) patients were male, and 17(53.13%) 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. fumigatus 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.](image1.png)

![Figure 2. DNA of Aspergillus spp was extracted, and polymerase chain reaction was performed using specific primers of HSP90 gene. M: Marker molecular weight 1000P; Line 1, 2, 5: HSP90 gene in resistant isolates (700 bp); Line 3, 4: absent of HSP90 gene in susceptible isolates; Line 6: Negative control.](image2.png)
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. fлавus 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 μg/mL) to amphotericin B; however, the precise mechanisms of sensitivity and resistance to AMB are not thoroughly observed (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) Mkg1, 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.

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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References

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of isolates</th>
<th>HSP90 expression in sensitive isolates (S)</th>
<th>HSP90 expression in resistant isolates (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>A. Fumigatus</td>
<td>12</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>A. Flavus</td>
<td>16</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>A. Terreus</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>19</td>
<td>0</td>
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