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Dysregulation of Key Proteinases in *Aspergillus fumigatus* Induced by Blood Platelets

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Abstract

Background: *Aspergillus fumigatus* is the most common species causing invasive aspergillosis (IA), a life-threatening infection with more than 80% mortality. Interactions between *A. fumigatus* and human blood platelets lead to intravascular thrombosis and localized infarcts. To better understand *A. fumigatus* pathogenesis, we aimed to analyze the genetic basis of interactions between the pathogen and blood platelets.

Methods: A bioinformatic pipeline on microarray gene expression dataset, including analysis of differentially expressed genes (DEGs) using Limma R package and their molecular function, as well as biological pathways identification, was conducted to find the effective genes involved in IA. In the wet phase, the gene expression patterns following fungal exposure to blood platelets at 15, 30, 60, and 180 min were evaluated by quantitative reverse transcriptase-PCR analysis.

Results: Three genes encoding aspartic endopeptidases including (Pep1), (Asp f 13), and (β-glucanase) were the standing candidates. The invasion-promoting fungal proteinase-encoding genes were down-regulated after 30 min of hyphal incubation with blood platelets, and then up-regulated at 60 and 180 min, although only Pep1 was greater than the control at the 60and 180 min time points. Also, the same genes were downregulated in more the clinical isolates relative to the standard strain CBS 144.89.

Conclusions: Our findings delineate the possible induction of fungal-encoded proteinases by blood platelets. This provides a new research line into *A. fumigatus'* molecular pathogenesis. Such insight into IA pathogenesis might also guide researchers toward novel platelet-based therapies that involve molecular interventions, especially in IA patients.

Keywords: *Aspergillus fumigatus*, Blood Platelets, Gene Expression, Microarray Analysis, Proteinases.

Introduction

Aspergillus fumigatus is the most common of the *Aspergillus* species that cause invasive fungal infection in immunocompromised individuals including organ transplant recipients, leukemia, AIDS, and covid-19 patients, and those receiving immunosuppressive therapy for autoimmune or neoplastic diseases (1, 2). Invasive aspergillosis (IA) is a life-threatening infection with a mortality rate of about 85% in untreated patients; adequate intervention increases patient survival (3-5). Thus, molecular biologists are trying to develop better diagnostic laboratory tests for IA patients than those currently

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available (6). In susceptible individuals, the most frequent manifestation is invasive pulmonary aspergillosis, in which inhaled conidia lodge in the lung and germinate into mycelia within the alveoli. One important process in IA is angioinvasion by fungal hyphae, which results in endothelial damage, local erosion, and deep tissue invasion. Consequently, interactions between *Aspergillus* and blood platelets lead to intravascular thrombosis and localized infarcts (7-9). The interplay between platelets and *Aspergillus* has been shown to be a multifactorial process (10). Human platelets exhibit specific features of classic cell-mediated immunity and play important roles in antifungal host defense in IA by preventing germination, hyphal elongation, viability (11, 12), and increased susceptibility to antifungal drugs *in vitro* (13-16). During exposure to *Aspergillus*, platelets interact directly with hyphae, aggregate, surround the fungus, and release stored serotonin from their dense granules within 30 min (13). *Aspergillus*-induced activation of platelets also causes tissue damage and thrombosis, a well-known histopathological hallmark in IA patients. Thrombosis results from the exaggerated release of inflammatory mediators (inflammatory necrosis) by the procoagulant-complement proteins, which promote vascular endothelial damage and subsequent release of tissue factor from the vessels following invasion by the fungal hyphae (8, 11, 17-19). However, some studies have shown that blood platelets contribute to hemostasis and inflammation during thrombosis. Moreover, they play an important role in antimicrobial host defenses (20). For example, they affect *Staphylococcus aureus* (21) and *Candida albicans* (22) viability. Also, platelets can internalize bacteria and viruses (20) through contact-dependent mechanisms or platelet microbial peptides (PMPs) (23). Recent data has demonstrated that resistance of *C. albicans* to PMPs increases the severity of experimental Candida endocarditis (24). It was observed that serotonin, stored in platelet granules, has fungicidal activity against *Aspergillus* species and reduces fungal virulence (25). On that basis, we attempted to determine the interaction between platelets and *Aspergillus* species in more detail. Information on the antifungal role of platelets against *Aspergillus* spp. is limited. One study showed that activated human platelets express the CD63 glycoprotein after interacting with *A. fumigatus*. Furthermore, platelets help polymorphonuclear leukocytes (PMNs) to attack aspergilli. Perkhofer et al., reported that platelet granules contain serotonin, which is immediately released after contact with *Aspergillus* spp. Thus, on *Aspergillus* exposure, platelets considerably affect germination and hyphal elongation (13, 14, 26).

Although previous reports have shown that study of aberrant gene expression and genetic locus association can be helpful in understanding diagnosis and/or treatment of infectious and non-infectious diseases, few reports address IA caused by *A. fumigatus* (27-30).

Several studies examined the transcriptional response of *Aspergillus* spp. exposed to human cells and reported various *A. fumigatus* genes expressed in response to neutrophils (31), monocyte-derived dendritic cells (32), and airway epithelial cells (33). Genome-wide microarray analysis identified the differential expression of almost 9500 genes in *A. fumigatus* following exposure to human platelets (34).

Better understanding of the interactions between platelets and *A. fumigatus* invasion factors might provide important insight into the pathogenesis of IA for patient treatment and the future design of novel diagnostic or prognostic kits. Using a bioinformatic analysis pipeline on microarray data to identify effective genes, and then using Q-RT-PCR analyses, the present study aimed to evaluate differential expression of *A. fumigatus* genes following exposure to human blood platelets after 15, 30, 60, and 180 min of contact.

Materials and Methods

Analysis pipeline on the gene expression microarray dataset

A microarray gene expression dataset of *A. fumigates* induced by human platelets *in vitro* (E-MTAB-3024) was retrieved from the Array Express database

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(https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3024). This dataset consists of triplicates for each of five groups including untreated fungi as the control, and after 15, 30, 60, and 180 min of exposure to human platelets.

Analysis of DEGs using Limma R package

Limma package was used in R.3.5.0 to identify the differentially-expressed genes (DEGs) between the following pairs: AF-15 min vs. AF-control, AF-30 min vs. AF-control, AF-60 min vs. AF-control, AF-180 min vs. AF-control, AF-30 min vs. 15 min, AF-60 min vs. AF-30 min and AF-180 min hours vs. AF-60 min. Applying≤ 0.01 filter for the P value and≥ |1| for logFC, the up and down-regulated genes were identified in each group.

Annotation, Visualization. and Integrated Discovery analysis of DEGs

The DEGs in the different pairs were then introduced to The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) to identify their molecular functions and biological pathways.

Aspergillus fumigatus culture

An *A. fumigatus* strain isolated from an IA patient was provided for the platelet-fungus exposure experimental. The isolate was grown on Sabouraud Dextrose Agar (SDA, Merck Co., Darmstadt, Germany) and incubated at 35 °C for four days. The conidial suspension was harvested by flooding each colony with 2 mL of RPMI-1640. The harvested conidia were counted on a hemocytometer and resuspended at $1 \times 10^5 - 2.5 \times 10^5$ colony forming units (CFUs)/mL. The size and viability of the inoculum were determined using quantitative colony counts. Hyphae were produced from germination of conidia incubated in RPMI-1640 at 37 °C for 16 h with shaking at 150 rpm. Under these conditions, almost 90% of the conidia formed hyphae (13).

Preparation and Storage of Platelet Concentrates (PCs)

Fresh PCs were sourced from the Central Blood Transfusion Organization of Tehran Province, Tehran. Platelets were collected from healthy donors and prepared by Random Donor Platelets. All the donors gave written informed consent to use the remaining PCs for research purposes. All residual PCs collected were de-identified and used anonymously. Platelets were stored at 22-24 ℃ for a maximum of two days, and platelet aggregation analyses were used for quality control. Platelets were used at 6×10^8 cells/mL**.**

Co-culture of platelets with fungal hyphae

Before exposure to platelets, the fungal hyphae were cultivated via conidial germination by incubation of conidial suspension in RPMI-1640 at 37 °C for 16 h. The platelet-fungus infection assays were performed in wells of six-well plates. The platelets were added to the hyphae at a ratio of 100:1, and plates were incubated for 15,30,60, and 180 min. Untreated hyphae served as control. All samples were assessed in triplicate. To confirm platelet-fungus infection, hyphae were incubated with platelets and examined at the 30 min timepoint (13, 35).

Clinical samples and standard strain CBS 144.89 as control

To practically confirm the platelets-fungus exposure, *A. fumigatus* clinical isolates from five IA patients were obtained from the reference culture collection of the Tehran Medical Mycology Laboratory (TMML). Strain CBS 144.89, isolated from an IA patient in France and obtained from the CBS-KNAW Fungal Biodiversity Centre or Centraalbureau voor Schimmelcultures (Central Bureau of Fungal Cultures), Utrecht, the Netherlands, was considered as standard. Of the five TMML strains, four were *A. fumigatus* isolated from bronchoalveolar lavage (BAL) samples and one from a pseudo mycetoma lesion with

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severe necrosis. All six isolates were grown on SDA (Merck Co., Darmstadt, Germany) at 35 °C for four days. The conidial suspension was harvested by flooding each colony with 2 mL of RPMI-1690. The conidia were counted on a hemocytometer and adjusted to a concentration of 1×10^5 colony-forming units/mL (CFUs/mL). For hyphal growth, conidia were incubated at 37 °C for 16 h with shaking.

RNA isolation

Total RNAs were extracted from all *A. fumigatus* strains by RNX-Plus solution (SinaClon) according to the manufacturer's instructions with chloroform, isopropanol, and 75% ethanol. RNA was treated with DNase I (CinnaGen Co, Iran) to remove any potential DNA contamination. For quality control, the RNA purity was measured at A260/A280 in a spectrophotometer. The ratios were generally greater than 1.6.

cDNA synthesis

First-strand cDNA was synthesized using an Easy™ cDNA Synthesis Kit (Parstous biotechnology, Iran) according to the manufacturer's instructions. For each sample, a no-RT control was used in parallel with the DNase-treated RNA to detect any potential non-specific genomic DNA amplification.

Quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR)

Q-RT-PCR was performed with the Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, USA) using a RealQ Plus 2x Master Mix Green kit (Pishgam, Iran). Pep1/aspergillopepsin F (Pep1), allergenic cerato-platanin (Asp f 13), and endo-1,3 beta-glucanase (β-glucanase) gene expression was evaluated in the *A. fumigatus* hyphae after exposure to platelets for the various incubation times. The same procedure was also performed on the five clinical isolates from TMML and a standard strain, CBS 144.89. All experiments were performed at least in duplicate using a thermal cycler under the following conditions: 95 °C for 15 seconds, 65 °C for 15 seconds, and 60 °C for 1 min. Melting curves were then determined with temperatures ranging from 65 to 95 °C. Specific primers for the genes were designed using AlleleID 6.0 (Premier Biosoft, Palo Alto, CA, USA) and Gene Runner software (Hastings Software, Inc., Hastings, NY, USA). Primer sequences for amplifying cDNA of Asp f 13, Pep1, β-glucanase, and β-actin (as an internal control) genes are listed in Table 1. The product lengths were 171, 158, 191, and 206 bp, respectively, and the annealing temperature was 58 °C. To compensate for variations in the amount of input RNA and the efficacy of reverse transcriptase, β-actin mRNA was quantified as an internal control and all gene expression was normalized to it. Non-template controls for each primer set were assayed for potential of DNA contamination or primer dimerization. To calculate the relative fold change of each gene, we used the $2^{-\Delta Ct}$ method.

Table 1. Sequence of forward (F) and reverse (R) primers used in this study for amplification of three genes encoding invasion-promoting fungal proteinases listed in the left column.

Genes	*Sequence of primers*
Pep1[a]	*F: 5'CCG TCA ATC TTC CAG CTG TCT3'* *R: 5'AGT CCA AGT TCA GGG TCG TTC3'*
Asp f 13[b]	*F: 5'TCA CCA CAC CCA TCT CTC TCA3'* *R: 5'GAG CAG GAA ACG TCG TTC ATC3'*
β-glucanase[c]	*F: 5'CTC ATC AGC AAC GCT CCC TAA3'* *R: 5'TGC TCT CAA TAC GGC CTG AAG3'*
ACT[d]	*F: 5'GTC ACT GTG CAG ATT GTC GC3'* *R: 5'TCA GGA TGA GGA AAG GGG GA3'*

[a] *Pep1:* Aspartic endopeptidase Pep1/aspergillopepsin F.
[b] *Asp f 13:* allergenic cerato-platanin.
[c] *β-glucanase*: endo-1,3 beta-glucanase.
[d] *ACT:* β-actin

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Statistical analysis

Data are presented as mean values and error bars indicated as ±S.E.M. The significance of differential expression was analyzed using the repeated-measures ANOVA. Q-RT-PCR data was adjusted based on the exact PCR efficiency. SPSS version 18 was used for all analyses. P< 0.05 was considered statistically significant.

Results

Candidate genes encoding aspartic endopeptidases and endo-1,3 beta-glucanase enzymes

The deregulated genes in all pairwise comparisons are shown in supplementary Table 1. Functional annotation of deregulated genes in different groups of pairwise comparisons guided us to select the candidate genes Pep1, Asp f 13, and β-glucanase, which were detected as deregulated. Their involvement in *A. fumigates* has been previously reported.

Platelet aggregation around the hyphae

Platelets were exposed to hyphae for the various incubation times. Untreated hyphae served as control. The platelet-fungus contact was evaluated using an inverted light microscope. Platelet aggregation was seen as early as 15 min and increased over time. After 180 min, 95% of the hyphae were surrounded by platelet aggregates (Figs. 1A-1D). The hyphae-platelet interactions can be clearly seen with higher magnification (Fig. 2).

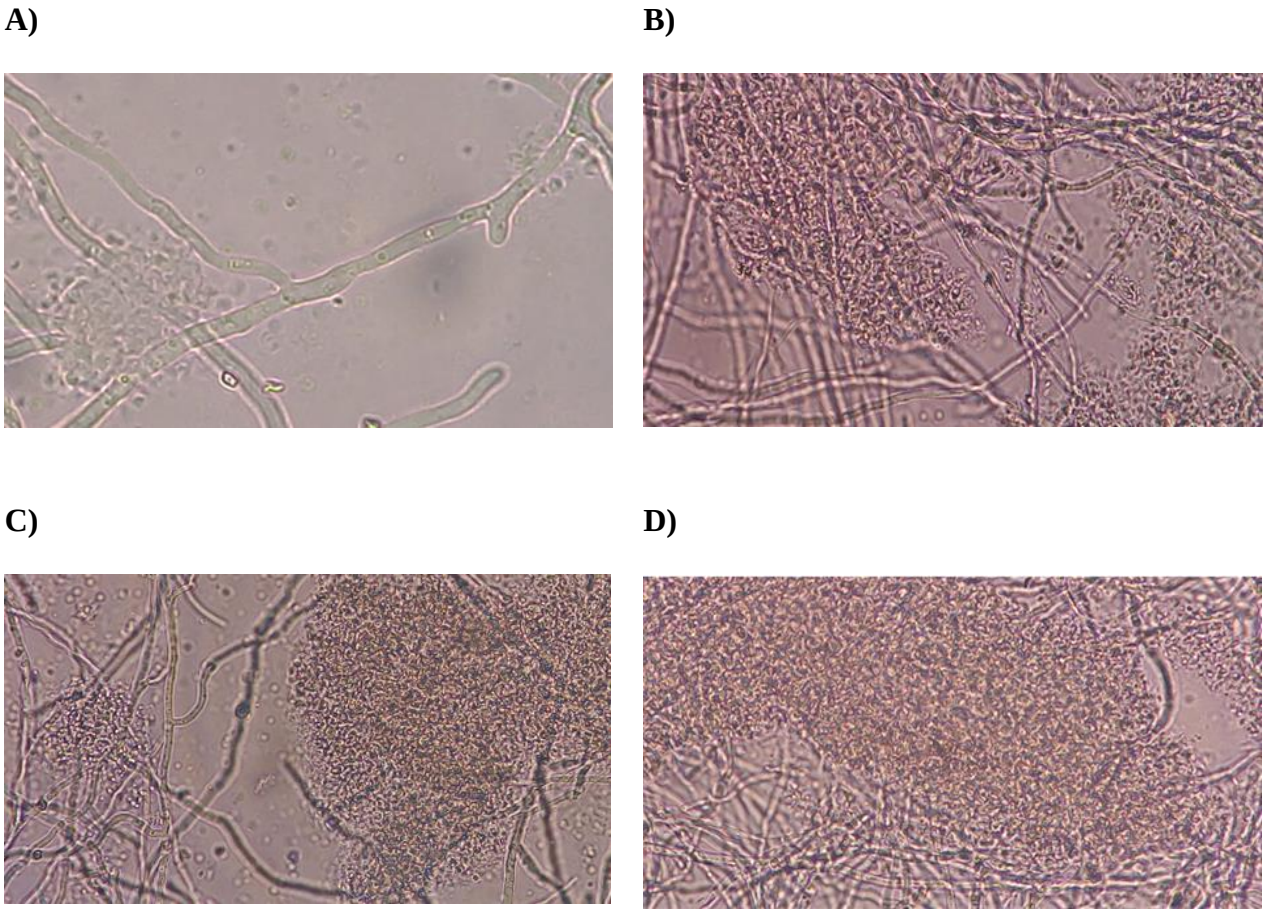

Fig. 1. *A. fumigatus* hyphae surrounded by platelet aggregates over time (A, 15 min, B; 30 min, C; 60 min, D; 180 min) identified by light microscopy (400x magnification). Fungal hyphae were cultivated via conidial germination by incubation of conidial suspension in RPMI-1640 at 37 °C for 16 h. The platelet-fungus infection assays were performed in wells of six-well plates. The platelets were added to the hyphae at a ratio of 100:1, and platelets were incubated for 15,30,60, and 180 min. Untreated hyphae served as control.

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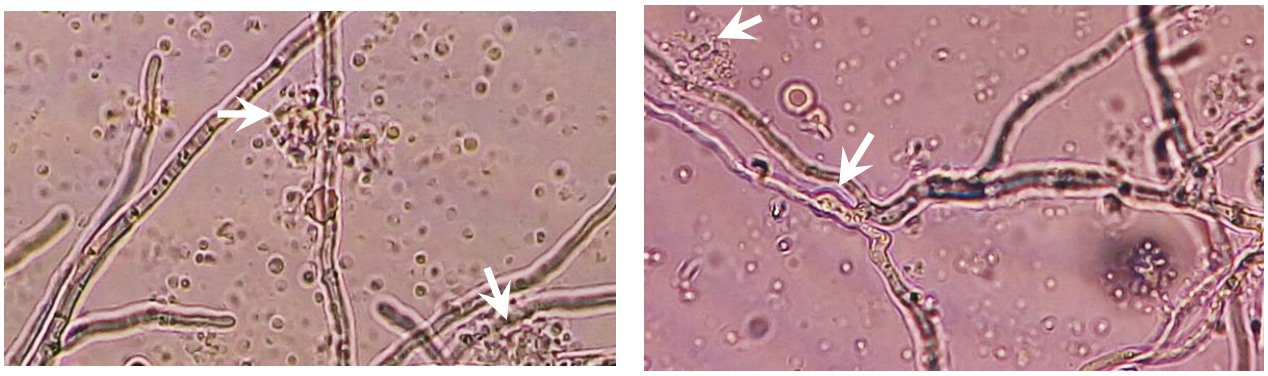

Fig. 2. Inverted light micrographs (400x magnification) showed hyphal-platelet interactions after 30 min (A) and 60 min (B). The assays were performed as in Figure 1.

Aberrant expression of DEGs

The relative expression of Asp f 13, Pep1, and β-glucanase in the hyphae was measured by Q-RT-PCR after exposure to platelets and compared with that of control hyphae. All three candidate genes were significantly down-regulated after 30 min of platelet-mycelia exposure (Fig. 3). Asp f 13-encoding gene expression was significantly less than control at 30 and 180 min; at no time point was expression significantly greater than the control; however, Asp f 13 gene expression was significantly greater at 60 than at 30 min. Pep1-encoding gene expression was significantly less than control at 30 min, but significantly greater at 60 min. Pep1 gene expression is also significantly greater at 60 and 180 than at 30 min. β-glucanase encoding gene expression was significantly less than control at 30 and 60 min, and was significantly greater at 60 and 180 min than at 30 min. We also evaluated the expression changes of Asp f 13, Pep1, and β-glucanase in the fungal cultures of the five TMML clinical isolates and standard strain CBS 144.89. Expression of these genes in four of the clinical isolates was not significantly different from the standard strain; however, expression of one bronchoalveolar lavage isolate, BAL4, was significantly greater than the standard for all three genes. (Fig. 4).

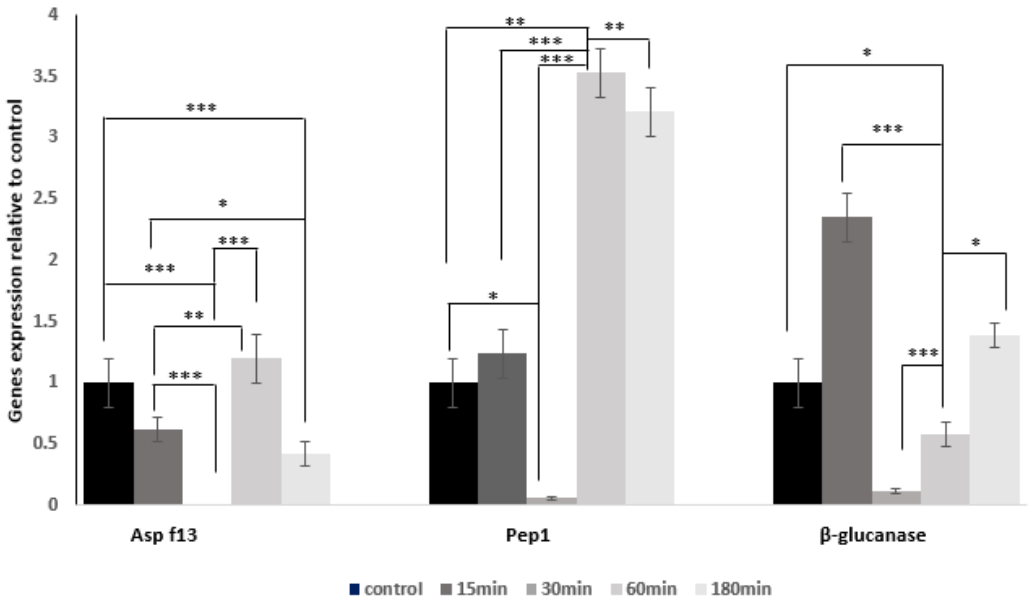

Fig. 3. Relative expression of the genes encoding the aspartic endopeptidases allergenic cerato-platanin (Asp f 13), aspergillopepsin F (Pep1), and endo-1,3 beta-glucanase (β-glucanase) in *A. fumigatus* hyphae infecting human blood platelets for 15,30,60, and 180 min. Controls were *A. fumigatus* hyphae with no platelets. All three genes were downregulated from control to 30 min. Then they were up-regulated from 30 min to 60 and 180 min; however, Asp f 13 and Pep1 down-regulated from 60 to 180 min, while endo-1,3 beta-glucanase expression was increased from 60 to 180 min.

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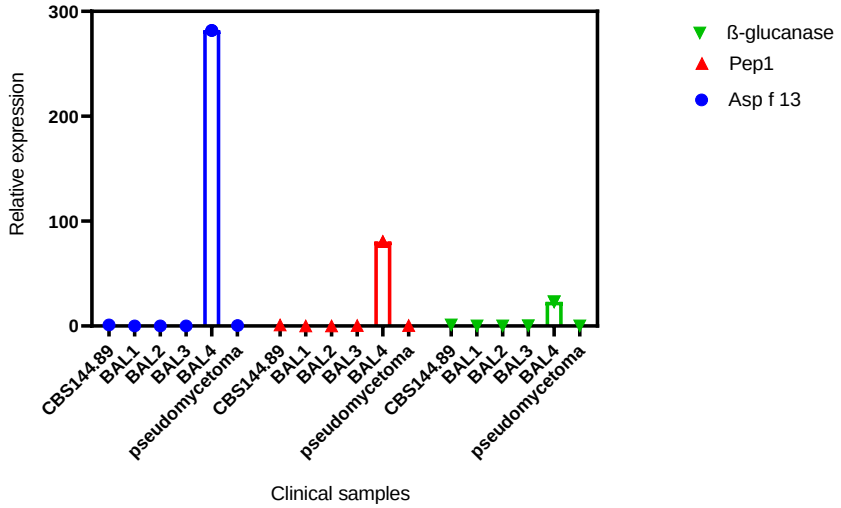

Fig. 4. Relative expression of Asp f 13, Pep1, and endo-β-1,3-glucanase genes in the five clinical isolates and standard strain CBS 144.89. All three genes in four of the five clinical isolates were expressed at the same low level as the standard strain CBS 144.89, but in one isolate, BAL4, all three genes were up-regulated relative to the standard strain.

Discussion

Few studies on the transcriptional response of *Aspergillus* spp. to human cells have been reported. The available studies identified DEGs in *A. fumigatus* conidia exposed to neutrophils (31), monocyte-derived immature dendritic cells (32), and 16HBE140 human bronchial epithelial cells (33). Based on our bioinformatics analyses, we chose three candidate genes that encode essential enzymes as thrombotic and angioinvasive components in IA: Aspartic endopeptidase Pep1/aspergillopepsin F, allergenic cerato-platanin Asp f 13, and endo-1,3 beta-glucanase (AFUA_5G13300, AFUA_2G12630, AFUA_6G14540). Perkhofer etal., studied the transcriptional response of *A. fumigatus* hyphae to human platelets in a time course and reported genes that were commonly identified in previous studies. Those genes were deregulated after exposure to platelets, and were associated with regulation of biological processes, molecular functions, pathogenesis, and cellular composition (34). In the present study, using experimental Q-RT-PCR, we validated the microarray dataset of the Perkhofer study to evaluate the expression changes of Pep1, Asp f 13, and β-glucanase genes in *A. fumigatus* co-cultured with human platelets, and also in the fungal cultures of five clinical isolates with a standard strain CBS 144.89. In this study, we aimed to gain a better understanding towards elucidation of probable interactions between platelets and fungal cells as they are involved in the thrombotic process. Because the fungal cell wall is an important defense against host immune machinery in IA, it might be a likely target of the host immune system and antifungal drugs (36, 37). Exposure of *A. fumigatus* to platelets significantly effects germination and hyphal elongation (13). Moreover, its interaction with platelets leads to the downregulation of some genes related to cell wall integrity, including cell wall galactomannoprotein (Afu4g00870), cell wall glucanase Utr2 (Afu2g03120), cell wall protein (Afu3g08110), and alpha-1,3-glucan synthase Ags2 (Afu2g11270). Galactomannoprotein and Utr2 were induced in the fungal infection (38). Utr2 and Afu3g08110 expression was increased in *A. fumigatus* exposed to human airway epithelial cells and human immature dendritic cells (32, 33). In *A. fumigatus*, β-1,3-glucan is an important constituent that confers rigidity to the cell wall. The β-1,3-glucan hydrolyzing enzymes are divided into exo- and endo-β-1,3-glucanases (39). The endo-β-1,3-glucanase cleaves inside a glucan chain somewhat randomly. β-1,3-glucanases, especially endo-β-1,3-glucanases, play a principal role in cell

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wall softening during germination and mycelial elongation (40). Endo-β-1,3-glucanases (AFUA_6G14540) gene expression might be expected change to maintain hyphal elongation during contact with platelets. In addition to cell wall enzymes, some important *A. fumigatus* extracellular enzymes produced in IA that can trigger thrombosis are aspartic proteases, which are named aspergillopepsin and alkaline serine proteases. Secreted aspergillopepsin (Pep1), which matches the known Asp F10 allergen as an aspartic protease, and Asp f 13/Alp2, an alkaline serine protease, have elastinolytic activities on blood vessel walls that can lyse human elastin, collagens, and fibronectin (4). This might be attributable to an initial rapid response to the hyphae-platelet contact, followed by a delayed response of secretory components released from platelet granules. The changes in gene expression between 15 and 180 min, may be due to the adaptation process of hyphae-platelet contact. However, we observed up-regulation of β-glucanase gene from 60 to 180 min, which might be due to continuing fungal growth. Also, Asp 1, 3 and Pep1 were down regulated over that time. According to a previous study, during fungal growth, soluble *A. fumigatus* proteases could induce platelet aggregation and granule release. It has been indicated that *A. fumigatus* is an effective inducer of platelets, which may be co-activated by thrombin stimulation (41). As described in the results, dysregulation of the candidate genes varied between the five TMML clinical isolates, with only one of them being up regulated (Fig. 4). This result may be due to patient differences including immune system suppression, neutropenic condition, antifungal therapy, and others. Histological features of tissue damage in invasive pulmonary aspergillosis differ between neutropenic and non-neutropenic patients; for example, inflammatory necrosis is significant in non-neutropenics, while angioinvasion is dominant in neutropenics (42). Also, studies have shown that platelets play a critical role in the pathogenesis of aspergillosis; thus, the infection might differ between patient groups according to the presence or absence of thrombocytopenia (43). Understanding the interactions between platelets and *A. fumigatus* invasion factors will provide insight into IA pathogenesis. Essential characteristics, such as immune cell activation, complement system recruitment, and mycocide component release have previously been elucidated and currently capture the attention of researchers; their results will direct future studies. A principal attribute in the bifunctional role of platelet-associated responses is that the useful antifungal effects and consequential disease burden reduction are offset by destructive outcomes including excessive inflammation and thrombosis. Moreover, the role of platelets in IA, if properly explored, may translate into new platelet-based therapies. We believe further studies are warranted to elucidate the overall significance of platelets in *Aspergillus* infection.

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The authors declare that they have no conflict of interest.

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