



Detection of Fungal Elements in Atherosclerotic Plaques Using Mycological, Pathological and Molecular Methods

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Abstract

Background: The aim of this study was to detect fungi in atherosclerotic plaques and investigate their possible role in atherosclerosis.

Methods: Coronary atherosclerotic plaques specimen were obtained from patients with atherosclerosis. Direct examination, culture, histopathology study, PCR and sequencing were performed to detect/identify the mycotic elements in the plaques. Age, sex, smoking, obesity, hypertension, hyperlipidemia, family history of heart diseases and diabetes were considered and data were analyzed using Chi Square test by SPSS version 15.

Results: A total of 41 specimens were analyzed. Direct examination for fungal elements was negative in all cases but in culture only one specimen grew as a mold colony. The presence of fungal elements were confirmed in 6 and 2 tissue sections stained by Gomori methenamine silver and Hematoxylin and Eosin methods, respectively. Using PCR, 11 cases were positive for fungi. The DNA sequence analysis of six positive specimens which were randomly selected revealed fungi as *Candida albicans* (n=3), *Candida guilliermondii* (n=2) and *Monilia* sp. (n=1).

Conclusion: A significant association between the presence of fungi in atherosclerotic plaques and severity of atherogenesis and atherosclerotic disease was not found. This could be due to limited numbers of patients included in our study. However, the presence of fungal elements in 26.8% of our specimens is considerable and the results does not exclude the correlation between the presence of fungi with atherosclerosis and coronary artery disease.

Keywords: Atherosclerosis, Coronary disease, *Candida*, Fungi

Introduction

Coronary heart disease (CHD) is one of the most common and dangerous human diseases. It is a major cause of disability and the most common cause of death; especially in industrialized societies. CHD is often a result of atherosclerosis (1). Atherosclerosis is a chronic inflammatory disease that many factors are involved in its pathogenesis (2). The classic risk factors include aging, obesity,

smoking, high blood pressure, diabetes, physical inactivity and stress (3). In addition to these factors, the role of inflammatory and infectious agents in pathogenesis of the atherosclerosis has been discussed (4, 5).

Several studies have been done in correlation between bacterial and viral pathogens such as *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Helicobacter*

pylori, herpes simplex virus and human cytomegalovirus with atherosclerosis and coronary artery disease (6-9). To the best of our knowledge, studies on the role of fungi in atherosclerosis are limited. A survey on fungal rDNA signatures in human coronary atherosclerotic plaques has been done and recently in another study the correlation of atherogenesis with *Candida albicans* infection has been reported (10, 11). Furthermore in an animal study, the role of *Cryptococcus neoformans* was investigated as a potential inducer of atherogenesis (12). The aim of the present study was to investigate the possible role of fungi in the atherosclerosis. In the study, to increase the accuracy of the results, we used mycological, pathological and molecular methods to detect presence of fungal elements in atherosclerotic plaques. Elimination of these infectious agents could be considered in prevention of coronary heart disease, if fungi have a role in atherogenesis.

Materials and Methods

During 2011 to 2012, a total of 41 patients with CHD hospitalized in Imam Khomeini and Mehr Hospitals in Tehran, Iran, included in our study. Artery occlusion was confirmed by angiography in all patients (more than 50% stenosis in at least three coronary arteries) then Coronary Artery Bypass Graft (CABG) as the sole treatment was performed. Patient's information such as age, sex, smoking, obesity, hypertension, hyperlipidemia, family history of heart diseases and diabetes were considered.

Informed consent to participate in this study was signed by all patients and Ethics Committee of Tehran University of Medical Sciences approved this study.

Atherosclerotic plaque specimens were obtained during surgery and aseptically divided to three pieces and separately were placed in sterile tubes containing normal saline and formalin used for culture and pathology, respectively. For molecular analysis DNA and RNA free tubes containing 70% alcohol were used and specimens were im-

mediately transported to the laboratory in a cool box.

Pieces of the specimens were cultured on CHROMagar Candida, Sabouraud dextrose agar, Sabouraud dextrose agar containing chloramphenicol, Brain heart infusion agar, Blood agar and Eosin methylene blue agar. Ten percent KOH preparation was used for microscopic direct examination of clinical specimens to seek for fungal elements. For pathological examination, tissue sections were stained by Gomori methenamine silver (GMS), Hematoxylin & Eosin (H&E), and Periodic Acid Schiff (PAS).

For molecular analysis, fungal DNA was extracted from the specimens using DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions and PCR performed for fungal rDNA detection using universal primers ITS1 (5-TCC GTA GGT GAA CCT GCGG-3) as forward and ITS4 (5-TCC TCC GCT TAT TGA TAT GC-3) as reverse primers. DNA amplification performed using one initial denaturation at 95°C for 5 min, followed by 35 cycles including 30 sec denaturation at 95°C, 1 min annealing at 49 °C and 90 sec extension at 72 °C and 1 cycle of 9 min at 72°C for final extension. Six positive specimens were randomly selected and the amplicons were subjected to sequencing and data were compared with reliable sequences deposited in GenBank using the basic local alignment search tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>).

Demographic data were analyzed using Chi Square test by SPSS (Chicago, IL, USA) version 15 and a *P* value <0.05 was considered as statistically significant.

Results

Direct examination with 10% KOH was negative for fungal elements in all specimens, but culture was positive in only one case and it was grown as a mold colony. The microscopic characteristics of this fungus in slide culture revealed hyaline, septate and branched mycelium with unicellular, spherical or cylindrical and hyaline conidia (Fig.1). Fungal elements were seen in tissue sections as well.



Fig. 1: Microscopic view of *Monilia* sp. isolated from a specimen of coronary atherosclerotic plaques

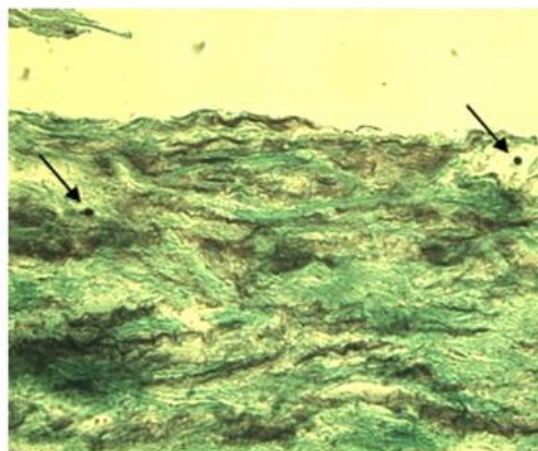


Fig. 3: Yeast cells in artery biopsy (GMS) ×1000

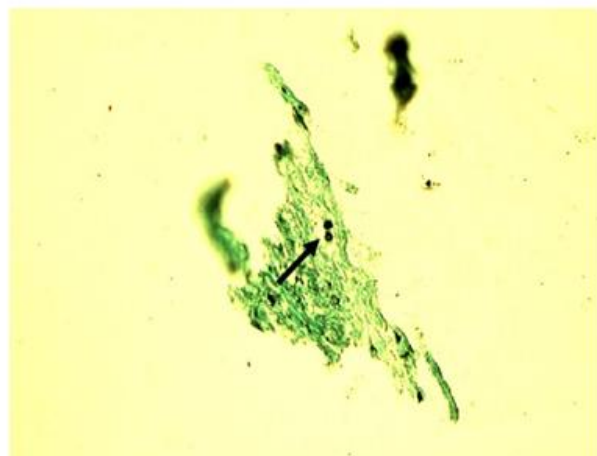


Fig. 2: Yeast cells in artery biopsy (GMS ×1000)

This isolate was microscopically identified as the *Monilia* sp. and confirmed based on PCR and sequencing. The presence of fungi were confirmed in tissue sections stained with GMS (Fig. 2, 3) and H&E, in 6 and 2 specimens, respectively. But fungal elements were not observed by PAS staining of tissue sections.

By performing PCR with fungal universal primers, 11 out of 41 specimens analyzed in this study, were positive for the fungal organisms. Blast analysis of the 6 sequences confirmed the amplicons as *C. albicans* (n=3), *C. guilliermondii* (n=2) and *Monilia* sp. (n=1) (Fig. 4).

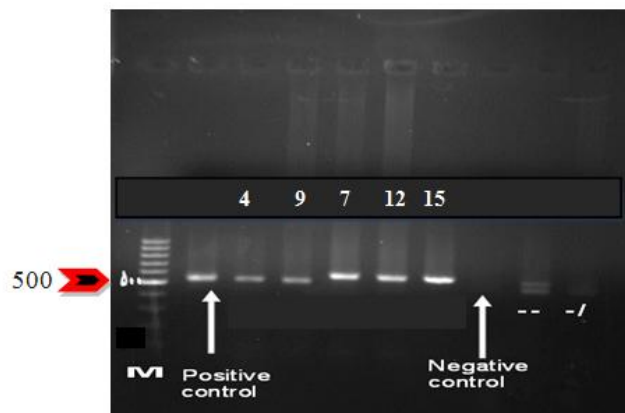


Fig. 4: An example of gel electrophoresis of PCR products of coronary atherosclerotic plaques. No. 4 and 9 are *C. albicans*, No. 7 is *Monilia* sp. and No. 12 and 15 are *C. guilliermondii*. Positive (standard *C. albicans*) and negative (sterile distilled water) controls are shown.

Discussion

In the recent years, the role of infectious agents in pathogenesis of atherosclerosis has supported by several studies. These studies have focused mainly on viral and bacterial pathogens and few of them have been done on the role of fungi in this process (2, 9). We studied the role of fungi in the pathogenesis of atherosclerosis in patients with angiographically proved coronary heart disease (more than 50% stenosis in at least three coronary

arteries) using direct examination, culture, histopathology and PCR-sequencing methods. Although fungi were found in atherosclerotic plaques in 26.8% of cases, our study did not demonstrate statistically significant association between the presence of fungi and atherosclerosis. Also, no significant association was found between age, sex, smoking, obesity, hypertension, hyperlipidemia, family history of heart diseases, and diabetes with the presence of fungal elements in atherosclerotic plaques. In a study conducted by Ott and colleagues, the presence of fungal organisms in 92.11% (35 of 38) of atherosclerotic plaque samples was confirmed. Therefore fungi were considered as a non-specific underlying factor in development of atherosclerosis (10). These results are not well consistent with the results of the present study. Difference in geographic area, patient groups and diagnostic method may be effective in different results of two studies. On the other hand results of our study are in agreement with results obtained by Maya J Nurgeldiyeva et al. that showed the presence of fungi of the genus *Candida* in 31.9% (15 out of 47) of atherosclerotic plaques (11).

The oral cavity and gastrointestinal tract could be the major sources of microbial agents present in atherosclerotic plaques (13, 14). Fungi, especially *Candida* species are a part of the mouth and gastrointestinal tract flora. Yeasts can enter the bloodstream through small wounds during brushing or by therapeutic interventions such as tooth extraction, root canal therapy and surgery and cause transient fungemia (2, 10, 14). Platelets immediately will cover fungi in circulatory system that forms platelet aggregation. This aggregation causes fungi clinging to the vascular endothelium, which can increase the risk of thrombosis and cardiovascular disease (15). Taheri and colleagues compared oral *Candida* flora of 90 patients with coronary atherosclerosis and 90 control subjects. Although no statistically significant difference was found between *Candida* colonization in patients and controls, the number of colonies and the intensity of *Candida* colonization were significantly lower in controls (16).

In our study, only patients with angiographically proved coronary heart disease were included. Application of different diagnostic methods for detection of fungi in atherosclerotic plaques is an advantage for our study that increased the accuracy of the results. However, the study population size was limited and this could be a disadvantage for the study.

Conclusion

Although, a significant association between the presence of fungi with atherogenesis and atherosclerotic disease was not found in this study, the presence of fungi in 26.8% of specimens is noteworthy and the results does not exclude the role of fungi in atherosclerosis and coronary artery disease. Therefore, more studies with more number of patients is recommended for a better evaluation of fungi role in atherosclerotic disease and CHD.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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