Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

Pharmacophore
an International Journal
ISSN 2229-5402

AN INVESTIGATION INTO DIFFERENT NUCLEOTIDE SEQUENCES OF THE ASPARTYL PROTEINASE GENE PROMOTER REGION IN THE CANDIDA ALBICANS ISOLATED FROM CLINICAL SAMPLES

Vahid Hasani[1*], Ayatollah Nasrollahi Omran[2], Ali Nazemi[3], Zahra Hamidi[4], Mohaddese Radmehr[5], Parisa Maleki[6]

1. Field of microbiology of Islamic Azad University of Tonekabon
2.3. Assistant Professor, Tonekabon, Islamic Azad University, Microbiology Department
4. Department of Nursing and Midwifery, Bojnourd Branch, Islamic Azad University, Bojnourd, Iran
5. Field of Microbiology, Islamic Azad University of Damghan.
6. Azad University of Damghan, Field of Biotechnology, Microbial Trend

ARTICLE INFO

Received:
03th Jun 2017
Accepted:
29th Nov 2017
Available online:
14th Dec 2017

Keywords: *Candida Albicans, Aspartyl Proteinase, Promoter*

ABSTRACT

Literature Review and Purpose: Candida Albicans is one of the most significant and prevalent opportunistic fungal diseases in human beings. Secretory Aspartyl Proteinases (SAPs) is the most important enzyme produced in Candida Albicans. The present study investigated the Aspartyl Proteinases Gene as a pathogen in Candida Albicans as well as investigating its different sequence of promoter region in the clinical samples.
Material and Method: In this study, 30 Candida samples provided from the clinical samples (at the research laboratory of Tonekabon Islamic Azad University) were incubated in the sabourd dextrose agar medium. Then, through microscopic tests as well as germ tube and chlamydospores diagnostic tests, only five Candida Albicans isolates were distinguished. The samples' DNAs were extracted using phenol-chloroform method. After that, promoter sequence was designed manually by means of specific primers. Finally and after PCR, the sequencing was conducted using DNA man software.
Findings: The five samples under investigation were sequenced. In the promoter region of the whole samples, the same mutation was seen in some situations. Also, a single mutation was seen in samples 3, 6, and 8.
Conclusion: In this study, promoter sequence of this gene was investigated in order to conduct some research on the investigation of the target site for the performance of new antifungal drug in case of drug resistance. The results indicated low protection in this region.

To Cite This Article: Vahid Hasani, Ayatollah Nasrollahi Omran, Ali Nazemi, Zahra Hamidi, Mohaddese Radmehr, Parisa Maleki, (2017), "an investigation into different nucleotide sequences of the aspartyl proteinase gene promoter region in the candida albicans isolated from clinical samples", ***Pharmacophore, 8(6S),*** *e-117383.*

Introduction

Candida Albican is a static natural fungus (flora) in humans and warm-blooded animals (2). This opportunistic two-shaped yeast (Dimorphism) (hyphae, yeast) often exists on mucosal surfaces in the form of natural flora. Depending on the host nature, this fungus creates two groups of infections: superficial infections (e.g. oral, vaginal, and cutaneous candidiasis) which are also seen in healthy individuals and deep infections (e.g. lung, gastrointestinal, urinary, candidaemia) which occur in individuals with severe immune disorders (6). These yeasts are usually opportunistic organisms (4). Controlling factors of the immune system such as chemotherapy in cancer patients, radiation therapy, using wide-ranged antibiotics, AIDS, diabetes mellitus, etc will turn these fungi invasive (3). Invasive candidiasis is one of the prevalent and potentially fatal side effects of

Corresponding Author: Vahid Hasani, Field of Microbiology of Islamic Azad University of Tonekabon, email: vahid_bienteha@yahoo.com

cancer and the related chemotherapy (7). Most candida infections are caused by candida albicans (8). Candida species accumulate and produce infection in nearly all cancer patients who undergo radiation therapy for head and neck. If this infection develops locally or generally, it will lead to the patient's death (10). Predisposing factors such as adhesion, biofilm formation, phenotype changes, and produced extracellular hydrolytic enzymes have been proposed for candida albicans pathogenicity. Extracellular hydrolytic enzymes, particularly secretory aspartyl proteinases (SAPs), are of several gene products having a direct role in candida albicans pathogenicity (14). Secretory aspartyl proteinases (SAPs) are the main extracellular hydrolytic enzymes produced in candida albicans. SAPs are coded sap_1 - sap_{10} by a gene family including at least 10 different genes. Sap_1 – Sap_3 are responsible for gene expression in the yeast phase, Sap_4 to Sap_6 for gene expression in the filamentous form, and Sap_9 to Sap_{10} for expression in both forms. It is indicated from the presence of 10 Sap genes, their activation time during separate infection phases, and their arranged composition for creating diseases that the members of this gene family play a significant role in the adaptive response by candida albicans to its environment and host. As an example, Sap_1 that codes an extracellular proteinase and had been simulated in 1999 was thought to be responsible for producing secretory aspartyl proteinase in candida albican pathogenicity (15). Shortly after discovering Sap_1, the majority of genes coding extracellular aspartyl proteinase were identified by PCR technique. RT-PCR method enables us to analyze gene expression in Sap_{1-10} in the isolated candida albicans directly through the human patients' mouth and vagina. As an example, it has specified that Sap_1 and Sap_3, in addition to Sap_6, and finally Sap_6 and Sap_8 are produced in the final phases of infection (17). The regulation of gene expression in transcription stage is accomplished by two groups of factors: the first group is the transcription factor and the second group is the regulatory areas. The transcription factor is divided into two groups: 1. general – in which all the genes are needed for transcription and that it helps RNA polymerase to connect to the promoter, and 2. Specific – which include specific genes (11). Regulatory areas also include promoter regions, enhancers, and silencers. The promoter where RNA polymerase ו and general transcription factors join and start transcription is the place for the upstream promoter (12). Of every 100 nucleotides present in our genomes, one is methylated. This process is conducted on cytosine and the enzyme involved in it is called DNA methyl transferase. There are genes that code this enzyme

(13). This process is related to gene silencing and takes place wherever DNA sequence occurs in the form of 5 – CpG – 3, i.e. the points where we have CG consecutively. If the gene promoters are methylated in these 5 – CpG – 3 areas, the gene will be silenced (16).

This study aimed at investigating different nucleotide sequences of the aspartyl proteinase gene promoter region in the candida albicans isolated from clinical samples, so that we might report the existing potential changes.

Material and Method

Identification of Candida Isolates

First, the fungus candida albicans provided at the research laboratory of Tonekabon Islamic Azad University was cultured on the sabourd dextrose agar (SDA) medium containing Chloramphenicol and kept in an incubator at 37 ˚C for 48 hours. After the incubation time passed, the created colonies were examined on the sabourd dextrose agar medium to identify the yeast type. The candida isolates were identified through germ tube formation test in horse serum, chlamydospore formation test on the cornmeal agar medium (DIFCO Laboratories, Detroit, Mich., USA) with 1% tween 80 (CMA_T_{80}), and finally culture on chromagar medium.

DNA Isolation

First, the monoculture of each candida albicans isolate was provided and incubated at 37 ˚C for 48 hours. DNA was extracted using phenol-chloroform method. In short, the process was as follows: a full loop of fresh candida albicans was suspended in 500 µl lysis buffer (10 mM EDTA pH 8, 100 mM NaCl, 1% SDS, 100 mM Tris pH 8, 20 Mm Pectinase X-100 2% Triton). Then we added 50 µl of proteinase k and put the samples in driver plate for 16 hours. Next, we added and mixed 60 µl of TAB and 140 µl of 5 M NaCl. After that, we added 750 µl of chloroform and mixed it again. After 10 minutes, the cellular debris was isolated for 5 minutes using a centrifuge with 10000 rpm and the top phase was transferred to the new Micro-tube. Then, we added 0.6 of isopropanol and 0.1 of 3 M sodium acetate. The samples were frozen for 10 minutes and then centrifuged with 14000 rpm for 10 minutes. In the last phase, DNA was washed with 500 µl of 70% ethanol, dried in air, and suspended in TE buffer (10 mM Tris-HCL, 1 mM EDTA pH: 8). DNA concentration and absorption ratio with the wavelengths of 260 and 280 Nms (A260/A280) were determined through the spectrophotometry method. The genomic of the isolated DNA was kept at -20 ˚C until the time of use.

PCR Method

Primer Design for the Proliferation of the Aspartyl Proteinase Gene Promoter Region

The primer of the Aspartyl Proteinase Gene was designed manually. This primer had a sequence as the following:

Promoter sequence: ATATCAAAAAAACAATATAAAAGGATAGATGATTTCCCTTGTTGTGGAGA

Forward primer: AACAATAACAAAGAGCACC 19-mer

Reverse primer: GTAGCATCGACTAATAAAGC 20-mer primer size: 220 bp

PCR was done with the total volume of 50 µl including 2.5 µl of buffer X 10, 14.5 µl of H_2O_2, 2 µl OF $MgCl_2$, 1 DNTP mix, 1 µl of primer mix, 1 µl of Taq, 3 µl of template DNA, and 25 µl of the total volume. Thermal cycles of the PCR reaction included initial denaturation at 95 ˚C for 4 minutes that worked with 35 cycles as follows: (denaturation: at 95 ˚C for 1 minute), (connectivity: at 51 ˚C for 1 minute), and (elongation: at 72 ˚C for 1 minute), and the final elongation was done at 72 ˚C for 10 minutes. In qualitative analysis, we put some of the polymerase reaction product along with deoxyribonucleic acid marker on 1% agarose gel and investigated them in terms of band strength and piece size.

Sequencing of PCR Products

Having conducted qualitative analysis, the remaining of the polymerase reaction product as well as the forward primer was sent to Macro Gene Company in South Korea for sequencing. Then, the obtained DNA sequences were analyzed using DNA man software.

Findings

Phenotypic Identification

5 isolates out of the 30 vaginal candidida isolates present at the research laboratory of Tonekabon Islamic Azad University were identified as germinated yeast cells, false and true mycelia under microscope. They produced light green colonies on chromagar medium, they were positive germ tube, and they were identified as candida albicans.

Figure 1. Candida yeast cells and their false myceli

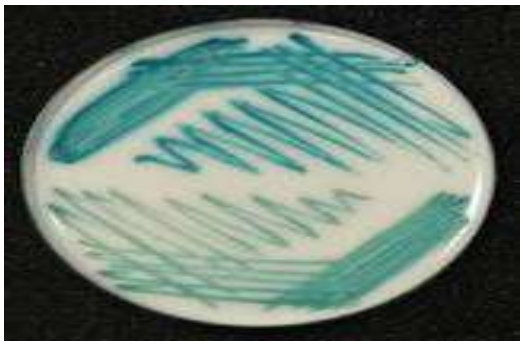

Figure 2. Candida albicans in chromagar medium

Molecular Methods for identifying Candida Albicans Isolates

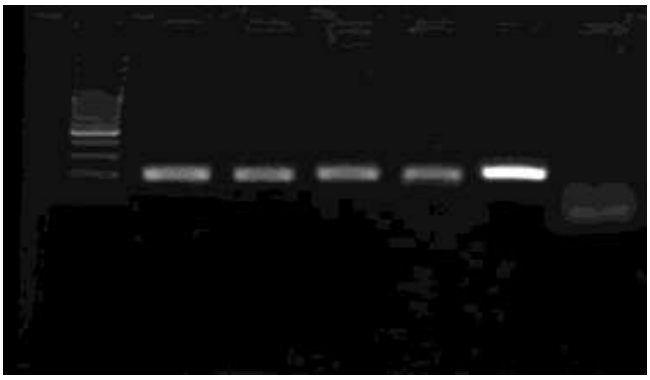

Figure 3. PCR products from 5 candida albicans DNAs that run on 1% agarose gel that produced band in 620 (pb) piece

Results for the Sequencing of PCR Products

For the sequencing to be done, 5 samples were investigated. 100% equal mutation was seen in positions 2،15،17،23،37،39،42،47،48 of the promoter region, 60% in position 4, 40% in position 14, and a single mutation was seen in positions 3،6،24، and 25.

Fig 4. Sequencing mutations

Discussion

The opportunistic fungus candida albicans produces SAP enzyme that enables this yeast to use environmental proteins as a source of nitrogen and causes yeast growth and pathogenicity (15). Biological substrates of SAP enzymes are abundant and include bovine serum albumin (BSA), creatine, collagen, Lactoferrin, and secretory immunoglobulins (IgA which is normally resistant to bacterial proteases). These enzymes are utilized by fungus to disintegrate outer tissues of the body, stop the host immune system, and achieve amino acids necessary for yeast metabolism. It has been proved in the previous studies that candida albicans needs SAP enzymes to create candidiasis. The presence of these enzymes is proved due to the different phases of penetration into the host tissue and consequently pathogenesis. Therefore, these enzymes can serve as good targets for developing medicines against this yeast (3). In this study, research on pathogenic strains of Candida albicans in the category of Sap enzymes and production of recombinant vectors was conducted for the first time. For this reason, the researchers aimed at discussing the most important enzyme of the SAP family in terms of pathogenicity in a suitable vector and purification of recombinant protein in order to achieve a permanent production source of the so-called enzyme so that they may use it in their further studies to provide vaccine and diagnostic kits, produce medicine against this yeast, and do further research in Iran (16). A great deal of research has been done concerning the biology of candida species. Since these factors are related to treatment, surgery, and other medical services, the results of these studies have been published in the form of hundreds of one-year scientific essays on candida albicans and yeast organisms. The use of molecular – biological laboratory methods has led to

increased potential opportunities for offering direct and indirect methods in diagnosing candida infections as well as determining serious antifungal target molecules and virulence factors in candida species (14).

The previous studies on SAPs mostly dealt with comparing SAP genes and their pathogenicity. Mohd Sajjad Ahmad Khan et al (2010) studied fungal virulence factors and found out that adhesion, two-shapedness, phenotype change, hydrolytic enzyme secretion (SAPS), biofilm formation, and adaptive capability in the host's body temperature can be in connection with candida albicans (1). Also, regarding aspartyl proteinase enzyme mutations, Yee-Chun Chen et al (2002) found out that Sap_2 plays a key role in candidia pathogenicity. (5) In another study by Lermann & Joachim (2008), it was claimed that deletion mutation for genes Sap_1 and Sap_2 or Sap_3 influences candidia vaginitis pathogenicity in mice, while this mutation in Sap_4 - Sap_6 will create no defect as virulent as candida in mice (9).

The present study investigated a part of aspartyl proteinases gene promoter region which has not been studied thus far. All the present research has dealt with aspartyl proteinase enzyme mutations in pathogenesis as well as important factors in the virulence of the fungus candida. Promoter is a region above the start codon that is attached to RNA polymerase. This allows DNA to start transcription at the proper place. This region also has protected regions in all eukaryotes.

Conclusion

The present study in which 5 samples were investigated for sequencing aimed at investigating the sequence of the aspartyl proteinase gene promoter region in candida. After the analysis of the results of sequencing in the 5 samples under study, aspartyl proteinase gene sequences were determined through sequencing. By mutation in position 2 of the promoter area in all of the samples, open thymine nucleotide replaced cytosine. In position 3 of sample 8, open adenine nucleotide replaced thymine. In position 4 of the promoter area 60% of the open thymine nucleotide replaced cytosine which was also seen in samples 3, 9, and 10. In position 5 of samples 3, 8 open thymine nucleotide replaced cytosine and in positions 6, 9, and 10 open adenine replaced cytosine. In positions 15, 39 of the promoter region, there was 100% of nucleotide mutation and open cytosine replaced thymine. Also, in positions 37, 42 of the promoter region, there was 100% of nucleotide mutation and open cytosine replaced thymine. 100% mutation was also seen in positions 17, 23; in position 23, open adenine replaced thymine and in position 17, open adenine thymine open guanine. Also, there was 100% mutation in positions 47, 48 where open thymine replaced open guanine in position 47, but it replaced open adenine in position 48. Single mutation was also seen in positions 6 and 24 of sample 3 where open thymine replaced open adenine. In positions 6 and 24 open thymine replaced open cytosine and a single mutation was also seen in position 25 where open cytosine had replaced open thymine in sample 8. In position 14 of the promoter region, 40% of nucleotide mutation was seen where open cytosine replaced guanine and it was also seen in position 11 of the promoter region. However, other mutations were also seen at the terminal areas of aspartyl proteinase gene in some of the samples which were beyond the scope of our research. Thus, mutation was seen in all of the samples and it was high in this region. As a result, it will not be a suitable site for medicinal treatment. Also, due to the impossibility to extract this enzyme from candida culture medium, we can investigate the sequence of this enzyme as well as its mutation rate as a means to study for providing vaccine.

REFRENSE

1. Ahmad Khan, M., Ahmad, I., Aqil, F., 2010. Virulence and Pathogenicity of Fungal Pathogens with Special Reference to Candida albicans.j clin microbiology. Med Oral Patol Oral Cir Bucal.35, 1695-16703.
2. Anassie, E.J, MacGinnid, M.R., Pfaller, M.A., 2003. Candida virulence factors. In: Anassie EJ, MacGinnid MR, Pfaller MA. Clinical New York. 203-24.
3. Bernardis, D.e., Sullivan, F., Cassone, P.A., 2001. Aspartyl proteinases of Candida albicans and their role in pathogenicity. Med Mycol. 39, 303-13.
4. Barki, M., 1993. Isolation of a Candida albicans DNA Sequence Conferring Adhesion and Aggregation on Saccharomyces cerevisiae, journal of bacteriology. 175(17), 5683-5689.
5. Chen,Y., Wu,Y., Lian,W., 2002. Differential secretion of Sap4–6 proteins in Candida albicans during hyphae formation. Rev Iberoam Micol. 148, 3743–3754.
6. Claude, G., Goldman, R., 1993. Stimuli that induce production of Candida albicans extracellular aspartyl proteinase. J Bacteriol. 139,1643-1651.
7. Dadgar, A., 2006. Comparison of methods for DNA extraction from Candida albicans. Department of Medical Biochemistry and Microbiology. 65-98.

8. Kalkanci, A., Bozdayi, G., Biri, A., Kustimur, S., 2005. Distribution of secreted aspartyl proteinases using a polymerase chain reaction assay with SAP specific primers in Candida albicans isolates. Folia Microbiol (Praha). 50(5), 409-13.

9. Lermann, U., Morschhauser, J., 2008. Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by Candida albicans. Med mycol. 154, 3281–3295.

10. Mirhendi, S.H., Makimura, K., 2003. PCR- Detection of Candida Albicans in Blood Using a New Primer Pair to Diagnosis of Systemic Candidiasis. Iranian J. Publ. Health. 32 (1),1-5.

11. Mullen, C.A., Abd El-Baki, H., Samir, H., Tarrand, J.J., Rolston, K.V., 2003. Non-albicans Candida is the most common cause of candidemia in pediatric cancer patients. Supportive Care Cancer. 11, 321-25.

12. Meurman, J., Siikala, E., Richardson, M., Rautemaa, R., 2007.Non-Candida albicans Candida yeasts of the oral cavity. Applied Microbiology. 55, 20–25.

13. Naglik, j., Moyes, D.J., Kanzaria, M., Tsichlaki, P., Weindl, G., Anwar, R., 2008. Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis.J Clin Microbiology. 154, 3266–3280.

14. Sanglard, D., Ischer, F., Marchettti, O., Entenza, J., 2003.CalcineurinA of Candida albicans: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. 48, 959–976.

15. Smolenski, G., Sullivan, P.A., Cutfield, S.M., Cutfield, J.F., 1997. Analysis of secreted aspartic proteinases from Candida albicans: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes. Microbiol. 143(P 2), 349-56.t

16. Tavanti, A., Pardini, G., Campa, D., Davini, P., Lupetti, A., Senesi, S., 2004. Differential Expression of Secretory Aspartyl Proteinase Genes (SAP1-10) in Oral Candida albicans Isolates with Distinct Karyotypes. Med mycol.1(4), 4726–4734.

17. White, T.C, Agabian, N., Candida albicans, White T.C., Agabian, N., 1995. Candida albicans secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J Bacteriol 177(18), 5215-5221.