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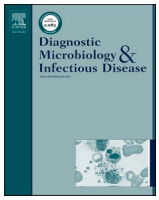

Candida albicans cDNA library screening reveals novel potential diagnostic targets for invasive candidiasis

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ABSTRACT

The detection of patterns associated with the invasive form of *Candida albicans*, such as *Candida albicans* germ tube antibodies (CAGTA), is a useful complement to blood culture for Invasive Candidiasis (IC) diagnosis. As CAGTA are detected by a non-standardisable and non-automatable technique, a *Candida albicans* cDNA expression library was screened with CAGTA isolated from serum of an animal model of invasive candidiasis, and five protein targets were identified: hyphally regulated cell wall protein 1 (Hyr1), enolase 1 (Eno1), coatomer subunit gamma (Sec21), a metallo-aminopeptidase (Ape2) and cystathionine gamma-lyase (Cys3). Homology with proteins from other organisms rules out Cys3 as a good biomarker while Sec21 results suggest that it is not in the germ tubes surface but secreted to the external environment. Our analysis propose Ape2, Sec21 and a region of Hyr1 different from the one currently being studied for immunoprotection as potential biomarker candidates for the diagnosis of IC.

1. Introduction

In critical patients, invasive fungal infections (IFI) rate is increasing, being one of the main causes of morbidity and mortality; most of these infections are caused by species of the genus *Candida* [1]. Invasive Candidiasis (IC) not only affects to critical patients; according to the review of Lamoth and collaborators [2], live threating *Candida* infections are the most common IFI in the majority of solid organ recipients, and the second cause in haematopoietic stem cells transplant recipients and in patients with haematological malignancies. IC is also the third cause of bloodstream infection in children, mainly affecting low birth weight newborns [3].

Although the global incidence of IC increased, this trend seems to be steadying in most of the countries [4,5]. In spite of this stabilization, it is estimated that, annually worldwide, there are 750,000 cases of IC with mortality rates over 40 % [6]. Regarding the causative species, despite an increasing rate of infections by non-albicans *Candida* species, *C. albicans* remains the most common [7].

The term IC comprises three types of pathologies: candidemia (bloodstream infection), deep-seated candidiasis and candidemia associated with deep-seated candidiasis [8].

Candidemia is the most frequent presentation of IC, and has an estimated attributable mortality between 10 and 47 % [9]. On the other hand, for intraabdominal candidiasis (IAC), which is the most common group of deep-seated candidiasis, mortality ranges between 13 and 88 % depending on the type of infection [10]. In addition, deep-seated candidiasis incidence could be underestimated because culture, the reference standard for diagnosis, often fails to detect this type of infection, detecting only 2-10 % of IAC [10].

The prognosis of these infections is strongly associated with early diagnosis and correct adherence to treatment [11]. However, diagnosis is challenging, as clinical manifestations are similar to some severe bacterial infections [12]. Besides the low sensitivity of the blood culture, it may take 24-48 hours to obtain the results, and even longer for the species

Abbreviations: BDG, β-D-glucan; CAGTA, *Candida albicans* germ tube antibodies; CAGTA-enr, CAGTA enriched fraction; CAGTA-pur, CAGTA purified fraction; Sec21, coatomer subunit gamma; Cys3, cystathionine gamma-lyase; *E. coli*-λ ads-CAGTA, *E. coli* lysed with non-recombinant Lambda ZAP II phage; Eno1, enolase 1; Hyr1, hyphally regulated cell wall protein 1; IIF, indirect immunofluorescence; IAC, intraabdomial candidiasis; IC, invasive candidiasis; IFI, invasive fungal infection; Ape2, metallo-aminopeptidase.

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identification and antifungal susceptibility assays. Consequently, empirical therapy is often applied in high-risk patients with some studies questioning the benefits of this practice [2] in addition to the associated costs.

Several alternative non-culture-based methods have been developed and some of the best studied are based on the detection of biomarkers, including β-D-glucan (BDG) as well as mannan and anti-mannan antibodies, but they still have limitations. For example, BDG is a panfungal marker implying that a negative result may rule out IC but a positive test result may be due to infection by other fungi, as well as to certain interfering medical procedures such as dialysis [13]. On the other hand, some studies suggest that the detection of mannan and anti-mannan antibodies has a low predictive value for deep-seated candidiasis [8] and fails to differentiate between *Candida* colonization and infection [14].

The detection of CAGTA (*Candida albicans* Germ Tube Antibodies) is a technique that was developed in our laboratory [15] and relies on the detection of antibodies that react with specific superficial antigens of the germ tubes of *Candida albicans* by indirect immunofluorescence (IIF). Several studies have proved the CAGTA title determination as a useful diagnostic and prognosis tool even though it has moderate sensitivity and specificity [8,16]. CAGTA detection can distinguish colonisation from invasive infection; however, IIF is a very subjective and non-automatable technique. For this reason, the identification of the specific targets of these antibodies would allow the development of techniques, such as ELISA or lateral flow test, that overcome these limitations. These techniques could complement blood culture to advance the results, together with other techniques that may indicate other characteristics of the microorganisms, such as antimicrobial resistance.

2. Materials and methods

2.1. Candida albicans cDNA library

The cDNA library used in this study was kindly provided by Dr. W. Fonzi (Georgetown University, USA) and Dr. P. Sundstrom (Ohio State Medical School, USA). It is a λ ZAP II (Stratagene) cDNA library prepared from mRNA of the *Candida albicans* strain SC5314 growing in the mycelial phase. The library was propagated in *E. coli* XL1-Blue MRF' (Agilent).

2.2. Serum quantification, fractionation and purification

Serum from a rabbit model of IC was used; this infection was carried out prior to this study following the protocol described by Sáez-Rosón et al. [17] with the approved file M20/2017/067 of the animal ethics committee of the University of the Basque Country and procedures were conducted following animal welfare policy. CAGTA levels were titrated by indirect immunofluorescence following the protocol described by Moragues et al. [18]. Serum fractions were obtained through incubation of the sera with heat-inactivated *C. albicans* blastospores to obtain CAGTA-enriched fraction (CAGTA-enr) that was subsequently incubated with *C. albicans* germ tubes. The germ tube attached antibodies were eluted to obtain the CAGTA-purified fraction (CAGTA-pur). Protocols were previously described by Sáez-Rosón et al. [17]. Lambda phage and *E.* coli cross-reactive antibodies were removed from the CAGTA-enriched serum fraction by absorption to nitrocellulose membranes embedded in *E. coli* lysed with non-recombinant Lambda ZAP II phage (*E. coli*-λ ads-CAGTA), following the protocol described by Sambrook and Russell [19].

2.3. Screening of the cDNA library

Screening of the phage library was performed following the protocols described by Sambrook and Russell [19]. Briefly, while growing, plates containing *E. coli* infected with the cDNA library phages were overlaid with 0.5M IPTG-soaked nitrocellulose filters to induce the expression of the *C. albicans* proteins that resulted blotted to nitrocellulose membranes when visible lysis plaques were developed. Membranes were lifted and blocked with TBS-BSA (Tris-Buffered Saline with 2.5 % (w/v) bovine serum albumin), then incubated with *E. coli*-λ ads-CAGTA and next incubated with an anti-rabbit IgG conjugated to alkaline-phosphatase. The positive plaques that were revealed with BCIP/NBT substrate in the first screening round were selected and re-screened at a lower plaque density to confirm the positive reaction with CAGTA-enr antibodies and to ensure the isolation of pure plaques.

Clones codifying for proteins that were considered of interest were mixed 1:5 with non-insert phages and rescreened with CAGTA-pur to further verify their CAGTA specificity.

2.4. Analysis of positive clones for protein identification and characterization

To obtain the sequence of interest from the positive clones a PCR was performed with T3 and T7 primers, whose sequences flank the *C. albicans* gene inserts in the phage genome. Amplicons were submitted to the Sequencing and Genotyping Service SGIker of the University of the Basque Country UPV/EHU and the resulting sequences were analysed with the BioEdit software. Protein identification was carried out with BLAST, and they were checked for similar sequences in other microorganisms. To determine whether the proteins were located on the surface of the organism, the presence of signal peptides, GPI anchor sites and transmembrane motifs was checked with the software programs SignalP [20], PredGPI [21] and TMHMM [22].

3. Results

3.1. Antigens recognized by the CAGTA-enriched fraction

The initial screening of 1.5×10^6 plaque forming units followed by several rounds of purification and re-screening with the rabbit *E. coli*-λ ads-CAGTA serum fraction allowed the selection of 53 positive clones that were checked to confirm that their inserts were in the correct reading frame to be expressed. The sequence analysis of the 53 clones showed identity with full or partial gene sequences codifying for five different *C. albicans* proteins (Table 1). Most clones (28) codified for different segments of the Hyr1 protein, derived from different *HYR1* gene sequences, comprised between nucleotides 113 and 2,760. Twenty one codified for the whole Eno1 protein while the remaining clones contained partial sequences of the *SEC21* (2 clones), *CYS3* and *APE2* genes.

Table 1
Number of positive clones analysed and *C. albicans* proteins identified in the phage library screening.

No. of clones	Gene name	Name of the protein	Nucleotide sequence contained in the phage coding for the *C albicans* protein	GenBank accession number [a]
28	*HYR1*	Hyphally regulated protein 1	Different segments between nt 113 and 2,760 (end of the CDS)	XM_717090.2
21	*ENO1*	Enolase 1	Whole protein (nt 1-1,323)	XM_706790.2
2	*SEC21*	Coatomer subunit gamma	Nt 934- 2,811 (end of the CDS)	XM_708476.2
1	*CYS3*	Cystathionine gamma-lyase	Nt 952-1,200 (end of the CDS)	XM_711148.2
1	*APE2*	Metallo-aminopeptidase	Nt 544-840	XM_019475095.1

[a] GenBank (NCBI) database accession number of the sequence used for identification.

3.2. Clones selection and reaction with purified CAGTA

One clone of each protein was selected as representative to confirm their reaction with CAGTA-pur. Since *HYR1* clones covered different segments of the gene sequence codifying for the Hyr1 protein, we selected a clone of 579 bp (from nucleotide 1,441 to 2,019), named D22b, because the alignment of most *HYR1* clones sequences shared this gene region.

The purified CAGTA from the rabbit serum recognized the selected clones that contained the sequences of Hyr1, Cys3 and Ape2. However, those expressing Eno1 and Sec21 did not show any specific reaction.

3.3. Protein analysis

In silico analysis of the five proteins predicted that only Hyr1 has a putative signal peptide and a GPI anchor point. Meanwhile, TMHMM detects, a signal peptide-like sequence in Ape2 between the amino acids 21 and 40 and, only in this case, a helix transmembrane motif.

According to BLAST, none of the identified proteins presents significant similarity with human proteins nor with any other higher eukaryotic organism. For Eno1, Sec21, Cys3 and Ape2 proteins, the alignments show high similitude (>80 %) with their correspondents of the genus *Candida* (especially *Candida africana* and *Candida dubliniensis*) and other yeasts of genera such as *Spathaspora* or *Scheffersomyces*. Ape2 showed around 50 % similarity with an *Aspergillus* peptidase and a hypothetical protein of *Penicillium*. Interestingly, Ape2, Eno1 and Cys3 showed high identity (80-90 %) with sequences of the Asgard archaea group proteins.

Concerning Hyr1 sequence, it only shows high homology with the hyphally regulated protein of *C. africana* (87.5 % identity with 65 % coverage) and mild similarity (40-50 % identity with 38-53 % coverage) with *C. dubliniensis, Candida tropicalis, Candida viswanathii* and *Candida maltosa* proteins. Hyr1 sequence also showed mild similarity with an Asgard archaea group hypothetical protein.

The analysis of the protein fragments codified by Sec21 and Ape2 phages that were recognized by the CAGTA-enr serum fraction, yielded equivalent rates of homology as for the complete protein. On the contrary, the subterminal section of the Hyr1 protein codified by the D22b phage showed only mild homology with a putative hyphally-regulated cell wall protein precursor of *C. dubliniensis* and the hyphally-regulated protein of *C. africana*. Finally, the peptide codified by the Cys3 phage showed high homology (85.7 %) with the cystathionine beta lyase of *Chlamydia*.

4. Discussion

The presence of *Candida* as a commensal in the human body and the lack of specific symptoms for IC, together with the low sensitivity of blood culture make it necessary to develop new diagnostic approaches, especially in the case of deep-seated candidiasis. CAGTA are specific for antigens located on the surface of the germ tubes of *Candida albicans*, morphology which is associated with the invasion [23] and has shown good diagnostic results for deep-seated candidiasis [24]. However, this technique has limitations, because it is subjective and requires training of the person in charge, so the identification of specific CAGTA targets would help to develop more sensitive, objective and automatable techniques. In this regard, Díez et al. [25] have had promising results with some of the proteins recognised by these antibodies, but the identification of new targets or specific protein fragments could improve or complement them.

In our study, we used a *C. albicans* cDNA phage library and serum from a rabbit model of invasive candidiasis to identify proteins susceptible to be biomarkers for IC. With this approach, a CAGTA-enriched fraction of the immune serum recognized five *C. albicans* proteins identified as: Hyr1, Eno1, Sec21, Cys3 and Ape2, but only three of them (Hyr1, Cys3 and Ape2) were confirmed with the purified CAGTA.

The in silico analysis of these proteins pointed only at Hyr1 as a conventionally secreted glycosylphosphatidylinositol (GPI) -anchor protein. GPI-anchor proteins are essential in cell wall integrity, morphogenesis and virulence of *C. albicans* [26]. An exposed location, as well as their role in pathogenicity, have led to study several proteins such as Als3, Hwp1 or Hwp2 as possible biomarker candidates, vaccine antigens and/or therapeutic targets [25,27-29]. Hyr1 has already been described as a GPI protein [30,31] associated to hyphal morphology of *C. albicans,* nevertheless its absence does not influence the morphological switching [30]. Luo and co-workers [32] showed that this protein is involved in resistance to phagocytosis and considered it an interesting vaccine candidate. In this respect, mice immunisation with Hyr1 induced protection not only against *C. albicans* infections, but also against other microorganisms such as *Acinetobacter baumannii* and *Klebsiella pneumoniae* [33] and, recently, the multi-resistant *Candida auris* [34]. Cases of cross immunization might be due to conformational features since the BLAST analyses did not reveal any similarities between Hyr1 and any protein of these microorganisms. While these studies focused on the N-terminal region of the protein (up to amino acid 350), the fragment proposed in our study is located in the medial-to-C-terminal region of the protein (amino acids 479 to 667), and covers part of the Ser/Thr-rich domain (amino acids 346 to 576), an area that is predicted to be heavily O-glycosylated. Glycosylation has been reported to be relevant for the particular conformation of other *Candida* proteins and therefore, essential for their correct function [35]. Moreover, glycosylation has a double role in the antibody-antigen recognition; on the one hand, it could mask some epitopes, while on the other hand, it could be indispensable for the correct exposure of the epitope [36] either because the oligosaccharide is directly recognised by the antibody or because it influences the proper presentation of the peptide epitope. In this regard, the protein fragments expressed in this lambda phage screening, were produced in *E. coli* and therefore without any eukaryotic post-translational modification, thus, the native conformation of this protein should also be studied.

Concerning the utility of Hyr1 for the diagnosis of invasive candidiasis, to our knowledge, there is only one report of our group that evaluated the detection of antibodies to the recombinant Hyr1 protein produced in *E. coli*, with moderate results of sensitivity and specificity, 58.3 % and 82.2 % respectively [37]. Aiming to improve the diagnosis of invasive candidiasis, in this work we propose the use of the mid-terminal segment of Hyr1 recombinantly expressed in a yeast expression model such as *Pichia pastoris*. The incorporation of glycosidic residues, as well as the correct folding of this Hyr1 fragment, would mimic the native protein, improving the detection of specific antibodies for diagnosis, as well as the development of a more specific response in immunisation models.

Although predictive tools differ on whether the metallo-aminopeptidase Ape2 has a signal peptide or not, Klinke et al. [38] isolated this protein from the *Candida* cell wall and observed that its gene sequence is likely to encode for two exons linked by an intron, with the first exon acting as a secretion signal that would explain how the protein reaches the surface. In addition, a posterior study [39] registered a significant increase in the expression of Ape2 during epithelial invasion, which would imply a role in the virulence of the fungus. With regard to a diagnostic application, El Moudni et al. [40] used a secreted aminopeptidase from *C. albicans* with good results, but the protein they studied seems not to be the one identified in our study [38]. Further studies may help to evaluate the utility of secreted aminopeptidases for diagnosis.

The in silico analysis of Eno1, Cys3 and Sec21 did not detect any secretion signal peptide for them, however, some *C. albicans* proteins without a signal peptide have been found in the cell surface as well as in the external medium after being released by alternative secretion processes [41–42]. Some of this non-signal peptide secreted proteins have a known function in the cytoplasm but are also involved in surface-specific processes. These so-called moonlighting proteins,

perform some additional function unrelated to their "conventional" function and usually occur in subcellular locations where they are not expected to be [43].

Eno1 is a highly conserved moonlighting protein found in a wide range of organisms [44]. Even though the role of this *C. albicans* glycolysis and gluconeogenesis enzyme in the fungal pathogenesis is not clear, it seems to be involved in the adhesion to the host [45], the spread of infection [46] and phenotypic changes [47]. Moreover, its diagnostic utility has already been confirmed in several studies, either by detecting the free antigen [48] or specific antibodies [49], based on the significant amounts of enolase that can be detected on the surface of filamenting *C. albicans*, and the strong reaction developed by patients with IC against this protein [44].

Regarding to the presence of the enolase 1 in the external environment, several hypotheses have been considered, however, Gil-Bona and collaborators [50] showed evidences that Eno1 was exported to the external medium via vesicle mediated transport. Later, Karkowska-Kuleta et al. [44] proposed that secreted Eno1 was reabsorbed and captured by the Als3 protein as a way of adhesion to the cell wall.

Cys3, a cytoplasmic enzyme involved in aminoacid biosynthesis, was detected in a recent study in external vesicles in both yeast and hyphae forms of *C. albicans* [51]. Even though hyphae shown an increased amount of this protein in the cytoplasm [52] and its gene is overexpressed during the biofilm formation [53] we have not found any studies showing evidence of this protein on the external surface, of *Candida spp.,* nor in other organisms. The reaction with the purified CAGTA fraction could be due to cross-reactivity with conformational epitopes from other proteins. In addition, the identified fragment would not be a good diagnosis marker of IC due to its high homology with the cystathionine beta-lyase from *Chlamydia*.

Finally, Sec21 has also been found in vesicles secreted by *C. albicans* [54]. Sec21 and other six proteins conform the coat complex protein I (COPI) of the vesicles responsible for retrograde transport from Golgi to the Endoplasmatic Reticulum [55] and it is therefore a cytoplasmic protein in origin. However, unlike Eno1 and Cys3, Martínez-López and collaborators [54] found that this protein and other COP proteins were detected only in the vesicles secreted to the external medium by *C. albicans* growing as mycelia but not in the yeast form. Although these proteins seem to be related to the morphological change, the induction of specific antibodies by IC patients would follow a similar pattern to Sec20, another structural protein involved in the retrograde transport [56,57].

Eno1 and Sec21 were recognized by the CAGTA enriched serum fraction of serum of a rabbit with invasive candidiasis but not by the purified CAGTA. In the case of Sec21, this could be due its presence in the external environment but not on the germ tubes surface. However, Eno1 can be in both yeast and hyphal surfaces, as well as secreted to the external medium [50,58]. Our results contrast with a previous study of our group where Eno1 was detected by the purified CAGTA of a similar animal model of invasive candidiasis but they used a more sensitive chemoluminiscent detection reaction [17]. These same authors [59] reported that CAGTA reaction with Eno1p was higher when the protein was purified from a cell wall extract than when it was produced recombinantly in *E. coli*; these antigenicity changes could be due to protein folding, post-translational modifications or, if the theory proposed by Karkowska-Kuleta [44] is correct, the link between Eno1 and a cell wall-binding protein that could alter its conformation, exposing some epitopes that could be buried in the soluble form of the protein. Further studies would be necessary to determine the Eno1-*C. albicans* cell wall interactions as well as antibody- antigen interactions for this protein.

5. Conclusions

In this study, five proteins have been identified through the screening of a *C. albicans* cDNA library with CAGTA: Eno1, Hyr1, Ape2, Sec21 and Cys3. Of them, only the moonlighting protein Eno1 has been extensively studied with diagnostic purposes. Hyr1, the most frequently identified protein in this study, and more precisely the N-terminal section of it, is being studied as a vaccine candidate with good results, but its application as a diagnostic tool is more limited. In contrast, as far as we know, the medium-to-C-terminal Hyr1 section that we are introducing in this study has not been studied for any of the mentioned purposes, and would avoid possible cross-reactivity derived from the inter-organisms highly conserved N-term end of the protein. Taking into account characteristics such as protein location and their role in virulence and/or yeast-to-hyphae transition, further studies are needed involving Ape2 and Sec21, together with the above mentioned subterminal fragment of Hyr1 as potential biomarkers for the diagnosis of invasive candidiasis.

CRediT authorship contribution statement

Marta Bregón-Villahoz: Investigation, Writing – original draft. **Pilar Menéndez-Manjón:** Methodology. **Giulia Carrano:** Methodology. **Ander Díez-Villalba:** Investigation. **Inés Arrieta-Aguirre:** Supervision, Writing – review & editing. **Iñigo Fernandez-de-Larrinoa:** Supervision, Funding acquisition. **María-Dolores Moragues:** Conceptualization, Visualization, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Transparency declarations

We declare that we have no competing interests.

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