Folia Microbiol (2011) 56:143–148
DOI 10.1007/s12223-011-0023-3

Differences in proteolytic activity and gene profiles of fungal strains isolated from the total parenteral nutrition patients

M. Sikora · M. Dabkowska · E. Swoboda-Kopec · S. Jarzynka · I. Netsvyetayeva · M. Jaworska-Zaremba · M. Pertkiewicz · G. Mlynarczyk

Received: 7 September 2010 / Accepted: 24 January 2011 / Published online: 1 April 2011

Abstract Fungal infections constitute a serious clinical problem in the group of patients receiving total parenteral nutrition. The majority of species isolated from infections of the total parenteral nutrition patients belong to *Candida* genus. The most important factors of *Candida* spp. virulence are the phenomenon of "phenotypic switching," adhesins, dimorphism of fungal cells and the secretion of hydrolytic enzymes such as proteinases and lipases, including aspartyl proteinases. We determined the proteolytic activity of yeast-like fungal strains cultured from the clinical materials of patients receiving total parenteral nutrition and detected genes encoding aspartyl proteinases in predominant species *Candida glabrata*—*YPS2*, *YPS4*, and *YPS6*, and *Candida albicans*—*SAP1–3*, *SAP4*, *SAP5*, and *SAP6*. *C. albicans* released proteinases on the various activity levels. All *C. glabrata* strains obtained from the clinical materials of examined and control groups exhibited secretion of the proteinases. All 13 isolates of *C. albicans* possessed genes *SAP1–3*. Gene *SAP4* was detected in genome of 11 *C. albicans* strains, *SAP5* in 6, and *SAP6* in 11. Twenty-six among 31 of *C. glabrata* isolates contained *YPS2* gene, 21 the *YPS4* gene, and 28 the *YPS6* gene. We observed that clinical isolates of *C. albicans* and *C. glabrata* differed in *SAPs* and *YPSs* gene profiles, respectively, and displayed differentiated proteolytic activity. We suppose that different sets of aspartyl proteinases genes as well as various proteinase-activity levels would have the influence on strains virulence.

Abbreviations

PCR	Polymerase chain reaction
SAP	Secreted aspartyl proteinase gene
Saps	Secreted aspartyl proteinases
TPN	Total parenteral nutrition
YPS	Yapsin-related aspartyl proteinase gene

Fungal infections constitute a serious clinical problem in group of patients after surgical procedures, receiving total parenteral nutrition (TPN). The majority of species isolated from infections of the TPN patients belong to *Candida* genus. Among the yeast-like fungal strains, *Candida albicans* is one of the microorganisms that cause infections the most frequently. The other species—*Candida parapsilosis*, *Candida glabrata*, and *Candida tropicalis*—are isolated from clinical materials less commonly (Slodkowski et al. 2004). The most important factors of *Candida* spp. virulence are the phenomenon of "phenotypic switching"—phenotypic changing, adhesins, dimorphism of fungal cells, and the secretion of hydrolytic enzymes, such as proteinases and lipases, including aspartyl proteinases (Saps). Saps had been recognized as the factors of virulence since the time they were discovered (Tavanti et al. 2004). Some species that are

M. Sikora (✉) · M. Dabkowska · E. Swoboda-Kopec · S. Jarzynka · I. Netsvyetayeva · G. Mlynarczyk
Department of Medical Microbiology,
Warsaw Medical University,
Chalubinskiego 5,
02004 Warsaw, Poland
e-mail: sikorka78@yahoo.com

M. Jaworska-Zaremba
Department of Prosthetic Dentistry, Warsaw Medical University,
Warsaw, Poland

M. Pertkiewicz
Department of Nutrition and Surgery,
W. Orlowski Clinical Hospital,
Warsaw, Poland

not involved in Sap proteinases secretion may produce enzymes of aspartyl proteinases activity, closely related to *Saccharomyces cerevisiae* yapsins. All these enzymes, named Yps proteinases, play an important role in *C. glabrata* virulence (Kaur et al. 2007; Krysan et al. 2005). Some species, including *C. albicans*, being dimorphic, can grow as yeast cells or in the form of pseudohyphae or hyphae (Wang 2009). This ability is connected with the expression of genes encoding proteinases. It is worth to notice that proteinases are responsible for infection development.

The aim of our study was to determine the proteolytic activity of yeast-like fungal strains cultured from the clinical materials of patients receiving total parenteral nutrition and the detection of genes encoding aspartyl proteinases in predominant species, isolated from examined and control groups. We have also attempt to divide all isolates into more and/or less virulent. The virulence genes for analysis were selected according to their role and importance in development of infections.

Material and methods

The clinical materials consisted of yeast-like 55 fungal strains isolated from 37 patients (hospitalized in Department of Nutrition and Surgery in W. Orlowski Hospital in Warsaw) with TPN from the various clinical materials isolated (Table 1; examined group). In the control group, 35 isolates cultured from the clinical materials of 30 patients without TPN support were analyzed (Table 2).

All clinical materials were cultured on Sabouraud agar plates with chloramphenicol and gentamicin (bioMérieux). After a 1- to 2-day incubation at 30°C, all isolates were identified using the ID 32 C test (bioMérieux). The proteolytic activity was determined on modified Staib's agar (in percent): agar, 2%; glucose, 2%; KH_2PO_4, 0.1%; and $MgSO_4$, 0.05%, with addition of 0.4 g bovine serum albumin (Biomed) to each 200 mL of the broth (Kurnatowska et al. 1999). For the analysis, the suspensions of 0.5 McFarland *Candida* cells from 2-day-old Sabouraud agar cultures were prepared. The 10-μL drops of the suspensions were put on the agar surfaces. All plates were incubated up to 7 days at 37°C. The proteolytic zone was observed and evaluated as follows: (++++)—proteolysis after 24 h, (+++)—after 48 h, (++)—after 72 h, (+)—clouding around the colonies, or no reaction (−) after 7 days as described previously by Nowakiewicz and Ziolkowska (2007).

The isolation of genomic DNA was done according to the EURx protocol enclosed to the yeast DNA isolation kit. Three-day-old yeast cultures drown in 1.5 mL Sabouraud liquid broth without any antibiotics addition were used. After removing the broth, the enzymic lysis of the cells was done in buffer containing proteinase K and ribonuclease. All obtained lysates were applied to the binding spin columns and washed twice with buffer to remove lipids and proteins. The elution of DNA bound to the membrane was done with Tris buffer (pH 8.0).

Genotypic analysis was based on the detection of chosen genes encoding fungal proteinases using standard PCR reaction. The analysis was performed for the genes considered to be most important in the development of virulence. The following genes were amplified from the

Table 2 Control group (fungal isolates cultured from the clinical materials of 30 patients without TPN support)

Species	Sputum	Urine	Swab from throat	Total
Candida albicans	19	2	2	23
Candida glabrata	1	4	–	5
Candida tropicalis	1	–	–	1
Candida parapsilosis	1	–	–	1
Candida inconspicua	3	–	–	3
Candida kefyr	1	1	–	2
Total	26	7	2	35

Table 1 Examined group (fungal isolates cultured from clinical material of 37 patients with TPN support)

Species	Blood	Urine	Stoma	Swab from anus	Sputum	Swab from oral cavity	Bronchial discharge	Swab from cervix	Total
C. albicans	3	1	1	2	3	1	2	–	13
C. glabrata	1	9	6	7	3	2	1	2	31
C. inconspicua	–	–	–	–	1	–	–	–	1
C. krusei	–	1	–	–	1	–	–	–	2
C. lusitaniae	–	1	1	–	–	–	–	–	2
C. parapsilosis	3	–	–	–	–	–	–	–	3
C. tropicalis	1	–	2	–	–	–	–	–	3
Total	8	12	10	9	8	3	3	2	55

genomic DNA of most isolates: *C. glabrata*—*YPS2*, *YPS4*, and *YPS6*, and *C. albicans*—*SAP1–3*, *SAP4*, *SAP5*, and *SAP6*. The reaction was conducted in DNA Engine thermocycler (MJ Research) under optimized conditions for *SAPs* genes: 95°C for 5 min, 30 cycles of 95°C for 30 s, 49°C for 45 s, 72°C for 1 min, and 72°C for 5 min; for *YPS* genes: 95°C for 5 min, 30 cycles 95°C for 1 min, 54°C for 45 s, 72°C for 1 min, and 72°C for −5 min. The method of the amplification generally followed protocols of Kaur et al. (2007) and Kalkanci et al. (2005). Table 3 shows the primer sequences and product lengths for analyzed genes. Reaction products were separated by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide under UV light.

Results

The secretion of proteinases in 19 strains isolated of study group (*C. albicans*—12, *C. parapsilosis*—2, *C. tropicalis*—2, *Candida krusei*—2, and *Candida lusitaniae*—1) was observed. In control group, 22 isolates of *C. albicans* released proteinases on the various activity levels. All *C. glabrata* strains of examined and control groups secreted proteinases (Table 4).

All 13 isolates of *C. albicans* in the study group possessed genes *SAP1-3*. Gene *SAP4* was detected in genome of 11 *C. albicans* strains, *SAP5* in 5, and *SAP6* in 11 strains. Simultaneous presence of all analyzed genes was detected in case of five *C. albicans* isolates. Two isolates revealed simultaneous deficiency of *SAP5* and *SAP6* genes, and two other simultaneously lack the *SAP4* and *SAP5* genes. In case of four isolates, the absence of *SAP5* gene was observed with simultaneous presence of other analyzed genes (see Table 4).

Twenty-six among 31 of *C. glabrata* isolates in study group possessed *YPS2* gene, 21 the *YPS4* gene, and 28 the *YPS6* gene. The presence of all analyzed genes was demonstrated in case of 21 *C. glabrata* strains. Three isolates of *C. glabrata* revealed the lack of *YPS2*, *YPS4*, and *YPS6* genes, two strains exhibited simultaneous deletion of *YPS2* and *YPS4*. The lack of *YPS4* gene with simultaneous presence of other genes was observed in case of five *C. glabrata* isolates (see Table 4). In control group, all studied *SAP* genes were detected in genome of 12 strains. Two of them demonstrated lack of the *SAP6* gene with simultaneous presence of *SAP1–3*, *SAP4*, and *SAP5*. In case of five isolates, *SAP5* gene was absent. Two isolates revealed the deletion of *SAP5* and *SAP6* genes, one isolate *SAP4* and *SAP5*. The deficiency of *SAP1–3*, *SAP4*, and *SAP5* genes was observed in case of one strain. Three isolates out of five strains of *C. glabrata* demonstrated simultaneous lack of *YPS2*, *YPS4*, and *YPS6* genes. In one isolate, single deletion of *YPS6* gene and, in another one, the double deletion of *YPS4* and *YPS6* were detected.

Discussion

Aspartyl proteinases of *Candida albicans* are encoded by the multigene family of at least ten different highly regulated genes (*SAP1–10*) (Tavanti et al. 2004). Those genes were classified into three separated subgroups on base of their amino acid sequences. Enzymes Sap1–3 formed the group with a 75% similarity in sequence

Table 3 Primer sequences and products size

Target gene	Primer sequence, 5′ → 3′		Amplicon, bp
Candida albicans			
SAP1–3	F	GCT CTT GCT ATT GCT TTA TTA	253
	R	CAT CAG GAA CCC ATA AAT CAG	
SAP4–6	F	GCT CTT GCT ATT GCT TTA TTA	–
SAP4	R	TAG GAA CCG TTA TTC TTA CA	394
SAP5	R	ACC TAA AAT ACC CTT ACG AG	578
SAP6	R	GGT AGC TTC GTT GGT TTG GA	605
Candida glabrata			
YPS2	F	CTA CAA TCC TGC ATT GAG TGA	621
	R	TCC TGG CTC ACC TTG TGG CAT	
YPS4	F	CAA CTT GCT GCC AAT GGC TC	565
	R	TCA ATG TCA TCT CTG GCT TGC	
YPS6	F	CTA TAA ATT CTA ACG GAA CTA CG	561
	R	GAC TCT GCA CCA CTT GGG ATG	

Table 4 Gene profiles and proteolytic activity of *C. albicans* and *C. glabrata* isolates from examined and control groups

Strain number[a]	*SAP1–3*	*SAP4*	*SAP5*	*SAP6*	Secretion of proteinases[b]	Clinical material
Candida albicans						
C2	+	+	+	+	+	Anus
C5	+	+	+	+	++++	Blood
C8/1	+	+	+	+	++++	Sputum
C9/1	+	+	−	+	++	Sputum
C13	+	+	+	+	++	Anus
C14	+	+	−	+	+++	Sputum
C16	+	+	−	+	++++	Blood
C19	+	−	−	+	−	Blood
C20	+	−	−	+	+++	Oral cavity
C24/2	+	+	−	+	+	Stoma
C25	+	+	+	+	+	Bronchial discharge
C39/1	+	+	−	−	++++	Urine
C42	+	+	−	−	+	Bronchial discharge
K1	+	+	+	+	+++	Sputum
K2	+	+	+	+	+	Sputum
K3	+	+	+	+	++	Sputum
K4	+	+	+	+	+	Throat
K5	+	+	+	−	−	Throat
K6	+	+	+	+	+	Sputum
K7/1	+	+	+	+	+	Sputum
K9	+	+	+	+	++++	Sputum
K10	+	+	+	−	+	Sputum
K11	+	−	−	+	++++	Sputum
K12	+	+	+	+	++	Sputum
K14	+	+	−	+	++	Sputum
K16	+	+	−	+	++	Sputum
K19	+	+	+	+	+	Sputum
K20	+	+	−	−	++	Sputum
K21	+	+	+	+	++	Sputum
K22	+	+	−	−	++++	Sputum
K23	+	+	−	+	++++	Urine
K24	+	+	+	+	++++	Sputum
K26/2	−	−	−	+	+	Urine
K27	+	+	−	+	++++	Sputum
K28	+	+	+	+	++++	Sputum
K30	+	+	−	+	++	Sputum
Strain number[a]	*YPS2*	*YPS4*	*YPS6*	Secretion of proteinases[b]	Clinical material	
Candida glabrata						
C3	+	+	+	+	Stoma	
C4	+	+	+	+	Cervix	
C7/1	+	+	+	+	Stoma	
C8/2	+	+	+	+	Sputum	
C9/2	+	+	+	+	Sputum	
C11	+	+	+	++	Urine	
C12	+	+	+	+	Stoma	
C15	+	+	+	+	Sputum	
C17	+	+	+	+	Stoma	
C21	−	−	−	+	Anus	

Table 4 (continued)

Strain number[a]	*SAP1–3*	*SAP4*	*SAP5*	*SAP6*	Secretion of proteinases[b]	Clinical material
C22	+	−	+	++		Oral cavity
C23	−	−	+	+		Anus
C26	+	−	+	+		Cervix
C28	+	+	+	+		Anus
C29	+	+	+	+		Anus
C30	+	+	+	+		Urine
C31	−	−	−	++		Urine
C34	+	+	+	+++		Urine
C35	+	+	+	++		Blood
C36	−	−	+	+		Urine
C38	+	−	+	+		Urine
C39/2	+	+	+	+		Urine
C40	+	+	+	++		Urine
C41	+	−	+	++		Oral cavity
C43	−	−	−	++		Stoma
C44	+	+	+	+		Bronchial discharge
C45	+	−	+	+		Anus
C46	+	+	+	+		Urine
C47	+	+	+	+		Stoma
C48	+	+	+	+		Anus
C49	+	+	+	+++		Anus
K13	−	−	−	+		Urine
K18/2	−	−	−	+		Sputum
K25	+	+	−	+		Urine
K26/1	+	−	−	++		Urine
K29	−	−	−	++		Urine

[a] C—strain from study group, K—strain from control group

[b] (++++) proteolysis after 24 h, (+++) proteolysis after 48 h, (++) proteolysis after 72 h, (+) cloud around the colonies, (−) no reaction after 7 days

whereas Sap4–6 with 90%. Proteinases Sap1–3 play an important role in mucosal infections while Sap4–6 in systemic infections (Felk et al. 2002). Kalkanci et al. (2005) proved that the deletion of *SAP4* to *SAP6* genes can lead to a virulence reduction and, in consequence, to attenuated development of disseminated fungal infections. *C. albicans* strains causing mucosal infection are less virulent than the strains that are the etiological factors of candidemia. *C. albicans* isolates analyzed in our study displayed differences in the gene set of encoded aspartyl proteinases. The detected deletions concerned genes *SAP4–6*, both in our examined and control groups. The analysis did not reveal mutants in *SAP1–3* genes. Considering the literature data, we suggest that strains possessing all analyzed genes (*SAP1–3*, *SAP4*, *SAP5*, *SAP6*) are able to develop systemic infections, whereas mutants in *SAP4–6* genes (as less virulent) would cause infections with milder course.

Protein products of the *SAP1–3* genes are secreted by yeast cells and by pseudohyphal form of *C. albicans*. Sap4–6 proteinases are mostly secreted by pseudohyphae. According to Felk et al. (2002), *C. albicans* strains that formed pseudohyphal forms but were deprived of accompanied proteinases encoded by *SAP6*, in particular, demonstrated reduced virulence. The double mutants *SAP4–6*, *SAP5–6*, or *SAP4–5* were characterized by very similar feature. Our results demonstrated that the majority strains in both examined and control groups possessed deletions of genes from the *SAP4–6* subfamily (single or double).

C. albicans is not a unique species of *Candida* genus secreting proteinases. Other pathogenic *Candida*, including *Candida dubliniensis*, *C. tropicalis*, and *C. parapsilosis*, possessed also the *SAP* genes. *C. tropicalis* has four *SAPs*, while *C. parapsilosis* has at least two (Naglik et al. 2003). The existence of cluster of 11 genes encoding aspartyl proteinases (*YPS*) was showed in the genome of *C. glabrata*. They play an important role in the maintenance of cell wall integrity, adherence to host cells, and survival of fungal pathogens in macrophages and in

virulence. During contact of *C. glabrata* cells with host macrophages, the transcription of *YPS2* gene and other *YPS* cluster genes, except *YPS3* and *YPS6*, increased. Genes *YPS3* and *YPS6* were expressed during incubation of yeast cells with macrophages, as well as during *Candida* growth on agar medium (Kaur et al. 2007). Similarly, we suggest that the strains with deletion of *YPS* genes could be characterized by modulated virulence level. In our study group, 5 strains of *C. glabrata* with deletion of *YPS2* gene, 10 with deletion of *YPS4*, and 3 lacking *YPS6* gene were detected. However, unambiguous correlation of gene deletions and strain proteolytic activities were not observed. *C. albicans* species displayed different proteolytic activity, and the majority of isolates secreted high levels of enzymes (Kurnatowska et al. 1999). On the other hand, in case of *C. glabrata* isolates, the activity was low or even very low, which agrees with the findings of Li et al. (2007) who suggested that *C. glabrata* exhibited extracellular proteinase activity but not on significant levels. The differences can be explained, e.g., by distinctive functions of corresponding enzyme in both species.

In conclusion, we confirmed the heterogeneity of gene encoding proteinases in *C. albicans* and *C. glabrata* clinical isolates and different *SAPs* and *YPSs* gene profiles, which can affect the strain virulence.

Acknowledgments This work was supported by the Polish State Committee for Scientific Research (grant no. NN404 093735).

References

Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, Sanglard D, Korting HC, Schäfer W, Hube B (2002) *Candida albicans* hyphal formation and the expression of the EFG-1-regulated proteinases SAP4 to SAP6 are required for the invasion of parenchymal organs. Infect Immun 70:3689–3700

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 50:409–413

Kaur R, Ma B, Cormack BP (2007) A family of glycosylphos phatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. Proc Natl Acad Sci USA 104:7628–7633

Krysan DJ, Ting EL, Abeijon C, Kroos L, Fuller RS (2005) Yapsins are a family of aspartyl proteases required for cell wall integrity in *Saccharomyces cerevisiae*. Eukaryot Cell 4:1364–1374

Kurnatowska AJ, Rozga A, Kurnatowski P (1999) Activity of aspartic proteinase of fungal oral isolates. (In Polish) Mikol Lek 6:21–25

Li L, Redding S, Dongari-Bagtzohlou A (2007) *Candida glabrata*, an emerging oral opportunistic pathogen. J Dent Res 86:204–215

Naglik RJ, Challacombe SJ, Hube B (2003) *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 3:400–428

Nowakiewicz A, Ziolkowska G (2007) Phenotypic characteristics of *Malassezia pachydermatis* strains. (In Polish) Medycyna Wet 63:448–454

Slodkowski M, Cebulski W, Deptala A, Krasnodebski IW (2004) Fungal infections in patients receiving parenteral and enteral nutrition. (In Polish) Zakazenia 4:45–49

Tavanti A, Pardini G, Campa D, Davini P, Lupetti A, Senesi S (2004) Differential expression of secretory aspartyl proteinase genes (*SAP-10*) in oral *Candida albicans* isolates with distinct karyotypes. J Clin Microbiol 10:4726–4734

Wang Y (2009) CDKs and the yeast–hyphal decision. Curr Opin Microbiol 12:644–649