Open Forum Infectious Diseases

BRIEF REPORT

Candida albicans Transcriptional Profiling Within Biliary Fluid From a Patient With Cholangitis, Before and After Antifungal Treatment and Surgical Drainage

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We used ribonucleic acid sequencing to profile *Candida albicans* transcription within biliary fluid from a patient with cholangitis; samples were collected before and after treatment with fluconazole and drainage. *Candida albicans* transcriptomes at the infection site distinguished treated from untreated cholangitis. After treatment, 1131 *C. albicans* genes were differentially expressed in biliary fluid. Up-regulated genes were enriched in hyphal growth, cell wall organization, adhesion, oxidation reduction, biofilm, and fatty acid and ergosterol biosynthesis. This is the first study to define *Candida* global gene expression during deep-seated human infection. Successful treatment of cholangitis induced *C. albicans* genes involved in fluconazole responses and pathogenesis.

Keywords. *Candida albicans*; cholangitis; fluconazole; RNA-Seq; transcriptional profiling.

Candida transcriptional responses to antifungal drugs have been described in vitro [1, 2]. In a recent study using a mouse model of hematogenously disseminated candidiasis (HDC), *Candida albicans* genes induced within kidneys 2 hours after a single caspofungin dose were largely different from those induced by the drug in vitro [3]. Only 248 genes were profiled in this study using a nanoString platform, and the design did not account for source control interventions that are crucial adjuncts to antifungal treatment of human invasive candidiasis [3]. Nevertheless, the study established (1) that *Candida* gene expression profiling after treatment in vivo is feasible and (2) that responses to antifungal agents cannot necessarily be extrapolated from in vitro systems.

Transcriptional profiling in vivo has also advanced understanding of the pathogenesis of invasive candidiasis. Using microarrays, investigators demonstrated that *C. albicans* induces genes involved in stress responses, adhesion, fatty acid utilization, and hyphal formation within mouse kidneys [4, 5]. These insights are important, but microarray profiling is limited by insensitivity for low-copy transcripts [6]. Ribonucleic acid-sequencing (RNA-Seq) offers improved sensitivity and the potential to define whole transcriptomes at sites of infection [6]. To date, genome-wide *C. albicans* transcriptional profiling has not been reported during deep-seated, invasive candidiasis of humans. We hypothesized that *Candida* gene expression patterns at sites of human infection may successfully distinguish treated from untreated invasive candidiasis. To test this hypothesis, we performed RNA-Seq on biliary fluid recovered from a patient with *C. albicans* cholangitis, before and after treatment with fluconazole and biliary tract drainage.

CASE REPORT

A 61-year-old man with pancreatic adenocarcinoma was diagnosed with acute cholangitis after developing fever and abdominal pain. Abdominal computed tomography scan revealed a mass in the pancreatic head and a hydropic gall bladder (GB) secondary to common bile duct (CBD) obstruction. Blood cultures were positive for *Escherichia coli*. Percutaneous cholecystostomy was performed, and piperacillin-tazobactam was administered. The patient initially improved, but he redeveloped symptoms 1 week later. Because the cholecystostomy was not providing adequate drainage, a transhepatic external-internal draining catheter was placed into the CBD (Figure 1). Budding yeast were identified by Gram-stain of biliary fluid from the newly placed external-internal drain (labeled as CBD-D1), and fluconazole was administered. Cultures of CBD-D1 and fluid from the cholecystostomy tube on day 1 (GB-D1) grew *C. albicans*. The patient responded within 24 hours to fluconazole and drainage, as evident by defervescence, resolution of abdominal pain, and improvement in biliary and peripheral neutrophil counts. Biliary fluid collected from the external-internal device on day 2 after a second dose of fluconazole (CBD-D2) remained *C. albicans* culture-positive, but Gram-stain was negative and fungal burdens were reduced (Supplemental Table 1). Beginning on day 3, cultures were negative. The patient completed a 2-week course of fluconazole without incident.

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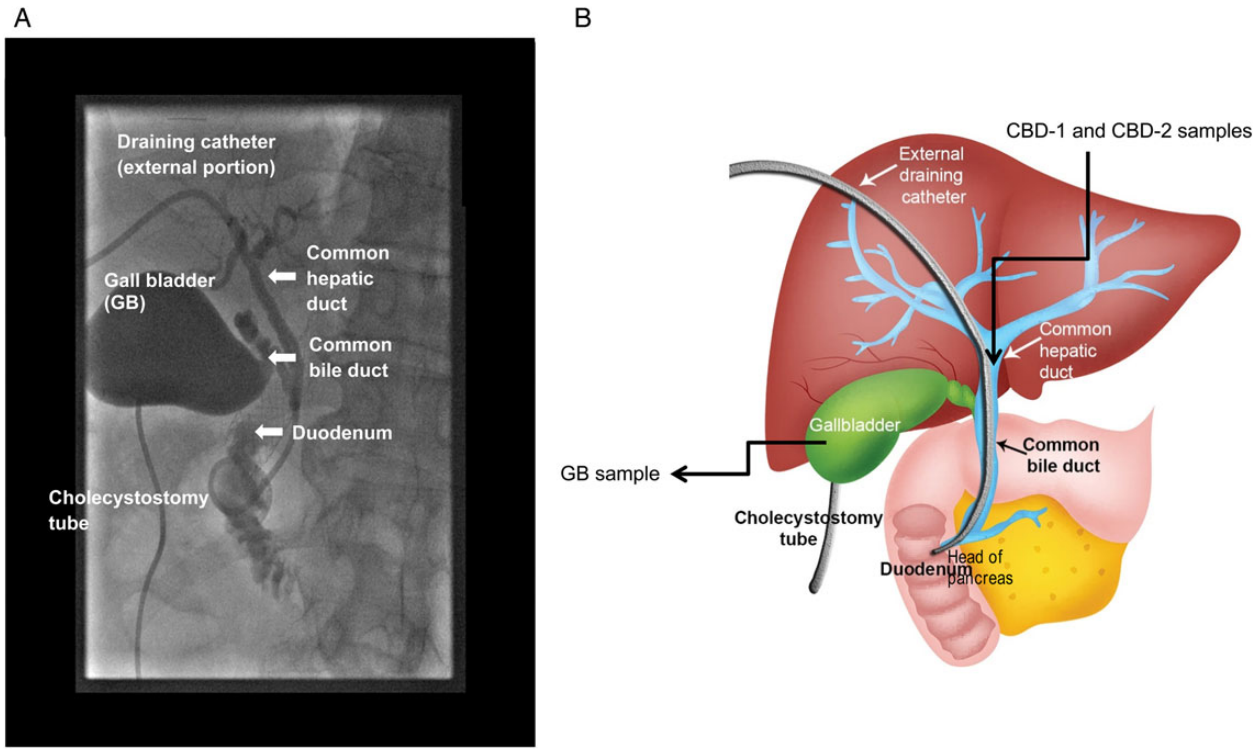

Figure 1. Cholecystostomy and drainage of the common bile duct (CBD) in a patient with *Candida albicans* cholangitis. Dye (appearing black) from a percutaneous transhepatic cholangiography (A) reveals the cholecystostomy tube, gall bladder (GB), external-internal draining catheter, and hepatobiliary anatomy in a 61-year-old man with *C. albicans* cholangitis. The patient originally presented with a malignant mass in the head of the pancreas that invaded the duodenum and caused an obstruction of the common bile duct. Percutaneous cholecystostomy was performed to decompress the GB. One week later, a transhepatic external-internal draining catheter was placed to relieve obstruction and provide adequate biliary drainage. The catheter was inserted through the right hepatic and common hepatic ducts, terminating in the common bile duct. A schematic figure (B) highlights the placement of the cholecystostomy tube and draining catheter as well as anatomic landmarks. The black arrows indicate collection sites for GB-D1, CBD-D1, and CBD-D2 samples.

METHODS

Samples

Informed consent was obtained. The GB-D1 was collected by aspirating from the cholecystostomy catheter, which continuously drained the GB. Common bile duct samples were obtained by aspirating from the external portion of the external-internal drain. The CBD-D1 was obtained at the time of drain placement. The GB-D1 was collected at the same time. On D2, biliary fluid in the external-internal drain and bag was discarded, the external portion of the drain was clamped for 5 minutes, and fresh fluid was aspirated (CBD-D2).

Ribonucleic Acid Sequencing

Ribonucleic acid was immediately extracted from samples using the RiboPure-Yeast kit (Ambion) and a bead beater. DNase-treated samples were enriched for poly(A) messenger RNA using oligo(dT) beads. Complementary deoxyribonucleic acid libraries were constructed from fragmented samples according to manufacturer's instructions (www.illumina.com) [7]. The RNA-Seq was performed using 1×100 base-pair runs on the Illumina MiSeq platform. A 90% cutoff was imposed for minimum similarity and length of reads. Nonspecific matches (reads with multiple hits) were ignored. Reads were mapped to *C. albicans* coding sequences (SC5314, assembly 19; www.candidagenome.org) using the RNA-Seq module of the CLC Genomics Workbench (www.clcbio.com). Expression values were calculated in RPKM (reads per kilobase exon model per million mapped reads), normalized for total exon-length and number of matches.

Data Analysis

Differentially expressed genes were identified using the EdgeR Bioconductor package [8]. The RPKM was normalized between samples and replicates using the trimmed mean of M-values method [9]. A gene was considered differentially expressed for false-discovery rate (FDR) and fold change <0.05 and >4, respectively. Gene ontology enrichment analysis was performed using resources at http://genecodis.cnb.csic.es/ [10]; hypergeometric P value <.05 (corrected by FDR) was significant. For comparative purposes, RNA-Seq data from biologic replicates of *C. albicans* under in vitro (YPD medium, 30°C, 48 hours), ex vivo (OKF6-TERT2 oral epithelial cells), and in vivo (mouse kidneys at 48 hours, and mouse tongues at 5 days) conditions were retrieved from the National Center for Biotechnology

Information's Gene Expression Omnibus repository (details in Supplemental Material) [11, 12].

RESULTS

Candida albicans Gene Expression in Patient Samples

There were 1.8–4.7 million *C. albicans* reads and ≤386 000 human reads in patient samples (Supplemental Table 1). The most highly expressed *Candida* genes in D1 samples were enriched in glycolysis, host defense response, gluconeogenesis, apoptosis, and response to oxidative stress (Supplemental Table 2). The most highly expressed *Candida* genes in the D2 sample were enriched for host defense response, adhesion, cell wall organization, hyphal growth, and pathogenesis.

Overall, 4538 *C. albicans* genes were expressed in both GB-D1 and CBD-D1 (>1 RPKM). Only 2% (n = 102) of genes were differentially expressed between samples, including 74 and 28 up-regulated in GB-D1 and CBD-D1, respectively (Supplemental Figure 1). The GB-D1 and CBD-D1 were treated as biologic replicates in subsequent analyses, because transcriptional profiles were broadly similar, relatively few genes were differentially expressed, samples were collected simultaneously, and biliary fluid from each sample originated from the same cells (hepatocytes).

Thirty-one percent (1131 of 4811) of *C. albicans* genes were differentially expressed in CBD-D2 (up-regulated, n = 434) and combined D1 samples (up-regulated, n = 697) (Supplemental Figure 1). Genes up-regulated on D1 were enriched in 1 carbon and galactose metabolism, pyrimidine salvage pathway, inosine monophosphate biosynthetic pathway, and response to oxidative stress (Table 1). Genes up-regulated in CBD-D2 were enriched in hyphal growth, pathogenesis, cell wall organization, adhesion, cellular response to drug, fatty acid biosynthesis, regulation of carbohydrate metabolism, ergosterol biosynthesis, actin organization, and response to oxidative stress and heat (Table 1).

Table 1. *Candida albicans* Gene Expression in Response to Treatment of Cholangitis[a]

Expression Pattern	GO Biologic Process	Cluster Frequency[a,b]	Background Frequency[b]	*P* Value[c]	Genes Annotated to GO Biologic Process
Up in D1 samples vs CBD-D2	One carbon metabolism	0.7% (5/688)	0.1% (10/6725)	.009	SHM2, GLY1, GCV3, GCV2, GCV1
	Pyrimidine salvage pathway	0.6% (4/688)	0.1% (7/6725)	.01	FCY2, URK1, orf19.1888, FUR1
	Galactose catabolism	0.4% (3/688)	0.06% (4/6725)	.01	PGM2, GAL10, GAL1
	De novo IMP biosynthetic process	0.6% (4/688)	0.12% (8/6725)	.02	ADE1, ADE4, ADE2, ADE13
	Response to oxidative stress	2.2% (15/688)	1.2% (82/6725)	.04	YFH1, TRX1, orf19.1340, CIP1, orf19.2299, GAD1, TSA1B, orf19.86, GAL10, TTR1, orf19.2262, CKA2, NCE103, orf19.3537, ALO1
Up in CBD-D2 vs D1 samples	Hyphal growth	8.2% (35/427)	3.1% (209/6725)	$6.9\ e^{-6}$	CCH1, SLA1, SFL1, CEK1, MSS11, CYR1, ERG3, ALS1, CLA4, RIM101, CHK1, VRP1, ERG24, RGA2, SSK1, NIK1, RFG1, MYO5, NRG1, ALS4, CLN3, RVS167, FLO8, KEL1, CPP1, OLE1, BNI1, CPH1, YCK2, SGS1, MDS3, MYO2, CST20, RGT1, EFG1
	Pathogenesis	8.4% (36/427)	4.4% (217/6725)	$1.1e^{-5}$	ALS3 CEK1 CYR1 LPD1 ERG3 ALS1 SKN7 CNH1 PHR2 CLA4 GSC1 RIM101 CHK1 ERG24 SSK1 NIK1 RFG1 NRG1 IFF4 RVS167 VPS27 FLO8 CPP1 SET3 HAP43 RBT4 DFG16 BNI1 CPH1 FAS2 RCK2 MDS3 TCC1 RFX2 CST20 EFG1
	Cell wall organization	5.2% (22/427)	1.9% (125/6725)	.0003	PGA62 CEK1 PHR2 GSC1 SAP9 SOG2 SIM1 SSK1 MYO5 ECM7 RVS167 SSR1 ECM25 CPH1 ECM17 RCK2 XOG1 CAS4 RLM1 MSB2 CST20 ECM3
	Adhesion	1.2% (5/427)	0.1% (8/6725)	.0006	ALS3, DEF1, ALS1, ALS7, ALS6
	Cellular response to drug	8.2% (35/427)	4.2% (285/6725)	.001	CCH1, PGA62, SFL1, CEK1, CYR1, ERG3, SKN7, orf19.6586, CLA4, GSC1, FAS1, RIM101, SOG2, RAD50, ZCF31, ERG24, UPC2, ECM7, TOR1, NRG1, CLN3, MNL1, AUR1, ADO1, SSR1, orf19.7029, RBT4, CDR11, KSP1, ERG6, YCK2, ZCF34, CAS4, orf19.7522, RLM1
	Fatty acid biosynthesis	1.2% (5/427)	0.2% (16/6725)	.006	ERG3, FAS1, ACC1, orf19.4812, FAS2
	Regulation of carbohydrate metabolism	0.7% (3/427)	0.1% (8/6725)	.02	GLC7, ADR1, KIS2
	Ergosterol biosynthesis	1.2% (5/427)	0.3% (23/6725)	.02	ERG3, ERG24, ERG10, UPC2, ERG6
	Response to oxidative stress	2.6% (11/427)	1.2% (82/6725)	.02	GLR1, orf19.3292, SKN7, RAD50, SSK1, PRX1, MNL1, DOT5, MSN4, RCK2, RFX2
	Actin filament organization	0.9% (4/427)	0.2% (17/6725)	.03	PRK1, orf19.1444, RGA2, SAC6
	Response to heat	0.7% (3/427)	0.2% (12/6725)	.049	MET6, SSK1, MNL1, RFX2, CST20

Abbreviations: CDB, common bile duct; GO, gene ontology; IMP, inosine monophosphate.

[a] A total of434 and 697 genes were ≥4-fold up-regulated genes in CDB-D2 and D1 samples, respectively (false-discovery rate, <0.05). Among 697 up-regulated genes in D1 samples, 9 did not show annotations in selected biological processes. Among 434 up-regulated genes in D2 samples, 7 did not show annotations in selected biological processes.

[b] Cluster frequency: (number of genes annotated to the GO term/number of genes with the expression pattern under consideration); denominator excludes differentially expressed genes that are not annotated to a GO term category. Background frequency: (number of genes annotated to the GO term/genes in *C. albicans* genome).

[c] Corrected hypergeometric *P* value.

Comparisons of *Candida albicans* Ribonucleic Acid-Sequencing Data From Patient and Experimental Samples

Gene expression in patient samples was compared with publically available RNA-Seq data for biologic replicates of *C. albicans* isolates under in vitro conditions, in the presence of epithelial cells ex vivo, and in vivo within mouse kidneys and tongues during HDC and oral candidiasis, respectively [11, 12]. Across patient and experimental datasets, expression data (>1 RPKM) were available for 5365 genes; 3406 genes were differentially expressed in ≥1 comparison. Hierarchical clustering and principal coordinate analysis revealed that replicates for each condition clustered most tightly (Supplemental Figure 2). In vitro and ex vivo replicates clustered more tightly than in vivo replicates. Expression patterns within mouse kidneys and tongues clustered more closely with one another than either did with patient samples. Finally, CDB-D2 was largely dissimilar from D1 samples.

Detailed comparisons of *C. albicans* gene expression in patient and experimental samples are presented in Supplemental Tables 3 and 4. Genes up-regulated in both D1 and D2 biliary fluid compared with in vitro growth were enriched in fatty acid β-oxidation, transmembrane transport (including carbohydrates, glucose, amino acids), thiamine biosynthesis, glyoxylate cycle, hyphal growth, and oxidation reduction. Genes up-regulated in biliary fluid compared with mouse kidneys were enriched in fatty acid β-oxidation, thiamine biosynthesis, amino acid transport, tricarboxylic acid cycle, and oxidation reduction.

DISCUSSION

To our knowledge, this is the first study to describe *Candida* global gene expression at the site of deep-seated human infection. We demonstrated that *C. albicans* transcriptional responses after initiation of therapy for cholangitis were highly dynamic, involving differential expression of >1000 genes. *Candida albicans* preferentially expressed genes involved in carbon, galactose, and nucleotide metabolism and in response to oxidative stress in D1 biliary fluid samples. This profile likely reflected active growth before treatment. After 2 doses of fluconazole and placement of a CBD drain, infectious burdens within D2 biliary fluid were reduced by >1-log. Not surprisingly, *C. albicans* induced ergosterol biosynthesis genes, consistent with previously reported transcriptional responses to fluconazole [2]. At the same time, *C. albicans* up-regulated numerous genes that were previously implicated in the pathogenesis of invasive candidiasis, even as fungi were being eliminated from the biliary tract. As hypothesized, *C. albicans* gene expression patterns within the biliary tract distinguished treated from untreated cholangitis.

Fluconazole exerts fungistatic effects on *Candida* by inhibiting lanosterol 14-α-demethylase, an enzyme in the ergosterol biosynthetic pathway. In microarray experiments, *C. albicans* exposed to inhibitory concentrations of fluconazole in vitro up-regulated >400 genes compared with unexposed cells [2]. As in CBD-D2, up-regulated genes in vitro were significantly enriched in ergosterol biosynthesis, pathogenesis, oxidation reduction, hyphal formation, and cell wall organization. Twenty-one genes that were up-regulated in common between CBD-D2 and in vitro datasets were enriched in ergosterol biosynthesis (n = 4), hyphal formation (n = 5), and oxidation reduction (n = 4). Therefore, stresses imposed by fluconazole's inhibition of ergosterol biosynthesis triggered a range of molecular and cellular responses by *C. albicans* in vitro and in vivo, including activation of biological processes that in other settings promote pathogenesis. Our results resemble those generated by a nanoString assay for *C. albicans* transcripts in mouse kidneys after caspofungin exposure, in that the functional spectrum of drug-induced genes was similar to in vitro conditions, but the specific genes induced in vivo were often different [3]. Taken together, the data indicate that it may be possible to define *C. albicans* gene expression patterns that represent markers for effective fluconazole treatment.

Candida albicans gene expression in CBD-D2 was also likely influenced by drain placement. In this regard, induction of biologic processes such as adhesion and biofilm formation may be viewed as an attempt by *C. albicans* to adapt to significant environmental changes introduced by the external-internal catheter. As is true for processes such as hyphal formation, cell wall regulation, oxidation reduction, and fatty acid biosynthesis, adhesion and biofilm formation make well characterized contributions to virulence in various mouse models of *Candida* infection [3]. In our case, however, the local conditions imposed by fluconazole activity and drainage were not conducive to maintenance or progression of invasive disease. Therefore, the data highlight that *C. albicans* virulence gene expression only results in disease in a conducive setting and that pathogenesis is dependent on both microbial virulence factors and a suitable host environment [13]. Furthermore, our findings suggest that widely divergent outcomes of *Candida* infection, such as successful treatment and eradication or tissue invasion and destruction, may be associated with overlapping transcriptional patterns.

CONCLUSIONS

Candida albicans gene expression during cholangitis in our patient differed significantly from patterns previously identified using RNA-Seq during growth in vitro, in the presence of oral epithelial cells ex vivo or within mouse kidneys and tongues. Indeed, the remarkable success of *C. albicans* as a commensal and an opportunistic pathogen is mediated by regulation of transcription in response to diverse environmental stimuli and niches [3, 14]. Just as gene expression during murine candidiasis cannot be extrapolated from other experimental systems [3, 14], our data suggest that transcriptional profiling at sites of infection in humans may offer unique insights into pathogenesis and treatment responses. Intra-abdominal candidiasis (IAC),

which comprises disease manifestations such as peritonitis, abscesses, and cholangitis, is particularly amenable to human transcriptional profiling studies because infections are localized, *Candida* are typically present at high concentrations in the absence of excess host RNA, and drainage procedures are routinely performed for diagnostic and therapeutic purposes [14, 15]. Our success in performing RNA-Seq establishes the feasibility of such studies. Building on this work, it should be possible to corroborate and extend our observations by performing RNA-Seq on samples from other patients with IAC. Reads for human gene expression were insufficient for detailed analysis in this study, but profiling of host transcriptional responses may also be feasible in certain clinical samples. In future studies, our objectives will be to define transcriptional responses to successful and unsuccessful treatment with various antifungals and to distinguish between gene expression during intra-abdominal disease versus colonization of a draining catheter. In addition to providing scientific insights, these studies may have value in developing prognostic or diagnostic markers for use in the clinic.

Supplementary Data

Supplementary material is available online at *Open Forum Infectious Diseases* online (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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