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Original Article

Fungal dysbiosis of the gut microbiota is associated with colorectal cancer in Chinese patients

Yu Wang[1*], Yahui Ren[2*], Yongming Huang[1], Xiangnan Yu[1], Yiming Yang[1], Di Wang[1], Liang Shi[1], Kaixiong Tao[1], Guobin Wang[1], Ke Wu[1]

Departments of [1]Gastrointestinal Surgery, [2]Pediatrics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China. [*]Equal contributors.

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Abstract: Changes in bacteria and virions are associated with colorectal cancer (CRC). However, the fungal microbiota in the intestines of CRC patients remains largely unexamined. We identified differences in the intestinal fungal microbiota between healthy persons and patients with colorectal polyps or CRC. Using second-generation sequencing technology, we sequenced and aligned the ITS1 regions of fungi collected from fecal samples. We found a significant increase in the *Candida albicans* levels in the guts of CRC patients. Dectin-1 is a C-type lectin receptor that recognizes β-1,3-glucan in the cell walls of most fungi and is expressed by many cell types, including dendritic cells, macrophages, and monocytes. However, the mechanisms controlling the expressions and functions of dectin-1 in intestinal epithelial cells (IECs) remain unclear. Furthermore, the putative effects of *C. albicans* on IECs are unknown. *C. albicans* induces the proliferation of IECs by activating the Wnt signaling pathway, and the Wnt pathway contributes to the development of CRC. Mice infected with *C. albicans* show an activation of the Wnt pathway. Therefore, IECs may recognize the activation of the Wnt pathway by *C. albicans* through dectin-1 to promote the development of CRC.

Keywords: Fungi, sequencing, *Candida albicans*, intestinal epithelial cells, proliferation, Wnt

Introduction

The gut microbiota is a complex ecosystem that plays an important role in the pathogenesis of colorectal cancer (CRC) [1]. Most studies have focused on the bacterial component of the microbiome, but the role of fungi in CRC has not been widely explored [2, 3]. Therefore, our understanding of the contribution of fungi to colorectal carcinogenesis is limited. Technological advances, particularly next-generation sequencing, have enabled the study and demonstrated the importance of fungi in the gut microbiota [4-6]. Recently, diversity in the intestinal fungal communities was found to be associated with human inflammatory bowel disease and cirrhosis [7, 8]. Differences in the fungal composition of tissues biopsied from 27 subjects with colorectal adenomas suggested that a host's fungal population is altered during the development of CRC [4]. The sequencing of the human intestinal fungal microbiota is needed to understand the composition and distribution of the fungal communities in the gut [9]. The identification of the pathogenic fungal species associated with gastrointestinal disorders is critical. Understanding the diversity and abundance of fungi in CRC patients is important for determining their contribution to tumorigenesis, particularly the development of CRC from intestinal polyps.

Fungi are typically recognized by their immune receptors. Dectin-1, a C-type lectin receptor that recognizes β-1,3-glucan in the cell wall of most fungi, contributes to phagocytosis and the killing of fungi [10, 11]. Dectin-1 was originally thought to be a dendritic cell-specific receptor and was designated dendritic cell-associated C-type lectin-1; however, it is now known to be expressed in many cell types, including macrophages, monocytes, neutrophils, and T cells [12-14]. At the portals of pathogen entry, such as the lungs and intestine, dectin-1 is expressed at high levels, which is consistent with its potential role as a trans-

membrane receptor in immune surveillance [13]. Various cellular functions are mediated by dectin-1, including fungal binding, uptake, and inducing the production of cytokines and chemokines [15]. Dectin-1 recognizes fungi in the mouse intestine, and dectin-1 appears to increase the susceptibility to experimental murine colitis [11]. The binding of fungal β-glucan to dectin-1 results in the phosphorylation of an immunoreceptor tyrosine-based activation motif-like sequence within the cytoplasmic domain. The subsequent binding of spleen tyrosine kinase (Syk) to the phosphorylated receptor induces the assembly of a scaffold consisting of the caspase recruitment domain 9 (CARD9) protein and adaptor proteins Bcl-10 and MALT1 [16, 17]. Dectin-1 induces the formation of the CARD9-Bcl-10-MALT1 scaffold via the classical nuclear factor (NF-κB) pathway, leading to the nuclear translocation of the p65 subunit of NF-κB [18]. This suggests that the p65 subunit of NF-κB interacts with β-catenin and inhibits its activity in human colon cancer cells [19], which impacts the Wnt pathway. In 2014, Cohen-Kedar showed that human intestinal epithelial cells (IECs) express functional dectin-1 and demonstrated that β-glucans induce the secretion of pro-inflammatory chemokines through dectin-1 and Syk activation [20]. This process proceeds through a signaling pathway comparable to the signaling pathway in myeloid lineage cells [20].

IECs express dectin-1, which has been implicated in the antifungal responses of myeloid cells as well as in intestinal inflammation. Dectin-1 induces β-catenin stabilization through Syk activation and the subsequent production of reactive oxygen species, which promotes Wnt5A expression in macrophages [21]. However, dectin-1 expression and its function in IECs remain unclear [22, 23]. Furthermore, the putative effects of *Candida albicans* on IECs remains unclear. We studied the intestinal fungal microbiota using fecal samples from CRC patients, intestinal polyps, and healthy volunteers. We used a second-generation sequencing technique to compare the internal transcribed spacer 1 (ITS1) regions of the fungi collected from the fecal samples. We anticipated finding different fungi in the intestinal tracts of each group. Additionally, we screened the fungi discovered to verify which could induce the proliferation of IECs by activating the Wnt signaling pathway of dectin-1 and promote the development of CRC.

Materials and methods

Samples collection

This study was approved by the Human Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Informed consent was obtained from all the patients in accordance with the Declaration of Helsinki and its subsequent amendments. All the patients were recruited from the Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) from 2015 to 2017. The human ethics approval authorization number is IEC-J (248). The participants were grouped according to their diagnoses: normal, intestinal polyps, and colorectal cancer. The diagnoses were determined using clinical, radiological, endoscopic, and histological criteria. The patients were not administered antibiotics or antifungal medications within the three months before diagnosis. None of them were administered a vegetarian diet, and none of them had undergone colorectal surgery before. The samples were collected separately from each group and the fecal samples were stored at -80°C until use.

DNA extraction and ITS1 gene amplicon sequencing

Total DNA Stool Mini Kits (Qiagen, Hilden, Germany) were used for the DNA extractions following the manufacturer's instructions. The preliminary analysis of the ITS region of the fungal genome was carried out using polymerase chain reaction (PCR) for a total of 55 cases which met the sequencing requirements. Based on the amplified ITS1 region characteristics, a small fragment library was constructed for double-end sequencing using the Illumina HiSeq sequencing platform (Illumina, San Diego, CA, USA) and using the paired-end method. Through the splicing and filtering of reads, the operational taxonomic units (OTUs) were clustered, and the species annotation and the abundance analysis were performed.

Bioinformatics and the statistical analysis

A rarefaction analysis was performed to assess the gene richness in the N, P, and T samples. The alpha diversity of the intestinal fungi in

each group was estimated using the Chao1, Simpson, and Shannon indices, and the sequence data were analyzed on several different scales. The fungal community compositions were examined at the phylum, class, order, family, genus, and species levels, and the overall differences in the fungal microbiota composition between the N, P, and T samples were assessed using paired t-tests. The OTU composition between the T, N, and P samples were analyzed using hierarchical clustering and using Spearman ranked distance and principal component analyses (PCA). Two-tailed t-tests were used to identify the OTUs with different abundances in each group's samples. Beta diversity analyses were performed to determine the differences between the samples. LEfSe analyses were conducted using LEfSe software; the default setting of the linear discriminant analysis score was 4.

Fungal strains and mice

Candida albicans (China Institute of Microbiology, SC5314) were obtained directly from yeasts cultured under aerobic conditions on Sabouraud dextrose broth (EMD Millipore, Billerica, MA, USA) at 26°C. All the animal studies were approved by the Institutional Animal Care and Use Committee of Tongji Medicine College. Six-week-old male C57BL/6J mice were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China) and raised under pathogen-free conditions in the Tongji Medicine School Animal Center. The IACUC number for the animal ethics approval is 2443. To eliminate intestinal fungi, fluconazole (0.5 mg/mL, Sigma, St. Louis, MO, USA) was added to their drinking water for 14 days. Next, to induce colitis, the mice were given drinking water supplemented with 2.5% (w/v) dextran sulfate sodium (DSS, MP Biomedicals, Solon, OH, USA) for 7 days. Upon the colitis induction, the mice were administered four doses (1×10^8 yeast/mouse/dose) of *C. albicans* every other day. A control group was administered phosphate-buffered saline (PBS). Each group contained five mice.

Cell lines

The human colon epithelial cell line NCM460 was obtained from INCELL (San Antonio, TX, USA) and cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Tianhang Biological Technology, Hangzhou, China). The cells were kept in a humidified incubator at 37°C with 5% CO_2.

Co-culture of C. albicans and the NCM460 cells

The fungal cells were collected using centrifugation and washed in PBS (Hyclone). The yeast cells were resuspended in an RPMI 1640 medium and the turbidity of the suspension (proportional to the cells' concentration) was assessed with a microplate reader (BioTek, Winooski, VT, USA) and adjusted to the desired value with an RPMI 1640 medium before adding the cells. The IECs were seeded into 6-well (5×10^5 cells/well) and 96-well (1×10^4 cells/well) polystyrene plates (Corning, Inc., Corning, NY, USA) and incubated overnight at 37°C in 5% CO_2. Where indicated, the anti-dectin-1 monoclonal antibody (mAb) GE2 (ab82888, Abcam, Cambridge, UK, 0.5 mg/mL) or a Wnt inhibitor (ICG-001, Selleck, Houston, TX, USA, 0.1 mg/mL) was added 30 min before adding the heat-inactivated *C. albicans* at a 10:1 fungal cell-to-host cell ratio. On the following day, the cells in the 6-well plates were collected for the protein extraction, and the cells in the 96-well plates were collected for the cell proliferation assays.

Cell lysis and western blotting

After the treatment, the cells were washed twice with cold PBS and lysed using a cell lysis buffer (PMSF: RIPA 1:100). The RIPA buffer and the PMSF were purchased from Sigma-Aldrich. The cell debris was removed using centrifugation (12,000×g, 15 min at 4°C), and the protein concentrations of the supernatant were measured with a bicinchoninic acid assay kit (Beyotime, Haimen, China). A gel sample buffer was added to the cell lysate, and the lysate was boiled for 10 min and stored at -20°C until use. Nuclear and cytoplasmic proteins from the mice's colorectal tissues were isolated using Nuclear and Cytoplasmic Extraction Reagents (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The proteins were analyzed using sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (10% Tris-glycine gels) and transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tween 20-Tris-buffered saline containing 5% skim milk for 1 h and incubated with the following antibodies according to the manufacturer's instructions: Ki-67 antibody

Table 1. Demographic and clinical details of the samples

Factor	Normal	Intestinal polyps	Colorectal cancers
Sample size	14	28	13
Age	53.57±18.05	56.19±10.18	57.17±16.64
Male/female	12/2	15/13	9/4
BMI	22.18±3.00	21.91±2.28	26.57±2.61

Table 2. The PCR Primers

Gene name	Primers
C. albicans-F	5'-CTGTTTGAGCGTGGTTTC-3'
C. *albicans*-R	5'-ATGCTTAAGTTCAGCGGGTAG-3'
Mouse GAPDH-F	5'-TGACAGTGACTTGGGACAAGG-3'
Mouse GAPDH-R	5'-GGAGTTGCTGTTGAAGAAGTCGC-3'

(catalog #PB0065, Boster Biological Technology, Wuhan, China), rabbit polyclonal human dectin-1 antibody (ab140039, Abcam), rabbit β-catenin monoclonal antibody (Ab32572, Abcam), rabbit c-myc monoclonal antibody (ab32072, Abcam), rabbit transcription factor 4 (Tcf-4) monoclonal antibody (ab130014, Abcam), GAPDH polyclonal antibody (G9545, Sigma). The membranes were then washed with Tween 20-Tris-buffered saline and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at 20-25°C. The immunoreactive bands were measured using the enhanced chemiluminescence method. The levels of the proteins of interest were normalized to those of GAPDH.

Cell proliferation assays

The cell proliferation was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sisco Research Laboratories, Mumbai, India). After the indicated treatments, the cell culture medium was removed, and freshly prepared MTT solution (5 mg/mL) was added to each well, and the plate was incubated for 4 h. Next, dimethyl sulfoxide was added to each well, and the absorbance was measured with a microplate reader (BioTek) at a wavelength of 570 nm. Five independent readings per time point were measured.

Immunofluorescence

The cells were seeded onto a coverslip in 6-well plates at a density of 3-4×10^5 cells/well. After the overnight incubation, the cells were fixed for 30 min with 4% paraformaldehyde, washed with PBS, and permeabilized with 0.1% Triton for 10 min at room temperature. The cells were incubated with the dectin-1 antibody (described above) overnight at 4°C and then with a secondary antibody (donkey anti-rabbit Alexa Fluor-555 [A31572], Invitrogen) for 1 h at room temperature. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 5 min. After washing with PBS (three times), the cells were visualized using a fluorescence microscope equipped with a digital camera (Nikon Eclipse 80i, Tokyo, Japan; ×20 magnification). To detect whether *C. albicans* administered by gavage colonized the intestines of the mice, we performed in situ staining of the intestinal fungi. The OCT-embedded intestinal specimens were sectioned, mounted on microscope slides, and incubated for 40 min in PBS containing 2% fetal calf serum. The intestinal sections were stained with an anti-*C. albicans* antibody (Abcam, ab53891). The slides were rinsed with PBS and stained for 5 min with 0.1 μg/mL DAPI and overlaid with a mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). The slides were examined using a fluorescence microscope equipped with a digital camera (Nikon Eclipse 80i). All the images were collected and processed identically.

cDNA synthesis and real-time PCR

Total RNA was isolated from the colorectal tissues using TRIzol (Invitrogen). The cDNA was synthesized with a cDNA Synthesis kit (Takara, Shiga, Japan). Quantitative real-time PCR was performed using SYBR Green with an ABI StepOnePlus system (Life Technologies, Carlsbad, CA, USA). The primers used are listed in **Table 2**. All the gene expression levels were normalized to the GAPDH levels.

Statistical analysis

The data are reported as the mean ± standard error of the mean (SEM). Student's t-tests were used for the statistical analysis of the two groups, and one-way ANOVA was used to analyze the differences among three or more groups (GraphPad Prism software, GraphPad, Inc., San Diego, CA, USA). We used T-tests, Wilcox rank-sum tests, and Tukey's tests (T-test

and Wilcox rank-sum tests when there are only 2 groups, Tukey and Wilcox rank-sum tests when there are more than 2 groups) to analyze the alpha diversity and the beta diversity. We used the MetaStat method to perform the hypothesis testing on the species abundance data between the groups to obtain the *p*-values. The LEfSe analyses were carried out using nonparametric factor Kruskal-Wallis rank-sum tests. Differences were considered significant when $P<0.05$. Specifically, the following conventions were used: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as indicated in the figure legends.

Results

Intestinal fungal ecological disorders related to CRC

Our study population consisted of 24 CRC cases, 31 intestinal polyp cases, and 18 healthy individuals. Through ITS1 sequencing, we evaluated the composition of the fecal fungal microbiota in each group: T, P, and N. The characteristics of the participants are presented in **Table 1**. First, the composition was examined at the fungal community level to identify the phylum, class, order, family, and genus in each group. According to the OTU clustering results, the species were annotated based on representative sequences in each OTU, and the corresponding species information and the relative abundance distributions of each sample or group at each classification level were obtained. After filtering, an average of 69,045 reads per sample was obtained (range 26,305 to 116,813). We first investigated the richness and uniformity of the three groups of intestinal fungi. The sequencing depth was checked by plotting the rarefaction curve for richness (**Figure 1B**). Most samples reached a plateau, indicating that the sequencing depth was adequate. In the fungal taxa, the phylum *Basidiomycota* dominated the mycobiota, and *Ascomycota* was the second most abundant phylum in the N, P, and T groups (**Figure 1A**). The fungal alpha diversities as measured by the Chao1 index significantly differed between the T versus the N and P groups (**Figure 1C**). The Simpson and Shannon diversities, however, were not significantly different between the T and the N and P groups. The above sequencing results showed that the richness of the intestinal fungal community in the T group was significantly higher than it was in the N and P groups. However, there were no significant differences in the diversity of the intestinal fungal communities in the three groups. Therefore, a richer fungal community is not a sign of a healthy gut microbiota but may indicate the excessive growth of various harmful fungi in cancer patients.

Taxonomic changes in the intestinal fungi in CRC

To assess the similarities and differences in the intestinal fungal communities in the N, P, and T groups, the fungal OTUs in the samples were compared and displayed via Venn diagrams (**Figure 2A**). The results showed that 269 OTUs were detected in all three groups. There were 544 OTUs detected in both the N and P groups compared to 468 OTUs in both the P and T groups. However, there were 339 OTUs in both the N and T groups. We also found that the number of OTUs appearing in the T group, but not in the P and N groups, was much greater than the number appearing in the P and N groups but not in the T group (1045 compared to 615 and 301). This indicates that the intestinal fungal communities in the T group were more complex than those in the P and N groups. To determine the taxonomic affiliation of the community members, all the qualified reads from each sample were assigned to their respective taxonomic ranks at the phylum to genus levels. The relative abundance of fungi in the three groups at the phylum level is shown in **Figure 2B**. *Basidiomycota* and *Ascomycota* were the two most abundant phyla in the three groups. At the genus level (**Figure 2C**), the two most predominant genera in the N group were *Lasiodiplodia* (14.96%) and *Paraphysoderma* (13.01%). The P group was dominated by *Lasiodiplodia* (6.65%) and *Microidium* (2.12%). In the T group, the distribution was *Candida* (6.22%) and *Microidium* (1.54%). To analyze the differences in the fungal community structures among the groups, we performed beta diversity index analyses among the three groups (**Figure 2D**). The results revealed a significant difference in intestinal fungal beta diversity among the three groups. Next, the overall differences were visualized using PCA and non-metric multidimensional scaling plots. The diversity described in the PCA plots by the top two principal coordinates was 75.86%

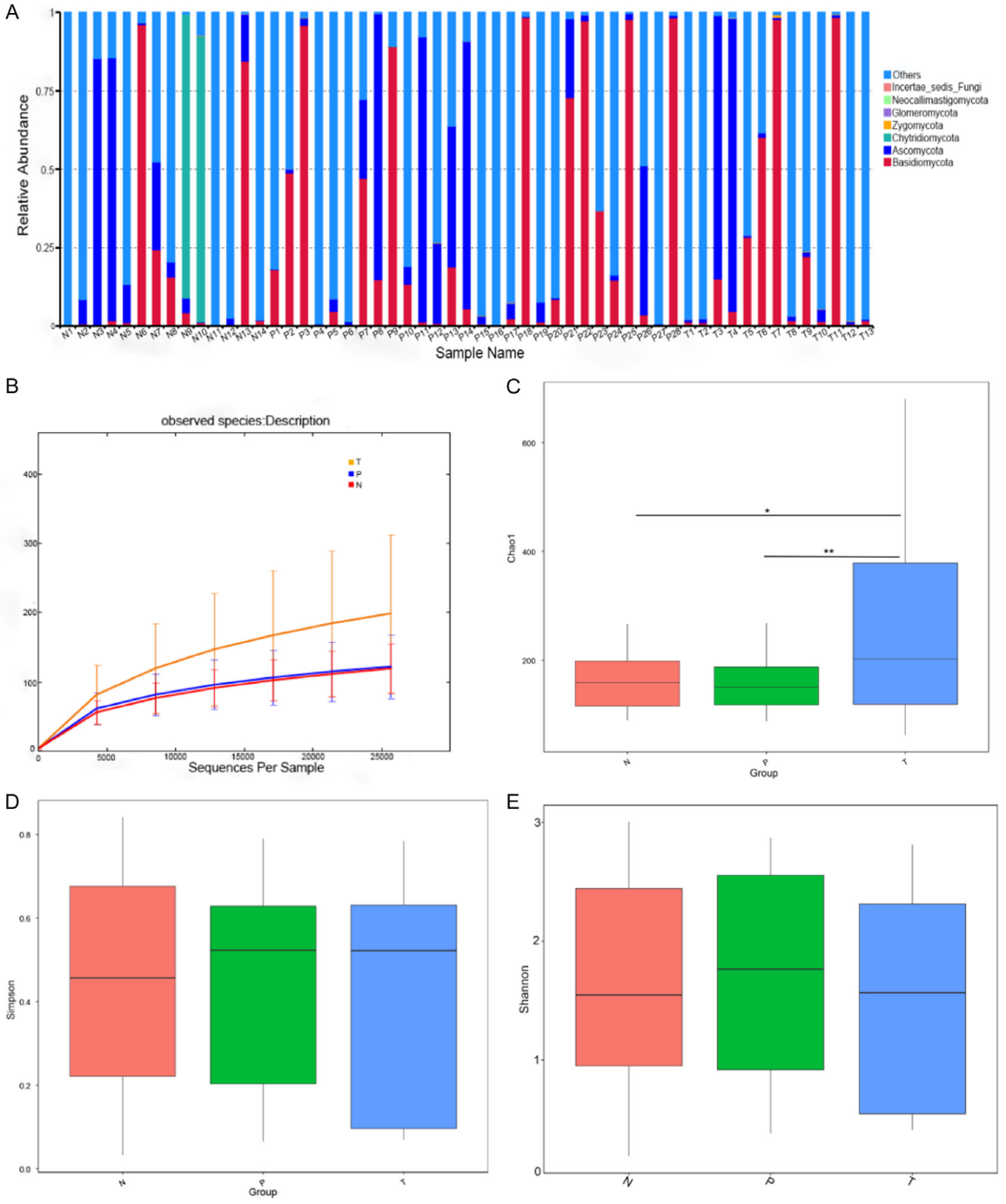

Figure 1. Intestinal fungal ecological disorders related to CRC. A. Relative abundance of dominant enteric fungi phyla in N (n=14), P (n=28) and T (n=13). The dominant phyla are *Ascomycota* and *Basidiomycota* in both groups. B. The rarefaction curves show that a sufficient sequencing depth was reached and that an increased number of intestinal fungal species were detected in the T group compared with the N and P groups. C. The alpha-diversity (Chao1 plot) of the gut fungus communities in the N, P, and T groups. D. The alpha-diversity (Simpson plot) of the gut fungus communities in N, P, and T groups. E. The alpha-diversity (Shannon plot) of the gut fungus community in N, P, and T groups.

based on the weighted UniFrac phylogenetic distance matrices. The N, P, and T samples were clustered separately, with a significant difference confirmed by an analysis of the

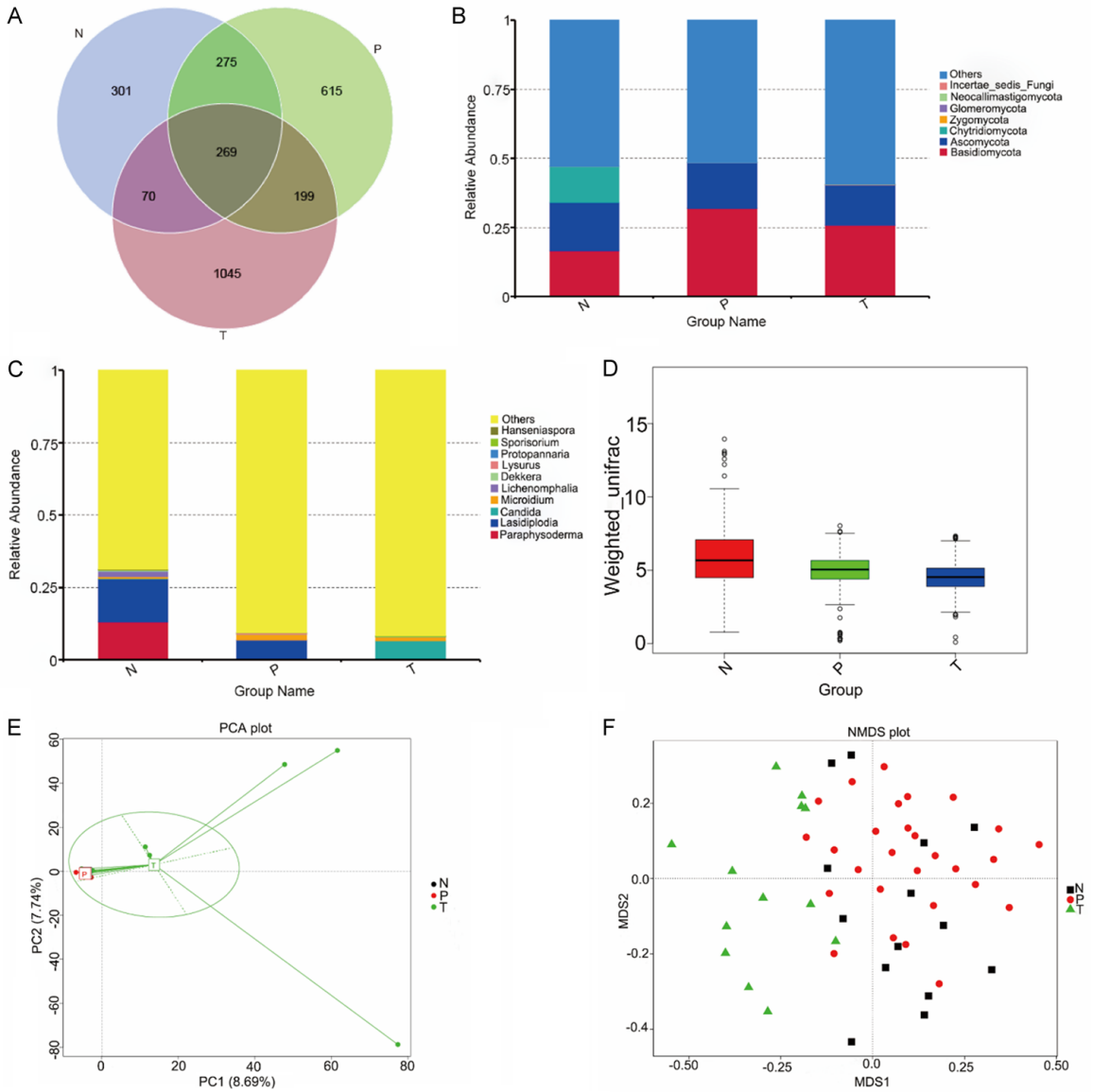

Figure 2. Taxonomic changes in the intestinal fungi in CRC. A. Venn diagrams were used to show the overlap of the OTUs of the study groups. The samples were divided into three groups: N, T, P. B. The fungal structures at the samples' phylum levels. Only the relative abundances of the 10 most abundant genera are plotted. C. The fungal structures at the samples' genus levels. Only the relative abundances of the 10 most abundant genera are plotted. The others are concatenated in the group named 'others'. D. β-diversity is represented by a weighted UniFrac distance between the intestinal fungal communities. Wilcoxon rank-sum test, *P<0.05, **P<0.01, ***P<0.001. E. PCA analysis on N, P, and T samples based on core OTUs. F. NMDS ordination analysis of the fungal OTU community distance based on the Bray-Curtis dissimilarity metrics of the relative abundance profiles.

similarities (P=0.048, **Figure 2E**). The results of the non-metric multidimensional scaling analyses also divided the samples into three separate clusters (**Figure 2F**), suggesting significant differences in the overall community structure of the intestinal fungi in the N, P, and T groups. The above results indicate taxonomic changes in the intestinal fungi in the N, P, and T groups.

The abundance of Candida albicans in the CRC is increased

To evaluate the fungal species showing significant differences between the groups, we performed a hierarchical clustering heat map analysis. The gut microbiota in the T group contained higher *Candida* levels than the other two groups at the genus level (**Figure 3A**).

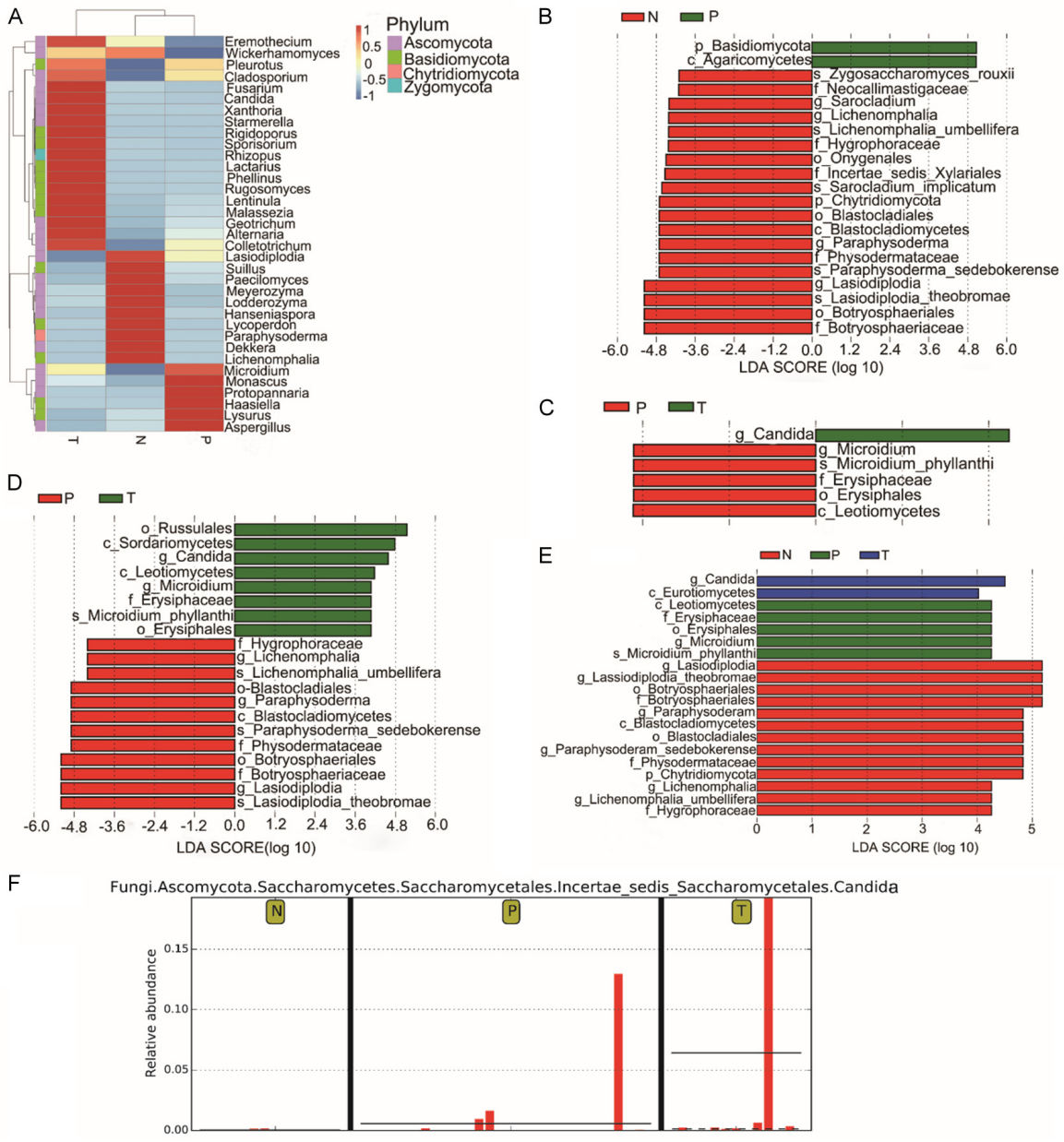

Figure 3. The abundance of *Candida* in the CRC is increased. A. A two-dimensional heatmap depicting the rank normalized abundances (scaled between -1 and 1) of the 35 fungal genera in the N, P, and T group patients of gut fungi. B. A linear discriminant analysis (LDA) revealed significant bacterial differences between the N and P groups. The LDA scores (log10)>2 and P<0.05 are listed. C. Our linear discriminant analysis (LDA) revealed significant bacterial differences between the P and T groups. The LDA scores (log10)>2 and P<0.05 are listed. D. Our linear discriminant analysis (LDA) revealed significant bacterial differences between the N and T groups. The LDA scores (log10)>2 and P<0.05 are listed. E. Our linear discriminant analysis (LDA) revealed significant bacterial differences between the N, P, and T groups. The LDA scores (log10)>2 and P<0.05 are listed. F. A histogram of the increased abundances of *Candida* in the T group patients compared with the N and P groups.

Next, to detect any significant alterations in the taxa abundance in the T group, we conducted LEfSe. *Basidiomycota* and *Agaricomycetes* were the most abundant fungal species (both have 5.0-fold enrichment) in group P, while *Botryosphaeiaceae, Botryosphaeriales, Lasiodiplodia theobromae*, and *Lasiodiplodia* were more abundant in group N (**Figure 3B**). Compared to the N and P groups, *Candida* was the most abundant fungal species (4.5-fold

enrichment) in group T (**Figure 3C-E**). However, the main fungi in the intestinal tract of group P were Leotiomycetes and *Erysiphaceae* (**Figure 3C-E**). The results (**Figure 3F**) also showed that the relative abundance of *Candida* in the T group of the intestinal tract was significantly increased compared to in the N and P groups. We then performed a tree classification analysis of the specific species, which showed that *C. albicans* was the most abundant intestinal fungus in the T group (**Figure 4A**). These sequencing results indicate that the abundance of *Candida* in the intestinal tract of CRC patients was increased. The anti-*C. albicans* immunofluorescence of the colorectal tissues from the patients in the N, P, and T groups showed the same result (**Figure 4B**). Moreover, growth conditions suitable for *C. albicans* are the most common cause of human fungal infections [24]. Thus, we chose *C. albicans* as a target to evaluate the effects of fungi on the dysregulation of the IECs.

The NCM460 human colon epithelial cells express dectin-1

Cohen-Kedar et al. demonstrated that HT-29 and SW480 cells (human IECs) express dectin-1 [20]. We measured the dectin-1 cell-surface expressions in the human IEC line NCM460 using immunofluorescence (**Figure 5A**). Of the eight dectin-1 isoforms, isoform B senses and signals the presence of *C. albicans*, which migrates as an approximately 23-kDa protein [25]. Our Western blot analysis showed that the NCM460 cells expressed the 23-kDa isoform of dectin-1 (**Figure 5B**).

Candida albicans induces the proliferation of the NCM460 cells through the Wnt/β-catenin signaling pathway

We next investigated the changes in the NCM460 cells, specifically the cell proliferation, upon exposure to *C. albicans*. Compared to the untreated cells, *C. albicans* significantly promoted the growth of the NCM460 cells after the treatment for 48 h (**Figure 5C**). We found that *C. albicans* induced the proliferation of the NCM460 cells, as determined using an MTT assay (**Figure 5D**). We confirmed increased cell proliferation by assessing the expression of Ki-67, a marker of cell proliferation, using western blotting (**Figure 5E**). The Wnt signaling pathway is associated with cellular proliferation, differentiation, apoptosis, motility, and polarization in invertebrates and mammals [26]. Therefore, we evaluated whether the Wnt signaling pathway is activated in cells exposed to *C. albicans* and found that the expressions of the Wnt pathway components were increased following treatment with *C. albicans* (**Figure 5E**). Additionally, we investigated the effects of a Wnt inhibitor (ICG-001) on the cells exposed to *C. albicans* (**Figure 5F**). The expressions of the Wnt-related proteins decreased after being treated with the Wnt inhibitor (**Figure 5G**). To determine whether *C. albicans* regulates the proliferation of human IECs through the Wnt signaling pathway, we investigated the proliferation of NCM460 cells exposed to *C. albicans* and treated with a Wnt inhibitor. The proliferation of the NCM460 cells treated with a Wnt inhibitor before their exposure to *C. albicans* was significantly lower than the proliferation of the NCM460 cells exposed to *C. albicans* and not treated with the Wnt inhibitor (**Figure 5G**). Taken together, these results suggest that the IEC response to *C. albicans* is mediated through the Wnt signaling pathway.

Blocking of dectin-1 reduces C. albicans-induced cell proliferation and dectin-1-independent Wnt signaling activation

Previous studies showed that β-glucan recognition is mediated by dectin-1 [20] and that β-glucans are important constituents of the cell wall of *C. albicans* [27]. Thus, we tested whether the observed IEC response to *C. albicans* was dectin-1-dependent. We used an anti-dectin-1 antibody to interfere with dectin-1 function [28]. Specifically, we incubated NCM460 cells with the anti-dectin-1 antibody in RPMI 1640 for 30 min. Thereafter, the cells were exposed to heat-inactivated *C. albicans* (10:1 ratio). Cells not treated with anti-dectin-1 antibody and not exposed to *C. albicans* were used as negative controls (NT). We investigated the cell proliferation by evaluating the expression of Ki-67 using Western blotting (**Figure 5I**) and the cell survival and proliferation using MTT assays (**Figure 5H**). The expression of Ki-67 decreased when the cells were pre-treated with an anti-dectin-1 antibody before *C. albicans* exposure. This treatment was also associated with decreased cell numbers. These data indicate that the IEC response to *C. albicans* is mediated by dectin-1. Because our results suggest that the effects of *C. albicans* on cell pro-

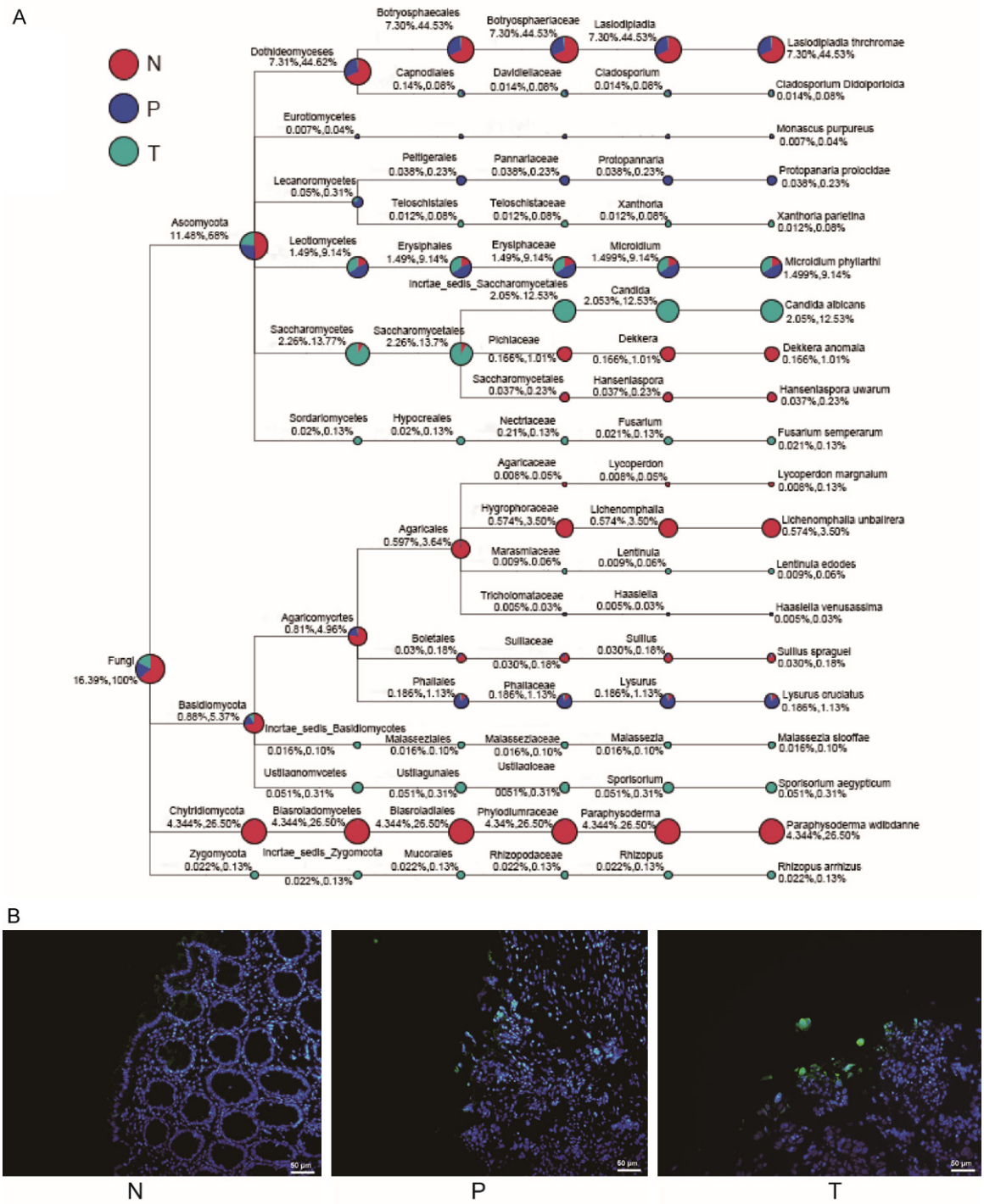

Figure 4. The abundance of *Candida albicans* in the CRC is increased. A. A comparative analysis of the phage taxonomy in the three group samples, N, P, and T. The phages identified from the samples are represented by different colors. B. Colon sections from the N, P, and T patients were stained with an anti-*Candida albicans* antibody (green) and counterstained with DAPI. The magnification is 200 times and the scale bar represents 50 microns.

liferation were mediated by Wnt signaling, we further explored whether the inhibition of dectin-1 affected the Wnt pathway. The pre-incubation of the cells with an anti-dectin-1 antibody was associated with a decreased expression of the Wnt-related proteins in the NCM460 cells exposed to *C. albicans* (**Figure 5I**). Thus, our results suggest that the *C. albicans*-induced IEC proliferation is mediated by the dectin-1 and Wnt signaling.

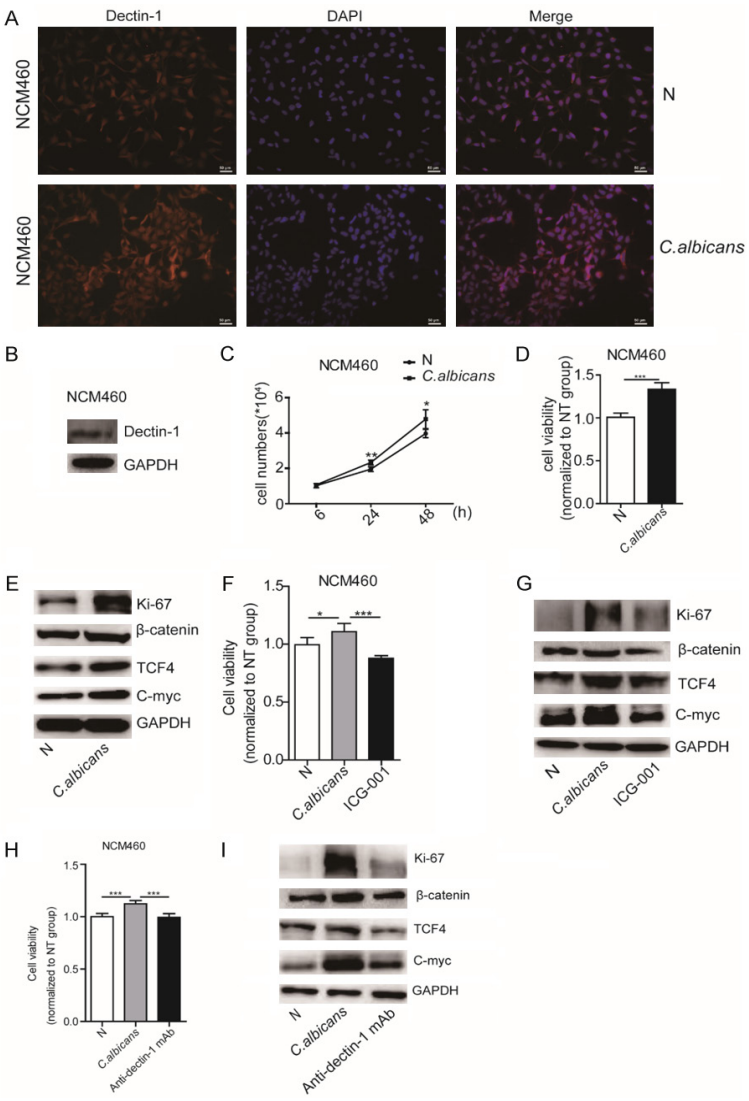

Figure 5. The IECs express Dectin-1, and *C. albicans* induces a proliferation of the NCM460 cells through the Wnt/β-catenin signaling pathway. A. The Dectin-1 expression counterstained with DAPI by NCM460 was measured using immunofluorescence. The magnification is 200 times and the scale bar represents 50 microns. B. Western blot analysis of the cell lysates from primary human NCM460 cells revealed signals referring to Dectin-1 at 23 KDa. C. NCM460 cells were incubated with PBS, or heat-inactivated *C. albicans* (10:1 ratio). The cell proliferation rates were evaluated using cell number counting after treatment at 6, 24, and 48 hours ($^{*}P<0.05$; $^{**}P<0.001$, unpaired Student t-test). D. NCM460 cells were incubated for 24 h in the absence (N) or presence of heat-inactivated *C. albicans* (10:1 ratio). The effect of cell proliferation was assessed using MTT assays. E. Western blots showed that *C. albicans* upregulates the expression of Ki-67 and activates the Wnt pathway activation in NCM460. F. The effect of *C. albicans* and the Wnt signaling inhibition (ICG-001) on cell proliferation was assessed using MTT assays. G. The expression of Ki-67 and the Wnt signaling-related proteins in the groups: N (not exposed ICG-001 or *C. albicans*), *C. albicans* (exposed to *C. albicans*) and ICG-001 (exposed to ICG-001 and *C. albicans*). H. NCM460 cells were incubated for 24 h in the absence (N) or presence of heat-inactivated *C. albicans* (10:1 ratio). The effect of the *C. albicans* and Dectin-1 inhibition on the cell proliferation was assessed using MTT assays. I. The expressions of Ki-67 and the Wnt signaling-related proteins in the groups: N (not exposed ICG-001 or *C. albicans*), *C. albicans* (exposed to *C. albicans*) and Anti-dectin-1 mAb (exposed to Anti-dectin-1 mAb and *C. albicans*).

The Wnt pathway is activated by C. albicans infection in mice colorectal tissues

Finally, we investigated the cell proliferation in the colorectal tissues upon exposure to *C. albicans*. To confirm our hypothesis, we administered DSS or PBS as a negative control for one week to 5- to 6-week-old wild-type mice that had been given fluconazole in their drinking water for two weeks. Upon the colitis induction, the mice were supplemented every other day with four doses (1×10^8 yeast/mouse/dose) of *C. albicans*. Another group was administered PBS as a control. The colorectal tissues from each group were stained with an anti-*C. albicans* antibody (red) and counterstained with DAPI. Our immunofluorescence analysis revealed a significant increase in colonic *C. albicans* in mice in the gavage group (CA) compared to the control group (N) (**Figure 6A**). Simultaneously, we detected fungal mRNA in the terminal colons of the mice using qPCR. The *C. albicans* levels were higher in the CA tissues than in the normal tissues (**Figure 6B**). Next, we examined whether the Wnt signaling pathway is activated in the colorectal tissues exposed to *C. albicans*. Using immunoblotting, we found that the Wnt signaling pathway was activated in the colorectal tissue of the CA group but not in the control group (**Figure 6C**). In summary, the Wnt pathway mediates the intestinal dysregulation of IECs induced by *C. albicans*.

Discussion

The ITS regions of fungi from the human fecal samples were analyzed to evaluate the intestinal fungal composition and

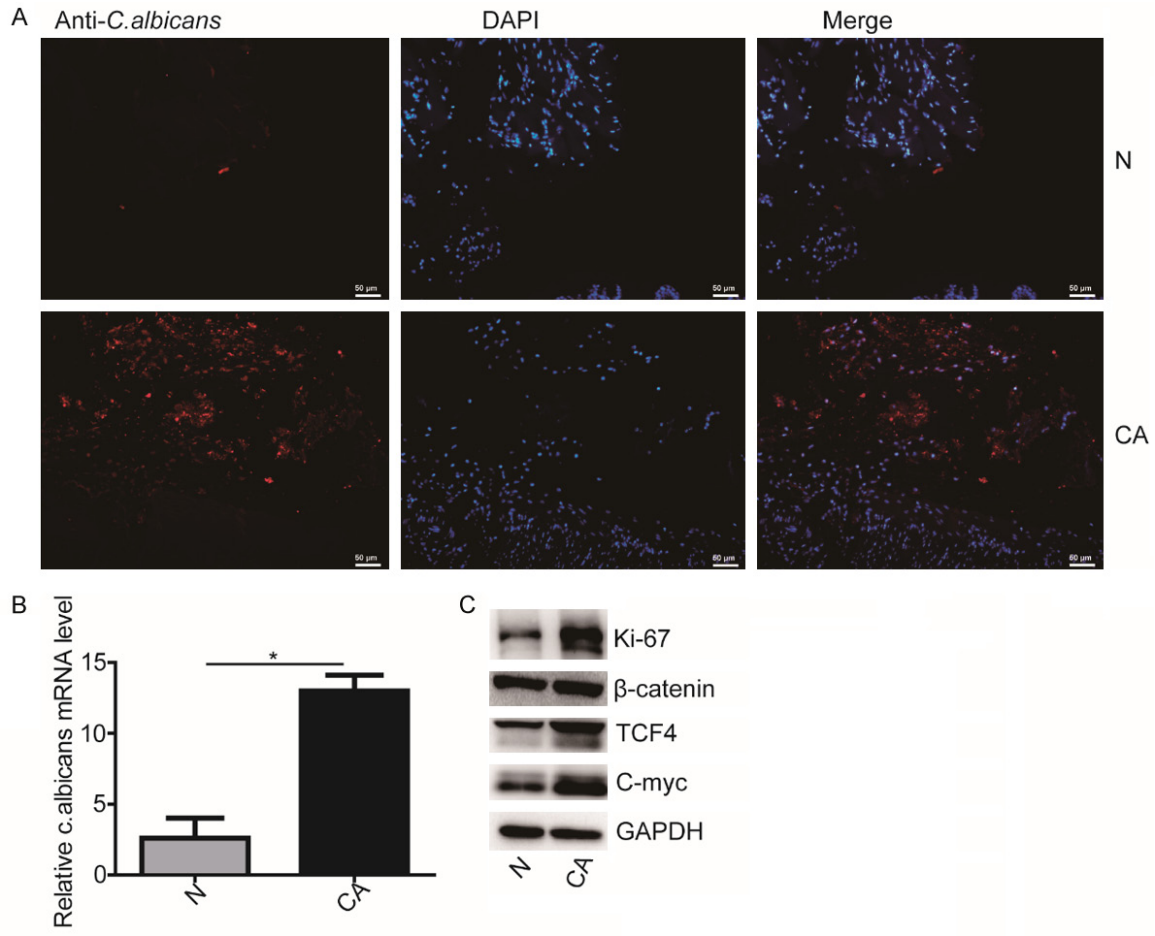

Figure 6. *C. albicans* induces the proliferation of colorectal tissues through the Wnt/β-catenin signaling pathway. A. Colorectal tissues from the WT mice (N) and the WT mice were administered stomah with *C. albicans* (CA) stained with an anti-*Candida albicans* antibody (red) and counterstained with DAPI. The magnification is 200 times and the scale bar represents 50 microns. B. The colorectal tissues from WT mice (N) and WT mice were administrated stomach (CA) with *C. albicans* for four doses, the mRNA expressions of *C. albicans* in the colorectal tissues were measured using qPCR. C. Western blots illustrated that *C. albicans* activated Ki-67 and the Wnt pathway activation in colorectal tissues.

microbiota changes associated with CRC. We demonstrated that the intestinal fungal characteristics in CRC (T) cases differed from those in the control (N) and patients with polyps (P). According to the reported intestinal fungal profile, both the CRC and control subjects' gut microbiota were dominated by Ascomycetes and Basidiomycetes, which is consistent with our findings [29, 30]. According to the intestinal mycobiome profiles, the relative abundance of *Fusobacterium nucleatum* was significantly elevated in the advanced stages of intramucosal carcinoma [31]. Our results revealed the destruction of the intestinal fungal communities in the CRC patients.

We identified changes in the composition of CRC-specific fungi by the enrichment of eight genera, focusing on the *Candida* genus. *C. albicans* is commonly found in clinical settings and is one of the leading causes of hospital-acquired infections [33]. *Candida,* isolated from infected medical devices, accounts for approximately 15% of hospital-acquired sepsis cases [32]. *Candida tropicalis* can induce myeloid-derived suppressor cell differentiation and activate the immuno-suppressive function of these cells [34]. At the species level, our study showed that the *C. albicans* index in the T group was significantly higher than it was in the other two groups. At the genus level, *Lasiodiplodia* was found to be depleted in the CRC patients. However, it was highly expressed in the gut of the patients in the N and P groups. The β-d-glucan derived from *Lasiodiplodia* has a direct anti-tumor effect and can indirectly

reduce tumor proliferation via an immunomodulatory reaction [35]. The elimination of *Lasiodiplodia* in the CRC patients highlights its potential beneficial effects in the intestine, which may be useful for preventing and treating CRC. The greater abundance of these genera and species in the CRC patients indicates their contributions to CRC pathogenesis; however, the mechanisms that govern this contribution require further research. Additionally, our sequencing results showed that among the normal and adenomatous polyp groups, the relative abundance distribution and fungal community diversity were significantly higher in the CRC group. Our statistical analysis of the fungi showed that *C. albicans* was more abundant in the CRC microenvironment.

IECs recognize fungal β-glucan and are subsequently induced to produce cytokines [20]. We hypothesized that IECs can recognize *C. albicans* and evaluated whether *C. albicans* can activate intestinal IECs. Our results show that dectin-1 is expressed in the IEC line NCM460; upon its interaction with dectin-1 in IECs, *C. albicans* promotes IEC proliferation in a dectin-1- and Wnt-dependent manner. Dectin-1 is a cell-surface molecule expressed by primary human IECs and IEC lines (such as HT-29 and SW480) and is involved in the β-glucan-induced responses in human mucosal surfaces [20]. Dectin-1 is also expressed by NCM460 cells. Thus, we hypothesized that dectin-1 participates in the *C. albicans*-induced response in this cell line. We found that *C. albicans* induces the proliferation of NCM460 cells and that this effect is significantly decreased upon pre-treatment of the cells with an anti-dectin-1 antibody, indicating that dectin-1 is necessary for *C. albicans*-induced cell proliferation. However, we cannot rule out the possibility that other receptors cooperate with dectin-1 in this process, as indicated by studies in human and mouse cells of the myeloid lineage [36-38]. In a different model system, it has been shown that dectin-1 can activate the Wnt pathway; indeed, dectin-1 induces the stabilization of β-catenin via Syk and reactive oxygen species, contributing to the expression of Wnt5A in macrophages [37]. Therefore, we investigated whether the Wnt signal, which is involved in multiple cellular processes including cell proliferation, differentiation, and apoptosis, affected our system. We demonstrated that *C. albicans* activates IECs and induces cell proliferation in a Wnt-dependent manner, as Wnt inhibitors abrogated most of the effects of *C. albicans*. Interestingly, pre-treatment with an anti-dectin-1 antibody inhibited the Wnt signaling pathway, further demonstrating that *C. albicans* activates the dectin-1/Wnt axis.

When colitis occurs, the mucosal barrier is destroyed, IECs undergo apoptosis, and crypt epithelial cells are induced to proliferate. Previous studies reported that *C. albicans* is involved in colitis in mice and that β-glucans promotes the release of the inflammatory factors that promote colitis. Here, we found that *C. albicans* promotes the proliferation of IECs through the dectin-1/Wnt pathway. We also prepared a mouse model infected with *C. albicans* in which the Wnt pathway was activated. Currently, we are establishing a mouse model of CRC induced by azoxymethane supplemented with dextran sodium sulfate. We hope to use this model to study the role of *C. albicans* in the development of CRC in more detail. Proliferation is important during the healing response after damage [39]. However, because *C. albicans* can promote colitis and the proliferation of IECs, and because colitis is a risk factor for CRC, *C. albicans* may be associated with CRC. Additional studies are required to evaluate this hypothesis.

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Disclosure of conflict of interest

None.

Address correspondence to: Ke Wu and Guobin Wang, Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1277 Jiefang Avenue, Jianghan District, Wuhan 430022, China. Tel: +86-13545248289; E-mail: wuke201288@126.com (KW); Tel: +86-13907129668; E-mail: wangguobinuh@126.com (GBW)

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