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Molecular Prevalence of Microsporidia Infection in Patients with Lung Cancer

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Abstract. Infections are still among the most important causes of morbidity and mortality in patients with lung cancer, which has the highest rate of cancer-related deaths in the world. Microsporidia, which are opportunistic parasitic fungi, primarily localize to the intestine by ingestion but can disseminate to the respiratory tract or can be acquired by spore inhalation. Cancer patients are at higher risk for microsporidia, a life-threatening infection, than the normal population is. We aimed to characterize the prevalence of microsporidia infection for the first time by evaluating the intestinal and respiratory tracts of patients with lung cancer. In this study, we investigated 98 patients with lung cancer and 103 healthy individuals for microsporidia infection and evaluated the clinical findings of patients who were found to be positive. Sputum and stool samples were tested by microscopic examination, in addition to pan-microsporidia and genus-specific polymerase chain reactions. Nine patients with lung cancer had positive results for microsporidia (9.2%), which was significantly higher than the rate in healthy individuals ($P = 0.008$), and most of them had clinical findings. Among these positive patients, polymerase chain reaction revealed microsporidia in the sputum samples of seven patients, the stool sample of one patient, and both the sputum and stool samples of one patient. *Encephalitozoon cuniculi* was identified as the predominant pathogen in 87.5% (7/8) of positive sputum samples. Microsporidia infection was significantly associated with advanced stages of cancer. However, in the control group, *Encephalitozoon intestinalis* was detected in the stool sample of an individual without clinical symptoms. Microsporidia, especially *E. cuniculi,* should be considered as a cause of respiratory tract infection as well as intestinal infection in cancer patients and should be screened in respiratory samples of these patients when they have pulmonary symptoms.

INTRODUCTION

Lung cancer is the third-most common cancer in the world and the leading cause of cancer-related deaths.[1] Patients with lung cancer are more prone to infections than the normal population because of the cancer itself (i.e., disruption of organ integrity); treatments such as surgical procedures, chemotherapy, and radiotherapy; and alterations in the host–humoral/cellular immune system.[2,3] Infections and their complications are still among the most important causes of morbidity and mortality in cancer patients, despite current developments in technologies in the field of cancer.[2,4–6] In addition, the diagnosis of infections can be complicated by the scarcity of symptoms or by atypical clinical findings. Therefore, detection of pathogens, including opportunistic or neglected microorganisms, is important in terms of reducing mortality.

Microsporidia are a diverse group of obligate eukaryotic intracellular pathogens that infect a wide range of hosts, including vertebrates and invertebrates.[7] Microsporidia are a phylum of fungi formerly classified as a parasite. They are considered opportunistic pathogens in immunocompromised individuals, causing a wide range of symptoms.[7–10] Species of the highest clinical importance for humans are *Encephalitozoon* (*Encephalitozoon cuniculi, Encephalitozoon intestinalis*, and *Encephalitozoon hellem*) and *Enterocytozoon bieneusi*. Although *E. bieneusi* and *E. intestinalis* are known to cause the most common intestinal infections, *E. cuniculi* and *E. hellem* are associated with central nervous system, ocular, respiratory, and urinary tract infections.[7–9,11]

Although the mechanism of transmission of microsporidia, which are abundant in the environment, is unclear, their spores can reach various organs or tissues by inhalation, the fecal–oral route, regurgitation, or hematogenous spread.[12,13] Inhaled spores, especially of *E. cuniculi, E. hellem*, and *E. bieneusi,* can invade the sinus or lung epithelium, leading to respiratory tract infections.[14] However, certain studies have suggested that pulmonary microsporidiosis is usually caused by dissemination of gastrointestinal or urinary tract infections rather than an external transmission.[15,16] Nevertheless, it was recently stated that *E. cuniculi* can cause a latent infection with no clinical signs in immunocompetent hosts that may be reactivated after immunosuppression.[17]

Microsporidia likely cause a self-limiting disease in immunocompetent individuals, whereas they may present as a life-threatening infection in immunocompromised patients.[18] Based on the literature, HIV-infected patients, children, and transplant recipients have been identified as specific population groups at risk for microsporidia.[10,16,19–24] However, studies reflecting the prevalence of microsporidia infection in humans are still scarce, and additional studies involving specific vulnerable groups are required. Likewise, studies presenting the prevalence of infection in cancer patients are still limited, although some case reports have shown microsporidia infections in these patients.[19,25]

Diagnosis of microsporidia and accurate characterization of their species are important in determining appropriate antibiotic therapy and predicting the probability of developing a localized or disseminated disease.[7,8,18] Therefore, use of sensitive and specific molecular methods such as polymerase chain reaction (PCR) allows both the diagnosis and species

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identification of microsporidia, and will provide the prevalence of infection and accurate diagnosis.[8,9,18,19]

The aims of this study were to determine the prevalence of microsporidia infection and the risk of respiratory microsporidiosis in patients with lung cancer, using both their sputum and stool samples, and to identify the species of microsporidia in infected cancer patients. To our knowledge, it is the first report describing the prevalence of respiratory microsporidiosis among patients with lung cancer.

MATERIALS AND METHODS

Participants. This study included 98 patients with lung cancer who were hospitalized between 2020 and 2022 in the Department of Pulmonology, Dr. Suat Seren Chest Diseases and Surgery Education and Research Hospital. Lung cancer was diagnosed pathologically by either a histological or a cytological approach in all patients. Patients with another cancer or neoplasm were excluded from the study. Some of the patients included in the study were under chemotherapy and/or radiotherapy, and some were newly diagnosed patients. Details of patient characteristics are given in Table 1. As a control group, we enrolled 106 individuals without cancer and with no family history of cancer, who were matched with cancer patients in terms of age, gender, and chronic disease. The study protocol was approved by the Ethics Committee of Suat Seren Hospital (2020-KAEK-139). In accordance with the Declaration of Helsinki, all individuals were verbally informed, and written consent forms were obtained. Demographic and clinical information on the patients was recorded. Stool samples, which were examined as part of standard care for these subjects, and sputum samples as part of the research study were collected.

Microscopic examination. Sputum samples were treated with Sputolysin as a mucolytic agent at a ratio of 3:1 (volume/weight) (Behring Diagnostics, San Jose, CA), incubated for 10 minutes at room temperature, and centrifuged at 5,000 rpm for 10 minutes. The resulting pellet was resuspended with phosphate buffered saline, and smear slides for staining were prepared. The stool samples were enriched with the formalin ether concentration method, and slides were prepared. All slide samples were fixed with methanol for 30 seconds and stained with the modified trichrome method.[26] The remaining portions of all samples were stored at −80 °C until DNA extraction for molecular analysis.

Real-time PCR. Microsporidia were investigated in stool and sputum samples by real-time quantitative polymerase chain reaction (qPCR). All samples were subjected to 95 °C for 5 minutes after −80 °C (repeated twice) and added to microcentrifuge tubes containing 0.5-mm glass beads. After samples were homogenized with Magna Lyser at 5,000 × rpm for 1 minute, DNA was isolated using sputum and stool isolation kits (Norgen Biotek Corp., Thorold, ON, Canada), according to the manufacturer's instructions. The concentration and purity of DNA were evaluated by spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Pan-microsporidian–specific primers were used for PCR, targeting the conserved region of the small subunit-ribosomal ribonucleic acid (rRNA) gene of *E. cuniculi*, *E. hellem*, and *E. intestinalis*. Approximately 250- to 280-bp fragments were amplified.[27] The PCR was performed using 5 μL DNA template in a final volume of 25 μL, including 0.4 μM of primers and 2× SYBR Green I Master (Roche). To avoid carryover contamination, 0.6 U Uracil-DNA Glycosylase (Roche) was also added in the PCR test. The PCR protocol was as follows: 10-minute preincubation at 95 °C and 10-second denaturation step at 95 °C, 10 seconds at 60 °C, and 20 seconds at 72 °C for 40 cycles on the LightCycler® 480 device (Roche).

A standard curve was generated with seven 10-fold serial dilutions of a plasmid-positive control, containing a small subunit-rRNA gene fragment (Sigma-Aldrich, St. Louis, MO). Each sample was run with and without the presence of plasmid control for testing PCR inhibition. To evaluate PCR specificity, *E. intestinalis*– and *E. cuniculi*–positive patient samples, negative controls, and positive patient samples with other parasites, including *Blastocystis hominis, Aspergillus* species, *Pneumocystis jirovecii*, *Cryptosporidium* spp., *Toxoplasma gondii,* and *Giardia intestinalis* were used. Positive samples were verified by sequencing pan-microsporidia PCR products commercially (MedSanTek Inc, Istanbul, Turkey).

For species identification in positive samples, PCR was carried out using species-specific primer sets of *Encephalitozoon* spp. (*E. cuniculi*, *E. hellem*, and *E. intestinalis*)[24,28] with the reaction protocol mentioned above. Primers are shown in Table 2. The qPCR protocol was as follows: 10-minute preincubation at 95 °C and 10-second denaturation step at 94 °C, 15 seconds at 55 °C, and 1 minute at 72 °C for 40 cycles. The products of amplification were visualized on a 1.5% agarose gel. All reactions were studied in triplicate.

TABLE 1
Characteristics of patients with lung cancer and healthy individuals

Examined parameter	LC patients (%) (*n* = 98)	Controls (%) (*n* = 103)
Age in years (range)	62.4 (42–81)	60.5 (38–82)
Female	38 (38.7)	42 (40.8)
Male	60 (61.2)	61 (59.2)
Smoking status		
Never	26 (26.5)	18 (17.5)
Previous	52 (53.1)	56 (54.4)
Current	20 (20.4)	29 (28.1)
Comorbid disease		
Cardiovascular disease	4 (4.1)	2 (1.9)
Hypertension	58 (59.2)	67 (65)
Diabetes mellitus	10 (10.2)	8 (7.8)
Tuberculosis	2 (2.0)	–
Staging according to TNM		
I	10 (10.2)	–
II	31 (31.6)	–
III	21 (21.4)	–
IV	36 (36.7)	–
Pathological classification		
Adenocarcinoma	72 (73.5)	–
Squamous carcinoma	26 (26.5)	–
Tumor location		
Left side	37 (37.8)	–
Right side	62 (62.2)	–
Anatomical type		
Peripheral carcinoma	81 (82.7)	–
Central carcinoma	17 (17.3)	–
Treatment		
Surgical resection	51 (52)	–
Chemotherapy	75 (76.7)	–
Radiotherapy	46 (46.9)	–
Laboratory test*		
Hemoglobin (g/L)	138.2 + 14.3	–
White blood cell count (×10^9/L)	6.4 + 1.6	–
Albumin (g/L)	41.6 + 4.3	–

LC = lung cancer; TNM = tumor, node, and metastasis classification.
* Values stated are standard deviations.

TABLE 2
Primers used in the diagnosis of microsporidia

Parasites	Primer (5′–3′)	Target gene	PCR product	Accession number	Reference
Microsporidia	F: CACCAGGTTGATTCTGCCTGA R: CCTCTCCGGAACCAAACCCTG	SSU-rRNA	250–280 bp	–	Joseph et al.[27]
Encephalitozoon hellem	F: TGAGAAGTAAGATGTTTAGCA R: GTAAAAACACTCTCACACTCA	SSU-rRNA	547 bp	L19070	Visvesvara et al.[24]
Encephalitozoon cuniculi	F: ATGAGAAGTGATGTGTGTGCG R: TGCCATGCACTCACAGGCATC	SSU-rRNA	549 bp	L17072	Visvesvara et al.[24]
Encephalitozoon intestinalis	F: TTTCGAGTGTAAAGGAGTCGA R: CCGTCCTCGTTCTCCTGC	SSU-rRNA929	520 bp	U09929	Da Silva et al.[28]

F = forward; PCR = polymerase chain reaction; R = reverse; SSU-rRNA = small sub-unit ribosomal ribonucleic acid.

Statistical analyses. Statistical analyses were performed using the SPSS software package for Windows (version 18.0, SPSS, Chicago, IL). The χ^2 or Fisher's exact test was used for categorical endpoints. Continuous endpoints were evaluated using Student's *t*-test or Mann–Whitney *U* test. Values are presented as number (percentage), mean ± SD, and median and interquartile range. The threshold for statistical significance was *P* value < 0.05. The sample size was calculated using G*Power V.3.1.9.4 based on the following criteria: medium effect size (w = 0.3), power of 0.80, and statistical significance of 95% (α = 0.05). The minimum number of subjects was considered to be 88.

RESULTS

The patient group had a median age of 62.4 years (range, 42–81 years) and consisted of 38 women and 60 men. The control group included 42 women and 61 men with a median age of 60.5 years (range, 38–82 years). We found no significant differences in age and gender between the lung cancer (*N* = 98) and control (*N* = 103) groups (Table 3). Although microsporidia were not detected in microscopic examination in both groups, the presence of microsporidia was detected by PCR in nine patients with lung cancer (9.2%) and one patient in the control group (0.97%). Among positive samples, microsporidia were found in the sputum samples of eight patients (8.4%) and in the stool sample of two patients (2%). In one patient, both sputum and stool samples were positive for microsporidia. In the control group, microsporidia were detected in the stool of only one individual, whereas it was negative in all sputum samples (Table 3). After amplicons obtained with pan-microsporidian primers were sequenced, BLAST analysis revealed 99–100% homology with *Encephalitozoon* sequences deposited in GenBank, with L17072, L39107, and X98470 of *E. cuniculi*, AF272836 of *E. hellem*, and OQ077202 of *E. intestinalis* (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences obtained in this study were submitted to GenBank with the number OQ248514, OQ248515, and OQ248516.

In patients with lung cancer, we evaluated associations between microsporidia positivity and clinical situations, including cancer stage, histological grade, and tumor location. All patients who were positive for microsporidia were in stages III and IV of lung cancer, according to the eighth edition of tumor, node, and metastasis (TNM) classification (*P* = 0.008). We found no significant associations between microsporidia positivity and other factors. However, most microsporidia were detected in samples obtained from male patients (eight of nine positive samples) (Table 4).

After specific PCR for species of microsporidia, we detected *E. cuniculi* in sputum samples of seven patients and *E. hellem* in the sputum of one patient. We evaluated most of them as having respiratory microsporidiosis due to clinical findings, such as fever, dyspnea, hemoptysis, cough, and wheezing.

TABLE 3
Comparison of the characteristics and frequencies of microsporidia in LC and control groups

Parameter	LC group (*n* = 98)	Control group (*n* = 103)	*P* value
Age in years (range)	62.4 (42–81)	60.5 (38–82)	0.281*
Female	38 (38.7%)	42 (40.8%)	0.775†
Male	60 (61.2%)	61 (59.2%)	
Microsporidia			
Positive	9 (9.2%)	1 (0.97%)	0.008†
Sputum	8	–	
Stool	2	1	
Negative	89 (90.8%)	102 (99.03%)	

LC = lung cancer.
* Mann–Whitney *U* test.
† Fisher's exact test; values indicate number of patients (%).

TABLE 4
Associations between microsporidia and various clinical parameters in patients with lung cancer (*N* = 98)

Parameters	Microsporidia, *n* (%) Negative	Positive	*P* value
Age in years (range)	62.5 (42–81)	61.5 (44–78)	0.265*
Female	37 (97.4)	1 (2.6)	0.074†
Male	52 (86.7)	8 (13.3)	
Smoking			
Previous	46 (88.4)	6 (11.5)	0.69†
Current	17 (85)	3 (15)	
Comorbid disease			
Hypertension	52 (90)	6 (10)	0.50*
Diabetes mellitus	7 (70)	3 (30)	
Tuberculosis	1 (50)	1 (50)	
Staging according to TNM			
I + II	41 (100)	0	0.008†
III + IV	48 (84.2)	9 (15.8)	
Pathological classification			
Adenocarcinoma	68 (94.4)	4 (5.6)	0.117†
Squamous carcinoma	22 (84.6)	4 (15.4)	
Tumor location			
Left side	34 (91.9)	3 (8.1)	0.626‡
Right side	58 (93.5)	5 (6.5)	
Anatomical type			
Peripheral carcinoma	73 (90.1)	8 (9.9)	0.513‡
Central carcinoma	16 (94.1)	1 (5.9)	
Treatment			
Surgical resection	46 (90.2)	5 (9.8)	0.1*
Chemotherapy	66 (88)	9 (12)	
Radiotherapy	39 (84.8)	7 (15.2)	

TNM = tumor, node, and metastasis classification.
* Mann-Whitney *U* test.
† χ^2 test.
‡ Fisher's exact test; values indicate number of patients (%).

Two of these microsporidia-positive patients were considered to have colonization because they had no clinical findings except weight loss and cycle threshold (Ct) values of these samples were slightly higher than others. In addition, the stool samples of two patients were positive for either *E. intestinalis* or *E. cuniculi.* Both had significant gastrointestinal findings, including fever, watery diarrhea, vomiting, and weight loss, indicating intestinal microsporidiosis (Table 5). In the control group, *E. intestinalis* was detected in the stool of an individual who had nausea and abdominal spasm.

DISCUSSION

Infection and its complications are still among the most important causes of morbidity and mortality in patients with lung cancer, despite therapeutic advances in recent years.[2,5,6] Because of impairments in immunity caused by either the neoplastic disease or oncological treatment, cancer patients are at a higher risk of infections and manifest more severe clinical symptoms than the normal population.[2–4]

Microsporidia are emerging pathogens and cause a wide range of symptoms in humans, with more severe symptoms observed in immunocompromised individuals.[10] The vast majority of human microsporidiosis cases have been reported over the past two decades, mainly in patients with HIV/AIDS; however, the infection has been described as relatively rare in immunocompetent individuals.[18,22,24] The present study showed microsporidia infections occurred significantly more often (9.2%) in patients with lung cancer, at the time of diagnosis, than in the control group. Of note, although intestinal microsporidia infections were higher (2.04%) than in the control group (0.97%), the microsporidia positivity rate was lower than previously reported.[19,25,29,30] The presence of microsporidia is based primarily on examination of intestinal samples. Studies in cancer patients have shown that the prevalence of intestinal microsporidia infections ranged from 5.6% to 69.9% and was higher than in the normal healthy population.[19,25,29,30] Wide variations in the prevalence of microsporidia may be due to differences in patient groups, the regions where the studies were conducted, or diagnostic methods.

Moreover, this study revealed that microsporidia were positive in 8.4% of respiratory samples from patients with lung cancer, whereas the samples of individuals without lung cancer were negative. To date, microsporidia infections with respiratory involvement have been reported very rarely. They have usually been shown in immunocompromised patients such as transplant recipients and patients with AIDS and cancer. Most of these patients died as a result of respiratory system complications and cardiorespiratory failure, suggesting that microsporidia infection of the respiratory tract can be a life-threatening disease.[10,20,22,23,31] Moreover, the only two data concerning the prevalence of microsporidia in respiratory samples showed that 14.2% of immunosuppressed patients[32] and 8.3% of renal transplant recipients[20] were positive for the pathogens. In this study, among patients with lung cancer, microsporidia infection was significantly associated with lung cancer stage and male gender, independent of age. This suggests that tissue disruption caused by tumor mass, types of cancer therapy, and alterations in the immune system may influence the dissemination of infection or increased susceptibility to respiratory infection.

TABLE 5

Characteristics of patients with positive microsporidia PCR results in sputum/stool samples

Case	Sample	Age in years	Gender	Smoking	Stage	Histology	CM	CT	RT	Signs	Radiological findings	Colonization or infection	Species of microsporidia
1	Sputum	56	Male	Former	IVB	AC	No	Yes	No	Dyspnea, sputum, cough with brown secretion	Pulmonary infiltrates	Infection	*Encephalitozoon cuniculi*
2		78	Male	Former	IVC	AC	DM, HT	Yes	Yes	Weight loss	Not determined	Colonization	*E. cuniculi*
3		59	Male	Current	IIIB	AC	HT	Yes	Yes	Vomiting, weight loss	Pulmonary infiltrates	Infection	*E. cuniculi*
4		61	Male	Former	IIIA	AC	HT	Yes	Yes	Dyspnea, fever, fatigue, cough, wheezing, chest pain	Diffuse pulmonary infiltrates	Infection	*Encephalitozoon hellem*
5		44	Male	Former	IIIA	SC	No	Yes	Yes	Fever, hemoptysis, dyspnea, vomiting, weight loss	Diffuse pulmonary infiltrates	Infection	*E. cuniculi*
6		58	Male	Current	IIIA	SC	HT	Yes	Yes	Cough with brown secretion, fatigue	Pulmonary infiltrates	Infection	*E .cuniculi*
7	Stool	69	Female	Former	IVA	SC	DM	Yes	No	Weight loss	Not determined	Colonization	*E. cuniculi*
8	Sputum	67	Male	Former	IVA	AC	HT, DM, TBC	Yes	Yes	Effort dyspnea, fever, cough with brown secretion, heezing, diarrhea, vomiting	Pulmonary infiltrates, pleural effusion	Infection	*E. cuniculi*
9	Stool	62	Male	Current	IVB	SC	HT	Yes	No	Fever, diarrhea, vomiting, weight loss	Not determined	Infection	*Encephalitozoon intestinalis*

AC = adenocarcinoma; CM = comorbidity; CT = chemotherapy; DM = diabetes mellitus; HT = hypertension; PCR = polymerase chain reaction; RT = radiotherapy; SC = squamous carcinoma; TBC = tuberculosis.

It is still unclear whether the pathogens are acquired by inhalation or are spread from another body site of infection. Therefore, both respiratory tract samples and stool samples were tested in patients in this study. The fact that one patient was positive for microsporidia in both stool and sputum samples suggests a possibility of dissemination, but it does not fully rule out the other. Although there are a few case reports, this study was the first in the world to evaluate the prevalence of microsporidia infections in patients with lung cancer, using both their stool and respiratory samples.

Based on the literature, animal models infected orally with *E. cuniculi* revealed that this pathogen persists in the kidney and lungs and, after the acute stage, can cause chronic infection in these organs.[12] In addition, *E. bieneusi* was detected in urine and bronchoalveolar lavage samples of one hematopoietic stem cell transplant recipient, although the stool sample of this patient was negative.[33] The absence of the pathogen in the patient's stool may indicate that the infection, acquired by inhalation and localized in the lung, spread hematogenously to the urine.[11,33] Considering both previous reports and the results of this study, the presence of *E. cuniculi* in both the intestine and respiratory tract of cancer patients suggests that a reactivation of latent infection acquired prior to immunosuppression and hematological spread cannot be ruled out.

In addition, this study revealed the presence of *E. cuniculi* in the majority of positive samples (8/10), especially in respiratory tract samples. Similarly, Kicia et al.[20] reported that seven of eight positive microsporidia respiratory tract samples of renal transplant recipients were *E. cuniculi*. Indeed, it has been stated that *Encephalitozoon* spp. are an important agent in most cases of respiratory microsporidiosis in immunosuppressed patients.[10,20,22,23] These findings further support that *E. cuniculi* may tend to infect the respiratory tract by inhalation, or to disseminate between organs.

Moreover, the diagnosis of respiratory microsporidiosis can be challenging, both clinically and diagnostically. Although respiratory symptoms are frequently observed, they are not specific for microsporidia. This pathogen, which is very common in the environment, is often neglected in the clinic and is not considered in the differential diagnosis.[9,13,18] In addition, traditional methods are generally insufficient in the diagnosis of microsporidia and species identification; therefore, the use of molecular techniques is necessary.[11,26,33] Polymerase chain reaction is more sensitive than microscopy in diagnosing microsporidia, as seen in our study, as all patients positive for microsporidia were negative by microscopy. The high Ct values obtained from PCR also suggest that the pathogen burden may be below the microscopy detection limit. Although significant progress has been made in developing molecular methods for the diagnosis of microsporidia in recent years, it is still difficult to diagnose microsporidia, and the tests need to be standardized.[26,34]

So far, microsporidia have been described in various biological specimens, primarily stool samples, nasal secretions, sputum, tracheobronchial aspirate, bronchoalveolar lavage, and lung biopsy specimens.[10,16,21,30,32,33] This study's findings show that sputum samples can be used in the diagnosis of microsporidia in cancer patients with pulmonary symptoms, especially when bronchoscopy is unavailable. Particularly in vulnerable patient groups and in the case of suspicious clinical findings, investigating the presence of microsporidia in other body samples as well as the stool by molecular techniques and considering microsporidia in the differential diagnosis will contribute to the treatment of these patients.

In conclusion, the high prevalence of microsporidia that we observed indicates that microsporidia infection is a significant risk for cancer patients. More importantly, respiratory microsporidiosis, symptoms of which often resemble those of other infective agents, should also be considered in cancer patients. Among microsporidia, *E. cuniculi* may be more localized in the airways or may be acquired by inhalation, resulting in respiratory microsporidiosis in immunocompromised patients. Microsporidia, which are life-threatening pathogens, should not be ignored, as they are significantly associated with cancer stage and male gender among cancer patients.

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