The First Evidence of Cryptosporidium meleagridis Infection in a Colon Adenocarcinoma From an Immunocompetent Patient

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Objectives: The potential linkage between Cryptosporidium spp. infection and colorectal human cancer was suggested by limited reports showing higher prevalence of C. parvum and C. hominis in patients with colon cancer. Here we conducted research concerning presence of Cryptosporidium spp. in malignant tissue collected from patients with colorectal cancer.

Methods: Cancerous colon tissue samples collected from 145 non-HIV infected patients with colorectal cancer were screened for Cryptosporidium spp. by immunofluorescence antibody test and genus-specific nested polymerase chain reaction followed by sequencing.

Results: Screened pathogen was found in cancerous tissue originating from immunocompetent man with colon adenocarcinoma. Genotyping revealed presence of Cryptosporidium meleagridis. The presence of Cryptosporidium life cycle stages (oocysts and endogenous stages) in colon carcinoma tissue was confirmed by genus-specific FITC-labeling.

Conclusions: Herein, we report on a C. meleagridis infection of a colon adenocarcinoma in an immunocompetent patient. This is the first report of C. meleagridis infection in the human colon and first evidence of active development of this species in cancer tissue.

Keywords: Cryptosporidium meleagridis, colon cancer, adenocarcinoma, colon infection, PCR, immunofluorescence labeling

INTRODUCTION

Cryptosporidiosis, a diarrheal disease caused by species of protist parasites in the genus Cryptosporidium, can be severe and life-threatening in those with a compromised or underdeveloped immune system. More than 90% of human cases are caused by C. hominis, a species that is generally restricted to humans, and C. parvum, a species that infects a broad range of mammals. Cryptosporidium meleagridis, a species that primarily infects birds, but which also infects...
various mammal species, is the third most common cause of cryptosporidiosis in humans (Xiao, 2010). Estimated prevalence of human cryptosporidiosis in Poland (1.4–2.3%) has been available from limited research studies and based on the data from the National Institute of Public Health, National Institute of Hygiene in Poland and 24 findings of Cryptosporidium spp. have been reported from 2007 to 2016 (Wolska-Kusnierz et al., 2007; Bajer et al., 2008; Wesolowska et al., 2016; Bednarska et al., 2018). The majority of infection cases was caused by \textit{C. parvum} and \textit{C. hominis}. To date, \textit{C. felis} infection has been detected in a child after liver transplantation (Bednarska et al., 2018) and \textit{C. meleagridis} infection has only been confirmed in three immunodeficient patients; two children, with CD40L primary deficiency (Bajer et al., 2008) and with X linked hyper-IgM syndrome type 1 (XHIM syndrome) (Wolska-Kusnierz et al., 2007), and in a woman with AIDS suffering from persistent diarrhea (Wesolowska et al., 2016). Human infection by \textit{C. meleagridis} is more frequent in some populations in Thailand or Peru. In the rest of the world, such cases have been linked to travels to endemic countries or to contact with poultry (Leoni et al., 2006; Cama et al., 2008; Elwin et al., 2012; Silverlas et al., 2012).

Worldwide, colorectal cancer is the second most frequently diagnosed cancer in females and the third in males. Major risk factors for colorectal cancer include age, personal or family history of chronic inflammatory bowel disease, gene mutations, high consumption of red or processed meat, smoking, physical inactivity, obesity and moderate to heavy alcohol consumption (Subramaniam et al., 2016). Other risk factors include bacterial, viral and parasitic infections (van Tong et al., 2017).

Epidemiological studies have shown an association between colorectal cancer and \textit{Cryptosporidium} spp. infection (Osman et al., 2018; Sułczyć-Bielska et al., 2018). Furthermore, a limited number of experimental studies have shown that \textit{C. parvum} induces neoplastic changes in immunocompromised animals and in cells cultured in vitro, consistent with a role in carcinogenesis (Benamrouz et al., 2012a,b).

The present study is aimed to determine the presence of \textit{Cryptosporidium} spp. infection in tissue samples from colorectal tumors. We describe an active \textit{C. meleagridis} infection in samples from malignant tissue of non-HIV infected immunocompetent male patient with colon adenocarcinoma. To our knowledge, this is the first report of the case of \textit{C. meleagridis} infection in the colon of a human.

**PATIENTS AND METHODS**

Samples of colic neoplasia obtained during colectomies of 145 patients, who were being treated for colorectal cancer at the First Department of Surgical Oncology, Lower Silesian Oncology Center in Wroclaw (Poland) between 2009 and 2010 were screened for \textit{Cryptosporidium} infection between 2017 and 2018. The inclusion criteria for of all of the patients in this study were a) primary sporadic CRC (no history of hereditary/familial CRC), (b) HIV negativity, (c) not receiving an immunosuppressive treatment, (d) no chemotherapy and/or radiotherapy before surgical resection.

Tissues of colorectal cancer from all patients were screened for the presence of specific DNA of \textit{Cryptosporidium} spp. using molecular methods and presence of \textit{Cryptosporidium} developmental stages using the immunofluorescence antibody test (IFA). Samples were collected intraoperatively and aseptically. All tissue samples were stored in RNAAlater™ Stabilization Solution (Thermo Fisher Scientific, Carlsbad, CA, United States) and immediately frozen at −70°C. A total of 200 mg of tissue was homogenized by bead disruption for 60 s at 5.5 m/s with 0.5 mm glass beads using a Precellys 24 Instrument (Bertin Technologies, France). Genomic DNA was subsequently isolated using the Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality was verified by NanoDrop (Thermo Fisher Scientific, Carlsbad, CA, United States) measurements and β-globin gene amplification (Pan et al., 2001). To detect \textit{Cryptosporidium} specific DNA, a nested protocol was used to amplify a partial region of the small ribosomal subunit rRNA (SSU; ∼830 bp) gene (Xiao et al., 1999; Jiang et al., 2003). Only samples that were positive for SSU were screened for the 60 kDa glycoprotein (gp60; ∼900 bp) gene (Glaberman et al., 2001; Alves et al., 2003). The PCR conditions were as previously described (Xiao et al., 1999; Glaberman et al., 2001). Negative (molecular grade water) and positive (DNA of \textit{C. proliferans}) controls were included with each PCR amplification. Secondary products were purified (QiAquick® Gel Extraction Kit, Qiagen, Hilden, Germany) and sequencing was carried out in both directions using an ABI 3130 sequencer (Applied Biosystems, Foster City, CA, United States). Amplification and sequencing of each locus were repeated three times. The nucleotide sequences in this study were manually edited using the program ChromasPro 2.1.4 (Technelysium, Pty, Ltd., South Brisbane, Australia) and aligned with previously published sequences. Sequences of SSU and gp60 derived in this study have been deposited in GenBank under accession numbers MK311181 and MK311182.

For IFA analysis, tissue samples were thawed at laboratory temperature, mechanically homogenized using a sterile mortar and pestle, and smeared on sterile slides (ten for each labeling). The presence of \textit{Cryptosporidium} oocysts was examined using differential interference contrast (DIC) and fluorescence microscopy following labeling with a fluorescein-tagged mouse monoclonal antibody that is specific for the \textit{Cryptosporidium} oocyst wall (\textit{Cryptosporidium} IF Test, Crypto cel, Cellabs Pty Ltd., Brookvale, Australia). Endogenous life stages were examined under DIC and fluorescence microscopy following labeling with fluorescein-tagged rat anti-\textit{Cryptosporidium parvum} sporozoite polyclonal antibody, which is specific for sporozoites, merozoites, and all other intracellular reproductive stages (A600FLR-20X Sporo-Glo™, Waterborne, INC. New Orleans, LA, United States).

This study was approved by the Human Research Ethics Committee of Wroclaw Medical University (agreement number KB-328/2009). Written informed consent was obtained from every participant prior to examination.
RESULTS

Among all patients (n = 145) the mean age was 65 ± 10.2 years, and ranged between 35–88 years. Overall, the male-to-female ratio was 77 (53%) to 68 (47%). The study group included 34 patients with proximal (right part of colon inclusive of transverse colon) and 111 patients with distal (left part of colon) tumor location. Cryptosporidium meleagridis DNA was confirmed in one patient and no other species of Cryptosporidium were found in the tumor tissues of the rest of study group.

Cryptosporidium-positive patient was a 71-year-old HIV-negative male who presented with a 1-year history of bloody stools, increased difficulty with defecation, abdominal pain, flatulence, increased fatigue and unintended weight loss. He had a 20-year history of regular smoking above 2 packs of cigarettes per week, seldomly drank alcohol (median 50 g of pure alcohol per week), and had no previous medical or surgical history of note. Additionally, he had no risk factors for, or blood result anomalies suggestive of, underlying immunodeficiency. A colonoscopy identified a lesion (pre-cancerous polyp) located 20 cm from ileocecal valve. Histopathological examination of biopsy tissue showed the presence of a stage IIA adenocarcinoma, according to the TNM Classification of Malignant Tumors (Tumor, Nodes, Metastasis, TNM) of staging as maintained by the American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) (Hari et al., 2013). The patient was admitted to the hospital to undergo a planned right hemicolectomy of an ascending adenocarcinoma.

The presence of Cryptosporidium life cycle stages (oocysts and endogenous stages) in colon carcinoma tissue was confirmed by genus-specific FITC-labeling (Figure 1). Phylogenetic analyses of SSU rRNA sequences revealed the presence of C. meleagridis that was identical to isolates previously reported in humans and birds (GenBank acc. Nos. KY166839 and KY352486). Subtyping of C. meleagridis isolates at the gp60 locus showed the presence of subtype family IIig previously reported in a human from Nepal and Sweden (GenBank acc. Nos. KU852730 and KJ210619) and birds from Algeria (GenBank acc. Nos. KY352480, KY352481, JX878610).

DISCUSSION

In this study, microscopic methods have demonstrated the presence of C. meleagridis endogenous stages (merozoites) in adenocarcinoma tissue in the colon. To our knowledge this is the first report of C. meleagridis in the human colon, and the identification of merozoites indicates an active infection. In the only other study to date in humans, C. meleagridis infection was localized to the ileocecal valve, a sphincter muscle that separates the small and large intestine, in a person who was immunocompromised following a hematopoietic stem cell transplantation (Kagawa et al., 2018). Minor C. meleagridis infection has been reported in the colon of birds (chickens and turkeys) and mice; however, the infection intensity was low relative to that in the small intestine, which was the major site of infection (Pavlásek, 1994; Akiyoshi et al., 2003). Several recent studies have shown an increased prevalence of Cryptosporidium in patients with intestinal cancers. For example, in Poland, Cryptosporidium coproantigen was significantly more prevalent in patients with colorectal cancer than in a control group without malignant changes (Sulzyck-Bielicka et al., 2018). Similarly, Osman et al. (2018) showed that DNA and oocysts of C. parvum and C. hominis were more prevalent in digestive biopsies of patients with diagnosed colon neoplasia/adenocarcinoma compared to patients without digestive neoplasia. Moreover, according to Berahmat et al. (2017) the rate of cryptosporidiosis in children with cancer undergoing chemotherapy was higher (3.8%) than the one in the control group (0%). Although, while using an immunosuppressed mouse model, chronic C. parvum infection has been reported to cause neoplastic changes in the small intestine, suggesting a potential role as a carcinogen (Benmarrouz et al., 2012a,b), the contribution of Cryptosporidium spp. to cancer etiology has been largely unexplored (Cheeseeman et al., 2016). Taking into account the fact that among 145 examined patients in our study only one was infected with C. meleagridis, we consider this infection as opportunistic. Considering that human adenocarcinoma usually takes above 10 years to be diagnosed (Subramaniam et al., 2016), it is more likely that the C. meleagridis infection in the present study has resulted from the tumor presence. Our patient infected with C. meleagridis...
subtype IIfg, has not reported direct contact with poultry and wild birds, and the source of infection remains unknown. Nevertheless, the occurrence of this subtype as well as the other C. meleagridis subtypes (IIBb, IICc, IId, IIIf, IIIfb and IIfi) in humans, indicates that they are susceptible to almost all subtypes of C. meleagridis (Abal-Fabeiro et al., 2013; Wang et al., 2013). Future studies should examine characteristics of carcinoma tissue that could increase susceptibility to Cryptosporidium infection, including altered mucin expression and loss of epithelial cell integrity (Nath and Mukherjee, 2014; Cheeseman et al., 2016).

Although our results have shown that C. meleagridis might inhabit pathologically changed tumor tissue in human colon, there is still no evidence that this apicomplexan parasite should be considered as carcinogenic agent to humans according to International Agency for Research on Cancer (IARC).

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AUTHOR CONTRIBUTIONS

ŽK and MKi designed research. ŽK, MKv, PK, PL, BS, and MKi performed research. ŽK, MKv, PK, AH, MS, PL, JM, and MKi analyzed data. ŽK, MKv, JM, and MKi wrote the manuscript. AH and MS revised the manuscript for important intellectual content.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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