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IDENTIFICATION OF *Blastocystis hominis* : RELATIONSHIP WITH IL-6 LEVELS IN COLORECTAL CANCER PATIENTS

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ABSTRACT

Blastocystis hominis is a protozoan commonly found in human feces, *Blastocystis hominis* has a wide distribution throughout the world. Prevalence of cancer cases reaches 15 - 20% caused by infections, including parasitic infections. *Blastocystis hominis* is a parasite suspected play a role in worsening colorectal cancer. Increased IL-6 triggered by *Blastocystis hominis* suspected plays an important role in angiogenesis, because IL-6 can increase vascular endothelial factor (VGEF) which cause new formation of blood vessels and provide nutrition to colorectal cancer cells and fight the apoptosis. *Blastocystis hominis* is suspected have important role in exacerbating clinical manifestations and worsening prognosis in colorectal cancer patients. This study aims to analyze relationship between the presence of *Blastocystis hominis* and interleukin-6 levels in colorectal cancer patients. This research uses analytical observation and cross sectional method design. 32 samples colorectal cancer patients at RSUP Dr. M. Djamil, Padang, divided into 2 groups, positive and negative *Blastocystis hominis*. PCR (Polymerase Chain Reaction) used to identify the presence of *Blastocystis hominis*, ELISA (Enzyme Linked Immunoassorbant Assay) used to analyze levels of Interleukin 6 and analyze association between presence of *Blastocystis hominis* and Interleukin-6 levels in colorectal cancer patients. Based on the findings, the mean level of interleukin 6 in the group of colorectal cancer positive *Blastocystis hominis* was lower than negative *Blastocystis hominis*, 47.8 pg/mL in the positive and 50.5 pg/mL, comparative T test results p=0.696 (p >0.05) It can be concluded that there is no association between *Blastocystis hominis* and IL-6 levels in colorectal cancer patients.

Keywords : Blastocystis hominis., Interleukin-6., Colorectal Cancer.

INTRODUCTION

Colorectal cancer is currently still a problem that attracts attention in the world. Colorectal cancer is ranked third in most cancer cases worldwide with 1,931,590 (10%)[1]. More than 1.9 million new cases of colorectal cancer in the world in 2020 and mortality rate from colorectal cancer worldwide was second ranked at 935,173 (9.4%) of the total 9,958,133 cases of death cause cancer in worldwide[2]. Based on the data above, it proves an increase the prevalence of cancer cases throughout the world. Prevalence of colorectal cancer cases in Indonesia is fourth rank in cancer cases with a total of 34,189 (8.6%)[3]. Meanwhile, prevalence of colorectal cancer cases at M. Djamil Hospital in Padang in 2019 was 200 cases, and in 2020 there were 167 cases. Based on this data, it proves the importance of preventive action to control the incidence of colorectal cancer cases.

Various studies have been carried out to control the incidence of colorectal cancer cases, one of which is the search for causes or etiology. As many as 15% - 20% of cancer cases are caused by infection[4]. Various studies have begun to link infection to cancer, one of which is the parasitic infectious agent responsible for infection of the digestive tract epithelium which is thought to be related to colorectal cancer, namely the parasite in the form of the protozoan *Blastocystis hominis*.

Blastocystis hominis. is one of the most common protozoan parasites found in the human digestive tract. *Blastocystis hominis* infection can cause gastrointestinal manifestations in the form of abdominal pain, vomiting and diarrhea.[5]. *Blastocystis hominis* is transmitted fecal - oral through contaminated food or drink.

The mechanism by which *Blastocystis hominis* infects humans is still debated, because there are some that cause clinical manifestations, but it is also found in hosts that do not cause clinical manifestations.[5]. Therefore, many studies have been carried out and it is reported that *Blastocystis hominis* causes pathological reactions in Malaysia as much as 20.4%.[6], Iraq 26%[7], and Phillipines 12,98%[8] which proves that *Blastocystis hominis* can cause oxidative stress reactions and an increase in pro-inflammatory cytokines, namely IL-6 cytokines.[9]. Research was carried out on the identification of *Blastocysis hominis* in patients with Irritable Bowel Syndrome, and it was found that increased levels of the cytokine IL-6 were found, which is a mechanical stimulator of the nerves in the gastrointestinal tract.[10].

In vitro test was carried out with *Blastocystis hominis* on HCT116 or colon cancer cells and it was reported that there was an increase in HCT116 with *Blastocystis hominis* and it was reported that *Blastocystis hominis* also has the ability to increase IL-6 levels[11]. It is known that IL-6 plays an important role in angiogenesis in colorectal cancer, because IL-6 stimulates an

increase in vascular endothelial factor (VEGF) resulting in the formation of new blood vessels, which will provide nutrients and oxygen to cancer cells, this is supported by IL-6 research related to VEGF was carried out in Saudi Arabia with samples of colorectal cancer patients and it was reported that there were increased levels of IL-6 and VEGF in colorectal cancer patients with or without metastases compared to the control group[12]. IL-6 is also responsible for fighting the process of programmed cell death, due to the inhibition of programmed cell death signaling (apoptosis) via the JNK pathway by IL-6 which causes cells to proliferate continuously without programmed cell death[12].

Various epidemiological studies were carried out on *Blastocystis hominis* in patients with various types of cancer. Research in Uzbekistan reported that there was a fourfold increase the parasite *Blastocystis hominis* (2.8%) in colorectal cancer patients compared to the control group[13]. Research in China reported that colorectal cancer had the third highest prevalence (8.1%) from 49 samples of colorectal cancer patients, followed by second place in gastric cancer (8.0%) from 88 samples of gastric cancer patients, and first place in lung cancer (8.9%) from 90 samples of lung cancer patients[14]. Research was also conducted in Poland and found Blastocystis hominis in 13 patients (12.15%) of 107 patients who had colorectal cancer, and found in 3 patients (2.42%) of 124 control patients who had colorectal cancer and had no history of other neoplasms, examination This was carried out using the ELISA and PCR methods with samples in the form of feces from colorectal cancer patients, with samples from patients who had just been diagnosed through colonoscopy and had not received any oncological therapy at all.[15].

Research was conducted in Egypt with samples of 200 feces and it was reported that *Blastocystis hominis* was identified with almost the same prevalence in colorectal cancer patients as much as 52% and 42% with the control group who did not suffer from colorectal cancer.[16]. This research was carried out with the aim of identifying the presence of *Blastocystis hominis* in colorectal cancer patients and testing IL-6 levels and conducting a comparative analysis of IL-6 levels with colorectal cancer patients who did not have the *Blastocystis hominis*.

MATERIALS & METHODS

This research has passed the ethical test from the ethics committee of the Faculty of Medicine, Andalas University No. 434/UN.16.2/KEP-FK/2023 This research is an analytical observational type. This research design uses Cross Sectional. The samples used were 32 fecal and serum samples from colorectal cancer patients diagnosed by digestive surgery specialists and examined at the biomedical laboratory of the Andalas University medical faculty.

Sampling criteria

The research was conducted in the surgical ward at hospital RSUP dr. M. Djamil Padang, with a sample of all colorectal cancer patients at RSUP dr. M. Djamil Padang, who was newly diagnosed by a surgeon in digestive surgery and based on the results of an anatomical pathology examination and has not had any surgical procedures performed and has complete medical record data.

Stool Sample Collection

Samples in the form of fresh feces were taken from colorectal cancer patients who were diagnosed by a digestive surgery specialist at hospital. RSUP dr. M.Djamil Padang in the form of biopsy results from the laboratory and no surgery has ever been carried out, and no chemotherapy has ever been carried out. The samples were stored in a feces pot and put in a cooling box and delivered to the Biomedical Laboratory, Faculty of Medicine, Andalas University for identification analysis of the *Blastocystis hominis*.

Samples in the form of feces were subjected to DNA isolation using a DNA stool mini kit [QIAGEN] according to the factory protocol. The DNA isolation results were used to analyze the presence of *Blastocystis hominis* using the polymerase chain reaction (PCR) method. PCR amplification was carried out using primers BL18SPPF1 (AGTAGTCATACG CTCGTCTCAAA) and BL185R2 (TCTTCGTTACCCGTTACTGC) targeting the SSU rRNA gene. The reaction was carried out with an initial denaturation of 95 ℃ for 3 minutes followed by 35 cycles of further denaturation of 95 ℃ for 30 seconds, annealing of 60 ℃ for 30 seconds, elongation 72℃ for
30 seconds and final elongation 72℃ for 5 minutes. PCR products are read by electrophoresis.

Serum Sample Collection

Apart from feces, patients who meet the criteria for taking fecal samples also have their blood taken using the phlebotomy technique, and the samples are transferred into a vacutainer with a yellow lid and put into a cooling box. The samples are taken to the Biomedical Laboratory, Faculty of Medicine, Andalas University to be carried out. analysis of IL-6 levels

Serum samples were used to analyze IL-6 levels using the enzyme linked immunoassay assorbant (ELISA) method using Human Interleukin 6, IL-6 BT-LAB kit with lot number 202305018 according to the manufacturer's protocol.

The results of the ELISA examination were read with a microplate reader and the IL-6 levels were obtained in pg/ml units.

Analysis of research results in colorectal cancer patients

After carrying out PCR examination on feces and ELISA on the serum of Colorectal Cancer patients. The samples were divided into two groups, namely the positive *Blastocystis hominis* group and the negative *Blastocystis hominis* group. A comparative test was carried out with the results data from the two groups and Interleukin 6 levels were analyzed using paired T Test data analysis.

RESULTS

The research was carried out at RSUP dr. M. Djamil Padang hospital and obtained 51 samples in the form of feces and serum from colorectal cancer patients in the surgical ward of RSUP dr. M. Djamil Padang hospital.

DNA Isolation Results

DNA isolation was carried out using the QIAmp Fast DNA Stool mini kit (Qiagen) with working procedures according to the instructions manual for the DNA isolation kit and the DNA purity measurement results were obtained in table 1.

Table 1. The results of measuring DNA concentration using nanodrops showed purity ranging from 1.33 to 4.70, with the lowest concentration of nucleic acid at 9.5 ng/µl and the highest concentration at 206.1 ng/µl

Sample Code	Nucleic Acid Conc. (ng/µl)	260/260
F1	82,1	2,22
F2	206,1	2,20
F3	180,9	1,94
F4	110,6	2,24
F5	52,1	1,33
F6	55,1	2,08
F7	87,9	2,02
F8	47,7	2,52
F9	71,8	1,64
F10	27	2,73
F11	18,8	2,41
F12	47	2,07
F13	29,2	2,58
F14	88,5	1,98
F15	152,3	1,95
F16	80,6	2,40
F17	46	2,30
F18	187,9	2,27
F19	33,1	1,87
F20	18,5	3,04
F21	10,2	3,70
F22	41,1	2,27
F23	38,4	2,30
F24	9,5	4,70
F25	176,5	2,19
F26	75,6	2,05
F27	26,7	2,35
F28	33,9	2,02
F29	69,3	1,96
F30	153,7	2,03
F31	125,9	1,76
F32	16,3	2,48
F33	105,8	2,26
F34	87,2	2,33
F35	144,4	2,16
F36	63,3	2,00
F37	14,7	2,83
F38	39,8	1,45
F39	34	2,30
F40	148,5	2,21
F41	146,8	2,17
F42	135	2,25
F43	47,8	2,33
F44	63,2	2,30
F45	22,4	2,92
F46	11,3	3,90
F47	140,9	2,03
F48	14,2	2,48
F49	128,1	1,93
F50	23,1	2,76
F51	17	2,86

Results of Identification of *Blastocytis hominis*

PCR testing was carried out on 51 stool samples from colorectal cancer patients in the surgical ward of RSUP dr. M. Djamil hospital,. The PCR test was carried out at the Biomedical Laboratory, Faculty of Medicine, Andalas University, Padang and the results can be seen in Figure 1.

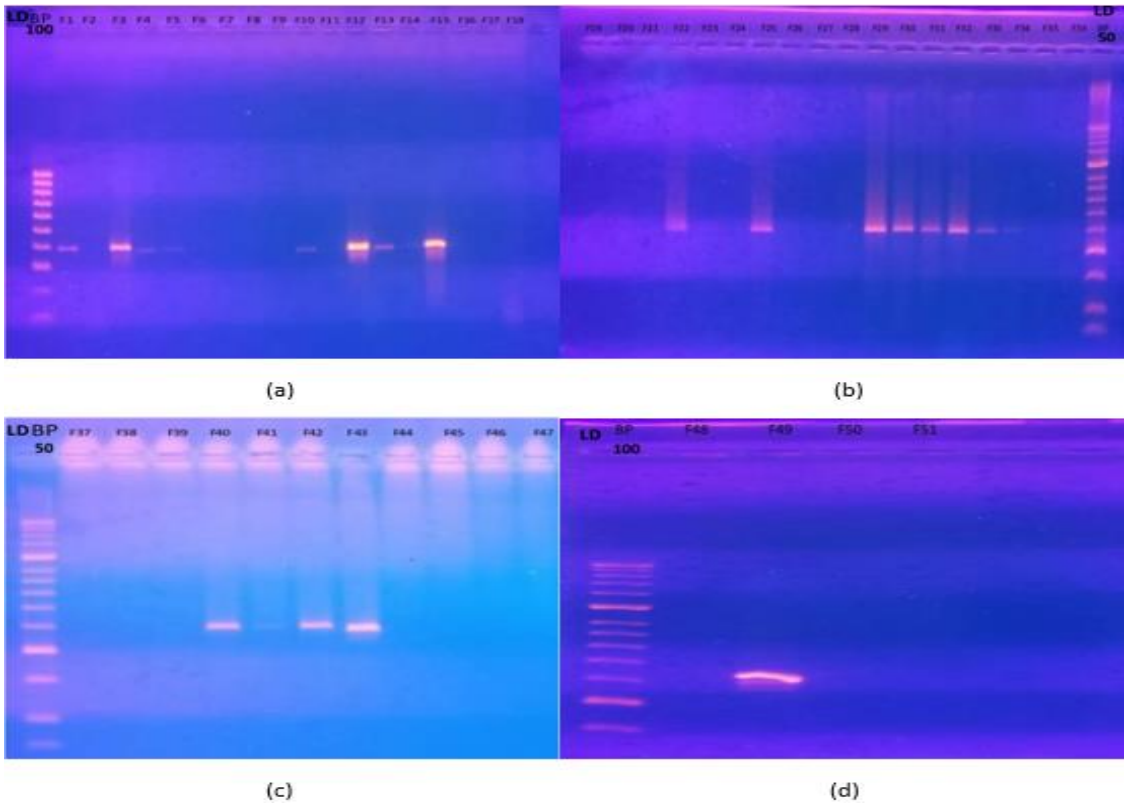

Figure 1. Results of identification of *Blastocystis hominis* using the PCR method, *8 Blastocystis hominis* detected from 18 samples (a), 8 *Blastocystis hominis* detected from 17 samples (b), 4 *Blastocystis hominis* detected from 10 samples (c), 1 *Blastocystis hominis* detected from 4 samples, with a total of 21 (41.2%) of colorectal cancer patients detecting *Blastocystis hominis*, and 30 (58.8%) of *Blastocystis hominis* not detecting, the DNA bands in the PCR products ranged from 320-340bp.

Determination of the number of samples and sample grouping after PCR

PCR examination was carried out on a total of 51 samples for the detection of *Blastocystis hominis*, 32 samples that met the inclusion criteria were taken, and the remaining 19 samples were considered for exclusion criteria. A total of 32 samples were taken and divided into 2 group where *Blastocystis hominis* was detected and the group where *Blastocystis hominis* was not detected. Sampling used convenience sampling techniques and determining the number of samples using the hypothesis testing formula for two independent groups from two nominal data with the calculation results obtained 16 samples in the positive *Blastocystis hominis* group and 16 samples in the negative *Blastocystis hominis* group with a total sample in both groups of 32 samples A PCR examination was carried out and it was found that 16 patients were positive for *Blastocystis hominis* and 16 patients were negative for *Blastocystis hominis.*

Results of IL-6 levels

The results of calculating IL-6 levels with the number of samples are in accordance with the provisions for number and grouping after PCR. Analysis of IL-6 levels carried out using the ELISA method can be seen in table 2.

Table 2. Results of IL-6 levels using the ELISA method, it was found that the lowest IL-6 level was 12,118 pg/ml and the highest IL-6 level was 93,247 pg/ml from 32 samples (16 positive for *Blastocystis hominis*, and 16 negative for *Blastocystis hominis*)

No	Sample Code	Interleukin-6 (pg/ml)
1	F13	74.502
2	F12	77.754
3	F34	65,629
4	F4	61.324
5	F43	12.118
6	F32	42.846
7	F33	55.924
8	F3	55.333
9	F31	41.76
10	F24	64.39
11	F42	48.4
12	F25	36.983
13	F10	22.883
14	F40	34.918
15	F22	54.745
16	F48	15.655
17	F17	40.683
18	F20	89.123
19	F37	24.702
20	F19	67.501
21	F18	37.505
22	F23	37.505
23	F9	50.107
24	F27	41.221
25	F14	58.905
26	F6	93.247
27	F26	56.516
28	F51	36.983
29	F44	27.035
30	F2	48.4
31	F39	61.324
32	F36	38.555

Results of analysis of the relationship between IL-6 levels and the presence of *Blastocystis hominis* in colorectal cancer patients

After obtaining the identification results for *Blastocystis hominis* using PCR, and grouping positive *Blastocystis hominis* and negative *Blastocystis hominis* was carried out and obtained 32 samples deemed to meet the inclusion criteria and 19 considered exclusion criteria by calculating the number of samples using the hypothesis test formula for two independent groups from two nominal data. . IL-6 levels were obtained using the ELISA method in 32 samples that met the previously determined inclusion criteria. An analysis of the relationship between IL-6 levels and the presence of *Blastocystis hominis* was carried out in colorectal cancer patients. The results were in the form of a box plot and tested using paired T test analysis, which can be seen in Figure 2 and Table 3.

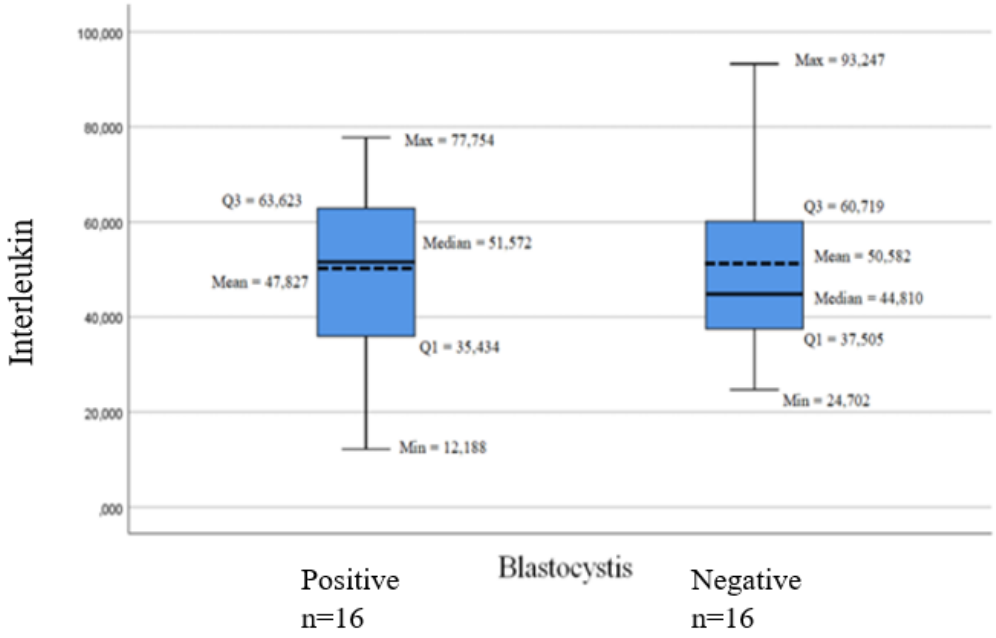

Figure 2. Boxplot of the relationship between *Blastocystis hominis* and IL-6 levels. In the ELISA examination, it was found that IL-6 levels in the *Blastocystis hominis* detected group had a minimum value of 12,188 pg.ml and a maximum value of 77,754 pg/ml with a median of 51,572, in the group not detected *Blastocystis hominis* had a minimum value of 24,702 pg/ml and a maximum value of 93,247 pg/ml. ml with a median of 44,810pg/ml.

Table 3. Paired t test analysis on the relationship between *Blastocystis hominis* and IL-6 levels, there is a difference in the mean levels of interleukin 6 in the colorectal cancer group where *Blastocystis hominis* was not detected, which is higher than in the group where *Blastocystis hominis* was detected. mean value of IL-6 levels in colorectal cancer patients who detected *Blastocystis hominis* and those who did not detect *Blastocystis hominis* was 47.827 pg/ml versus 50.582 pg/ml with a p value = 0.696 (p> 0.05). It can be concluded that there is no significant relationship between *Blastocystis hominis* with IL-6 levels in colorectal cancer patients, p=0.696 (p>0.05).

Blastocystis hominis	IL-6		p value*
	N	*Mean* ± SD (pg/ml)	
Positive	16	47,827 ± 19,704	0,696
Negative	16	50,582 ± 19,829	
Total	32		

*Pair T-Test

DISCUSSION

Interleukin-6 is a pleiotropic cytokine that is thought to promote cancer growth. There is increased expression of IL-6 in colorectal cancer patients. IL-6 activates STAT3 to facilitate tumor growth. IL-6 stimulates VGEF to form new blood vessels that provide nutrients to tumors and IL6/STAT3 also inhibits the apoptosis process which causes cancer cells to proliferate continuously[12]. Colorectal cancer cells also secrete IL-6 which will exacerbate the inflammatory response and increase tumor aggressiveness [17].

Blastocystis hominis is a parasite that is commonly found in humans and animals, this parasite is more often found in areas with low levels of hygiene, contact with animals, and poor sanitation, but the main cause of how *Blastocystis hominis* can infect humans is still under debate[18]. It is known that there are 17 subtypes of Blastocystis hominis, and it is reported that 9 subtypes occur in humans, but the most frequently found are the ST1, ST2, ST3, and ST6 subtypes[19]. Due to the diversity of subtypes of *Blastocystis hominis*, until now whether *Blastocystis hominis* is a potential pathogen is still under debate and still needs further investigation[20].

Some Blastocystis hominis cause pathological reactions, and some do not cause pathological reactions [5]. There is supporting research that analyzes the relationship between the presence of *Blastocystis hominis* subtype ST2 and patients who have pathological and non-pathological reactions and there is no significant relationship p=0.044 (p>0.05) and says that *Blastocystis hominis* subtype ST2 is a genotype of *Blastocystis hominis* that is non-pathogenic[15]. In *Blastocystis hominis*, the infection mechanism starts from the development of *Blastocystis hominis* in the large intestine and reproduces by binary fission.

The attachment of *Blastocystis hominis* to intestinal mucin will secrete Cysteine Protease which through degradation of secretory IgA, mediated by Rho/ROCK, increased secretion of pro-inflammatory cytokines mediated by NF-κB along with apoptosis of host cells[20]. Cysteine protease which is triggered by the attachment of *Blastocystis hominis* will increase paracellular permeability which will cause clinical manifestations. *Blastocystis hominis* can also stimulate the production of pro-inflammatory cytokines such as IL-6 which will sensitize colonic afferent and enteric nerves to stimulate bowel movement and cause a pathological response[15].

Research was conducted on the activity of Systein Protease in various subtypes of *Blastocystis hominis* obtained from fecal samples of patients who caused pathological symptoms and did not cause pathological symptoms. It was found that *Blastocystis hominis* subtype ST2 from samples with pathological symptoms had the lowest levels of Systein Protease, even subtype ST2 in samples without pathological symptoms did not have any Systein Protease activity at all with a level of 0.00 mU/mL, but ST1, ST3, and ST6 had Systein Protease activity in both patient samples that had pathological symptoms or not, with a range of 0.11 mU/ mL to 0.21 mU/mL, then the levels of pro-inflammatory cytokines in the form of IL-6 for each subtype were examined, and there was no significant relationship between cysteine protease activity in *Blastocystis hominis* and IL-6 levels[21].

Supporting research conducted in Mexico which compared patients with the presence of *Blastocystis hominis* with patients who did not find *Blastocystis hominis* and was associated with pro-inflammatory cytokines, there was no significant relationship between the presence of *Blastocystis hominis* and IL-6 levels with a value of p=0.549 (p> 0.05)[22].

CONCLUSIONS AND SUGGESTIONS

The finding of *Blastocystis hominis* is quite high. There is no association between *Blastocystis hominis* and IL-6 levels in colorectal cancer patients, this is due to the diversity of subtypes, *Blastocystis hominis* ST2 has low Cysteine Protease activity, and there is an increase in IL-6 from the colorectal cancer cells themselves.

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