Parasitol Res (2013) 112:3551–3555
DOI 10.1007/s00436-013-3538-5

ORIGINAL PAPER

Blastocystis sp. subtype 3 triggers higher proliferation of human colorectal cancer cells, HCT116

Vinoth Kumarasamy · Umah R. Kuppusamy · Chandramathi Samudi · Suresh Kumar

Received: 16 April 2013 / Accepted: 9 July 2013 / Published online: 10 August 2013

Abstract *Blastocystis* sp. is a commonly found intestinal microorganism and was reported to cause many nonspecific gastrointestinal symptoms. Various subtypes have been previously reported, and the pathogenicity of different subtypes of *Blastocystis* is unclear and remains as a controversial issue. A recent study has shown that the *Blastocystis* antigen isolated from an unknown subtype could facilitate the proliferation of colon cancer cells. Current study was conducted to compare the effect of solubilized antigen isolated from five different subtypes of *Blastocystis* on colon cancer cells, HCT116. A statistically significant proliferation of these cells was observed when exposed to 1.0 μg/ml solubilized antigen isolated from subtype 3 *Blastocystis* (37.22 %, $p<0.05$). Real-time polymerase chain reaction demonstrated the upregulation of Th2 cytokines especially transforming growth factor beta in subtype 3-treated cancer cells ($p<0.01$, 3.71-fold difference). Of interest, subtype 3 *Blastocystis* antigen also caused a significantly higher upregulation of cathepsin B (subtypes 1 and 2, $p<0.01$; subtypes 4 and 5, $p<0.001$; 6.71-fold difference) which lead to the postulation that it may enhance the exacerbation of existing colon cancer cells by weakening the cellular immune response. The dysregulation of IFN-γ and p53 expression also suggest *Blastocystis* as a proponent of carcinogenesis. Therefore, it is very likely for subtype 3 *Blastocystis* to have higher pathogenic potential as it caused an increased propagation of cancer cells and substantial amount of inflammatory reaction compared to other subtypes.

Vinoth Kumarasamy, Umah R. Kuppusamy, and Chandramathi Samudi contributed equally to this work.

V. Kumarasamy · C. Samudi · S. Kumar (✉)
Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
e-mail: suresh@um.edu.my

U. R. Kuppusamy
Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

Introduction

Blastocystis is an anaerobic microorganism which is commonly found in the human stool sample (Windsor et al. 2002). It is known to cause many nonspecific symptoms such as diarrhea, abdominal pain, and flatulence (Suresh et al. 2009) which may be observed in immunocompromised and immunocompetent hosts (Doyle et al. 1990; Cirioni et al. 1999). High rates of *Blastocystis* infection are found in developed countries (Wong et al. 2008). To date, nine different subtypes of *Blastocystis* have been reported in human based on genomic studies (Yoshikawa et al. 1998, 2000, 2004). The pathogenesis of *Blastocystis* remains as a controversial issue, as it cannot be clearly assigned to certain genotype. Recently, the solubilized antigen of *Blastocystis* was shown to facilitate the in vitro proliferation of human colorectal carcinoma cells (HCT116) (Chandramathi et al. 2010). It was also observed to cause oxidative damage in rats inoculated with human-derived *Blastocystis* isolate (Chandramathi et al. 2009). However, these studies were limited to only one single isolate. Therefore, the present study attempts to evaluate the effect of solubilized antigen isolated from five different subtypes of *Blastocystis* on colon cancer cells, HCT116 proliferation. In addition to that, we also compared the gene expression of cytokines, nuclear transcription factors, and apoptotic genes (Table 1) in colon cancer cell line in the presence of *Blastocystis* (Figs. 1, 2, 3, and 4).

Methods

Preparation of solubilized antigen from *Blastocystis*

The axenic *Blastocystis* was collected using the Ficoll-Paque density gradient centrifugation method as described

Table 1 Inventoried primers used in quantitative real-time RT-PCR analysis (TaqMan® Gene Expression Assays, Applied Biosystem)

Gene symbol	Assay ID	Description	Reference sequence
Th2 cytokines			
IL6	Hs000174131_ml	Interleukin-6	NM_000600.2
IL8	Hs000174103_ml	Interleukin-8	NM_000584.2
TGF-β	Hs00998130_m1	Transforming growth factor beta	NM_000660.4
Th1 cytokines			
IFN-γ	Hs00989290_ml	Interferon gamma	NM_000619.2
TNF-α	Hs99999043_ml	Tumor necrosis factor alpha	NM_000594.2
Nuclear transcription factor			
NF-κB	Hs00231653_ml	Nuclear transcription factor enhancer of activated B cells	NM_003998.2
Apoptotic genes			
p53	Hs00153340_ml	Tumor suppressor gene	NM_000546.4
CTSB	Hs00157194_ml	Proapoptotic gene	NM_001908.3

previously by Chandramathi et al. (2010) and Chan et al. (2012). Sonication was carried out at a frequency of 60 Hz and 0.5 amplitude for 10 cycles. The lysate was observed under a microscope to ensure that a complete lysis had taken place. The sonicated samples were left overnight at 4 °C and were centrifuged at 60,000×*g* for 15 min. The supernatants were filter sterilized, and the protein concentrations were determined using the Bradford assay (Bio-Rad, USA).

Human colorectal carcinoma cell line, HCT116

Human colon cancer cell line HCT116 was obtained from the American Type Culture Center and grown at 37 °C in a humidified atmosphere containing 5 % CO_2 in 5 ml RPMI 1640 growth medium supplemented with 5 % (*v*/*v*) fetal bovine serum, L-glutamine (2 mM), penicillin–streptomycin (100 U/ml), and Fungizone (2.5 μg/ml) in a 25-cm^3 culture flask. The cells were detached by trypsinization. The detached cells were washed once with PBS and then resuspended in the RPMI 1640 medium prior to the introduction of antigen.

Proliferation assay

HCT116 cells were harvested and seeded into 96-well plates (1×10^3 cells per well) and grown in a 100-μl growth medium. The cells were incubated overnight at 37 °C in a CO_2

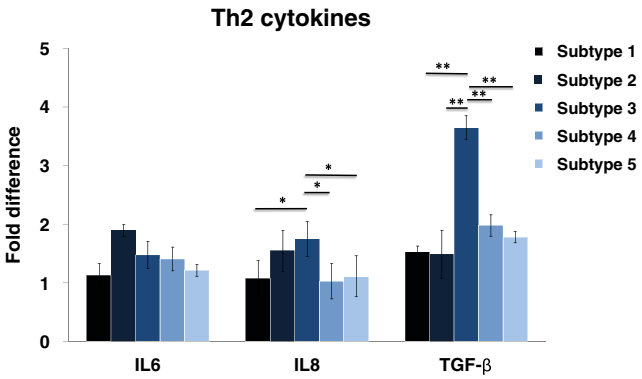

Fig. 1 Gene expression profiles of Th2 cytokines in HCT116 cells upon exposure to 1 μg/ml of different subtypes of *Blastocystis*. Values are presented in fold difference observed in comparison with respective control (untreated samples) and normalized against endogenous gene (human β-actin). *Error bars* represent the relative quantity ± SD ($n=3$). A positive fold difference refers to upregulation and vice versa. The significant difference between ΔC of different subtypes is represented by $*p<0.05$, $**p<0.01$, and $***p<0.001$ using one-way ANOVA (SPSS version 17). All the studied genes used inventoried primers sourced from *Homo sapiens* using probes labeled with 6-carboxyfluorescein (FAM) dye and minor groove binder (MGB) quencher (Applied Biosystems)

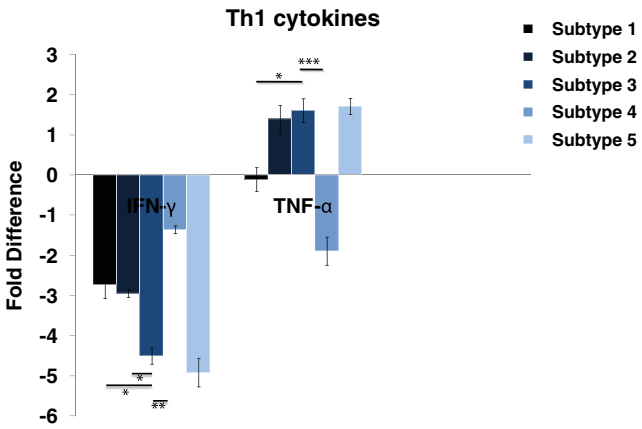

Fig. 2 Gene expression profiles of Th1 cytokines in HCT116 cells upon exposure to 1 μg/ml of different subtypes of *Blastocystis*. Values are presented in fold difference observed in comparison with respective control (untreated samples) and normalized against endogenous gene (human β-actin). *Error bars* represent the relative quantity ± SD ($n=3$). A positive fold difference refers to upregulation and vice versa. The significant difference between ΔC of different subtypes is represented by $*p<0.05$, $**p<0.01$, and $***p<0.001$ using one-way ANOVA (SPSS version 17). All the studied genes used inventoried primers sourced from *H. sapiens* using probes labeled with FAM dye and MGB quencher (Applied Biosystems)

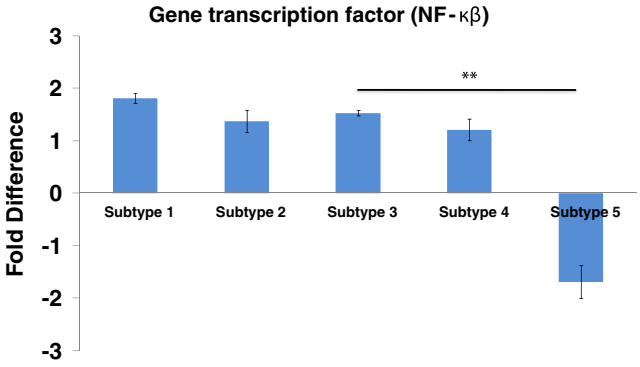

Fig. 3 Gene expression profiles of gene transcription factor in HCT116 cells upon exposure to 1 μg/ml of different subtypes of *Blastocystis*. Values are presented in fold difference observed in comparison with respective control (untreated samples) and normalized against endogenous gene (human β-actin). *Error bars* represent the relative quantity ± SD (n=3). A positive fold difference refers to upregulation and vice versa. The significant difference between ΔC of different subtypes is represented by *p<0.05, **p<0.01, and ***p<0.001 using one-way ANOVA (SPSS version 17). All the studied genes used inventoried primers sourced from *H. sapiens* using probes labeled with FAM dye and MGB quencher (Applied Biosystems)

incubator containing 5 % CO_2 before introducing the antigen with concentrations ranging from 0.01 to 10 μg/ml. A preliminary test was conducted to determine the optimum concentrations of antigen to be added into each well. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed after incubating cells for 48 h to determine cell proliferation in vitro (Mosmann 1983). This assay measures the capacity of mitochondrial succinate dehydrogenase enzymes in living cells which reduces MTT to purple-colored formazan. The intensity of the color was measured spectrophotometrically at 595 nm.

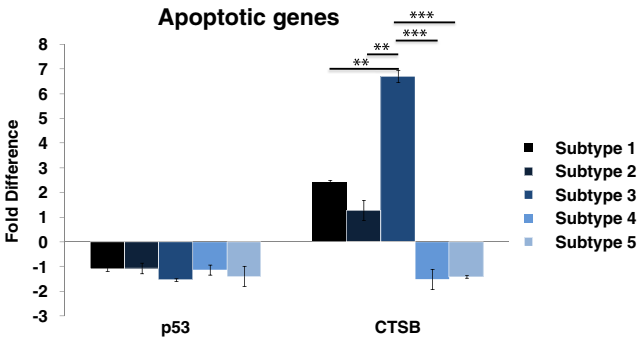

Fig. 4 Gene expression profiles of apoptotic genes in HCT116 cells upon exposure to 1 μg/ml of different subtypes of *Blastocystis*. Values are presented in fold difference observed in comparison with respective control (untreated samples) and normalized against endogenous gene (human β-actin). *Error bars* represent the relative quantity ± SD (n=3). A positive fold difference refers to upregulation and vice versa. The significant difference between ΔC of different subtypes is represented by *p<0.05, **p<0.01, and ***p<0.001 using one-way ANOVA (SPSS version 17). All the studied genes used inventoried primers sourced from *H. sapiens* using probes labeled with FAM dye and MGB quencher (Applied Biosystems)

Real-time polymerase chain reaction analysis

Optimum concentration (1 μg/ml) of *Blastocystis* antigen (which gave a maximum proliferation of HCT116 cells) was introduced into a culture flask containing 5 ml medium and 1×10^5 cells. Jones' medium was introduced instead of antigen for control experiments. Total RNA was extracted from pelleted HCT116 cells via the Ambion RNAqueous®-4PCR Kit (Ambion, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized from total RNA samples extracted from HCT116 cells using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Real-time PCR was performed with the Applied Biosystems StepOne™ System. The threshold cycle (C_T) value is defined as the cycle number at which the fluorescence crosses a fixed threshold above the baseline. For a relative quantification, fold changes were measured using the $\Delta\Delta C_T$ method. The C_T value of each sample was measured and compared to endogenous gene, β-actin. Relative transcripts were determined by the formula $2^{-\Delta\Delta C_T}$.

Statistical methods

The level of significance between the various subtype-induced cell proliferations was determined using one-way ANOVA. MTT tests were performed in triplicate. In all cases, three independent experiments were carried out. Results are presented as the mean value ± standard error of the mean. The level of gene expression difference between treated and non-treated (control) samples were assessed using one-way ANOVA. A p value of 0.05 was considered as the minimum threshold of significance. All statistical analyses were performed using the SPSS Software version 17.0.

Results

The proliferation of colon cancer cells was initially assessed after 24 h of incubation with solubilized antigen. The optimum concentration of the antigen for the proliferation of colon cancer cells was at 1.0 μg/ml. Table 2 shows the proliferations of cancer cells in the presence of various *Blastocystis* subtypes. Out of the five different *Blastocystis* subtypes tested, subtype 3 showed a statistically significant proliferation of HCT116 (Table 2). *Blastocystis* antigen caused the upregulation of Th2 and Th1 cytokines (Figs. 1 and 2) and downregulation of IFN-γ and p53 in HCT116 cells (Figs. 1 and 4) as determined by real-time polymerase chain reaction (RT-PCR) analysis. It is noteworthy that subtype 3 *Blastocystis* antigen caused a significantly higher stimulation of cathepsin B (CTSB) and TGF-β gene expression (Figs. 1 and 4).

Table 2 Proliferation of HCT116 upon exposure to 1.0 μg/ml *Blastocystis* antigen

Subtypes	Sample size, *n*	Proliferation (%)
1	4	16.69±4.03*
2	2	11.55±4.64*
3	6	37.22±2.86
4	2	12.43±8.30*
5	2	4.47±2.94**

Values are presented in mean ± SEM

*$p<0.05$; **$p<0.01$; ***$p<0.001$ (levels of significance of the mean comparison between cell proliferation in the presence of subtype 3 *Blastocystis* antigen and other subtypes)

Discussion

Study has already been conducted previously to correlate *Blastocystis* and HCT116, but the *Blastocystis* isolate used was devoid of any molecular characterization (Chandramathi et al. 2010). In another study, the pathogenic potential of symptomatic *Blastocystis* was reported to be higher compared to asymptomatic isolate when introduced to colorectal cancer cells (Chan et al. 2012). It is important to assess if different subtypes can influence cancer cells proliferation, especially when there have been conflicting reports on the subtypes of *Blastocystis* and its pathogenicity role (Kaneda et al. 2001; Tan et al. 2008). Therefore, the present study exploits the fact that *Blastocystis* stimulates proliferation of cancer cells and is being used in this study to assess if subtype variation of *Blastocystis* can influence its pathogenic potential towards cancer cells. Colon epithelial cells which are found at the surface of gastrointestinal tract forms a mechanical barrier and play an important role in gut mucosal immune response and defense mechanism against the invading pathogens. In this study, we hypothesized that in *Blastocystis*-infected colon cancer patients, the expression of many immunological components by these cells may probably contribute to pathophysiological changes in the host. In a previous study, the inflammatory cytokine responses were observed to be modulated in human epithelial cells in the presence of *Blastocystis* (Long et al. 2001). It was also speculated that *Blastocystis* has the ability to downregulate the host immune response at the beginning stage of colorectal cancer to improve its survival (Long et al. 2001). In this study, RT-PCR work has revealed that colon cancer cells express significantly higher levels of IL-6 and IL-8 in the presence of *Blastocystis* antigen (Fig. 1), providing a possible explanation for the observed proliferation. Therefore, IL-6 and IL-8 probably can be associated with cellular immune response activation towards *Blastocystis* antigen. Similar results were observed in HCT116 before incubation with a solubilized antigen of symptomatic *Blastocystis* isolate (Chan et al. 2012). However, the mean value of IL-8 expression is considerably high for subtypes 2 and 3 (Fig. 1). We speculate that the release of inflammatory cytokines such as IL-6 and IL-8 together with reactive oxygen species contribute to pathogenesis of carcinogenesis. Previously, IL-6 has been associated with proliferation of colon carcinoma in a number of studies (Galizia et al. 2002; Chung and Chang 2003; Becker et al. 2005). Of interest is the high expression of CTSB detected in response to subtype 3 *Blastocystis* antigen (Fig. 4). Cathepsin B has been widely associated with carcinogenesis and highly expressed in colorectal cancer (CRC) patients (Herszenyi et al. 2008). It has also been shown to enhance the motility of cancer cells and has tumor-promoting ability via upregulation of TGF-β (Elliott and Blobe 2005). RT-PCR analysis also showed downregulation of IFN-γ and p53 expression which was more significant in subtype 3-treated HCT116 cells (Figs. 1 and 4). This indicates *Blastocystis* infection, probably reducing the apoptosis in colon cancer cells. p53 is known to play a vital role in CRC and is known as a tumor suppressor gene, and its dysregulation has been widely accepted as a carcinogenesis proponent. Furthermore, elevated p53 levels have been reported in stage III colorectal tumors (Adrover et al. 1999).

Conclusion

There has been far no information on whether different subtypes of *Blastocystis* can influence proliferation of colon cancer cells. Therefore, this study basically provides a better understanding of how proliferation of HCT116 can be possibly affected by the solubilized antigen isolated from different subtypes of *Blastocystis* in vitro. The proliferation analysis and gene expression findings in the present study implicate a possible pathogenic role for subtype 3 *Blastocystis*. The data obtained also suggest the importance of screening *Blastocystis* in patients with an early diagnosis of CRC which may help to prevent the worsening of this condition.

Acknowledgments This study was funded by University of Malaya High Impact Grant (UM.C/625/1/HIR).

References

Adrover E, Maestro M, Sanz-Casla M, Del Barco V, Cerdán J, Fernández C, Balibrea J (1999) Expression of high p53 levels in colorectal cancer: a favourable prognostic factor. Brit J Cancer 81:122

Becker C, Fantini M, Wirtz S, Nikolaev A, Lehr H, Galle P, Rose-John S, Neurath M (2005) Extra views IL-6 signaling promotes tumor growth in colorectal cancer. Cell Cycle 4:217–220

Chan KH, Chandramathi S, Suresh K, Chua KH, Kuppusamy UR (2012) Effects of symptomatic and asymptomatic isolates of

Blastocystis hominis on colorectal cancer cell line, HCT116. Parasitol Res 110:2475–2480

Chandramathi S, Suresh K, Anita Z, Kuppusamy U (2009) Elevated levels of urinary hydrogen peroxide, advanced oxidative protein product (AOPP) and malondialdehyde in humans infected with intestinal parasites. Parasitology 136:359–363

Chandramathi S, Suresh K, Kuppusamy UR (2010) Solubilized antigen of *Blastocystis hominis* facilitates the growth of human colorectal cancer cells, HCT116. Parasitol Res 106:941–945

Chung YC, Chang YF (2003) Serum interleukin 6 levels reflect the disease status of colorectal cancer. J Surg Oncol 83:222–226

Cirioni O, Giacometti A, Drenaggi D, Ancarani F, Scalise G (1999) Prevalence and clinical relevance of *Blastocystis hominis* in diverse patient cohorts. Eur J Epidemiol 15:387–391

Doyle P, Helgason M, Mathias R, Proctor E (1990) Epidemiology and pathogenicity of *Blastocystis hominis*. J Clin Microbiol 28:116

Elliott RL, Blobe GC (2005) Role of transforming growth factor beta in human cancer. J Clin Oncol 23:2078–2093

Galizia G, Orditura M, Romano C, Lieto E, Castellano P, Pelosio L, Imperatore V, Catalano G, Pignatelli C, De Vita F (2002) Prognostic significance of circulating IL-10 and IL-6 serum levels in colon cancer patients undergoing surgery. Clin Immunol 102:169–178

Herszenyi L, Istvan G, Cardin R, De Paoli M, Plebani M, Tulassay Z, Farinati F (2008) Serum cathepsin B and plasma urokinase-type plasminogen activator levels in gastrointestinal tract cancers. Eur J Cancer Prev 17:438–445

Kaneda Y, Horiki N, Cheng X, Fujita Y, Maruyama M, Tachibana H (2001) Ribodemes of *Blastocystis hominis* isolated in Japan. Amer J Trop Med Hyg 65:393

Long H, Handschack A, König W, Ambrosch A (2001) *Blastocystis hominis* modulates immune responses and cytokine release in colonic epithelial cells. Parasitol Res 87:1029–1030

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Method 65:55–63

Suresh K, Venilla GD, Tan TC, Rohela M (2009) In vivo encystation of *Blastocystis hominis*. Parasitol Res 104:1373–1380

Tan T, Suresh K, Smith H (2008) Phenotypic and genotypic characterisation of *Blastocystis hominis* isolates implicates subtype 3 as a subtype with pathogenic potential. Parasitol Res 104:85–93

Windsor J, Macfarlane L, Hughes-Thapa G, Jones S, Whiteside T (2002) Incidence of *Blastocystis hominis* in faecal samples submitted for routine microbiological analysis. Brit J Biomed Sci 59:154–157

Wong KH, Ng GC, Lin RT, Yoshikawa H, Taylor MB, Tan KS (2008) Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore. Parasitol Res 102:663–670

Yoshikawa H, Abe N, Iwasawa M, Kitano S, Nagano I, Wu Z, Takahashi Y (2000) Genomic analysis of *Blastocystis hominis* strains isolated from two long-term health care facilities. J Clin Microbiol 38: 1324

Yoshikawa H, Abe N, Wu Z (2004) PCR-based identification of zoonotic isolates of *Blastocystis* from mammals and birds. Microbiology-Engl Tr 150:1147

Yoshikawa H, Nagano I, Wu Z, Yap EH, Singh M, Takahashi Y (1998) Genomic polymorphism among *Blastocystis hominis* strains and development of subtype-specific diagnostic primers. Mol Cell Probe 12:153–159