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A possible role of *Bacteroides fragilis* enterotoxin in the aetiology of colorectal cancer

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ABSTRACT

The prevalence of enterotoxigenic *Bacteroides fragilis* (ETBF) was investigated in stool specimens from 73 patients with colorectal cancer and from 59 control patients. Stool specimens were cultured on Bacteroides Bile Esculin agar and *B. fragilis* was identified by conventional methods. After DNA extraction, the enterotoxin gene (*bft*) was detected by PCR in 38% of the isolates from colorectal cancer patients, compared with 12% of the isolates from the control group (p 0.009). This is the first study demonstrating an increased prevalence of ETBF in colorectal cancer patients.

Keywords *Bacteroides fragilis*, *bft* gene, colorectal cancer, enterotoxin, fragilysin, PCR

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INTRODUCTION

Bacteroides fragilis is an obligate anaerobe that is found in the colon flora of healthy humans and animals, and is the *Bacteroides* species isolated most frequently from clinical specimens as an aetiological agent of endogenous suppurative infections. The pathogenicity of *B. fragilis* is related to its carbohydrate capsule, outer-membrane proteins and production of specific enzymes, including a recently recognised enterotoxin called fragilysin [1,2]. Fragilysin-producing *B. fragilis*, termed enterotoxigenic *B. fragilis* (ETBF), has been associated with diarrhoea in humans and young farm animals [3,4]. A significant correlation has been found between the presence of ETBF in patient stool specimens, or the toxin gene in colonic biopsy specimens, and the presence of active inflammatory bowel disease [5,6].

Koshy *et al*. [7] demonstrated that treatment of human colonic epithelial cells (HT29/C1) with *B. fragilis* toxin (BFT) resulted in time- and concentration-dependent redistribution of actin microfilaments (F-actin), as well as an increase in cell volume, without causing cell injury. It was suggested that these changes in F-actin and cell volume may lead to an alteration in tight-junction function in the intestinal epithelium, contributing to the pathogenesis of diarrhoea in ETBF infection. Another study from the same centre indicated that the target of fragilysin was the cell surface protein E-cadherin, which is the principal structural component of the zonula adherens and is responsible for cell-to-cell adhesion. Fragilysin, causing cleavage of the extracellular domain of E-cadherin, leads to complete degradation of this protein [8]. Following BFT treatment of HT29/C1 cells, it was also suggested that loss of membrane-associated E-cadherin triggered the nuclear localisation of β-catenin, which in turn, after binding with T-cell factor-dependent transcriptional activators, induced *c-myc* transcription and translation, resulting in persistent cellular proliferation [9].

Although the effect of enterotoxin has been demonstrated in patients with diarrhoea and inflammatory bowel disease, no previous study has investigated ETBF colonisation in patients

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with colorectal cancer. In order to investigate a possible association between the production of toxin and tumour development, the present study investigated the prevalence of ETBF in stool samples from patients with colorectal cancer in comparison with patients with no personal or familial history or diagnosis of colorectal disease.

MATERIALS AND METHODS

Patients

Stool specimens were collected from 73 consecutive colorectal cancer patients who were admitted to the Departments of General Surgery at Marmara University Hospital and Gulhane Military Medical School Hospital, Istanbul, Turkey. All data concerning age, gender, stage of the cancer and American Society of Anaesthesiology score (which reflects the general health status) were retrieved prospectively. During the same period, 59 healthy subjects, with no personal or familial history of colorectal disease, whose age and gender matched those of the cancer patients, were included in the study as controls. A recent history of diarrhoea was considered to be an exclusion criterion for recruitment as a control. Informed consent was obtained and the Marmara University Ethics Committee approved the study protocol.

Bacterial strains

The stool samples were inoculated on Bacteroides Bile Esculin Agar (Becton Dickinson, Sparks, MD, USA) plates. After incubation at 37°C for 2–4 days in an anaerobic jar, several colonies from each plate were tested for aerotolerance. Strict anaerobes were chosen for identification by conventional methods on the basis of colonial and cellular morphologies. Briefly, isolates were identified presumptively as *B. fragilis* if they were catalase-positive, were indole-negative and did not ferment arabinose, rhamnose, trehalose, salicin and xylan [10]. Identifications were then confirmed using the API-20A system (bioMérieux, Marcy l'Etoile, France).

DNA extraction

B. fragilis colonies were picked from culture plates and suspended in 500 μL of TES (50 mM Tris, pH 8, 50 mM EDTA, 50 mM NaCl) buffer. In brief, the cells were lysed by adding 10 mg lysozyme/mL and incubating at 37°C for 30 min, and then adding 10 mg proteinase K/mL and incubating at 65°C for 45 min. SDS 20% w/v was added and the solution was incubated until it became translucent. Following three treatments with phenol–chloroform, the aqueous phase was collected. DNA was recovered by ethanol precipitation and resuspended in water, essentially as described previously [11].

PCR analysis

The enterotoxin gene was amplified by PCR using forward primer BF3, 5′-GAGCCGAAGACGGTGTATGTGATTTGT, and reverse primer BF4, 5′-TGCTCAGCGCCCAGTATATGACCTAGT [12]. The expected size of the amplification product was *c.* 400 bp. PCR was performed in 50-μL volumes containing 25 μL of 2 × PCR Master Mix (Fermentas, Burlington, Canada), 22 μL of nuclease-free water, 50 pmol of each primer and 100 ng of DNA template. PCR conditions comprised 94°C for 1 min, followed by 40 cycles of 45 s at 94°C, 45 s at 52°C, and 45 s at 72°C, followed by 7 min at 72°C, using a modification of the protocol described by Pantosti *et al.* [13]. Positive and negative controls were the enterotoxigenic strain NCTC 11295 and the non-toxigenic strain ATCC 25285. Amplification products were separated by agarose gel electrophoresis, visualised by UV illumination following staining with ethidium bromide, and identified by comparison with reference markers.

Statistics

Pearson's chi-square or Fisher's exact test were used as appropriate; $p < 0.05$ was considered to be significant. Relative risk calculation with 95% CI was performed only for 2 × 2 tables.

RESULTS

In total, 73 cancer cases (with a male:female ratio of 33:40) and 59 age-matched controls (male: female ratio of 26:33) were included in this prospective study. The median age of the total cohort was 63 years (range 24–90 years). All cases and controls were of the same ethnic origin (Caucasian). The stages of cancer were as follows: 29 (40%) patients in stage I; 22 (30%) in stage II; and 22 (30%) in stage III. None of the cases or controls had a previous history of diarrhoea.

B. fragilis was isolated from the stool specimens of 56 (77%) cancer patients and from 40 (68%) healthy controls. Thus, the isolation rate was not statistically different between the two groups ($p > 0.05$). In contrast, the *bft* gene was detected among the *B. fragilis* isolates from 21 (38%) of the 56 cancer patients, compared with five (12%) of the 40 controls. Thus, the rate of *bft* gene detection in the *B. fragilis* isolates from colorectal cancer patients was significantly higher than in the isolates from the controls (relative risk 4.16, 95% CI 1.39–12.43; p 0.009; Table 1). American Society of Anesthesiology scores and the stage of the cancer were not significant factors for *bft* gene positivity among the cancer cases (Table 2).

DISCUSSION

Analyses of possible relationships between BFT and colon cancer to date have been performed using HT29/C1 cell lines [8,9]. In the present study, the rate of colonisation with ETBF in patients with colorectal cancer was compared with that in subjects with no personal or familial history or diagnosis of colorectal disease. ETBF

Table 1. Presence of the *bft* gene in *Bacteroides fragilis* isolates from cancer patients and control groups

Group (*B. fragilis*-positive)	*bft*-positive isolates *n* (%)	*bft*-negative isolates *n* (%)	RR (95% CI)	p
Cancer patients ($n = 56$)	21 (37.5)	35 (62.5)	4.16 (1.39–12.43)	0.009
Controls ($n = 40$)	5 (12.5)	35 (87.5)		

RR, relative risk.

Table 2. Presence of the *bft* gene in *Bacteroides fragilis* isolates from cancer patients with respect to disease status

Disease status	*bft*-positive isolates *n* (%)	*bft*-negative isolates *n* (%)	p
Stage of cancer			
I	7 (32)	15 (68)	
II	7 (41)	10 (59)	0.65
II	7 (41)	10 (59)	
ASA			
I	6 (32)	13 (68)	
II	9 (47)	10 (53)	0.87
III	6 (33)	12 (67)	

ASA, American Society of Anaesthesiology score.

strains were isolated more frequently from colorectal cancer patients than from the matched controls (38% and 12%, respectively; p 0.009). The stage of the cancer and the general health status of the cancer patients did not correlate with the isolation of *bft*-positive *B. fragilis*. The general health status of the entire cohort was not compared, since the matched controls were chosen from healthy subjects and it was predictable that the health status of the control subjects was superior to that of the cancer patients. However, when the American Society of Anesthesiology scores of the cancer patients were assessed, no difference was found between ETBF-positive and -negative cases (Table 2).

ETBF strains are emerging enteric pathogens associated with diarrhoeal disease in humans and animals [2–4,14–16]. Recent data have suggested that ETBF is also associated with recurrence of inflammatory bowel disease [5,6]. The virulence of ETBF is related to its production of fragilysin, a zinc-containing metalloprotease with a molecular mass of 20 kDa. Fragilysin is encoded by the *bft* gene and has tight-junction specific effects, which result in rounding and swelling of surface epithelial cells of the animal intestine, and in alterations of the morphology of intestinal epithelial cell lines *in vitro*. It thus contributes to the pathogenesis of diarrhoea by stimulating the secretory response [2,17]. BFT also stimulates intestinal epithelial cells (HT29, T84 cells and Caco-2) to secrete interleukin-8 (IL-8), which initiates the recruitment of polymorphonuclear leukocytes to the intestinal sub-mucosa, resulting in an inflammatory response that increases intestinal fluid secretion [18,19]. It has been demonstrated that IL-8 secretion is induced in intestinal epithelial cells in a concentration-dependent manner by biologically active BFT, and that induction of IL-8 mRNA expression occurs rapidly and ceases 6 h after BFT treatment, whereas IL-8 secretion continues for ≥18 h. Transcription of the IL-8 gene in response to BFT stimulation is thought to be regulated via the nuclear factor kappa-B activation pathway [19,20].

In addition to its diarrhoeagenic and inflammatory effects, the role of BFT in multifactorial processes in carcinogenesis has also been studied, since it cleaves E-cadherin bound intracellularly to β-catenins. Accumulation of free catenins in the cytosol can lead to transcription of the oncogene *c-myc*; thus, the stability of β-catenins within the cell is critical, and is provided by certain tumour suppressors, such as the adenomatous polyposis coli protein (APC) [21]. The tumour-suppressing function of APC resides in its capacity to properly regulate intracellular β-catenin levels. In contrast, free β-catenin, after translocating into the nucleus and complexing with members of the T-cell factor/lymphoid enhancer factor family, functions as a signalling molecule for activation of these transcriptional activators. This results in increased expression of Tcf-target genes, including *c-myc*. Mutations either in the APC gene or in genes encoding β-catenins (providing resistance to binding to APC proteins) induce accumulation of free catenins in the cytoplasm, which leads to increased transcription of oncogenes in the nucleus.

Another pool of β-catenins in the cytoplasm is that linked to the intracellular domain of E-cadherin. E-cadherin can also be regarded as a tumour suppressor, since it stabilises the second pool of intracellular β-catenin. However, it is not clear whether the consequence of E-cadherin inactivation is similar to that of APC inactivation [22,23]. Wu *et al*. [9] demonstrated that fragilysin, which causes loss of membrane-associated E-cadherin, triggered the nuclear localisation of β-catenin in HT29/C1 cells within 3 h of toxin treatment, with subsequent induction of *c-myc* transcription and stimulation of cellular proliferation. On the basis of these results, it was

concluded that fragilysin is the first known bacterial toxin to activate Tcf-dependent β-catenin nuclear signalling in intestinal epithelial cells, and it was suggested that *B. fragilis* may have the potential to contribute to oncogenic transformation in the colon. The statistically significant rate of ETBF colonisation in colorectal cancer patients observed in the present study is in agreement with this suggestion.

There were several limitations to the present study. First, the size of the cohort was small. Second, data concerning several environmental factors of potential relevance were not collected from the patients included in this study. Since carcinogenesis is multifactorial, factors such as diet, habits and environmental exposure may have roles in the process. To go beyond the present tentative conclusion, all possible causative factors would have to be taken into consideration. Third, the development of colorectal cancer is a lengthy process. Clarifying the possible role of microorganisms such as *B. fragilis* during pathogenesis therefore requires an observational, longitudinal population-based study. Clearly, this was not the design of the study presented here, and additional investigations are required to substantiate the present findings that indicate a possible correlation between the presence of the *bft* gene and colon cancer, as well as the suggestion that ETBF may have a role in carcinogenesis.

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REFERENCES

1. Jousimies-Somer HR, Finegold SM. Anaerobic gram-negative bacilli and cocci. In: Balows A, Hausler WJ, Herman KL, Isenberg HD, Shadomy HJ, eds, *Manual of clinical microbiology*, 5th edn. Washington, DC: American Society for Microbiology, 1991; 538–553.
2. Sears CL, Myers LL, Lozenby A, Van Tassel RL. Enterotoxigenic *Bacteroides fragilis*. *Clin Infect Dis* 1995; **20**(suppl): 142–148.
3. Myers LL, Shoop DS, Firehammer BD, Border MM. *Bacteriodes fragilis*: a possible cause of diarrhea in newborn lambs. *Infect Immun* 1984; **44**: 241–244.
4. Myers LL, Shoop D, Stackhouse L *et al.* Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhoea. *J Clin Microbiol* 1987; **25**: 2230–2233.
5. Prindiville T, Sheikh R, Cohen S, Tang Y, Cantrell M, Silva J. *Bacteroides fragilis* enterotoxin gene sequences in patients with inflammatory bowel disease. *Emerg Infect Dis* 2000; **6**: 171–174.
6. Basset C, Holton J, Bazeos A, Vaira D, Bloom S. Are *Helicobacter* species and enterotoxigenic *Bacteroides fragilis* involved in inflammatory bowel disease? *Dig Dis Sci* 2004; **49**: 1425–1432.
7. Koshy SS, Montrose MH, Sears CL. Human intestinal epithelial cells swell and demonstrated actin rearrangement in response to metalloprotease toxin of *B. fragilis*. *Infect Immun* 1996; **64**: 5022–5028.
8. Wu S, Lim KC, Huang J, Saidi RF, Sears CL. *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc Natl Acad Sci USA* 1998; **95**: 14979–14984.
9. Wu S, Morin PJ, Mauyo DJIK, Sears C. *Bacteroides fragilis* enterotoxin induces *c-myc* expression and cellular proliferation. *Gastroenterology* 2003; **124**: 392–400.
10. Murray PR, Citrol DM. General processing of specimens for anaerobic bacteria. In: Balows A, Hausler WJ, Herman KL, Isenberg HD, Shadomy HJ, eds, *Manual of clinical microbiology*, 5th edn. Washington, DC: American Society for Microbiology, 1991; 488–504.
11. Lawson PA, Gharbia SE, Shah HN, Clark DR. Recognition of *Fusobacterium nucleatum* subgroups Fn-1, Fn-2 and Fn-3 by ribosomal RNA gene restriction patterns. *FEMS Microbiol Lett* 1989; **531**: 41–45.
12. Moncrief JS, Obiso R, Barroso LA *et al.* The enterotoxin of *Bacteroides fragilis* is a metalloprotease. *Infect Immun* 1995; **63**: 175–181.
13. Pantosti A, Malpelli M, Wilks M, Menozzi MG, D'Ambrosio F. Detection of enterotoxigenic *Bacteroides fragilis* by PCR. *J Clin Microbiol* 1997; **35**: 2482–2486.
14. Sack RB, Albert MJ, Alam K, Neogi PKB, Akbar MS. Isolation of enterotoxigenic *Bacteroides fragilis* from Bangladeshi children with diarrhea: a controlled study. *J Clin Microbiol* 1994; **32**: 960–963.
15. Pantosti A, Menozzi G, Frate A, Sanfilippo L, D'Ambrosio F, Malpeli M. Detection of enterotoxigenic *Bacteroides fragilis* and its toxin in stool samples from adults and children in Italy. *Clin Infect Dis* 1997; **24**: 12–16.
16. Kato N, Liu C, Kato H, Nakamura H, Iwai N, Ueno K. Prevalence of enterotoxigenic *Bacteroides fragilis* in children with diarrhea in Japan. *J Clin Microbiol* 1999; **37**: 801–803.
17. Sears CL. Toxins of *Bacteroides fragilis*. *Toxicon* 2001; **39**: 1737–1746.
18. Sanfiloppo L, Li CK, Seth R, Balwin TJ, Menozzi MG, Mahida YR. *Bacteroides fragilis* enterotoxin induces the expression of IL-8 and transforming growth factor beta by human colonic epithelial cells. *Clin Exp Immunol* 2000; **119**: 456–463.
19. Kim JM, Oh YK, Oh HB, Cho YJ. Polarized secretion of CXC chemokines by human intestinal epithelial cells in response to *Bacteroides fragilis* enterotoxin: NF-κB plays a major role in the regulation of IL-8 expression. *Clin Exp Immunol* 2001; **123**: 421–427.
20. Wu S, Powell J, Mathioudakis N, Kane S, Fernandez E, Sears CL. *Bacteroides fragilis* enterotoxin induces intestinal epithelial cell secretion of interleukin-8 through mitogen-activated protein kinases and a tyrosine kinase-regulated nuclear factor-κB pathway. *Infect Immun* 2004; **72**: 5832–5839.
21. Hardy RG, Meltzer SJ, Jankowski JA. ABC of colorectal cancer: molecular basis for risk factors. *BMJ* 2000; **321**: 886–889.

22. Kinzler KW, Vogelstein B. Colorectal tumors. In: Kinzler KW, Vogelstein B, eds, *The genetic basis of human cancer*, 2nd edn. New York: McGraw-Hill, 2002; 583–612.

23. Fearon ER. Tumor-suppressor genes. In: Kinzler KW, Vogelstein B, eds, *The genetic basis of human cancer*, 2nd edn. New York: McGraw-Hill, 2002; 197–206.