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ORIGINAL ARTICLE

Retrospective Study

Association of *Fusobacterium nucleatum* infection with colorectal cancer in Chinese patients

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Abstract

AIM: To investigate *Fusobacterium nucleatum* (*F. nucleatum*) abundance in colorectal cancer (CRC) tissues and its association with CRC invasiveness in Chinese patients.

METHODS: The resected cancer and adjacent normal tissues (10 cm beyond cancer margins) from 101 consecutive patients with CRC were collected. Fluorescent quantitative polymerase chain reaction (FQ-PCR) was applied to detect *F. nucleatum* in CRC and normal tissues. The difference of *F. nucleatum* abundance between cancer and normal tissues and the relationship of *F. nucleatum* abundance with clinical variables were evaluated. Fluorescence *in situ* hybridization (FISH) analysis was performed on 22 CRC tissues with the highest *F. nucleatum* abundance by FQ-PCR testing to confirm FQ-PCR results.

RESULTS: The median abundance of *F. nucleatum* in CRC tissues [0.242 (0.178-0.276)] was significantly higher than that in normal controls [0.050 (0.023-0.067)] ($P < 0.001$). *F. nucleatum* was over-represented in 88/101 (87.1%) CRC samples. The abundance of *F. nucleatum* determined by $2^{-\Delta CT}$ was significantly greater in tumor samples [0.242 (0.178, 0.276)] than in normal controls [0.050 (0.023, 0.067)] (P < 0.001). The frequency of patients with lymph node metastases was higher in the over-abundance group [52/88 (59.1%)] than in the under-abundance group [0/13 (0%)] (P < 0.005). No significant association of *F. nucleatum* with other clinico-pathological variables was observed (P > 0.05). FISH analysis also found more *F. nucleatum* in CRC than in normal tissues (median number 6, 25^{th} 3, 75^{th} 10 *vs* 2, 25^{th} 1, 75^{th} 5) (P < 0.01).

CONCLUSION: *F. nucleatum* was enriched in CRC tissues and associated with CRC development and metastasis.

Key words: Colorectal cancer; *Fusobacterium nucleatum*; Metastases; Fluorescent quantitative polymerase chain reaction; Fluorescence *in situ* hybridization

Core tip: In this study, we demonstrated that *Fusobacterium nucleatum* (*F. nucleatum*) was significantly enriched in resected colorectal cancer (CRC) compared with adjacent normal tissues. *F. nucleatum* infection was associated with CRC development and metastasis. Our results were consistent with two previous reports. To our knowledge, this is the first report on the relationship between *F. nucleatum* and CRC in Chinese patients.

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INTRODUCTION

Colorectal cancer (CRC) ranks as the third most common cancer and the second most common cause of cancer-related mortality worldwide[1]. The etiology of CRC is still not fully understood. As a huge number of microbial communities are continuously colonized in the gut, some harmful microbiota may play roles in the development of CRC[2,3]. Likewise, the link of some pathogens to cancers, *e.g.*, *Helicobacter pylori* to gastric cancer, human papilloma virus to cervical cancer, and hepatitis B and C virus to hepatocellular carcinoma, has been completely established. These infections are in charge of 18% of cancers[4]. Although the precise mechanisms of the carcinogenesis remain unclear, it is rational to reduce the risk of these cancers by targeting the pathogens. Efforts have been made to explore the possible pathogens involved in CRC development. Once they are identified, it would lead to a breakthrough in the prevention and treatment of CRC.

Fusobacterium nucleatum (*F. nucleatum*) is an anaerobic oral commensal. Besides periodontal disease, it has been documented to be involved in a wide spectrum of disorders, including gastrointestinal diseases, respiratory tract infections, cardiovascular diseases, rheumatoid arthritis, Lemierre's syndrome, and Alzheimer's disease[5]. Currently, accumulating evidence has indicated that a larger number of Fusobacterium spp., especially *F. nucleatum*, is present in tumor tissues and stool samples of CRC patients versus normal controls. A limited number of studies have suggested a positive association of *F. nucleatum* with CRC invasiveness, *e.g.*, lymph node metastasis, but the finding has not been confirmed[6,7]. In the present study, we applied fluorescent quantitative polymerase chain reaction (FQ-PCR) and fluorescence *in situ* hybridization (FISH) to observe the presence of *F. nucleatum* in CRC tissues and to explore the clinical correlation of *F. nucleatum* to CRC invasiveness in Chinese patients.

MATERIALS AND METHODS

Patients

In total, 101 consecutive patients with histologically confirmed colorectal adenocarcinoma (males 55 and females 46, age range 36-82 years) undergoing surgical resections at the Department of General Surgery of Guangzhou First People's Hospital between November 2012 and November 2013 were recruited. The patients who had colorectal tumors other than adenocarcinoma, who received chemotherapy or radiotherapy before operation, and who had comorbid malignancies from other organs were excluded. Fresh CRC and adjacent non-tumor tissues (10 cm beyond cancer margins) from each subject were collected. Samples were snap frozen in liquid nitrogen and then stored at -80 ℃ until use. Samples for histological and FISH examination were fixed in 10% formalin and embedded in paraffin. The stages of CRC were assigned according to TNM and Dukes grades[8]. All 101 patients were enrolled into the FQ-PCR study, among which 22 patients with the highest *F. nucleatum* abundance by FQ-PCR testing were included in microbial FISH examination.

The study protocols complied with the Declaration of Helsinki and were approved by the ethics committee of Guangzhou First People's Hospital affiliated to Guangzhou Medical University. Written consent was obtained from each participant.

Taqman probe-based qPCR assay

Taqman probe-based qPCR was performed on Stepone Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, United States) to determine *F. nucleatum* levels in both cancer and matched normal tissues. Levels of 18s rRNA gene in *F. nucleatum* and internal control were measured simultaneously from the same DNA preparation. DNA was isolated with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The primer and probe sequences for *F. nucleatum* were as follows[9-11]: Forward primer, 5′-CAACCATTACTTTAACTCTACCATGTTCA-3′; Reverse primer, 5′-GTTGACTTTACAGAAGGAGATTATGTAAAAATC-3′, Probe, 5′FAM-GTTGACTTTACAGAAGGAGATTATGTAAAAATC-TAMRA3′. *F. nucleatum* probes and primers were synthesized by Life Technologies Company (Carlsbad, CA, United States). Ten microliters of reaction mixture for detection of *F. nucleatum* consisted of 20 ng template DNA, 400 nmol/L of primer set, 400 nmol/L of probe, and 5 μL TaqMan® Universal PCR Master Mix II (Applied Biosystems). The reaction conditions were 10 min at 95 ℃, 40 cycles of 15 s at 95 ℃, and 1 min at 60 ℃. Standard strain of *F. nucleatum* (ATCC10953) and of Escherichia coli (ATCC8739) provided by Microbial Culture Collection Center of Guangdong Institute of Microbiology, China was used as positive and negative controls, respectively. Taqman Ribosomal RNA Control Reagents, including primers and probes (Applied Biosystems) were used to quantify the human endogenous 18s rRNA gene according to the manufacturer's instructions. Amplicons of *F. nucleatum* and 18 s rRNA gene were cloned into pMD 18-T Vector (Takara Biotechnology Co. Ltd, Dalian, China) according to the kit protocol and then over-expressed in *Escherichia coli*-Trans5α (Dongsheng Biotech Co. Ltd, China). Standard curves were constructed with serial 10-fold dilutions of recombinant plasmids. In order to effectively normalize the qPCR data to tissue size, *F. nucleatum* levels were given as $2^{-\Delta CT}$, and the fold changes of *F. nucleatum* abundance in cancer tissue over matched normal colorectal tissue were calculated as $2^{-\Delta\Delta CT}$[6,7]. The relation of *F. nucleatum* infection to the clinical variables (gender, age, histological type, Dukes stage, location, and lymph node metastasis) was evaluated.

FISH analysis

FISH was performed with FISH pharmDx kit (Abbott Vysis Laboratories, Abbott Park, IL, United States) on 22 pair sections of CRC and matched non-cancerous tissues, which were formalin-fixed and paraffin-embedded. Cell nuclei were stained with DAPI. An Oregon-Green 488-conjugated "universal bacterial" probe (EUB338, pB-00159, green) binding 16s rRNA gene at bacterial conserved regions and a Cy3-conjugated Fusobacterium probe (FUSO, pB-0078, red) binding 16s rRNA gene at Fusobacterium specific regions were applied. The sequences of the probes were referred to probeBase (http://www.microbial-ecology.net/probebase/)[12,13]. Probe hybridization was performed at the following conditions: probe concentration 10 μL (5 ng/μL), denaturation at 75 ℃ for 5 min and hybridization at 37 ℃ for 18 h. Slides were imaged on a microscope (Carl Zeiss, Oberkochen, Germany), and the number of *F. nucleatum* was counted. Five random × 40 fields were chosen for evaluation by two pathologists blind to tumor/normal status. The selection criteria of mucosal tissue depth were used, and a minimum of five bacteria visualized by the EUB338 probe per field was required.

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software package for Windows (SPSS Inc. Chicago, IL, United States). Continuous data were expressed as median (25th percentile, 75th percentile) and examined by the Wilcoxon rank sum test of independent or paired samples. Categorical variables were analyzed with χ^2 test or McNemar test. Bonferroni corrections were applied for multiple comparisons. A *P* value < 0.05 (two tails) was considered statistically significant.

RESULTS

FQ-PCR assay

According to the standard curves, the amplification efficacy of *F. nucleatum* and 18 s rRNA gene was 99.05% and 94.56%, respectively. Compared with the matched normal tissues, *F. nucleatum* load was significantly over-represented in 88 out of 101 (87.13%) CRC samples (Figure 1). The median abundance of *F. nucleatum* determined by $2^{-\Delta\Delta CT}$ was significantly greater in the tumor samples [0.242 (0.178, 0.276)] than that in the matched normal controls [0.050 (0.023, 0.067)] (P < 0.001).

Association of F. nucleatum infection with clinicopathologic features of CRC patients

The association of between the clinicopathologic variables of patients and *F. nucleatum* infection is summarized in Table 1. In total, 52 out of 101 (51.5%) CRC patients had regional lymph node metastases. *F. nucleatum* level, expressed as fold changes ($2^{-\Delta\Delta CT}$, cancer versus normal, in the lymph node metastases group [1538.05 (479.21, 2643.12)] was significantly higher than that [212.87 (37.25, 257.37)] in the non-metastases group (P < 0.005). Lymph node metastases were present in 52 out of 88 (59.1%) patients with *F. nucleatum* over-abundance (fold changes > 1), and in 0 out of 13 (0%) patients with *F. nucleatum* under-abundance (fold change < 1) (P < 0.005). No significant association of *F. nucleatum* infection with other clinicopathological variables, *e.g.*, patient's gender, age, cancer stages, location, infiltration depth, and pathological differentiation, was observed (P > 0.05) (Table 1).

FISH detection

F. nucleatum was determined in 22 paired specimens of CRC and matched non-cancerous controls by FISH. *F. nucleatum* stained with FUSO probe (in red) was

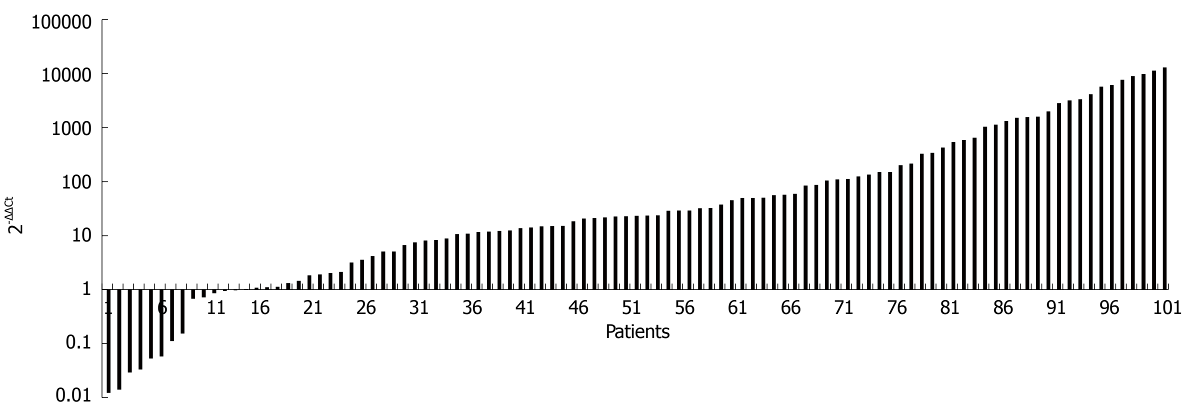

Figure 1 Fold change of *Fusobacterium nucleatum* abundance in colorectal cancer *vs* matched normal tissues (*n* = 101). $2^{-\Delta\Delta CT}$ represented the fold change of *Fusobacterium nucleatum* load in cancer tissue over matched normal tissues. The numbers in Y-axis indicated the $2^{-\Delta\Delta CT}$ value of each patient.

Table 1 Association of *Fusobacterium nucleatum* infection with clinicopathologic variables (*n* = 101)

	n	Cancer/normal tissue fold changes [$2^{-\Delta\Delta CT}$(Median)]	*P* value
Gender			
Male	55	1029.19 (418.27, 6517.82)	
Female	46	7348.74 (529.05, 8827.37)	0.539
Age (yr)			
< 65	52	1100.68 (671.32, 5624.36)	
≥ 65	49	677.03 (502.86, 4946.93)	0.375
Location of CRC			
Rectum	59	1045.11 (824.66, 7567.37)	
Colon	42	6844.72 (1735.91, 8141.03)	0.456
Differentiation			
Moderately and high	59	524.95 (345.19, 2341.54)	
Low	42	1158.67 (432.61, 1988.30)	0.412
Tissue infiltration			
T1 + T2	25	1029.20 (213.76, 1365.41)	
T3 + T4	76	837.89 (196,36, 2679.33)	0.676
Lymph node metastasis			
N0	49	212.87 (37.25, 257.37)	
N1 + N2	52	1538.05 (479.21, 2643.12)	0.005
Duke stages			
A	12	739.50 (132.24, 1615.65)	
B	36	837.46 (264.33, 1489.65)	0.368
C	46	1132.31 (289.73, 1688.72)	
D	7	1356.05 (311.03, 2321.73)	

found to be enriched within the colonic mucosa of CRC (median number 6, 25th 3, 75th 10) compared to the normal control tissues (median number 2, 25th 1, 75th 5) (Figure 2) ($P < 0.01$).

DISCUSSION

The human gastrointestinal lumen is harbored by more than 1000 species of microorganisms, which bring about beneficial and deleterious effects on the host. High abundance of Enterococcus, Escherichia/Shigella, Klebsiella, Streptococcus, Peptostreptococcus, Bacteroides-Prevotella, and low abundance of Lachnospiraceae/Roseburia, clostridia have been found in the gut compartment of CRC patients compared with normal controls[14-17]. For a long time, it has been believed that the dysbiosis in the gut results in a variety of colorectal diseases, including CRC, but no specific bacterium has been confirmed[14-17]. Recently, sequence-based investigations have provided us much valuable information in this field. In 2012, two North American studies published in *Genome Research* declared that a periodontal pathogen, *i.e.*, *F. nucleatum,* was overabundant in CRC tissues. Using a whole-genome sequencing technique, Kostic *et al*[18] identified the enrichment of DNA sequences of Fusobacterium spp. in CRC compared with control samples. Among them, *F. nucleatum* was the most dominant phylotype. Using RNA-sequencing approaches, Castellarin *et al*[7] also observed a large amount of Fusobacterium spp. in CRC versus normal tissues. In addition, they found a positive association of *F. nucleatum* over-abundance with lymph node metastasis. Afterwards, several studies confirmed overload of *F. nucleatum* in CRC tissues[3,6,19,20]. Currently, two studies revealed *F. nucleatum* to be a poor prognostic factor of CRC patients. Using molecular pathological epidemiology database of 1069 CRC patients, Mima *et al*[21] revealed a link between high *F. nucleatum* DNA load in CRC tissue and shorter survival of patients. Flanagan *et al*[6] found a significantly longer survival time in CRC patients with low *F. nucleatum* levels than those with moderate and high levels.

A persuasive interpretation of the above data is an important issue. Firstly, is *F. nucleatum* a cause or a consequence of CRC? So far, increasing evidence is in favor of the "cause" hypothesis, as emerging data have demonstrated that *F. nucleatum* at first induced precancerous lesions (*e.g.*, hyperplastic polyps and adenomas), which eventually progressed to CRC[6,10,22,23]. In addition, a number of pathogenetic studies supported the carcinogenic roles of *F. nucleatum*. As the mucosa adherent bacteria, *F. nucleatum* had ability to adhere to and invade epithelial cell[7,23-26]. *F. nucleatum* shuttled non-invasive bacteria, in particular Campylobacter spp. and Streptococcus into the host cell as mixed

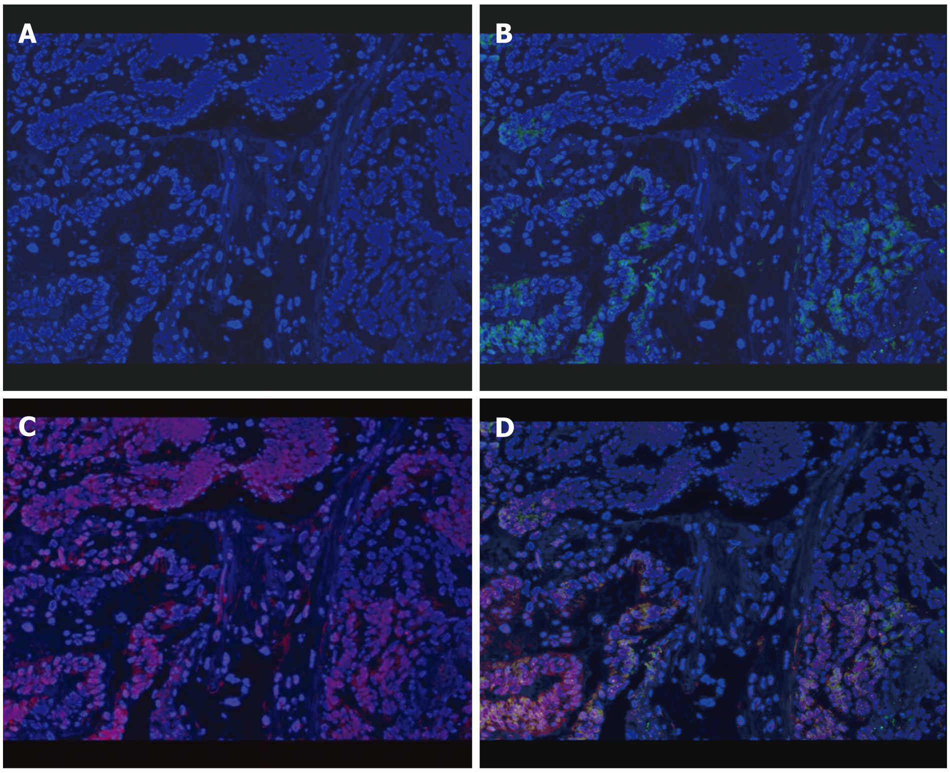

Figure 2 Enrichment of *Fusobacterium nucleatum* in colorectal cancer detected by FISH. Graph A: Colorectal cancer (CRC) specimen stained with DAPI. Cell nuclei were stained in blue; B: CRC specimen stained with both DAPI and universal bacterial probe (EUB338). Bacterial conserved regions were stained in green; C: CRC specimen stained with both DAPI and Fusobacterium specific probe (FUSO). The *Fusobacterium nucleatum* (*F. nucleatum*) specific regions were stained in red; D: CRC specimen triply stained with DAPI, EUSO and EUB338. Cell nuclei (in blue), bacterial conserved regions (in green) and *F. nucleatum* specific regions (in red) were clearly visible (all × 400).

species synergism[27]. By binding to E-cadherin on the epithelial cell, *F. nucleatum* stimulated FadA, which activated β-catenin signaling pathways and up-regulated oncogene expression[17]. *F. nucleatum* induced inflammation by releasing RNA into the host cell and activating nuclear factor kappa B[17]. *F. nucleatum* increases cytokines, in particular tumor necrosis factor-α and interleukin (IL)-10[20]. *F. nucleatum* induced a number of pathogenesis-related events, including CpG island methylator phenotype (CIMP), microsatellite instability (MSI), and genetic mutations in *BRAF*, *KRAS*, *TP53*, *CHD7*, and *CHD8*[28]. *F. nucleatum* reduced CD3+ T cell density in CRC tissues[29]. *F. nucleatum* regulated the tumor immune microenvironment through E-cadherin/β-catenin signaling[30]. Secondly, are gut microbiota other than *F. nucleatum* also enriched in CRC tissues and do they enhance CRC invasiveness? In our ongoing study, the five most suspected bacteria related to CRC in literature[14-17,31] were quantified in the same CRC population. *F. nucleatum*, Enterotoxigenic Bacteroides fragilis (ETBF), and *Enterococcus faecalis* (*E. faecalis*) were significantly enriched in CRC compared to the matched non-cancerous tissues, while no difference were found in enteropathogenic *Escherichia coli* and *Streptococcus gallolyticus* (*S. gallolyticus*). The positive rate of *F. nucleatum* overload (87.13%) was much higher than those of ETBF (61.67%) and *E. faecalis* (55.04%) (unpublished data). Our study was consistent with a recent cohort enrolling six bacteria, which demonstrated that *F. nucleatum* was the only bacterium significantly overabundant in CRC compared with normal tissues, and *F. nucleatum* and ETBF were associated with the clinicopathological features of patients[31]. Based on above observations, we infer that *F. nucelatum* is specifically enriched in CRC and enhances CRC invasiveness.

Recently, the emerging science of molecular pathways has shed light on the pathogenesis of CRC. MSI, chromosomal instability, and CIMP are common genetic changes, while microRNAs, DNA methylation, and histone modifications are analyzed in epigenesis. Interactions between genetic and epigenetic factors are involved in the carcinogenesis of *F. nucleatums*[32-34]. Environmental risk factors, *e.g.*, changes in diet and lifestyle, may affect nuclear receptors on the gut microbiota and induce carcinogenesis through molecular pathways[35]. Furthermore, molecular pathological epidemiology (MPE), a multidisciplinary investigation into the relationship among genetic factors, molecular signatures, and disease progression, provides insights into the pathogenetic process as well as therapeutic optimization[35-37]. Using a MPE database of 1069 CRC patients, Mima *et al*[21] successfully uncovered the association of *F. nucleatum* DNA load with the prognosis of patients.

There are a few limitations of this study. Firstly, we have not used the innovative concept of molecular sciences to design the study. Secondly, we have not included more intestinal bacterial species in addition to *F. nucleatum*. On the whole, our results still reflected the main features in Chinese people. Further larger studies are needed to examine these findings.

In conclusion, our results demonstrated that *F. nucleatum* was enriched in CRC tissue, and *F. nucleatum* abundance was positively associated with lymph node metastasis of CRC patients. There were no geographical and ethnic differences in the association. To our knowledge, this is the first report of its kind in Chinese patients.

COMMENTS

Background

Increasing evidence suggests that *Fusobacterium nucleatum* (*F. nucleatum*) infection in the colon plays a role in the pathogenesis of colorectal cancer (CRC). The clinical correlation of *F. nucleatum* load with CRC progression has not been fully established, especially in Chinese patients.

Research frontiers

This study investigated the relationship between *F. nucleatum* infection and the presence of CRC and the association of *F. nucleatum* abundance with CRC invasiveness in Chinese patients.

Innovations and breakthroughs

In this study, the authors demonstrate that *F. nucleatum* is significantly enriched in CRC tissues from surgical resections compared with adjacent normal tissues. *F. nucleatum* abundance is associated with CRC metastasis. These results are consistent with previous reports and confirm that there is no geographical and ethnic difference in the association. This is the first report of this important data in Chinese patients.

Applications

As *F. nucleatum* is identified as a pathogen of CRC, it may be a new drug target for the future prevention and treatment of CRC.

Terminology

CRC is one of most common cancers worldwide. The etiology of CRC is still not fully understood. *F. nucleatum* is an anaerobic oral commensal. Currently, increasing evidence suggests that *F. nucleatum* is involved in a wide spectrum of illness, including CRC. Fluorescent quantitative polymerase chain reaction and fluorescence *in situ* hybridization are laboratory techniques commonly used in practice.

Peer-review

The authors of this study have investigated the relationship between *F. nucleatum* infection with colorectal cancer in Chinese patients.

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