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A pilot study on clinicopathological features and intestinal microflora changes in colorectal cancer patients born over a nine-year period encompassing three years before and after the Great Chinese famine

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ABSTRACT

Background: Exposure to energy restriction during childhood is associated with a lower risk of developing colorectal cancer (CRC). To date, the association between this critical period of growth and prognosis of CRC has rarely been investigated. Changes in microbiota and epigenetic dysregulation may be key underlying mechanisms.

Methodology: Tissues collected from patients born between 1956 and 1964 were grouped based on time-period. The differences in overall survival among patients from the three time-periods were examined via univariate analysis. The 16S rRNA gene sequencing approach was to determine differences in microbiota among the groups. Samples were randomly selected to detect BRAF mutations, microsatellite instability (MSI) and promoter CpG island methylator phenotype (CIMP) status. The chi-square test was to assess the relationship between alterations in these molecules and microbiota differences.

Results: Patients from the three groups differed in terms of location of CRC (P = 0.034) and carcinoembryonic antigen (CEA) level (P = 0.036). A survival advantage was observed in the famine group compared with the other two groups. *Fusobacterium nucleatum*, *Bacteroides fragilis* and *Escherichia coli* were more abundant in the two comparing groups. Abundance of *B. fragilis* was associated with BRAF mutations, microsatellite instability (MSI) and abundance of *E. coli*. Moreover, the incidence of CIMP and MSI was higher in patients with greater abundance of *F. nucleatum*.

Conclusions: Limitation of energy intake during childhood may affect the composition of gut microbiota, resulting in persistent epigenetic changes that subsequently influence the prognosis of patients with CRC.

1. Introduction

Colorectal cancer is the fourth most commonly diagnosed cancer worldwide [1], resulting in 700,000 annual deaths in both men and women [2]. This cancer type represents a heterogeneous disease involving different sets of somatic molecular alterations that are influenced by diet, environment, microbial exposure and host immunity [3–5].

Several preliminary studies have shown that energy restriction protects against the development of CRC in animals. Consistently, observational research suggests that energy restriction during childhood and adolescence exerts a strong protective effect against CRC development and mortality [6,7]. Two earlier studies from the Netherlands additionally reported that experiences during early life trigger persistent epigenetic changes in humans, such as aberrant regulation of CIMP and MSI [8], but did not elucidate the reasons for these alterations and whether they could affect patient prognosis.

Researchers have proposed a plethora of potential reasons on why

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individuals develop CRC. In recent years, prevailing use of the16SrRNA gene sequencing approach has facilitated effective analysis of the microbial community. The microbiota is currently the subject of extensive research debate. Accumulating evidence indicates positive associations of *Fusobacterium* (a group of non-spore forming, anaerobic gram-negative bacteria) with status of CIMP and MSI [9].

Using a patient population from the period of the "Great Famine" in China, we investigated the associations between energy restriction in childhood and CRC prognosis as well as risk of developing CIMP[-positive] tumors in later life. Since CIMP[-positive] tumors are associated with MSI, which is also related to CRC patient prognosis [10,11], the relationship between energy restriction during childhood and development of tumors characterized by MSI was additionally examined.

In the current study, Kaplan-Meier analysis was applied to assess overall survival (OS) among CRC patient groups from three time-periods (born three years before, during and up to three years after the Great Chinese famine). Microbiota compositions and epigenetic alterations were additionally examined among the different patient groups, with a view to establishing the influence of energy restriction during childhood on prognosis.

2. Methods

2.1. Study population and design

In total, 179 individuals scheduled for colorectal resection at Qingdao University, Qingdao, China, were recruited for study. All patients born between 1956 and 1964 (three years before, during and after the China Famine) with stages I–III cancer who received standard curative surgery were recruited, while stage IV CRC tissues were collected from patients who received palliative surgery to relieve serious cancer-related contraindications. Individuals were not treated with antibiotics for one month prior to surgery but administered antibiotics intravenously a few hours before the procedure. Patients that had used or were regularly using non-steroidal anti-inflammatory drugs, statins or probiotics were excluded. Further criteria for exclusion included chronic bowel disease, other signs of infection, food allergies and dietary restrictions. Surgeries were performed at the General Surgery department of the Affiliated Hospital of Qingdao University between 2010 and 2012, and subsequent treatments carried out as recommended by the National Comprehensive Cancer Network Guidelines.

2.2. Assessment of energy restriction

It is widely accepted among Chinese researchers that the three-year famine in 1959–1961 was triggered by a series of events, including several waves of accelerated agricultural collectivization, nationwide establishment of the commune system, and especially the "Great Leap Forward" movement that began in 1958 [12,13]. The famine provides unique cases for studying the long-term effects of famine in general. The uniqueness of this famine not only lies in its long lifespan, unprecedented severity and scope of incidence, but also its substantial variation across regions. Numerous reports support the possibility that prenatal famine exposure has a positive or plasticity effect on health [14,15].

The effects of the famine were significantly more devastating in rural than urban areas. However, exposure to famine also varied greatly across provinces due to variations in population density, exposure to bad weather and provincial response to food shortage, among other contributory factors. Individual food intake data for cohort members were not available for the three periods and therefore proxy variables were used to describe exposure to energy restriction. We selected the place of residence during every period as an indicator for severe energy restriction. During the great leap forward famine (GLFF) period, food rationing was introduced, and owing to poorer food availability in rural areas, nutritional differences emerged between rural and urban residents [16].

2.3. Sample preparation

Tumor specimens were obtained from 179 patients subjected to proctocolectomy born between 1956 and 1964. CRC and adjacent normal tissue samples (at least 5 cm from the tumor site) of these patients were obtained from the gastrointestinal cancer specimen bank of Affiliated Hospital of Qingdao University, Qingdao, China. Specifically, surgically resected specimens were collected immediately after tumor removal and stored at −80 °C until use. TNM staging was determined according to the American Joint Committee on Cancer system and all specimens were graded histologically based on World Health Organization classification criteria. Written informed consent for joining the specimen bank was obtained from all patients before surgery. All protocols were approved by the Ethics Committee of Affiliated Hospital of Qingdao University. Clinical and pathologic data were reviewed from the gastrointestinal cancer database of the Affiliated Hospital of Qingdao University, Qingdao, China. For detection of the associations between bacterial species among the three periods, 57 patients corresponding to the Before famine group (born 1956–1958), 62 to the GLLF group (born 1959–1961), and 60 to the After famine group (born 1962–1964) were examined.

2.4. DNA extraction

We employed the cetyl trimethylammonium bromide (CTAB) method with minimal modifications to extract DNA from all tumor samples. DNA concentrations were measured using a fluorometer or microplate reader, and sample integrity determined via agarose gel electrophoresis (1% agarose gel; 150 V; 40 min electrophoresis time). All DNA samples were stored at −20 °C until use.

2.5. PCR and sequencing analysis

The V4 region of the bacterial 16S rRNA gene was amplified via polymerase chain reaction (PCR) using the universal primers 319 F and 806R. The reaction mixture consisted of Phusion High-Fidelity PCR Master Mix (NEB, Ipswich, MA, USA) and the appropriate primer/probe pairs. The PCR conditions used were as follows: denaturation at 98 °C, 30 cycles of 45 s at 95 °C (denaturation) in 3 min, annealing at 55 °C and 45 s at 72 °C (extension) in 45 s, with a final extension step at 72 °C for 7 min. Amplified products were purified with AMPure XP beads (Agencourt Bioscience) to remove non-specific products prior to library construction. The library was quantitated in two ways: average molecule length was determined using the Agilent 2100 bioanalyzer instrument (Agilent DNA 1000 Reagents) and quantified via real-time quantitative PCR (qPCR; EvaGreen TM). Sequencing of qualified libraries was performed by the BGI-Huada Genomics institute in Shenzhen using the MiSeq System, with the sequencing strategy PE250 (PE251 + 8 + 8 + 251) or PE300 (PE301 + 8 + 8 + 301) (MiSeq Reagent Kit).

2.6. Bioinformatics analysis

Sequences were clustered into operational taxonomic units (OTU) with a 97% threshold using USEARCH (v7.0.1090) [17], and OTU unique representative sequences obtained. Chimeras were filtered out using the UCHIME (v4.2.40) algorithm [18]. Representative OTUs were aligned to the optimized sequences and abundance of OTUs per sample measured for further analysis. Ribosomal Database Project (RDP) Classifier v.2.2 was applied for taxonomic classification of OTU representative sequences in the following databases: Greengene V201305 [19] and RDP (Release9 201203) [20].

2.7. Detection of BRAF mutations

Based on the BRAF V600E region, specific primers and probes were designed and an optimal real-time fluorescence PCR method established. Melanoma and normal clinical samples and BRAF V600E mutant plasmids were used for evaluation of accuracy, sensitivity and specificity of the method. "Mutation Target Primers" were designed specifically in the 3' direction of the Amplification Refractory Mutation System (ARMS) for the human BRAF gene V600E mutation type based on the principle of base complementarity. The primers were found only when the gene was c.1799 T > A mutation to match the BRAF mutant gene and specifically amplified by DNA polymerase. Internal control primers and probes were designed for the exon parts of the human BRAF gene. The probe reporter group was marked with HEX fluorescence' group and the signal truly reflected sample DNA quality to avoid false detection results.

2.8. Promoter methylation analysis

CpG island promoter hypermethylation was defined in cases positive for at least 2 out of 5 methylation markers (CACNA1G, CDKN2 A, NEUROG1, CRABP1and MLH1) as proposed by Weisenberger et al. [21]. The methylation status of these markers, used to create a methylation index with CIMP markers, was determined via bisulfite modification of 500 ng genomic DNA using a commercially available kit (Zymo Research) and subsequent methylation-specific PCR (MSP) [22,23]. MSP was employed to detect CIMP, since it is effective, specific and does not require specialist equipment. Additionally, no differences in performance have been reported between MSP and other technologies, such as MethyLight [24].

2.9. Microsatellite instability analysis

As a second-generation genetic marker, MSI is widely used in tumor gene diagnosis and analysis owing to a high level of polymorphism, stability and Mendelian co-dominant inheritance. MSI was determined via multiplex fluorescent PCR combined with capillary electrophoresis (CE) using the specific markers NR-21, BAT-26, NR-27, BAT-25, NR-24 and MONO-27, as described in detail by Suraweera et al. [25]. This method shows high efficiency, stability and sensitivity and generates reliable results. After amplification via multiplex fluorescent PCR, the CE method using dual internal standards (molecular weight markers shorter and longer than the PCR fragment of interest) was applied to measure microsatellite length. To further validate the use of CE to detect microsatellite instability, human tumors and matching control DNA specimens were used from patients.

2.10. Statistical analysis

Metastats (http://metastats.cbcb.umd.edu/) and R (v3.0.3) were used to determine the taxonomic groups that were significantly different between groups of samples. We adjusted the P-value obtained using Benjamini-Hochberg false discovery rate (FDR) correction (function 'P.adjust' in the stats package of R (v3.0.3)) [26]. Continuous data are presented as means ± standard deviation, unless otherwise stated. The P-values for Bray-Curtis and Weighted Unifrac distance were calculated via Analysis of similarities between groups (ANOSIM). Differences between groups were analyzed using the Student's *t* test (two-tailed). Associations between clinicopathological variables and differences among the three groups were examined with the χ 2 test. The Kaplan–Meier method was used to compare the overall survival (OS) curves. P-values were two-sided, and data were considered statistically significant at $P < 0.05$. Data analyses were performed with SPSS software 19.0.

Table 1
Clinicopathological characteristics of enrolled patients.

		the period			
	all patients	BF	GLLF	AF	p value
Place of residence					0.007*
rural area	66	20	20	26	
urban area	113	37	42	34	
Father's work					0.300
Farmer	98	34	36	28	
non-Farmer	81	23	26	32	
Gender					0.32
Male	109	36	41	32	
Female	70	21	21	28	
Location					0.034*
Colon	72	24	22	26	
Rectum	107	33	40	34	
Tumor size					0.369
< 5cm	100	32	39	29	
> = 5 cm	79	25	23	31	
CEA					0.036*
Normal	109	27	43	39	
Elevated	70	30	19	21	
Grade of differentiation					0.144
Well	134	37	49	47	
Poor	43	20	13	10	
Depth of invasion					0.112
T1 and T2	54	22	16	26	
T3 and T4	125	35	46	34	
Lymph node metastasis					0.893
Negative	101	31	34	36	
Positive	78	26	28	24	
Remote Metastasis					0.388
Negative	172	55	58	59	
Positive	7	2	4	1	
TNM stage					0.691
I and II	94	32	30	32	
III and IV	85	25	32	28	

* Statistically significant, $P < 0.05$.

3. Results

3.1. Correlation of specific time-periods of birth with clinicopathologic features of CRC

In total, 179 patients were enrolled (among whom 57 were assigned to the "Before famine" group, 62 to the "GLFF" group and 60 to the "After famine" group) and clinical characteristics compared (Table 1). We observed no significant differences with regard to place of residence ($P = 0.070$), father's work ($P = 0.300$), gender ($P = 0.320$), tumor size ($P = 0.369$), grade of differentiation ($P = 0.144$), depth of invasion ($P = 0.112$), lymph node metastasis ($P = 0.893$), remote metastasis ($P = 0.388$) and TNM stage ($P = 0.691$), while location of CRC ($P = 0.034$) and CEA levels ($P = 0.036$) were significantly different among the groups.

3.2. Differences in prognosis among the three groups

Kaplan-Meier analysis and log-rank test were employed to analyze the relationship between specific time-periods and patient survival. Notably, patients born within the famine period had higher 3-year and 5-year OS, although the differences were borderline significant (Fig. 1).

Sequencing of libraries of 16S rRNA V4 region amplicons from 179 CRC tumor samples led to the generation of a total of 8091485 high-quality and classifiable reads, with an average of 45,204 reads per sample. At a 3% dissimilarity level, a total of 2891 OTUs in all samples and an average of 231 OTUs per sample were identified.

The Good's coverage value for each group was over 99%. The estimators of community richness (observed species and Chao indices) and diversity and evenness (Shannon and Simpson indices) were

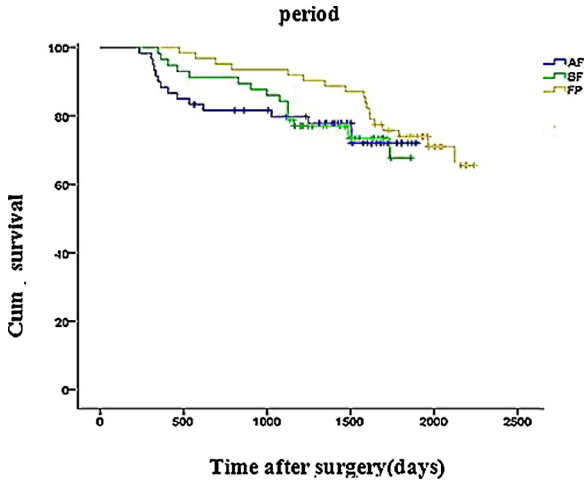
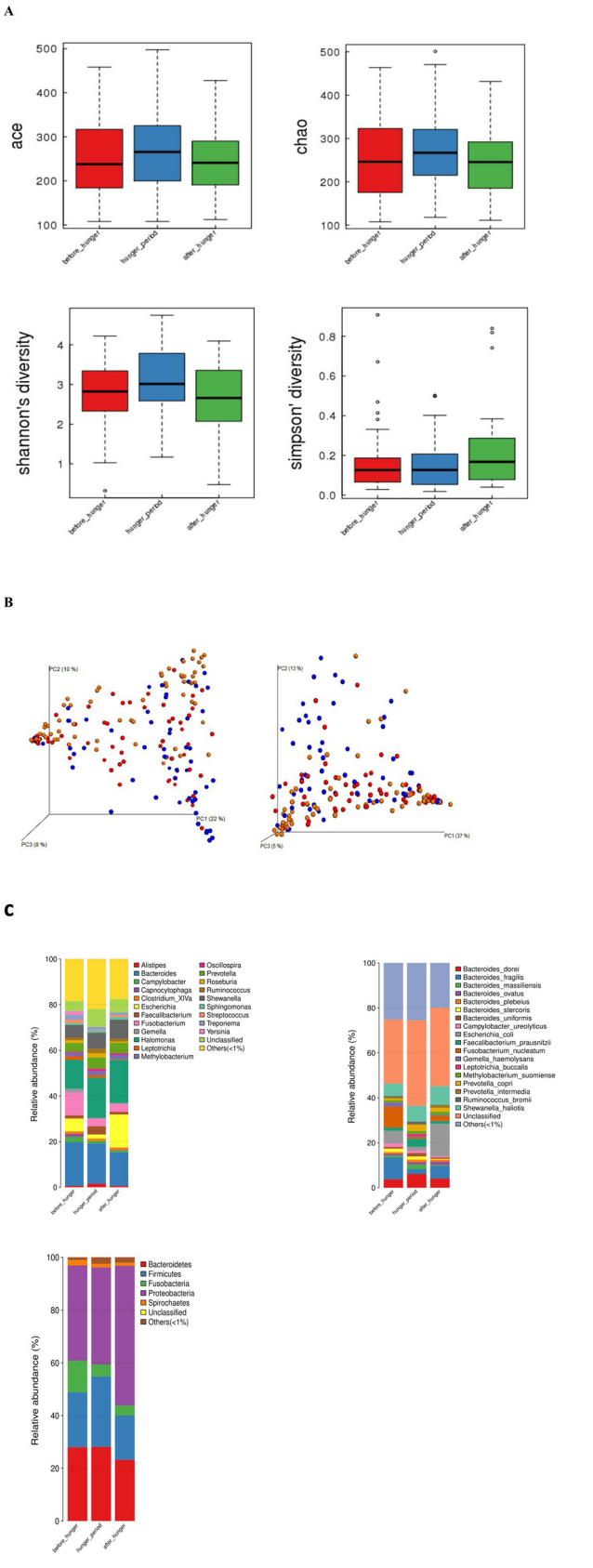


Fig. 1. Kaplan–Meier survival curves for overall survival (OS) in relation to the three periods (P values obtained via log-rank test). Significant differences in mucosal microbiota throughout the colorectal region between patients during the China Great Leap Forward Famine (GLFF) and controls.

examined (Fig. 2A). Significant differences were detected among the three groups in Shannon and Simpson diversity indices (Shannon, 2.82 ± 0.82 vs. 3.76 ± 0.23 vs. 2.64 ± 0.83, P = 0.02; Simpson, 0.17 ± 0.17 vs. 0.22 ± 0.18 vs. 0.21 ± 0.17 P value), with highest diversity determined for the GLFF group.

For beta diversity analysis, microflora and compositions were analyzed and compared based on the relative abundance of OTUs using Bray-Curtis distance and weighted Unifrac distance matrix for each group. Differences in bacterial community composition among groups were detected in subsequent results of principal coordinates analysis. The first three principal component scores of Bray-Curtis distance matrix and weighted Unifrac distance matrix were 22%, 10%, 8% and 42%, 14%, 4%, respectively (Fig. 2B). Significant differences were detected in Bray-Curtis distance (P = 0.012), indicative of distinct community memberships of each group. The microbial composition was different at the genus level among groups. *Streptococcus* (Before famine vs. GLFF: 1.40% vs. 0.07%, FDR = 0.025), *Faecalibacterium* (GLFF vs. After famine: 3.24% vs. 1.11%, FDR = 0.005) and *Escherichia coli* (GLFF vs. After famine: 13.01% vs. 1.73%, FDR = 0.005), which constitute over 1% of total bacteria in the survival group, exhibited relatively higher abundance in the Before famine and After famine groups, while *Fusobacterium* (Before famine vs. After famine: 9.25% vs. 3.23%, FDR = 0.008; Before famine vs. GLFF: 9.25% vs. 3.64%, FDR = 0.027) was relatively more abundant in the Before famine group compared with GLFF and After famine groups, although these changes were borderline significant.

At the species level, we observed higher abundance of *B. fragilis* (Before famine vs. GLFF: 9.59% vs. 2.16%, FDR = 0.004; GLFF vs. after famine 2.16% vs. 5.11%, FDR = 0.028) in patients born before and after the famine period, while *F. prausnitzii* (Before famine vs. GLFF: 1.33% vs. 3.25%, FDR = 0.004) was more abundant in the GLFF group. Moreover, borderline differences were observed in *F. nucleatum* levels among the three groups (Before famine vs. GLFF: 8.64% vs. 0.69%, FDR = 0.004; GLFF vs. After famine 0.68% vs. 2.21%, FDR = 0.094), with greater abundance in patients born before and after famine than in the GLFF period. The composition pattern of *E. coli* (GLFF vs. after famine 13.01% vs. 1.73%, FDR = 0.005) was similar to that of *F. nucleatum* and *B. fragilis* (Fig. 2C).



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3.3. CpG island methylator phenotype (CIMP) and microsatellite instability (MSI)

Sixty patients were randomly selected for detection of CIMP and MSI. Among these, 14 (23%) were MSI-high, 13 (21%) were CIMP-high (≥2/5 methylated CIMP-specific markers, i.e., CACNA1G, CDKN2 A, CRABP-1, MLH-1 and NEUROG-1), and 15 (25%) contained a mutation

Fig. 2. Diversity and structural changes of tumor microbiota among the Before-Period Before famine (n = 58), GLLF (n = 62), and After-Period After famine (n = 60) groups. **A.** Alpha-diversity distances calculated using phylotype relative abundance measurements among groups. *: statistically significant $P < 0.05$. **B.** Principal coordinates analysis (PCoA) scores plot of Bray-Curtis distance matrix and weighted Unifrac distance matrix based on the relative abundance of OTUs (97% similarity level). Each symbol represents a sample. Colors signify different groups. **C.** Dominant phyla, genera and species of the groups.

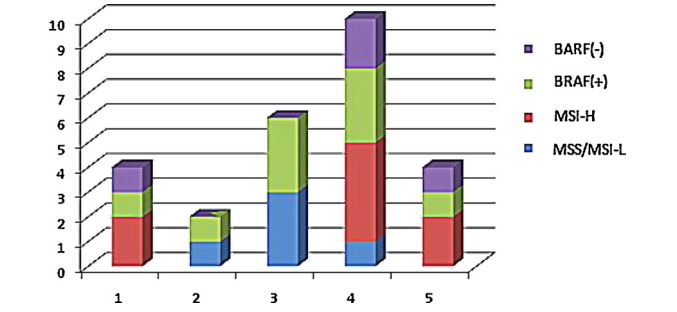

Fig. 3. Distribution of colorectal cancer, BRAF mutations and status of MSI according to number of methylated CIMP markers. Bimodal distribution of tumors is evident. BRAF mutations are common in highly methylated tumors. MSI-H status is common in tumors with more methylated markers.

in BRAF. Tumors were distributed bimodally according to the number of methylated CIMP markers (Fig. 3), and BRAF mutations and MSI-H state (> 2/5 methylated markers) were common in CIMP-high tumors'.

3.4. Comparison of molecular features in patients from the three groups

After detection of BRAF mutations and status of MSI and CIMP, we compared these molecular features in relation to bacterial abundance. Based on the relative abundance of each microbiota in tumor samples, patients were divided into high and low bacterial subgroups with median relative abundance as the cut-off (*B. fragilis* high vs. *B. fragilis* low, *F. prausnitzii* high vs. *F. prausnitzii* low, *F. nucleatum* high vs. *F. nucleatum* low, and *E. coli* high vs. *E. coli* low). We observed correlations of *B. fragilis* and *F. prausnitzii* with BRAF mutation. Higher incidence of MSI was observed in patients with greater abundance of *E. coli* and *F.nucleatum*, with significant differences. We observed significant differences in CIMP in patients with varying *F. nucleatum* abundance. Moreover, MSI status differed among patient groups from the three periods (P = 0.018) (Table 2).

Table 2
Correlations between the molecules examined and microbiota in the three groups.

	MSI			CIMP			BRAF		
	MSS/MSI-L	MSI-H	*P* value	(-)	(+)	*P* value	(-)	(+)	*P* value
BF			0.180			0.105			0.036*
BFH	21	9		21	9		19	11	
BFL	25	5		26	4		26	4	
FP			0.180			0.266			0.015*
FPH	25	5		25	5		27	3	
FPL	21	9		22	8		18	12	
FN			0.031*			0.029*			0.500
FNH	19	11		20	10		23	7	
FNL	27	3		27	3		22	8	
EC			0.002*			0.500			0.276
ECH	18	12		23	7		21	9	
ECL	28	2		24	6		24	6	
Period			0.018*			0.675			0.208
Before famine	11	9		15	5		13	7	
GLLF	18	2		17	3		17	3	
After famine	17	3		15	5		16	4	

* Statistically significant $P < 0.05$.

4. Discussion

In this study, we compared the prognosis of colorectal cancer patients born during the “Great Chinese Famine” with those born within three years before or after this period. Our data suggest that patients who experienced energy restriction during early life had better overall survival. We hypothesized that the early life limitation of energy intake affects the formation of microflora in early childhood, leading to persistent alterations in intestinal microbiota. Changes in specific bacterial levels during this process could affect MSI, CIMP and BRAF gene expression patterns, in turn, influencing prognosis of colorectal cancer in adult life.

The goal of the "Great Leap Forward" in China was to accelerate the pace of industrialization and urbanization by mobilizing rural surplus labor to participate in labor-intensive non-agricultural productive activities, such as making iron and steel using back yard blast furnaces [27]. However, the results turned out to be disastrous. Much of the iron and steel produced in back yards was useless, grain production declined partly because of a shortage of agricultural labor, and the rapidly expanding urban nonagricultural population resulting from rural-to-urban migration exacerbated the food supply problem. Beginning from 1959, the second year of the Great Leap Forward movement, China experienced a severe famine affecting the whole country [28]. Over 30 million people died from starvation or severe malnutrition and about 33 million births were either lost or postponed during the three-year period from 1959 to 1961 [29]. In the current study, we additionally examined the influence of location of the patient's mother (i.e., residence in the city (16%), town (22%), or village (62%)). Our data indicate that people living in cities were better protected from famine than those in towns and villages, while those in towns were better protected than villagers [30]. Moreover, in 1958, large-scale communes were formed as part of the nationwide movement. Contrary to expectations, however, agricultural production plunged dramatically for three successive years and widespread famine ensued [31]. During 1959-61, the death rate per thousand people increased dramatically while the crude birth rate per thousand declined equally precipitously [32]. Children born in this period also experienced extreme hunger and suffering. Ideally, the severity of malnutrition would be better measured by calorie intake at an individual level. Unfortunately, these data are unavailable.

Limited studies on famine exposure in cancer etiology are documented in the literature, which have mainly been conducted by European countries. For instance, earlier epidemiological investigations on famine survivors of the Dutch hunger in 1944–1945, a unique period of Dutch history, provided rare observational evidence suggesting that severe energy restriction during adolescence may lower CRC risk at a number of tumor sub-sites, particularly in men. These results provide further insights into the role of energy intake during this period of life in CRC development. The group showed that exposure to famine conditions during adolescence is inversely associated with the risk of developing CIMP[-positive] tumors in later life, leading to the conclusion that adolescence is a critical period of development of epigenetic changes that can influence CRC risk. These two studies revealed differences in MSI and CIMP findings, but did not explain how early life energy limitations affect MSI and CIMP status. More recent studies have shown that these types of changes affect prognosis of patients, but the underlying reasons are not discussed [33–35].

Earlier research comparing the relationship between famine exposure and cancer risk in early life demonstrated increased mortality

rates in gastric cancer [36]. A study focusing on the Shanghai area reported a diverse incidence rate of malignant tumors in different organs. For example, the incidence of gastric cancer and famine was shown to be positively correlated, while colorectal, liver, pancreatic and other cancer types were negatively correlated. In terms of gender, female groups exhibited higher susceptibility than men, which presents another potential key area for future research [37].

The most direct effect of dietary or energy intake is on intestinal microbiota, and breast milk is essential for the formation of intestinal microecology at a very early age [38,39]. Under conditions where mothers experience food shortage, the quality and volume of breast milk declines, which affects gut microbiota of offspring. Studies to date have shown that rapid expansion of bacterial diversity observed in infancy is significantly slower during early childhood (between 0 and 5 years of age), followed by relatively stable intestinal microbial diversity in adults [40]. Given the significant associations of gut microbiota with GI tract cancer, MSI and CIMP status [34,41,42], we aimed to ascertain whether early life energy restrictions affect colorectal cancer status in adulthood via influencing formation of microbiota.

To validate our hypothesis, we used data from 179 patients with colorectal cancer treated at the Department of General Surgery in Qingdao Affiliated Hospital from 2010 to 2012. Specifically, we compared clinical pathology and survival of patients who experienced three years of natural famine with those who were not subjected to energy restrictions. Notably, patients subjected to early life energy restrictions had an advantage in terms of survival relative to patients in the other groups, although these differences were only marginally significant. The 16S DNA sequencing method was further applied to detect differences in gut microbiota among the three groups. We focused on *F. nucleatum*, *B. fragilis*, *F. prausnitzii* and *E. coli*, since these bacteria have relative high abundance and are significantly associated with colorectal cancer [43–45].

The specific mechanisms by which the gut microbiota affects the development of CRC are not well understood at present. One of the most promising theories is effects through alterations in genetics and epigenetics. Interestingly, *F. nucleatum*, *B. fragilis*, *F. prausnitzii* and *E. coli* are key players in modifying CIMP, MSI and other genetic sites in CRC. Our data suggest that *F. nucleatum* is associated with MSI and CIMP. *F. nucleatum* is reported to enhance production of ROS and inflammatory cytokines in colorectal cancer. Inflammation and ROS cause epigenetic silencing of the mismatch repair protein, MLH1, leading to MSI. *F. nucleatum* exerts immunosuppressive effects by inhibiting human T-cell responses and modulating the tumor immune microenvironment in a suppressive manner. MicroRNA-21 enhances the levels of IL-10 and PGE2, which suppress antitumor T-cell-mediated adaptive immunity in the tumor microenvironment [46]. According to our results, patients who did not experience early life energy restriction showed higher abundance of *F. nucleatum*, which may explain the high MSI observed in the before and after GLFF groups.

In the study by Boleij et al. investigating expression of the *Bacteroides fragilis* gene (BFT) in colonoscopic samples from 49 healthy individuals and 49 colorectal cancer patients, the gene was detected more frequently in samples from colorectal cancer patients. Upon comparison of early- and late-stage cancer patients, BFT gene expression was more prevalent detected in late-stage cancer patients. Further research revealed an association between *B. fragilis* and BRAF [9], consistent with our findings. The potential association between *B. fragilis* and BRAF and its link with CRC is worth exploring in future studies.

In addition, studies have reported a correlation between pathogenic *Escherichia coli* and MSI, which can downregulate the mismatch repair component of intestinal epithelial cell lines in vitro via transcriptional mechanisms that rely mainly on the type III secretion system bacterial effect of EspF [47]. EspF can regulate several cellular processes, including phagocytosis, mitochondrial function and maintenance of cell integrity. During EspF-mediated regulation of mitochondria, the protein consumes the MMR proteins MSH2 and MLH1, leading to increased frequency of spontaneous mutations in infected cells at sites of microsatellite instability and presence of MSI in CRC. This finding may provide a clue to account for high level of microsatellite instability in CRC [48] and explain why we are more likely to find high MSI in the high *E. coli* abundance group.

MMR protein expression is positively correlated with that of MSI. In this study, specific bacteria were positively correlated with MMR, which may explain the variations in probability of MSI within patients from the GLFF group.

F. prausnitzii enters a vigorous breeding period under conditions of diet and energy restriction, and short-chain fatty acids (SCFA) produced from *F. prausnitzii* could reduce the possibility of MMR. Butyrate is reported to accumulate in cancerous murine CECs, supporting its function as a histone deacetylase (HDAC) inhibitor [49]. Inhibition of HDACs suppresses mRNA expression of the cell cycle regulators p21 and p27, and enhances transcription of the proapoptotic gene Fas, contributing to reduced cell proliferation and increased apoptosis [50].

Additionally, it is important to address some unavoidable limitations of our study. Information on individual food intake for the cohort was not available for the exposure periods, and therefore, precise measurements could not be used to determine energy restriction during GFLF. However, it was possible to use information on the place of residence during this period and father's work to reflect similar situations.

In conclusion, our study makes an assumption that components of the microbiota, such as *B. fragilis* and *F. prausnitzii*, participate in influencing the course/progression of CRC in patients subjected to energy restriction in their early childhood and further validates the association of *F. nucleatum* with epigenetic changes and gene mutations. Unique evidence from patients born during the GLFF period in China shows that exposure to periods of severe transient energy restriction during childhood is inversely associated with the risk of developing CIMP-positive tumors in later life. Our collective findings provide insights into how energy restriction in early life influences the development of colorectal cancer and further suggest that energy intake of individuals during this time is associated with epigenetic changes that subsequently influence CRC development.

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest regarding the manuscript's content.

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Author statement

Manuscript title:

I have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND

I have drafted the work or revised it critically for important intellectual content; AND

I have approved the final version to be published; AND

I agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All persons who have made substantial contributions to the work reported in the manuscript, including those who provided editing and writing assistance but who are not authors, are named in the

Acknowledgments section of the manuscript and have given their written permission to be named. If the manuscript does not include Acknowledgments, it is because the authors have not received substantial contributions from nonauthors.

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