Alteration of the abundance of *Parvimonas micra* in the gut along the adenoma-carcinoma sequence

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Abstract. *Parvimonas micra* (*P. micra*) is reported to be associated with colorectal cancer (CRC). However, its association with colorectal adenoma (CRA) and its role in the initiation of colorectal tumors remain unknown. The present study aimed to clarify the relationship between *P. micra* and CRA and CRC by exploring the changes of *P. micra* abundance in an adenoma-carcinoma sequence in a new cohort and 4 public sequencing datasets. To investigate the alterations of *P. micra* abundance in the gut along the adenoma-carcinoma sequence, quantitative PCR (qPCR) was conducted to measure the relative abundance of *P. micra* in fecal samples from 277 subjects (128 patients with CRA, 66 patients with CRC and 83 healthy individuals, as controls) who underwent colonoscopy as outpatients. Then, the relative abundance of *P. micra* was analyzed in fecal samples from 596 subjects (185 healthy controls, 158 CRC, 253 CRA) in four public 16S rRNA sequencing datasets. The qPCR results demonstrated that the CRA group had an abundance of *P. micra* (P=0.2) similar to that of the healthy control group, while the CRC group had a significantly increased abundance ($P=8.2x10^{-11}$). The level of *P. micra* effectively discriminated patients with CRC from healthy controls, while it poorly discriminated patients with CRA from healthy controls; with an area under the receiver operating characteristic curve of 0.867 for patients with CRC and 0.554 for patients with CRA. The same pattern of the alteration of *P. micra* abundance, which was low in healthy controls and patients with CRA but elevated in patients with CRC, was found in all four public sequencing datasets. These results suggested that *P. micra* was closely associated with, and may serve as a diagnostic marker for, CRC but not CRA. Moreover, it was indicated that *P. micra* may be an opportunistic pathogen of CRC, which may promote CRC development but serve a limited role in tumorigenesis.

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Introduction

Colorectal cancer (CRC) is the third most common cancer in the world with >1.3 million cases diagnosed every year, and the incidence of CRC worldwide is predicted to increase to 2.5 million new cases a year in 2035 (1). Furthermore, CRC accounts for ~10% of all annually diagnosed cancer types and cancer-related mortalities worldwide (1,2). Several of the risk factors of CRC, such as obesity, physical activity, smoking and alcohol use, easily affect the metabolic environment of the host, leading to alterations in the intestinal microbial community that may directly or indirectly cause gut microbiota dysbiosis and trigger the development of adenoma and CRC (3-6). It has been reported that ~10^{14} bacteria live within the human intestinal tract, which maintain a healthy gastrointestinal system for regulating processes such as immune regulation, microbial metabolism and host-derived chemical productions (5,7). Compared with healthy controls, patients with CRC have an abnormal gut microbiome structure (8). For example, patients with CRC can be distinguished from healthy individuals using specific microbial markers, including *Fusobacterium nucleatum (F. nucleatum)*, *Peptostreptococcus stomatis*, *Parvimonas micra* (*P. micra*) and *Solobacterium moorei* (2). It has also been revealed that transplanting fecal bacteria from patients with CRC into sterile mice results in the formation of tumors (9). Therefore, these studies suggest a causal relationship between the presence of specific microorganisms and the development of cancer.

P. micra is a fastidious, anaerobic, gram-positive coccus that is found in healthy human oral and gastrointestinal flora (10). Previous studies have reported that *P. micra* is involved in lung abscesses, iliopsoas abscesses, gastric carcinogenesis and infections of the periodontal area, soft tissue, bone and joints (11-14). Currently, based on metagenomic or 16S RNA sequencing analysis, numerous studies have

revealed the relationship between *P. micra* and CRC (15-17). By analyzing the 16S rRNA gene sequence data of 509 fecal samples from ethnically different cohorts, including those from China and Austria, Yu *et al* (2) observed that the detection rate and abundance of *P. micra* were significantly higher in patients with CRC compared with controls, and these results were further validated using quantitative PCR (qPCR) in 309 subjects (18). By analyzing the metagenomics sequencing results from 778 (including 386 samples from patients with CRC and 392 controls) and 969 (meta-analysis of five publicly available databases and two new cohorts with validation of the findings of two additional cohorts) stool samples, two research groups discovered that CRC-related microbial markers, including *P. micra*, could be consistently detected among different populations, regardless of the detection techniques, diet, geographical environment, genetics and other factors (19,20). These results demonstrate that *P. micra* has an important relationship with CRC, and may be involved in the development of CRC.

Most cancer types arise from adenoma, and colorectal adenoma (CRA) is a critical precursor of CRC (21,22). The process of CRC development begins with an aberrant crypt, which evolves into a polyp or adenoma and eventually progresses to CRC over an estimated 10-15 year period (1). Currently, the microbiota associated with CRA have not been consistently identified, and the association between *P. micra* and CRA remains elusive (23-25). Therefore, the present study aimed to investigate the association between *P. micra* and CRA by measuring the changes in the relative abundance of *P. micra* in stool samples obtained along the adenoma-carcinoma sequence using a qPCR method. Furthermore, the alteration pattern of the relative abundance of *P. micra* were evaluated in patients with CRC or CRA by analyzing four public 16S rRNA datasets.

Patients and methods

Patient recruitment and sample collection. An observational case-control study was conducted between January 2017 and March 2019 at The First Affiliated Hospital of Soochow University. Stool samples were collected prior to colonoscopy. All patients with CRC (37 males and 29 females) and CRA (66 males and 62 females) were first diagnosed via colonoscopy screening, and the diagnosis was later confirmed by pathology. The pathological diagnosis was performed by two professionals. Inclusion criteria were as follows: i) Age ≥18 years old; and ii) colonoscopy. The exclusion criteria for all participants included the use of the following medicines: Antibiotics within 1 month of study participation, non-steroidal anti-inflammatory drugs or probiotics. Individuals who reported chronic bowel disorders, food allergies or dietary restrictions were also excluded from the study. Additional exclusion criteria for patients with CRC included chemotherapy or radiation treatment prior to surgery. All patients were categorized according to histopathological features on the basis of the TNM classification of malignant tumors after surgery (26). A total of 83 healthy subjects (43 males and 40 females) were selected as controls by volunteering during a physical examination, and none of the healthy subjects had gastrointestinal tract disorders or any antibiotics treatments in the 3 months before sample collection. The clinical variables included age, sex and BMI (kg/m^2). All 277 participants (age range, 26-88 years) had been local residents of Suzhou city for >5 years prior to the study. In total, one fecal sample was self-collected prior to bowel preparation the day before colonoscopy from each patient or healthy subject. Samples were transported to the laboratory within 24 h after collection.

All individuals provided written informed consent prior to participating in the study. All procedures were performed in accordance with, and were approved by, the ethical standards of the institutional and/or the national research committee [the Ethics Committee of The First Affiliated Hospital of Soochow University; approval no. + 056 (2016)], and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Nucleic acid extraction and storage. Stool samples were immediately frozen in liquid nitrogen and stored at -80˚C. DNA was extracted using a TIANamp Stool DNA kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocols (27). The integrity of DNA was measured via 2% (w/v) agarose gel electrophoresis. Purified nucleic acids were quantified using a Qubit 3.0 instrument (Thermo Fisher Scientific, Inc.), and stored at -80˚C. Nucleic acids were extracted from all stool samples in a single batch by one operator to avoid inter-batch variation.

qPCR. All reactions were performed in a 96-well optical PCR plate. Each reaction contained 40 ng extracted fecal DNA, 250 nM primers and 2X ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) in 20 μl reaction volume. Amplification and detection of DNA was performed with the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following reaction conditions: Initial denaturation at 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The primers sequences (V3-V4) were as follows: *P. micra* forward, 5'-GTCACTACG GAAGAATTTGTC-3' and reverse, 5'-GGCTTGAGCGAT AATAACTTC-3'; and total bacterial DNA forward, 5'-GTG STGCAYGGYTGTCGTCA-3' and reverse, 5'-ACGTCRTCC MCACCTTCCTC-3'. Each sample was assayed three times. Results were analyzed using $2^{-\Delta\Delta Cq}$ method (28).

Meta-analysis of datasets from publications. A systematic PubMed search with the terms 16S, colorectal cancer or adenoma and gut microbiome was performed to identify studies involving 16S rRNA sequencing of stool samples from patients with CRC or CRA, and healthy controls. Available data was only found in four studies: Zeller *et al* (29) (accession no. ERP005534); Zackular *et al* (8) (http://www.mothur.org/MicrobiomeBiomarkerCRC); Baxter *et al* (15) (accession no. SRP062005); and Mori *et al* (30). All four datasets were obtained from samples from patients with CRA or CRC, and healthy subjects as controls (Table I).

Bioinformatics and sequence analysis. During data processing, short overlapping forward and reverse reads from the same fragment were joined together using PANDAseq (v0.21.1) to form overlapping sequences of the

Table I. Characteristics of the datasets included in this study.

Named	Author	Country	Healthy	CRA	CRC	Region of 16S rRNA	Seq platform
crc2	Zeller *et al* (29)	France	50	38	41	V4	Illumina MiSeq
crc4	Zackular *et al* (8)	USA	30	30	30	V4	Illumina MiSeq
crc45	Baxter *et al* (15)	USA+Canada	87	147	79	V4	Illumina MiSeq
crc49	Mori *et al* (30)	Italy	18	38	8	V4	Illumina MiSeq

CRC, colorectal cancer; CRA, colorectal adenoma.

V3-V4 16S region (31). After joining, the resulting fragments were trimmed using Trimmomatic (v0.30) (32). The average probability of a base being called in error was <0.01, and the minimal length of a fragment was 100 bp. Next, the chimeric sequences were removed using Vsearch (v1.9.6) (33). The samples were uniformly subsampled at a rarefaction level of 30,000 sequences per sample, to mitigate bias of the analyses due to differences in sampling depth. Samples with <30,000 reads were removed, and a collection of sequences suitable for further Quantitative Insights Into Microbial Ecology (QIIME v1.9) analysis was thus obtained (34). The sequences were then clustered into operational taxonomic units (OTUs) using a 99% similarity cutoff, and the relative abundances were calculated for the OTUs in each sample. The OTUs were classified using the assign_taxonomy.py script in QIIME using UCLUST (v1.2.22) (35) as an assignment method, and the Silva 99% OTU database, which was a modified version of Silva v132 (36), with the removal of uncultured or unclassified entries and the addition of extra entries from CORE database (37).

Statistical analysis. All statistical analyses were conducted in R software (version 2.15.3; R Foundation for Statistical Computing). For the qPCR method, the abundance (A) of *P. micra* in a sample was calculated as the ΔCq relative to the total bacterial DNA in the sample, and the relative abundance was calculated as ln (A x 10^9+1). For 16S rRNA sequencing data, the A of an OTU in a sample was calculated as the ratio of the sequence count of the OTU relative to the total number of sequences in the sample, and the relative abundance of the OTU was determined as ln (A x 10^6 +1) (38). Differentially abundant OTUs were selected with the Wilcoxon rank-sum test for the comparison of healthy subjects with the CRC or CRA group (39). The Benjamini-Hochberg procedure was used to calculate the false discovery rate according to the adjustment of the P-values obtained from the Wilcoxon rank-sum test.

Comparisons between groups were performed with unpaired Student's t-tests and χ^2 tests for quantitative and categorical variables, respectively. Variables that followed a Gaussian distribution were compared with one-way ANOVA. The correlation between the quantity of the *P. micra* and the characteristics of patients such as age, BMI, sex and tumor progression were calculated using Kendall, Pearson or Mann-Whitney analysis. As the majority of the datasets did not meet the assumptions of a normal distribution, non-parametric Dunn's tests with Kruskal-Wallis tests or the Mann-Whitney U test were used, where applicable. P<0.05 was considered to indicate a statistically significant difference. Meta-analysis was performed using the meta for package (v2.4-0) (40). The DESeq2 package (v1.28.1) was used to conduct the difference analysis on the OTUs of crc2, crc4, crc45 and crc49, and the log2 fold change of each OTU in each sample was obtained (41). Receiver operating characteristic (ROC) curves were drawn using the pROC package (v1.16.2) (42). Other diagrams were generated using the ggplot2 (v3.3.2) and ggpubr packages (v0.4.0) (43,44).

Table II. Demography of patients.

Group	Healthy	CRA	CRC	P-value
Sample	Stool	Stool	Stool	
Sex				0.882
Male	43	66	37	
Female	40	62	29	
Age, years	55.2±3.7	56.8±10.9	59.2±10.4	0.196
Height, cm	165.6±7.8	163.8±8	164.2±8.7	0.443
Weight, kg	63.9±16.3	74.2±28	65.4±16.7	0.026
BMI, kg/m^2	22.7±3.1	24.1±3.8	23.6±3	0.069

Data are presented as the mean ± SD. CRC, colorectal cancer; CRA, colorectal adenoma.

Results

Evaluation of P. micra using qPCR. To investigate the associations between *P. micra* and CRA and CRC, qPCR was performed to detect the relative abundance of *P. micra* in the fecal samples of 277 subjects (including 83 healthy controls, 128 patients with CRA and 66 patients with CRC) recruited from Suzhou (Table II). The results demonstrated that the relative abundance of *P. micra* in patients with CRC was significantly higher compared with the healthy controls and CRA (CRC vs. control, P=8.2x10^{-11}; CRC vs. CRA, P=4.9x10^{-8}; Fig. 1A), while the relative abundance in patients with CRA was not different from the healthy controls (P=0.2; Fig. 1A). Then, 80% of the samples were used as the training set and the rest as the test set to establish a prediction model, and it was found that CRC samples could be distinguished from healthy control samples with an area under the curve (AUC) of 0.867 and a cutoff of 8.589 (Fig. 1B). The model performed well for the test set with an FPR (false positive rate) of 0.053 and an

Table III. Diagnostic performance of *Parvimonas micra*.

Value	Group	CRC vs. healthy	CRA vs. healthy
Actual value			
	Healthy	19	19
	CRC/CRA	10	26
Predicted value	Healthy	18	17
	CRC/CRA	7	10
False positive rate		0.3	0.615
False negative rate		0.053	0.105

CRC, colorectal cancer; CRA, colorectal adenoma.

Table IV. A correlation between the relative abundance of *Parvimonas micra* and the characteristics of patients.

Factors	r	P-value	Method
Age, years	0.2812	0.00006	Pearson
BMI, kg/m^2	-0.0319	0.6759	Pearson
Sex		0.4845	Mann-Whitney
Tumor stage, I, II, III, IV	-0.0720	0.5383	Kendall

r: 0-0.3, uncorrelated; 0.3-0.5, weakly correlated; 0.5-0.8, moderately correlated; >0.8, strongly correlated.

FNR (false negative rate) of 0.3 (Table III). However, the CRA samples were poorly distinguished from the healthy control samples (AUC, 0.554 at a cutoff of 8.311) with an FPR of 0.105 and an FNR of 0.615 (Fig. 1C; Table III). These results suggested that *P. micra* may serve as a diagnostic marker for CRC, but not for CRA.

It was also identified that *P. micra* was predominantly enriched in stages I/II and III/IV of CRC compared with the healthy controls (Fig. 2A), and the relative abundance of *P. micra* was not affected by the sex, BMI or the site of cancer origin (right and left) of the patients but was affected by age when a Pearson correlation was used (Fig. 2B and C; Table IV).

Meta-analysis of 16S rRNA sequencing datasets. The association between *P. micra* and CRC was analyzed in four public datasets. A total of 596 samples, including 158 CRC, 253 CRA and 185 healthy control samples, were included in the analysis after quality filtering. Compared with the healthy controls, the relative abundance of *P. micra* in patients with CRC was significantly higher in all four datasets (crc2, P=0.001; crc4, P=0.023; crc45, P=0.0001; crc49, P=0.036) but was not different in patients with CRA (P=0.18-0.94) (Fig. 3A). Furthermore, there were significant increases in the fold changes in the relative abundance of *P. micra* in the CRC group compared with the healthy control group in all four datasets, while there were few changes between the healthy control and CRA groups (Fig. 3B and C).

Discussion

The present study used qPCR to measure the relative abundance of *P. micra* in healthy individuals, patients with CRA and patients with CRC, and demonstrated that the relative abundance of *P. micra* was similar in the healthy and CRA groups, but significantly increased in the CRC group. The fecal level of *P. micra* could effectively distinguish patients with CRC from healthy controls (AUC, 0.867) but could only poorly distinguish patients with CRA from healthy controls (AUC, 0.554). The same alteration pattern in fecal *P. micra* abundance, which was low in healthy controls and patients with CRA, but elevated in patients with CRC, was identified in all four public 16S RNA sequencing datasets. These results suggested that *P. micra* was closely associated with, and may serve as a diagnostic marker of, CRC but not CRA. To the best of our knowledge, the present study was the first to demonstrate the association of *P. micra* with CRC but not CRA in a large number of cases and using two different methods. The present study had some limitations, such as the mechanism of *P. micra* in CRC initiation and development was not clear and whether changes in its abundance are influenced by a number of host extrinsic factors, including diet medications and other lifestyle components, such as exercise, smoking, and sleep cycles was not assessed. The aforementioned points should be explored in future studies.

Early screening is essential for the prevention of CRC and the survival of patients with CRC, as the 5-year survival rate >90% if CRC is detected at an early stage but decreases to 10% if it is discovered at an advanced metastatic stage (24). Currently, the methods for CRC screening are the fecal occult blood test (FOBT), fecal DNA test, detection of tumor markers and colonoscopy. However, these methods suffer from high costs, invasiveness and/or low sensitivity (8,25). The FOBT is currently the standard non-invasive screening test, which has limited sensitivity and specificity for CRC and does not reliably detect precancerous lesions (29). A previous study indicated that the accuracy of fecal microbiota detection was similar to that of the standard FOBT, and when both approaches were combined, the sensitivity can be ≤45% while maintaining the specificity of FOBT (29). In addition, combining a fecal immunochemical test (FIT) with the detection of diagnostic markers, such as *F. nucleatum*, *Peptostreptococcus anaerobius* and *P. micra*, can significantly increase the detection rate for CRC with a sensitivity of 92.3% and a specificity of 93.0% (18). The combined test identifies >75% of the CRC samples missed by the stand-alone FIT (18). Similarly, the present results suggested that the fecal level of *P. micra* can effectively distinguish patients with CRC, indicating that *P. micra* can be used as a diagnostic marker for CRC screening.

To evaluate the role of the gut microbiota in CRC initiation and development, researchers have proposed a number of models (45-47), including the 'driver-passenger' model, first suggested by Tjalsma *et al* (48). In the 'driver-passenger' model, the 'drivers' are defined as microbial species that increase in abundance in the early stage of CRC, such as adenoma, while the 'passengers' are defined as those species that increase in abundance in the late stage of CRC (46). Drivers are the primary pathogens that cause the initiation

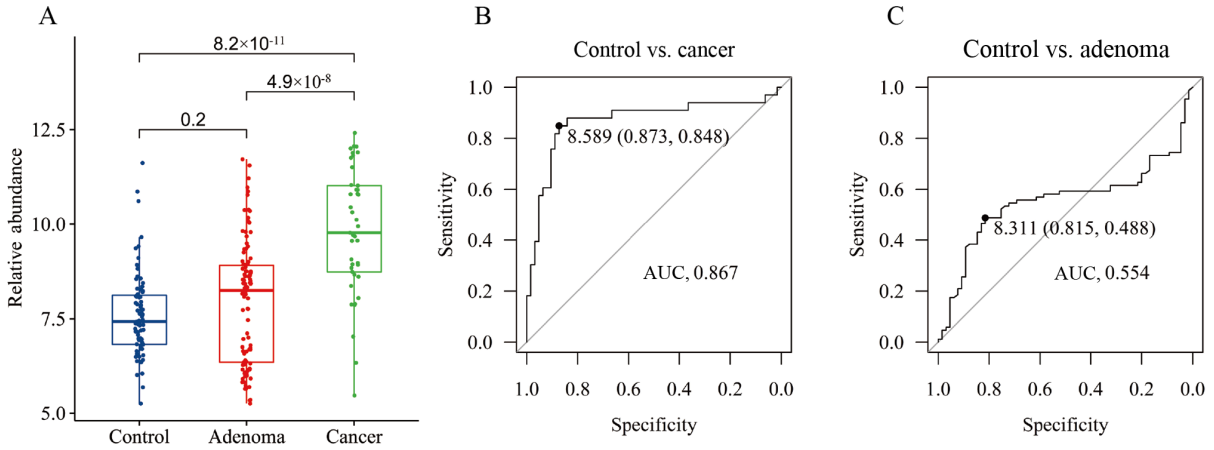

Figure 1. Quantitative detection of fecal *P. micra* in samples from healthy controls, patients with CRC and patients with CRA in the Suzhou cohort. (A) Boxplot of *P. micra* relative abundances in the healthy control, CRA and CRC groups. Receiver operating characteristic curve of *P. micra* for the discrimination of patients with (B) CRC and (C) CRA from healthy control subjects. AUC, area under the curve; *P. micra*, *Parvimonas micra*.

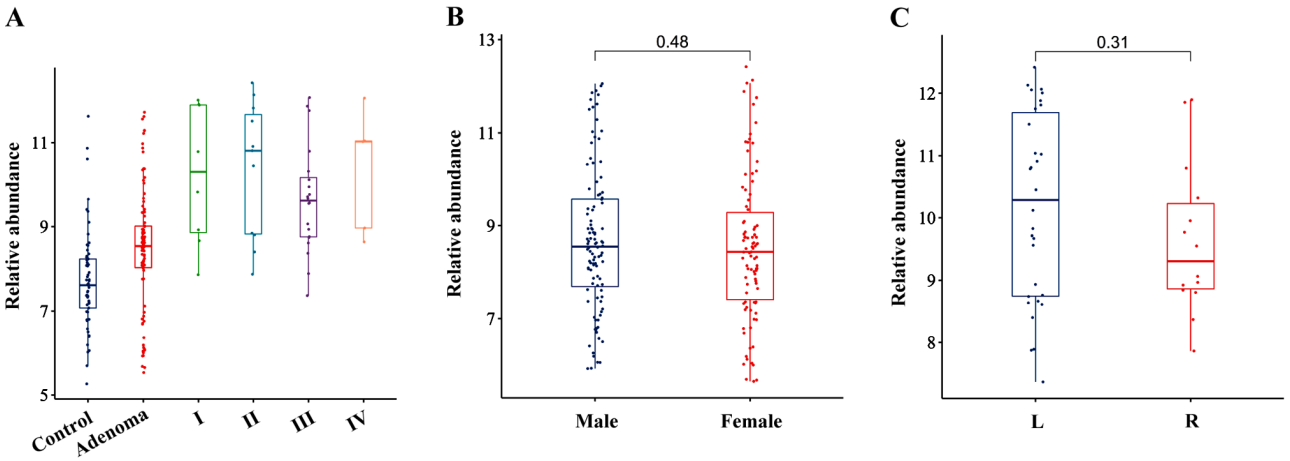

Figure 2. Association between the relative abundance of Parvimonas micra and the characteristics of patients. Association between the relative abundance of *Parvimonas micra* and (A) cancer progression, the (B) sex of patients and the (C) site of cancer origin. L, Left; R, Right.

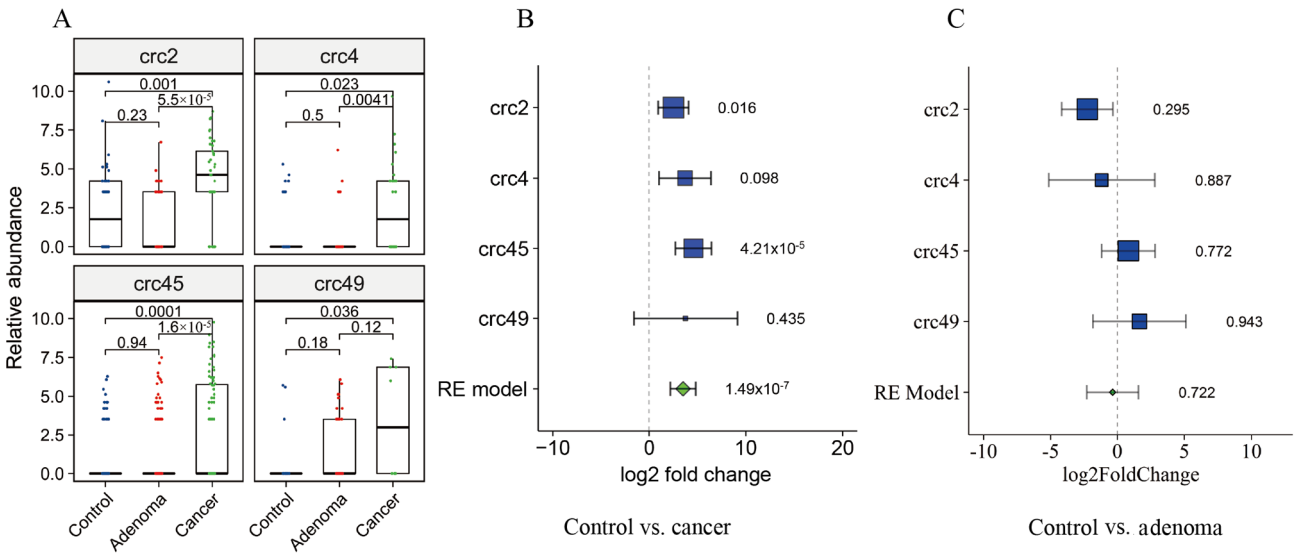

Figure 3. Meta-analysis of *P. micra* relative abundance in four publicly available datasets. (A) Boxplot of the relative abundance of *P. micra* in healthy control, CRA and CRC samples. Forest plot of the fold changes in the *P. micra* relative abundance in the form of the ratios of the values for (B) patients with CRC over healthy controls and those for (C) patients with CRA over healthy controls. *P. micra*, *Parvimonas micra*; CRC, colorectal cancer; CRA, colorectal adenoma; RE model, random effect model.

of tumors, and passengers are more suited to survive in the gut microenvironment resulting from tumorigenesis (46). An example of a passenger is *F. nucleatum*, which is enriched in CRC but not in CRA cases (49). The present study identified a significant elevation of *P. micra* in CRC but not in CRA cases. Consistent with these findings, it has been shown that *P. micra* is predominantly enriched in stages I/II and III/IV, and its abundance is decreased after tumor resection, indicating that *P. micra* is not the cause of carcinogenesis but is adapted to the CRC microenvironment (50,51). Therefore, *P. micra* may be a passenger in the driver-passenger model.

P. micra is a component of the healthy commensal flora of the gastrointestinal tract, and an opportunistic pathogen (10). As types of periodontal bacteria, *P. micra* and *F. nucleatum* have synergistic effects on biofilm formation, which is important for the colonization by these two species of apical periodontitis lesions (52). *P. micra* significantly enhances the activity of gingipains, which are virulence factors in *Porphyromonas gingiva* that are important in periodontal disease (53). *P. micra* may also promote cancer development, although the exact mechanism it yet to be fully elucidated. Moreover, *P. micra* may contribute to the pathogenesis of periodontitis by stimulating Toll-like receptor 4, nucleotide binding oligomerization domain containing (NOD)1 and NOD2 (54). It has also been reported that *P. micra* may be involved in gut bacterial translocation and the upregulation of interleukins in the tumor microenvironment (55). A previous study demonstrated that *APC* $^{Min/+}$ mice gavaged with *P. micra* exhibited a significantly higher tumor burden and tumor load, and cell proliferation was significantly higher in the colon tissues of *P. micra* gavaged germ-free mice compared with control mice (56). Furthermore, the tumor promoting effect of *P. micra* has been reported to be associated with altered immune responses and increased inflammation in the gut (50,56). These findings indicate that *P. micra* is primarily adapted to the CRC microenvironment and could contribute to a pro-tumoral inflammatory environment in patients susceptible to developing CRC.

In conclusion, the present study identified that *P. micra* was associated with CRC and may serve as a diagnostic marker for CRC. In addition, *P. micra* was not enriched in patients with CRA, suggesting that it serves a limited role in the tumorigenesis of CRA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JX, MY and SY analyzed and interpreted the patient data from patients with CRC. DW, YZ and WC wrote the manuscript. JX, MY and DW participated in the experimental study and data analysis. JX, MY and SZ participated in data collection and statistical analysis. JX, YZ and WC conceived the idea of, and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All individuals provided written informed consent prior to participating in the study. All procedures were performed in accordance with and were approved by the ethical standards of the institutional and/or the national research committee [the Ethics Committee of The First Affiliated Hospital of Soochow University; approval no. + 056 (2016)], and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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