Fecal *Enterotoxigenic Bacteroides fragilis*- *Peptostreptococcus stomatis*-*Parvimonas micra* Biomarker for Noninvasive Diagnosis and Prognosis of Colorectal Laterally Spreading Tumor

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Research

Keywords: laterally spreading tumor, enterotoxigenic *Bacteroides fragilis*, *Peptostreptococcus stomatis*, *Parvimonas micra*, noninvasive biomarker, diagnosis and prognosis

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Abstract

Background

Up to now, non-invasive prediction of laterally spreading tumor (LST) and adenoma recurrence after LST resection is inevitable. The purpose of this study was to identify a microbiome signature with a high predictive effect on LSTs and a microbiome with a high predictive effect on adenoma recurrence after LSTs colectomy.

Methods

We performed 16S rRNA sequencing in mucosal samples with 5 healthy controls (HC), 8 colorectal adenoma (CRA) patients and 11 LST patients. The differentiating microbiota in fecal samples was quantified by qPCR in 475 cases including 113 HC, 208 CRA patients, 109 LST patients and 45 colorectal cancer (CRC) patients. We applied linear discriminant analysis effect size analysis to identify taxa differentially abundant between cases and controls. ROC curve was used to evaluate the diagnostic value of bacterial candidates. Pairwise comparison of AUCs was performed using the Delong's test. Mantel-Haenszel hazard models were used to determine the effects of microbiota composition on recurrence free survival.

Results

LST microbial dysbiosis was characterized by relative high abundance of the genus *Bacteroides*-*Streptococcus* and the species *enterotoxigenic Bacteroides fragilis* (*ETBF*)-*Peptostreptococcus stomatis* (*P. stomatis*)-*Parvimonas micra* (*P. micra*). The abundance of *ETBF*, *P. stomatis* and *P. micra* were steadily increasing in LST and CRC groups. *P.stomatis* behaved stronger value on diagnosis of LST than the other two bacteria (AUC 0.887, 95%CI 0.842-0.931). The combination of *P.stomatis*, *P.micra* and *ETBF* (AUC 0.922, 95%CI 0.887-0.958) revealed strongest diagnostic power with 88.7% sensitivity and 81.4% specificity. *ETBF*, *P. stomatis* and *P. micra* were related to the malignant LST ($P_{P. stomatis}=0.0015$, $P_{P. micra}=0.0255$, $P_{ETBF}=0.0169$) and the abundance of IL-6. The relative high-abundance of *P. stomatis* were related to the adenoma recurrence after LST resection (HR = 3.88, $P=0.008$).

Conclusions

Fecal microbiome signature (*ETBF-P. stomatis-P. micra*) can diagnose LSTs with high accuracy. *ETBF*, *P. stomatis* and *P. micra* were related to the malignant LST and *P.stomatis* exhibited a high predictive effect on the adenoma recurrence after endoscopic resection of LSTs. The signature bacteria of LST may provide a noninvasive alternative to early detect LST and predict the adenoma recurrence risk after resections.

Background

Colorectal cancers (CRC) ranked third in incidence and second in mortality among all cancers globally in 2020(1). Almost 90% of cases of colorectal cancer develop from adenoma, especially advanced colorectal adenoma, through a series of genetic changes, known as the adenoma-carcinoma sequence, over 10 years(2, 3). Adenoma is the primary precursor lesion of CRC and can be removed by colonoscopic resection to avoid the carcinogenesis progress. Laterally spreading tumors (LSTs) are known for primary precursor lesion of CRC(4, 5). LSTs are at least 10 mm in size and are characterized by horizontally extending growth patterns(6). It is categorized into two types: LST-Gs with nodules or granules distributed evenly or not on the surface of the lesion and LST-NGs with a smooth surface without nodules or granules(6). LST-Gs are further classified into homogeneous (LST-G-H) and nodular mixed (LST-G-M) subtypes, and LST-NGs comprise flat-elevated (LST-NG-FE) and pseudo-depressed (LST-NG-PD) subtypes. The detection rate of LST is extremely low due to the special growth pattern and morphology. Thus, there is a need to sensitively detect LSTs to prevent and reduce CRC. Moreover, adenoma recurrence after endoscopic resection of LSTs is frequent(7, 8). Despite the widespread use of endoscopic resection, few examined the risk factors for adenoma recurrence after LST resection. Many LST patients without adenoma recurrence are subjected to frequently and ultimately unnecessary surveillance procedures. Hence, a non-invasive, economic and convenient marker that can sensitively detect LSTs and predict the adenoma recurrence probability of LSTs yields to be explored.

The colon and rectum interact with approximately 3×10^{13} gut microorganisms with which the intestinal epithelium has a constant crosstalk(9). The roles of gut microbiota in the carcinogenesis colorectal cancer has received much attention in the past 5 years. Human studies generally showed that compared with healthy individuals, the gut microbiota related to CRC patients was significantly different. It showed lower abundance of potentially protective taxa, a higher species richness and increased abundance of procarcinogenic taxa (such as *Porphyromonas*, *Escherichia*, *Bacteroides*, *Fusobacterium*)(10-12). These data pinpoint a potential core set of microorganisms that might be carcinogenic. Quantifying and detecting the fold-changes in relative abundance of these bacteria forms the principle of using the microbiota as a CRC biomarker. The increasing number of studies provide a rich source for developing fecal microbial markers for CRC diagnosis. Existing studies have utilized the abundance of multiple bacterial, viral, fungal species to distinguish patients with CRC from healthy individuals and these studies exerted fairly high sensitivity and specificity(12-17). Apart from their diagnostic potential, associations between bacterial biomarkers and clinical outcomes of CRC have raised the possibility of using them as prognostic markers. Several studies have showed the relationships between tumoural amounts of bacteria in tissue with CRC survival(18-20). The microbiota may be an effective, noninvasive, economic and convenient biomarker for prognosis of CRC.

LST, an important precursor of CRC, was hardly endoscopic recognized and resected for exhibiting flat appearance. Understanding the microbiome signature of LST could give rise to new strategies to detect, reduce, or slow the progression of LST. Current noninvasive stool-based screening tests have low sensitivities for detecting LST. While microbiome signature for LSTs and noninvasive stool-based screening tests remain rare-reported. For population-wide screen and surveillance of LSTs, non-invasive,

cost-effective and convenient biomarkers are urgently desired, since these features are obviously beneficial to the compliance of patients.

In this study, we identified a microbiome signature highly predictive of LSTs in differentiating from healthy controls and colorectal adenoma. Moreover, we identified the microbiota with high accuracy on predicting the adenoma recurrence after colonoscopic resection of LSTs.

Materials And Methods

Study design and participants

This retrospective cohort was done at the Endoscopy Center, Renji Hospital, Shanghai Jiao Tong University. Approval for this study was obtained from the ethics committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University. The study complied with the Helsinki Declaration of 1975. A total number of 475 patients was consisted with 113 healthy controls, 208 CRA patients, 109 LST patients and 45 CRC patients (Work flowchart see Fig.1). To avoid potential alternation of the gut microbiota, excluding criteria were established. The exclusion criteria included: 1) with history of upper GI tracts surgery; 2) with a history of familial adenomatous polyposis(FAP), hereditary non-polyposis colorectal cancer(Lynch syndrome) or Peutz-Jeghers syndrome or uncontrolled diabetes, hypertension or other chronic metabolic disorder; 3) with history of uninterested gastrointestinal tract neoplasia; 4) with active GI tracts bleeding in recent 6 months; 5) using any of the following medicine: nonsteroidal anti-inflammatory drugs, immunosuppressor, antibiotics or probiotics at least 1 month prior to enrollment; 6) with eating habits changes in recent 4 weeks(17).

Enrolled subjects were divided into four groups: healthy control (HC) group, CRA group, LST group, CRC group. The clinical phenotype was set by the endoscopic and pathological diagnosis. Tumors in the caecum, ascending colon, hepatic flexure, transverse colon or splenic flexure were considered to be proximal ones, while distal tumors included those in the rest of colon or rectum. Conventional-type colorectal adenomas include tubular adenoma, tubulovillous adenoma and villous adenoma. Serrated polyps include hyperplastic polyps and serrated adenoma. The early stage CRC(S0) as well as stage 0/pTis CRC were defined as intramucosal carcinoma and adenoma with high-grade dysplasia. Adenoma recurrence was endoscopically confirmed under 3 to 36 months' surveillance. Recurrence at each period of surveillance was defined as detection of the recurrent adenoma in patients who had no evidence of recurrence at any previous surveillance. Late recurrence was defined as adenoma at the site of previous resection at ≥ 12 months' surveillance in patients who had no evidence of recurrent adenoma at the previous surveillance.

Stool and mucosal samples collection

All 475 stool samples (113 HC, 208 CRA patients, 109 LST patients, 45 CRC patients) were collected in the special germ-free containment before bowel preparation for endoscopy. All stool samples were moved to -20 °C for temporary preservation and transferred to -80 °C for long-term storage within 48h. All 24

mucosal samples (5 HC, 8 CRA patients, 11 LST patients) were collected in the special germ-free containment after endoscopic resection and were moved to -80 °C for storage immediately.

DNA extraction, 16S rRNA gene sequencing and real time quantitative polymerase chain reaction

QIAamp DNA Stool Mini Kit and QIAamp DNA Tissue Mini Kit were used according to manufacturer's instructions (Qiagen, Hilden, Germany). All extracts were preserved at -20 °C before 16S rRNA sequencing and subsequent qPCR amplification. We performed 16S rRNA sequencing in mucosal samples. The 16S rRNA V3-V4 region was amplified by PCR and the sequenced using Miseq platform (Illumina, San Diego, California, USA). The primers sequences were described in Supplementary Table 1. All primers were synthesized and purified by Sangon Biotech Shanghai. 10 µl SYBR Green II was used as qPCR reaction system by TAKARA cooperation with SYBR®Premix Ex TaqTMII (TliRNaseH Plus). Stepone®plus by ABI company was used in qPCR with all the operation and configuration according to the manufacture's instruction with 40 cycles of 95 °C denaturation for 5s, and 60 °C annealing and extension for 30s in total after a 30s of pre-denaturation at 95 °C.

Statistical analysis

The 16S rRNA sequencing data were analysed using Quantitative Insights Into Microbial Ecology (QIIME2 V.2018.2). DADA2 software, wrapped in QIIME2, was used to filter the sequencing reads and construct feature table. We applied linear discriminant analysis effect size (LEfSe) analysis to identify taxa differentially abundant between cases and controls. This method first uses the non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance and then uses linear discriminant analysis (LDA) to calculate the effect size of each feature. Other statistical analysis included Fisher's exact test, Kruskal-Wallis test and partial Spearman's rank correlation (PResiduals package).

All qPCR samples were in triplicates. Average Ct value from the triplicates was calculated and the relative abundance of the target gut microbiota was based on the Δ Ct value defined as the target Ct value subtracted Ct value for 16S rRNA, which means the all the relative abundance was normalized by the abundance of 16S rRNA(21). As for statistical analysis, values were all expressed as mean $\pm$ SD. Mann-Whitney U test and Kruskal-Wallis test were used in comparison for continuous and nonparametric analyses respectively. Receiver Operating Characteristic (ROC) curve was used to evaluate the diagnostic value of bacterial candidates in distinguishing LST and CRA and to determine the best cut-off values that maximized the Youden index ($J = \text{Sensitivity} + \text{Specificity} - 1$). Pairwise comparison of areas under ROC (AUCs) was performed using the Delong's test. Recurrence-free survival (RFS) was defined as the time from diagnosis to recurrence of colorectal adenoma in 3 years. Patients who did not experience recurrence were censored at date of last follow-up. Mantel-Haenszel hazard models were used to determine the effects of microbiota composition on RFS. All tests were done by SPSS statistical software (version 23.0, IBM Corp, Armonk, NY, 2012) and R statistical software (version 3.6.0, 2019).

Results

Bacteria composition was significantly different between LSTs and controls

A total number of 475 patients were divided into 113 healthy controls, 208 CRA patients, 109 LST patients and 45 CRC patients (basic demographic characteristics see Supplementary Table 2; Work flowchart see Fig.1). To examine the bacteria composition in LSTs, The 16S rRNA sequencing was performed in 24 mucosal samples (5 HC, 8 CRA patients, 11 LST patients). Bacteria compositions in HC, CRA patients and LST patients were compared. In typing analysis, it was found that bacteria were significantly different in family, genus and species levels among the above three groups (Fig.2a). *Bacteroides* and *Streptococcus* were found to be abundant in LST group compared to the other two groups (Fig.2b-e). The potential differential bacterium patterns among the three groups was defined by Kruskal-Wallis test and LEfSe algorithm. *Lactobacillus johnsonii* and *Bacteroides fragilis* were significantly abundant in LST group (Fig.3a,b). By analyzing the GMrepo bacteria database, the abundance of *Peptostreptococcus stomatis* and *Parvimonas micra* were found to be positively relevant to the abundance of *Bacteroides fragilis* (Fig.3c). Moreover, *enterotoxigenic Bacteroides fragilis* (ETBF) was reported to be driver bacteria in colorectal cancer development.

The abundance of ETBF, P. stomatis and P. micra were increased in LST and CRC groups

ETBF, *P. stomatis*, *P. micra* and *Lactobacillus johnsonii* were also tested in fecal samples. An increase in the relative abundance of ETBF, *P. stomatis* and *P. micra* in LST patients over HC and CRA patients was observed. Moreover, there was an increase of the above microbiota in CRC patients over LST patients, as well (Figure 4A). Comparing LST patients with healthy controls, ETBF increased by 9.26 folds ($P < 0.0001$), *P. stomatis* increased by 14.79 folds ($P < 0.0001$) and *P. micra* increased by 10.17 folds ($P < 0.0001$). Comparing CRA patients with LST patients, ETBF increased by 2.95 folds ($P < 0.0001$), *P. stomatis* increased by 8.76 folds ($P < 0.0001$) and *P. micra* increased by 6.65 folds ($P < 0.0001$). Comparing LST patients with CRC patients, ETBF increased by 3.00 folds ($P = 0.0327$), *P. stomatis* increased by 15.31 folds ($P = 0.0058$) and *P. micra* increased by 6.41 folds ($P = 0.0413$). Further, no discrepancy was found for the relative abundance of three intestinal bacteria among distal and proximal colon for LSTs (Supplementary Figure 1A). In terms of topography and histopathology, no distinction showed between LST-G and LST-NG, as well as traditional adenoma type and serrated polyp type (Supplementary Fig.1b,c). In addition, the dysplasia degree led to totally different clinical decision and prognosis. Accordingly, the three bacteria in terms of dysplasia degree reported by pathologist were also tested. The S0 LST had higher abundance of fecal bacteria ($P_{P. stomatis} = 0.0015$, $P_{P. micra} = 0.0255$, $P_{ETBF} = 0.0169$) (Fig.4b). The inflammatory cytokines (IL-1 β , IL-6, IL-8, IL-10, TNF- α) data of LST patients further showed that *P. stomatis*, *P. micra* and ETBF significantly increased IL-6 secretion (Fig.4c) and the abundance of *P. stomatis* ($P_{P. stomatis} = 0.0024$) and *P. micra* ($P_{P. micra} = 0.0077$) positively correlated with the abundance of ETBF (Fig.4d).

As for *Lactobacillus johnsonii*, no significant changes have been revealed among CRA and LST groups (Supplementary Fig.2). Therefore, *ETBF*, *P. stomatis* and *P. micra* were selected as non-invasive candidate biomarkers for detecting LSTs.

The predictive values of *ETBF*, *P. stomatis* and *P. micra* for the occurrence of LSTs

The relative abundance of the *ETBF-P.stomatis-P.micra* microbiome showed an increasing in the LST group as compared to HC group and CRA group as described above (Fig.4a). In comparison between HC group and LST group, the ROC curves were performed. In terms of AUCs, *P.stomatis* behaved stronger than the other two bacteria (AUC_{*P.stomatis*} 0.887, 95%CI 0.842-0.931) (Fig.5a). These three contributing markers were therefore united as an independent model. Combination of *P.stomatis-P.micra* and *P.stomatis-P.micra-ETBF* revealed stronger diagnostic potential over the single *P.stomatis* ($P_{P.stomatis-P.micra} = 0.0376$, $P_{P.stomatis-P.micra-ETBF} = 0.00930$) (Fig.5b). The combination of the three bacteria showed the maximum AUC (AUC 0.922, 95%CI 0.887-0.958) and Youden index with 88.7% sensitivity and 81.4% specificity (Table 1).

Table 1. AUC and diagnostic value of different biomarkers for predicting LST

		AUC	95% CI	Sensitivity	Specificity	Youden Index
Detection LST from HC	ETBF	0.795	0.735	0.855	0.752	0.797
	Ps	0.887	0.842	0.931	0.936	0.726
	Pm	0.842	0.790	0.893	0.706	0.850
	ETBF+Ps	0.904	0.864	0.944	0.899	0.788
	ETBF+Pm	0.880	0.835	0.925	0.844	0.823
	Ps+Pm	0.909	0.871	0.947	0.862	0.814
	ETBF+Ps+Pm	0.922	0.887	0.958	0.881	0.814
Detection LST from CRA	ETBF	0.645	0.584	0.707	0.725	0.620
	Ps	0.807	0.760	0.854	0.844	0.678
	Pm	0.787	0.735	0.840	0.706	0.769
	ETBF+Ps	0.808	0.761	0.855	0.835	0.688
	ETBF+Pm	0.791	0.739	0.842	0.633	0.841
	Ps+Pm	0.835	0.790	0.879	0.762	0.808
	ETBF+Ps+Pm	0.835	0.790	0.879	0.762	0.808

HC= Healthy control. CRA= Colorectal adenoma. LST= Laterally spreading tumor. *ETBF*=

Enterotoxigenic Bacteroides fragilis. *Ps*= *Peptostreptococcus stomatis*. *Pm*= *Parvimonas micra*.

To distinguish LSTs from CRAs, the ROC curves were made. The result showed that *P.stomatis* exhibited higher AUCs than the other two bacteria (AUC_{*P.stomatis*} 0.807, 95%CI 0.760-0.854) (Fig.5c). Nonetheless, the diagnostic combination of *P.stomatis-P.micra* and *P.stomatis-P.micra-ETBF* showed higher AUCs than the single *P.stomatis* ($P_{P.stomatis-P.micra} = 0.0411$, $P_{P.stomatis-P.micra-ETBF} = 0.0416$) (Figure 5D). In terms of Youden index, the *P.stomatis-P.micra* marker was equal to the *P.stomatis-P.micra-ETBF* marker with 76.2% sensitivity and 80.8% specificity (Table 1).

***P. stomatis* were positively related to adenoma recurrence after resection of LSTs**

Considering the relationship between LST signature bacteria and tumoral development, the next step of this study aims to determine if there were differences in the above three bacteria composition between recurrence or non-recurrence adenoma after resection of LSTs. After 36 months' follow-ups, there were 23 LST patients with adenoma recurrence among 92 LST patients. To this end, the distribution of *ETBF*, *P. stomatis* and *P. micra* among the recurrence group and non-recurrence group was first evaluated. The recurrence group exhibited a predominant abundance of *P. stomatis* ($P < 0.0001$). Besides, no difference of the other two bacteria between the two groups were detected (Fig.6a) and *P. stomatis* presented strong value on predicting the adenoma recurrence (AUC 0.800, 95%CI 0.678-0.915, $P < 0.0001$). Based on these results, the enrolled patients were divided into high abundance of *P. stomatis* group and low abundance of *P. stomatis* group. Univariate analysis showed the dysplasia type of LST and the abundance of *P. stomatis* were related to the adenoma recurrence, while gender, age, history of hypertension or diabetes mellitus, size, location, topography, histopathology failed (Supplementary Table 3). The survival curve showed the S0 LSTs (HR = 2.78, $P = 0.026$) and high-abundance of *P. stomatis* (HR = 3.88, $P = 0.008$) were related to the adenoma recurrence event (Fig.6b). Furthermore, the abundance difference of *P. stomatis* in early-recurrence group and late-recurrence group was also tested to be no significant difference between the above two groups (Supplementary Fig.3).

Discussion

There is growing evidence that alteration in the intestinal microbiome correlate with colorectal neoplasms(12, 22, 23). However, LST, as a principal precursor lesion of CRC, was never reported about the intestinal microbiota signature. Moreover, early detection and predicting the risk of adenoma recurrence after LST resection were essential to prevent CRC. Herein, the gut microbiota structure of microbiome in LST cohort by means of 16S rRNA gene sequencing was delineated in this study. The results demonstrate that LST microbial dysbiosis was characterized by relative high abundance of the genus *Bacteroides-Streptococcus* and the species *ETBF-P. stomatis-P. micra*. Based on the microbial signature, fecal microbiobial biomarkers *ETBF-P. stomatis-P. micra* were defined as early detection of LST. It shows that disease-associated bacteria may form a synergistic network which result in diseases. Moreover, *P. stomatis* behaved high accuracy on predicting adenoma recurrence after LST resections.

ETBF has been known as a contributor to chronic colonic diseases, including oncogenic transformation, intestinal inflammation, chronic colonic dysfunctions and colorectal precancerous and cancerous lesions(24-28). Combined action of the *ETBF* and IL-17 on colonic epithelial cells promoted the differentiation of monocytic-myeloid-derived suppressor cells(MO-MDSCs) and suppressed T-cell proliferation(27). *Bacteroides fragilis*-associated lncRNA1 (BFAL1) is a mediator of *ETBF*-induced carcinogenesis and may be a potential therapeutic target for *ETBF*-induced CRC(28). The recent observation of *ETBF*-bearing biofilms in colon biopsies from humans with colon cancer susceptibility loci strongly suggests that *ETBF* plays one key role in colorectal cancer(29). *P. stomatis* and *P. micra* are gram-positive anaerobic bacterium and part of the oral and gut commensal flora(30, 31). The organisms have

been implicated as a causative agent of several diseases(12, 13, 30, 32-34), including oral squamous cell carcinoma(32) and apical abscess(33). A meta-analysis of 526 fecal shotgun metagenome datasets identified a microbial core of seven enriched bacteria in CRC(22). This core set included: *ETBF*; four oral bacteria of *Fusobacterium nucleatum*(35, 36), *P.micra*, *Prevotella intermedia*(37) and *Porphyromonas asaccharolytica*(38); and two other bacteria, *Thermanaerovibrio acidaminovorans*(22) and *Alistipes finegoldii*(39). Yachida et al(23) collected stool samples from 616 participants and found these bacteria were apparent in cases of multiple colorectal adenomas and intramucosal carcinomas. Stage-specific analyses showed two patterns of significant species elevation: the first increased across early to later stages, whereas the second was elevated only in the early stages. The former was characterized by *F. nucleatum*, *Solobacterium moorei*(37), *P.stomatis*, *Peptostreptococcus anaerobius*(40, 41), *Lactobacillus sanfranciscensis*, *P.micra* and *Gemella morbillorum*(42). *ETBF*, *P.stomatis* and *P.micra* were crucial to colorectal carcinogenesis while they were rarely reported to be biomarkers to detect precancerous lesions.

Several studies have utilized the abundance of multiple bacterial species to distinguish patients with CRC from healthy individuals. Among several bacterial candidates, *F. nucleatum* emerged as a key marker either when being quantified alone(16, 21) or combined with other bacteria(13, 17, 43), specifically *Clostridium symbiosum*(17). These findings provide support for an accurate stool-based diagnostic test using markers targeting a limited number of microbial species. In this study, the fecal abundance of the three bacteria *ETBF-P. stomatis-P. micra* displayed considerably high sensitivity and specialty in detecting LST, especially *P. stomatis*. While combinations of *P.stomatis-P.micra-ETBF* revealed higher diagnostic potential compared with the single biomarker *P.stomatis* and combined biomarkers of *P.stomatis-P.micra*, *P.stomatis-ETBF*, *P.micra-ETBF*. The wide application of the above bacterial markers in LST detection could be rather feasible since qPCR detection of fecal bacterial DNA in stool is technically more reliable and cost-effective than endoscopic screening.

Adenoma recurrence after endoscopic resection of LSTs is frequent(7, 8, 44, 45). Despite the widespread use of endoscopic resection, few examined the risk factors for recurrence. LSTs with cancerous histology were reported associated with the adenoma recurrence after endoscopic therapy(46). Some studies showed that independent risk factors recurrent adenoma were piecemeal resection, adjunctive argon plasma coagulation, lesion occupying 75% of the luminal circumference, lesion size >60 mm, and high grade dysplasia of LSTs(8, 46, 47). Furthermore, adenoma recurrence surveillance requests regular endoscopy screening. Many patients not developing adenoma recurrence have to subjected to frequently and ultimately unnecessary surveillance procedures. Carefully installed devices, well-trained technician and inconvenient bowel preparation before endoscopy are resource-consuming and may limit the use for population-wide screening. Based on this study, it is worth noting that the relative high abundance of *P.stomatis* positively correlated with adenoma recurrence after LST resection. Application of the biomarker *P.stomatis* to predict adenoma recurrence was therefore promising and feasible.

Furthermore, *ETBF-P. stomatis-P. micra* modulation, especially *P. stomatis* may be important for LST development, adenoma recurrence after LST resection and even colorectal carcinogenesis. Efforts need

to find the best interventional approaches to manipulate the microbiota. It will necessitate concerted efforts from researchers and industrial partners to translate it into a clinical product.

However, there is no clear answer about the role of *ETBF-P. stomatis-P. micra* in LST or in CRC. The process is intricate and influenced by genetic and environmental factors. Mechanisms include immune regulation, metabolism of dietary components, genotoxin production and inflammation(48-50). There remained a question what are the driver and passenger bacteria in LST development? As described above, the three bacteria increased IL-6 secretion in LST group. It can be hypothesized that the three bacteria induce inflammatory environment to accelerate the development of LSTs. While it deserves in-depth studies about carcinogenesis as well as cancer therapy responses.

The strengths of the present study were as follows. First, we reported for the first time that the bacteria signature of LST. Second, we found fecal microbiome signature (*ETBF-P. stomatis -P. micra*) can effectively predict the occurrence of LSTs. Moreover, *ETBF*, *P. stomatis* and *P. micra* were related to the S0 stage LST. Third, our study provided new evidence that *P.stomatis* exhibited the high accuracy on predicting the adenoma recurrence after endoscopic resection of LSTs.

Although clinical data for endoscopic resections was collected prospectively and all consecutive patients were included, this study was limited by its retrospective design, as all these results were from a single tertiary center and may not be generalizable. In future, studies in large cohorts enrolling more patients need to be carried out, which can derive the best diagnostic algorithm across populations.

Conclusion

In conclusion, we observed, for the first time, fecal microbiome signature (*ETBF-P. stomatis -P. micra*) can effectively predict the presence of LSTs. Moreover, *ETBF*, *P. stomatis* and *P. micra* were related to malignant LST and *P.stomatis* exhibited the high accuracy on predicting the adenoma recurrence after endoscopic resection of LSTs. Thus, the signature bacteria of LST can provide a noninvasive method to early detect LST and predict the adenoma recurrence risk after resections.

Abbreviations

LST: Laterally spreading tumor

HC: Healthy control

CRA: Colorectal adenoma

CRC: Colorectal cancer

ETBF: Enterotoxigenic *Bacteroides fragilis*

P. stomatis: *Peptostreptococcus stomatis*

P. micra: *Parvimonas micra*

LEfSe: Linear discriminant analysis effect size

LDA: Linear discriminant analysis

qPCR: Real time quantitative polymerase chain reaction

ROC: Receiver operating characteristic

AUC: Areas under receiver operating characteristic

RFS: Recurrence-free survival

Declarations

Ethics approval and consent to participate

This study has been conducted in accordance with ethical standards and according to the Helsinki Declaration of 1975. The study protocol was approved by the ethics committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing Interests

The authors declare that there are no competing interests.

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Authors' Contributions

Xiaonan Shen analysed the data and wrote the manuscript. Xiaonan Shen, Jialu Li, Jiaqi Li, Yao Zhang, Xiaobo Li, Qinyan Gao conceived the study and collected samples. Yun Cui and Xiaoyu Chen performed the pathological diagnosis. Yingxuan Chen and Jingyuan Fang revised the manuscript. Jingyuan Fang designed and supervised the study. All authors read and approved the final manuscript.

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Figures

Figure 1

Work flowcharts. HC=healthy control. CRA=colorectal adenoma. LST=laterally spreading tumor. CRC=colorectal carcinoma. qPCR= real time quantitative polymerase chain reaction.

Figure 2

Variations of mucosal microbiota composition in LST. a Typing analysis on family, genus and species groups in the HC(n=5), CRA(n=8) and LST group(n=11). b Distance heatmap in samples on genus level. c Heatmap in the three groups on genus level, Bacteriodes and Streptococcus were significantly over presented in LST group. d Cicros analysis on genus in three groups. e Tertiary analysis on genus level in three groups. HC=healthy control. CRA=colorectal adenoma. LST=laterally spreading tumor. CRC=colorectal carcinoma.

Figure 3

Variations of mucosal microbiota composition on species level in LST. a Kruskal-Wallis test analysis on species level in the three groups, Lactobacillus johnsonii and Bacteroides fragilis presented high abundance in the LST group. b LDA analysis on species level in the three groups, Lactobacillus johnsonii and Bacteroides fragilis significantly over-presented in the LST group. c The abundance of Bacteroides fragilis was positively related with the abundance of Peptostreptococcus stomatis and Parvimonas micra. LDA = linear discriminant analysis. HC=healthy control. CRA=colorectal adenoma. LST=laterally spreading tumor.

Figure 4

The fecal relative abundance of *ETBF*, *P. stomatis* and *P. micra* among groups. a The fecal relative abundance of *ETBF*, *P. stomatis* and *P. micra* was stepwise increased in the three groups. b The fecal high abundance of *ETBF*, *P. stomatis* and *P. micra* was relevant with S0 stage LSTs. c The expression of IL-6 was positively related with the abundance of the three bacteria. d The abundance of *P. stomatis* and *P. micra* increased with the increasing abundance of *ETBF*. HC=healthy control. CRA=colorectal adenoma. LST=laterally spreading tumor. CRC=colorectal carcinoma. *ETBF*= Enterotoxigenic *Bacteroides fragilis*. *P. stomatis*= *Peptostreptococcus stomatis*. *P. micra*= *Parvimonas micra*. P=LSTs with hyperplastic type or low-grade dysplasia. S0= LSTs with intramucosal carcinoma or high-grade dysplasia. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

Figure 5

The diagnostic performance of marker ETBF, *P. stomatis*, *P. micra* and their combined test indicated by the receiver operating characteristic (ROC) curve analysis a Differentiating LST from HC, the ROC curves and AUCs (Delong's test) of the three bacteria. b Differentiating LST from HC, the ROC curves and the AUCs (Delong's test) of the promising diagnostic bacterial models. c Differentiating LST from CRA, the ROC curves and AUCs (Delong's test) of the three bacteria. d Differentiating LST from CRA, the ROC curves and the AUCs (Delong's test) of the promising diagnostic bacterial models. HC=healthy control. LST=laterally spreading tumor. ETBF = Enterotoxigenic *Bacteroides fragilis*. Ps= *Peptostreptococcus stomatis*. Pm= *Parvimonas micra*. AUC=area under the receiver-operating characteristic curve. * $P < 0.05$. ** $P < 0.01$.

Figure 6

Performance of ETBF, *P. stomatis* and *P. micra* on adenoma recurrence after resection of LST. a The fecal relative abundance of *P. stomatis*, *P. micra* and ETBF among non-recurrence group and recurrence group. *P. stomatis* was related with adenoma recurrence. b Kaplan-Meier estimates for survival probability based on the abundance levels of *P. stomatis* in LST and histopathology of LST. High abundance of *P. stomatis* and S0 stage LST indicated adenoma recurrence. *P. stomatis*= *Peptostreptococcus stomatis*. *P. micra*= *Parvimonas micra*. ETBF = *Enterotoxigenic Bacteroides fragilis*. P=LSTs with hyperplastic type or low-grade dysplasia. S0= LSTs with intramucosal carcinoma or high-grade dysplasia. **** $P < 0.0001$.

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