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Peptostreptococcus anaerobius is a potential diagnostic biomarker of colorectal cancer

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ABSTRACT

Accumulating evidences have shown that *Peptostreptococcus anaerobius* (P.a) is abundantly enriched in the fetus of colorectal cancer (CRC) patients. P.a is reported able to invade colorectal tissues. This study intends to uncover the clinical significance of P.a in CRC. Mucosal tissues collected from CRC cases (n=109) and precancerous healthy ones (n=65) were subjected to the determination of the absolute copy number of P.a by droplet digital PCR. The positive rate of P.a in mucosal tissues of CRC and healthy ones was 79.8% (87/109), and 55.4% (36/65), respectively. The average absolute copy number of P.a in them was 2.3 copy/ng DNA, and 0.32 copy/ng DNA, respectively. The abundance of P.a in mucosal tissues of CRC, and age and TNM staging of CRC cases were correlated to its survival. The abundance of P.a in CRC cases was remarkably correlated to the relative level of SQLE. The abundance of P.a can be monitored to predict the prognosis of CRC.

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Introduction

Colorectal cancer (CRC) is the third most prevalent cancer throughout the world. The annual death of CRC each year ranks third in cancer mortality, and it is becoming younger (1). The pathogenesis of CRC has not been fully elucidated yet. Recent studies have highlighted the significant function of the intestinal flora in the development of CRC (2). It is reported that the xenografted CRC is hard to be induced in sterile or antibiotic-treated mice, whilst a gavage of fecal samples from CRC patients in mice obviously triggers the proliferation of mouse colorectal epithelial cells (3).

Currently, a close relationship between certain bacteria and the incidence of CRC has been identified through comparing fecal microflora in CRC patients and healthy subjects, such as *Fusobacterium nucleatum* (F.n) and *Escherichia Coli* (E.Coli). They are able to activate cancer-associated pathways and thus accelerate the development of CRC (4,5). The invasion of bacteria is a vital event during the development of CRC (6). It is generally considered that the mucous membrane of the intestines isolates intestinal flora and colorectal epithelial cells. Once the mucous membrane impairs, invaded bacteria in the mucous membrane would further invade epithelial cells, thus leading to an inflammatory response and creating a favorable environment for the carcinogenesis of CRC (7,8).

P.a is a Gram-positive anaerobic bacterium that usually lives in the mouth and intestines (9). It is reported that P.a is enriched in the fetus of CRC patients. It increases the number of AOM-induced CRC tissues in mice and accelerates the proliferative ability of intestinal epithelial cells by inducing cholesterol synthesis (10). The latest study demonstrated that intestinal epithelial cells can absorb P.a and further trigger the growth of CRC through regulating local immunity (11). However, potential diagnostic and prognostic values of P.a in CRC are unclear. This study intends to evaluate the clinical significance of P.a in CRC through comparing P.a abundance in mucosal tissues of CRC and healthy ones.

Materials and Methods

Clinical mucosal tissues

A total of 109 primary CRC patients operated for the first time in our hospital were recruited. Patients with a medication history for the treatment of CRC were excluded. Meanwhile, 65 cases of healthy mucosal tissues were collected. Tissue samples were immediately preserved at -80°C. CRC patients were followed up until June 2018. TNM staging and tumor size of CRC patients were recorded. Informed consent was obtained prior to sample collection. This study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University.

DNA extraction

DNAs from CRC and healthy tissues were extracted using the AllPrep DNA/RNA Mini Kit (QIAGEN, Hilden, Germany), which were quantified by Nanodrop determination.

RT-PCR

Total RNAs from tissues were extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA). After quantification, 1 μg RNA per sample was reversely transcribed

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to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas), which was amplified using the PowerUp™ SYBR™ Green Master Mix. Gene expression was calculated by the $2^{-\Delta\Delta ct}$ method.

Quantification of cholesterol

A total of 2 mg tissue was collected for quantification of cholesterol using the Cholesterol/Cholesteryl Ester Quantification Kit (ab65359, Abcam, Cambridge, MA, USA).

Quantification of the absolute copy number of P.a

Droplet digital PCR was conducted to quantify the absolute copy number of P.a (12). A total of 20 ng DNA was subjected to RT-PCR. A 22-µl system was prepared, including 1×ddPCR Supermix (0.125 µmol) and forward (CAACGGCTCCGGCATGTG) and reverse primers (0.25 µmol) of P.a (CTTGCTCTGGGCCTCGTC). An automated droplet generator was used to produce the droplet.

Statistical analysis

GraphPad 5 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) were used for statistical processing. Potential influences of different factors on the abundance of P.a were compared by the paired Student's t-test. The difference in the abundance of P.a between mucosal tissues of CRC and healthy ones was compared by the Mann-Whitney U test. Kaplan-Meier method and log-rank test were applied to assess the survival significance of P.a in CRC patients. A significant difference was set at $P<0.05$.

Results

Abundance of P.a in mucosal tissues of CRC

Baseline characteristics of recruited CRC patients, and their potential influences on the absolute copy number of P.a were listed in Table 1. The positive rate of P.a in CRC and healthy tissues was 79.8% (87/109), and 55.4% (36/65), respectively. The absolute copy number of P.a in CRC and healthy tissues was 2.3 copy/ng DNA, and 0.32 copy/ng DNA, respectively (Figure 1). Notably, a higher abundance of P.a was detected in stage III+IV CRC than that in stage I+II (Table 1). It is indicated that P.a could stimulate the development of CRC.

Prognostic potential of P.a in CRC

To estimate the influence of P.a on the survival of CRC patients, they were assigned into two groups with the median level of the absolute copy number of P.a (2.3 copy/ng DNA) as the cut-off value. During the follow-up period, 35 (32.1%) CRC patients survived and 74 (67.9%) died. Two non-cancer-relevant deaths were excluded. As Kaplan-Meier curves revealed, worse overall survival was detected in CRC patients with a higher abundance of P.a (Figure 2). We believe that P.a may be a novel prognostic factor of CRC. In addition, multivariate Cox regression

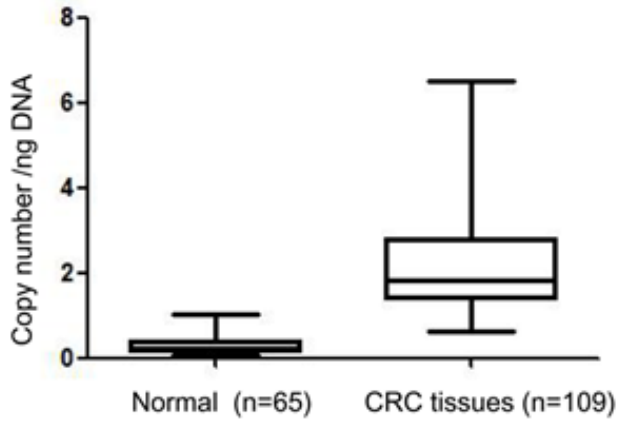

Figure 1. Aabsolute copy number of P.a in CRC and healthy tissues.

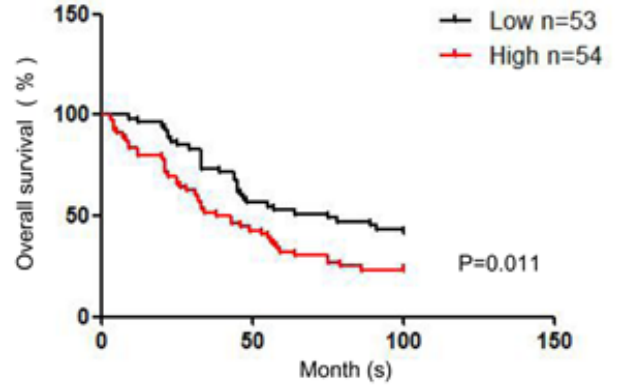

Figure 2. The overall survival via Kaplan-Meier analysis in CRC patients with high and low abundance of P.a.

Table 1. Clinical variables and their impact on P.a copy.

Variables	Average P.a copy/ng DNA	P value
Age		
≥61.2 (n=59) (54.1%)	2.2	0.591
<61.2 (n=50) (45.9%)	2.3	
Gender		
Male (n=68) (62.4%)	2.4	0.473
Female (n=41) (37.6%)	2.2	
Differentiation		
High and moderate (n=63) (57.8%)	2.5	0.442
Low (n=46) (42.2%)	2.1	
TNM stage		
I+II (n=59) (54.1%)	1.7	0.005**
III+IV (n=50) (45.9%)	2.8	
Metastasis		
Yes (n=43) (39.4%)	2.2	0.884
No (n=66) (60.6%)	2.3	

Table 2. Cox regression analysis for survival.

Variables	Multivariate analysis Relative risk (95%CI)	P value
Age		
≥61.2 (n=59) (54.1%)	1	0.018*
<61.2 (n=50) (45.9%)	3.236(1.955-5.878)	
Gender		
Male (n=68) (62.4%)	1	0.651
Female (n=41) (37.6%)	1.014(0.721-1.539)	
Differentiation		
High and moderate (n=63) (57.8%)	1	0.435
Low (n=46) (42.2%)	1.275(1.049-1.930)	
P.a copy		
Low (n=53) (49.5%)	1	0.046*
High (n=54) (50.5%)	2.036 (1.237-3.025)	
TNM stage		
I+II (n=59) (54.1%)	1	0.008
III+IV (n=50) (45.9%)	3.011(1.913-5.926)	
Metastasis		
Yes (n=43) (39.4%)	1	0.599
No (n=66) (60.6%)	1.264 (0.849-1.493)	

analysis showed that P.a was not the only factor influencing the survival of CRC. Age and TNM staging of CRC patients also affected the prognosis (Table 2).

A close correlation between P.a and SQLE

It is reported that P.a can stimulate the proliferative capacity of intestinal epithelial cells by inducing the synthesis of cholesterol. Here, a higher content of cholesterol was detected in CRC tissues than in normal ones (Figure 3A). SQLE is a vital rate-limiting enzyme in the sterol biosynthesis pathway. Our results identified a positive correlation between the absolute copy number of P.a and SQLE level (r=0.3035, P=0.0013) (Figure 3B). It is suggested that P.a could be involved in the synthesis of cholesterol.

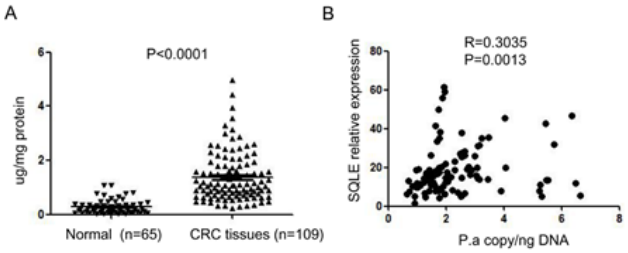

Figure 3. Correlation analysis between P.a and SQLE. (A) The content of cholesterol in CRC and normal tissues; (B) The absolute copy number of P.a was positively correlated with SQLE level (r=0.3035, P=0.0013).

Discussion

The critical function of the intestinal flora in CRC has increasingly emerged. The invasion of bacteria damages intestinal integrity (13,14). Nevertheless, how the intestinal flora causes the carcinogenesis of CRC remains unclear. A relevant study proposed that pathogenic bacteria are enriched, while probiotics decline during the development of CRC in a mouse model (2). In sterile mice of antibiotics-treated mice, a single pathogenic bacterium triggers the proliferative potential of intestinal epithelial cells and enhances the number of AOM-induced CRC lesions (2). P.a is abundantly enriched in fecal samples and mucosal tissues of CRC patients, which is considered as a proliferation stimulator. In the present study, the abundance of P.a was remarkably higher in CRC tissues than in controls, which was consistent with previous findings. P.a was confirmed to be a pathogenic bacterium that invades intestinal tissues. Furthermore, a higher abundance of P.a was detected in CRC patients with a worse overall survival, suggesting the prognostic potential of P.a in CRC.

The synthesis of cholesterol is a key event for P.a-induced stimulation of intestinal epithelial cell proliferation (10). Here, the content of cholesterol was much higher in CRC tissues than in normal ones. SQLE is a key enzyme during cholesterol synthesis, which serves as an oncogene in multiple types of human cancers (15-18). We identified a positive correlation between P.a abundance and SQLE level, confirming the function of P.a in regulating cholesterol metabolism. There are many genetic, biochemical, physiological, and epigenetic findings related to cancers (19-28).

Taken together, highly abundant P.a in CRC tissues is able to influence cholesterol metabolism, which is a promising prognostic indicator of CRC.

Conflict of Interests

The authors declared no conflict of interest.

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