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Dysbiosis of Cultivable Aerobic Microbiota Tightly Associated with Colon Cancer Epithelial Cells.

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***A*BSTRACT**

Modern researches focus on metagenome, metatranscriptome, and metaproteome of microorganisms associated with the incidence of colorectal cancer (CRC). Based on assays of fecal samples, it is difficult to obtain unambiguous conclusion about contribution of certain microorganisms to CRC. Bacteria tightly associated with colon mucosa could provide more data into CRC mechanisms and give a basis of future approaches for microbiota modulation and new therapies development. Here we characterize aerobic cultivable bacteria isolated from biopsy samples of human neoplastic and normal colon epithelia, to compare the spectrum and some physiological features of bacterial habitants of the biological niche, which nearby parts differ on a level of oncogenic transformation. 98 cultured bacterial strains from 28 patients with CRC were isolated, cultivated and identified using MALDI-TOF mass spectrometry. From colon biopsies cultivable Escherichia coli, Serratia marcescens and representatives of the genera Bacillus, Pseudomonas, Klebsiella, Enterobacter, Aeromonas were identified. No significant differences were observed in total number and antibiotic resistance of bacteria from malignant and non-malignant epithelium. We found RNase activity of E.coli strains isolated from malignant epithelium to be higher than in same bacteria from normal tissue. While some RNases are known as antitumor agents, activation of extracellular RNase synthesis could be a part of protective microbiota mechanism.

Keywords: colorectal cancer, microflora, antibiotic susceptibility, secreted RNase activity

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INTRODUCTION

Microbial composition of the human intestine is highly interrelated to health status. Intestinal microbiota plays a major role in the residual food fermentation, modulation of immune system function and high protection against pathogenic microorganisms and diseases [1, 2]. However, the state of the normal microbiota of the intestine can be affected by many endogenous (infectious and somatic diseases, congenital and acquired immunodeficiency) and exogenous (nutritional, medicinal, post-operative infection, stress, and other related environmental) factors. The human colonic microbiota has emerged as a major environmental factor that appears to modulate the risk of colonic cancer, and dysbiosis in gut microbiota is now believed to be an underlying factor in the development of diseases.

Colorectal cancer is the third most common cancer cases and the fourth most common cause of cancer deaths globally, which accounts 1.2 million new cases and 600000 deaths per year. The highest incidence is reported in countries of Europe, North America, and Oceania, whereas incidence is lowest in some countries of south and central Asia and Africa [3-5]. One of the most important endogenous risk factors in the development of CRC is thought to be chronic intestinal bowel inflammation and its severity has been directly correlated to CRC risk [6, 7] and is supported by the fact that colon cancer risk increases with longer duration of colitis, greater anatomic extent of colitis, the concomitant presence of other inflammatory manifestations [**7**] and family history of CRC [8].

From the beginning of human life, gastrointestinal tract (GIT) is inhabited with morphologically, physiologically and structurally different microorganisms; predominantly bacteria. Even a part of large intestine colon alone contains around 10^{14} bacteria [9]. These microflora are very essential in food digestion and absorption, metabolism of bile contents & xenobiotics, modulation of immune system of the GIT and control of intestinal hemostasis, development and health status [11] In recent years, a number of research works emphasizing the potential influence of intestinal microflora towards colon carcinogenesis and their outputs have explained different mechanisms for the contribution of intestinal dysmicrobiota in the development of colorectal cancer. It includes: liberation of carcinogenic compounds, production of toxic compounds to cellular genes, activation of immune system inducing cell proliferation and inhibition of different signaling pathways responsible to trigger cell apoptosis [12].

MATERIALS AND METHODS

Isolation of cultivable microflora from biopsies

We analyzed rectal mucosa biopsies of 28 patients with surgical resection of the colon after confirmed colorectal cancer diagnosis. Biopsy patterns were taken from transformed zone directly and adjacent portions of non-malignant epithelium. Tissue samples were obtained and investigated in accordance with a permission of the Ethical Committee of Kazan State Medical Academy (protocol No. 4 on May 7, 2009), given us on the cooperation basis with surgeon Prof. Gataullin I.G. and physician coloproctologist Maltsev P.V. Biopsy patterns with average 5-7 × 5-7 mm dimensions were intensively washed with 5 ml of PBS (Sigma, USA). Then 20µl of suspension was plated on petri dishes containing MPA and Endo culture media for isolation of cultivable microflora.

Isolated bacteria identification

Bacterial colonies isolated from 28 colorectal biopsies were directly placed on target MALDI-TOF plates, then α-cyano-4-hydroxycinnamic acid (CHCA, MW189.04 Da) matrix solution was added. The plates were subjected to MALDI-TOF mass spectroscopy (Bruker Daltonik) equipped with a 337-nm nitrogen laser, and microbial species were identified by pattern matching with the libraries in BioTyper 2.0 software. MALDI-TOF mass spectroscopy is a rapid, simple, accurate and high-throughput proteomic technique for the identification of a variety of bacterial species [13].

Evaluation of bacterial RNase activity

With a classical method [14], bacterial RNase activity was measured using high-polymorphic RNA as a substrate in a dense medium without phosphate source under incubation temperature of 37 ° C for 8 and 24

hours. The width of this clear zone correlated to the amount of extracellular enzyme, produced by separate colony of tested microorganism.

Antibiotic susceptibility test

The simplest and most reliable susceptibility testing method is the disk diffusion or Bauer-Kirby procedure [15] which is performed by applying a standardized inoculum of 1–2x10^8 CFU/ml to the surface of a large (150-mm diameter) Mueller-Hinton agar plate. Eight commercially prepared, fixed-concentration filter-paper antibiotic disks were placed on the inoculated agar surface with pre-isolated bacteria from colorectal cancer biopsies.

Statistical Analysis

For the statistical analysis of data, the range of the measured variable means and standard deviations (SD) were calculated, using Microsoft Excel 2010. The data are presented as a median or mean ± SD values. P values less than 0.05 were considered statistically significant.

RESULTS

Cultivated bacteria isolated from colorectal biopsies

Ninety eight cultivated microbial isolates were identified from twenty eight epithelial biopsies of normal and onco-transformed rectums. Analysis of microflora from epithelial biopsies of cancer patients allowed us to identify the dominant group of bacteria which are typical for the area adjacent to the carcinoma and its zone of intact epithelium. On average 10^3 to 10^5 microorganisms were grown per 1 ml of PBS washed biopsy suspension. Malignant epithelial biopsies were shown to have more Gram-negative microorganisms in comparison with normal epithelium (Table 1). From colon biopsies cultivable *Escherichia coli*, *Serratia marcescens, Klebsiella pneumoniae* and representatives of the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, Aeromonas were identified with MALDI-TOF mass spectrometry.

Table 1: Gram stain characterization of bacteria isolated from epithelial biopsies of the rectum.

Type of Epithelium	Gram Reaction of Isolated Bacteria, %*	
	Positive	Negative
Apparently Healthy	20.8 ± 2	79.2 ± 2
Malignant	13.3 ± 0.9	86.7 ± 0.9

* The total number of morphologically different isolates from a specific type of epithelium was taken as 100%.

Ribonuclease activity of bacterial isolates

We measured ribonuclease activity of all cultivated bacterial isolates using a method described above. We noted that the higher RNase activity (Table 2) was characterized for both Gram-positive and Gram-negative microflora of malignant epithelium.

84% isolates from malignant epithelium and only 28% isolates from healthy epithelium showed significant extracellular ribonuclease activity after 8 hours of cultivation on phosphor-lacking synthetic medium supplemented with RNA. 90% of bacteria isolated from healthy epithelial tissue showed a similar level of activity only after 24 hours of cultivation. Moreover, 10% of the isolates from healthy epithelium did not show any measurable extracellular RNase activity

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Table 2: Proportion of RNase activity secreted by microorganisms isolated from colorectal epithelium.

Type of Epithelium	Bacterial secreted RNases, %			
	8 hrs incubation		24 hrs incubation	
	Gram Reaction			
	Positive	Negative	Positive	Negative
Apparently Healthy	33	26	83	91
Malignant	75	85	100	100

In vitro antibiotic susceptibility of isolated bacteria

Test results for in vitro susceptibility of the 10 selected isolates of malignant and nonmalignant colorectal biopsies are presented in Table 3. Only three antibiotics - Gentamycin (90%), Ciprofloxacin (70%) and Chloramphenicol (70%) - showed susceptibility to test bacteria, whereas five antibiotics - penicillin G (100%), Nalidixic acid (R & I = 70%), Erythromycin (50%), Trimethoprim-sulfamethoxazole and Tetracycline (each 40%) - showed significant resistance.

Obtained data completely coincides with Gomi H. results [16] that ciprofloxacin was highly active in vitro against enteropathogens, while traditional antimicrobials such as ampicillin, trimethoprim, and trimethoprim/sulfamethoxazole showed high level and frequency of resistance.

Table 3: In vitro antibiotic susceptibility trend of 10 randomly selected bacterial isolates from colorectal cancer resection.

Isolated organism	Antibiotic							
	C [a]	SXT	TE	CIP	E	NA	CN	P
Malignant [b]								
K. pneumoniae	30 [c]	33	32	40	32	18	31	6
E. coli	23	0	10	0	0	0	17	0
E. coli	24	20	21	24	21	18	20	0
E. coli	0	0	17	23	00	18	18	0
S. marcescens	26	27	20	23	07	21	18	0
Nonmalignant								
E. coli	23	25	28	31	28	18	27	0
E. coli	20	0	9	0	0	0	13	0
E. coli	33	40	36	44	37	38	34	0
E. coli	22	23	10	18	24	0	12	8
S. marcescens	0	0	20	25	0	15	20	0

[a] C- Chloramphenicol; SXT- Trimethoprim-sulfamethoxazole; TE- Tetracycline; CIP- Ciprofloxacin; E- Erythromycin; NA- Nalidixic acid; CN- Gentamicin; P- Penicillin G.

[b] Tissue samples from transformed and adjacent untransformed intestinal wall.

[c] Diameter of antibiotic inhibition zone in mm.

DISCUSSION

Isolated cultivated bacteria and their RNase activity

In previous studies [17], patients with colorectal cancer exhibited disbiotic changes of the colon microflora: for instance, 82.7% of patients showed reduction in amount of bifidobacteria, 71.1% - in lactobacilli, 48% - in enterococci and 50% in *E. coli*. Thus, recorded changes in microbial community composition of rectum might put their own print in malignant transformation of colorectal epithelium development. However, we did not reveal a substantial difference in bacteria total number between cultured samples of transformed and normal colorectal epithelial tissues. It coincides with Sobhani and his colleagues report [18].

In the present study only 10^3 to 10^5 microorganisms were grown per 1ml of PBS washed biopsy suspension which are much lower in comparison with qPCR fecal analysis [19]. Thus the low quantitative

characteristic of the intestinal epithelium microflora might be associated with preoperative cleansing of patients bowels as well as the fact that the majority of patients before surgical operation were subjected to radiotherapy. The current data showed an increased proportion of Gram-negative microorganisms from malignant epithelial biopsies compared to normal epithelium. This coincides with the data obtained by Marchesi *et al*. [20] stated a tendency of more Gram-negative Bacteroidetes and less Firmicutes to be found on'ON-tumor' tissue than on 'OFF-tumor' mucosa.

Thus can be assumed that transformed epithelium microflora might have special physiological and biochemical properties which is different from the flora residing on the surface of normal intestinal epithelium. Microbial RNase is known to be secreted not only to provide a host cell with available phosphorus source in terms of phosphate starvation formed by the decomposition of RNA nonviable cells, but also can serve as a cellular weapon [21, 22**]** in competition with other microorganisms in the ecological niche. The biological functions of microbial ribonucleases (RNases) directly correlated with the regulation of gene expressions, growth and development of cells, cellular defense from pathogenic microorganism and selective induction of programmed cell death [23]. So it draws interest of many researchers because of possibility to develop new therapeutic agent, which is possible to control various types of human malignancies. This phenomenon would be aggravated in the gastrointestinal wall of human being inhabited with a number of different microbial cells. Therefore, differences in ribonuclease activity of intestinal bacteria may reflect the feature of colonized sites on colorectal area wall. Our experimental data indicated the level of ribonuclease activity of microflora to be depended on the extent of cancer lesion.

More than 40% of randomly selected isolates were found to be multidrug resistant. All selected bacterial isolates from colorectal resections were resistant to penicillin G. The inflammatory process in gut is prone to progress to untreatable chronic intestinal inflammation which might predispose the epithelium to carcenogenesis if identification and the subsequent testing for antibiotic susceptibility of the causative agent are not performed at the early stage of the disease.

SUMMARY

- 98 cultivable bacterial isolates were obtained and identified from 18 biopsies of normal and onco-transformed rectum epithelium. There were no significant differences in amount of cultivated bacteria isolated from normal and malignant colon biopsies.
- Vast majority of bacteria from malignant epithelium and only small part of bacteria from apparently healthy epithelium possessed a considerable RNase activity.
- Isolated bacteria were susceptible to gentamicin, ciprofloxacin, chloramphenicol and resistant to penicillin, nalidixic acid, trimethoprim-sulfamethoxazole and tetracyclin.
- 80% of them displayed α- and β-hemolysis on blood agar medium.

CONCLUSION

In this study we compared the microbial composition of malignant and healthy intestinal epithelial biopsies and analyze fecal samples of patients with confirmed diagnosis of colorectal cancer. Majority of bacteria from malignant epithelium and only small part of bacteria from healthy epithelium possessed a considerable RNase activity. Isolated bacteria were susceptible to gentamicin, ciprofloxacin, chloramphenicol and resistant to penicillin, nalidixic acid, trimethoprim-sulfamethoxazole and tetracyclin.

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