Research Article

Genotoxigenic isolates of microorganisms detected in patients with colorectal cancer

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ABSTRACT

Aim: In our study, we spent molecular-genetic analysis of microbial cultures isolated from patients with colorectal cancer according to genetic determinants controlling adhesive, hemolytic (hlyA), and toxigenic activity. **Methods:** Genomic DNA extracted from samples of bacterial cultures, isolated from malignant tumor of the rectal mucosa as well as from normal mucosa colorectal of cancer patients was investigated using molecular genetic studies. **Results:** We found presence of colibactin (clb) and cytotoxic necrotizing factor 1 (CNF1) toxigenes using polymerase chain reaction and sequencing analysis of genetic determinants controlling an adhesive (papC, papH, afa, eae, and bfpA), hlyA , and toxigenic (lthB, sta, stx, clb, cnfI, cdtB, cif, and colE) activity of microbial cultures test samples. clb -producing microorganisms (*Escherichia coli* and *Klebsiella pneumonia*), isolated from the patients' rectal mucosa, as well as *E. coli*, are known to be producents of cancer-causing toxin (CNF1). **Conclusion:** They also could be considered as etiologic agents of colorectal cancer, which is also desirable to be taken into account in the development of laboratory diagnostic and treatment-and-prophylactic measures.

KEY WORDS: Colibactin, Colicins, Colorectal cancer, Cytotoxic necrotizing factor-1, *Escherichia coli, Klebsiella pneumonia*, Polymerase chain reaction, Sequencing, Single nucleotide polymorphism

INTRODUCTION

The connection of human colon microflora with colorectal cancer accidence and its mechanisms was highlighted in studies all over the world,[1,2] indicating, the carcinogenic effect of a number of bacterial toxins such as colibactin (clb) and cytotoxic necrotizing factor 1 –(CNF1).[1,2]

Representatives of Enterobacteriaceae family - strains of *E. coli*, *Klebsiella pneumonia*, *Enterobacter aerogenes*, *Citrobacter koseri*, etc., are capable to produce clb, which can lead to oncogenic mutations due to double-stranded ruptures in damaged DNA.[3]

Nuclease activity that leads to DNA degradation in the cytoplasm is also possessed by colicins capable of killing normal and malignant eukaryotic cells.[4,5]

The ability of adhesive-invasive strains of *E. coli* to colonize the large intestine mucosa in patients with colorectal cancer was described in literature.[2,6]

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According to Starostina,[7] opportunistic microflora was shown to increase in 48% of patients with colorectal cancer. *K. pneumoniae* was isolated in 13.5% of colorectal patients, *Enterobacter* spp. - 7.6%, and *Citrobacter* spp. - in 3.8%.

According to Lai,[8] the presence of clb in genotoxigenic *K. pneumoniae* strains could be considered as a molecular basis for the epidemiological connection between this pathogen and colorectal cancer, using genetic mechanisms which allow this species of bacteria to colonize the intestinal mucosa.[9]

Hence, the main goal of our work was to spend molecular genetic analysis of microbial cultures isolated from patients with colorectal cancer (from a malignant tumor and normal rectum mucosa both) according to genetic determinants controlling the adhesive, hemolytic (hlyA), and toxigenic activity.

MATERIALS AND METHODS

The selection of biopsies was carried out in accordance with the Permission of the Ethical Committee of the Kazan State Medical Academy (Protocol No. 4 of May

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7, 2009). Selected biopsies were incubated for 1 h in 1 ml of sterile saline at 37°C followed by sowing 100 µl of the solution onto meat-peptone agar and growing until colonies appeared.

Genomic DNA analysis with subsequent molecular genetic studies of detection of genetic determinants controlling the adhesive (papC, papH, afa, eae, and bfpA),[10-13] hlyA[14] and toxigenic (lthB, STa, Stx, clb, cnfI, cdtB, and cif)[2,4,15] activity were studied in six samples of microbial cultures isolated from three patients with colorectal cancer, from malignant tumor of the rectal mucosa (Tru26o, Shi1o, and Ef15o) and normal mucosa (Tru26n, Shi1n, and Ef15n samples), both.

The list of oligonucleotide primers for polymerase chain reaction (PCR) used in the work is presented in Table 1.

PCR was performed on the MJ Mini Gradient Thermal Cycler (Bio-Rad, USA). The PCR results were detected by horizontal electrophoresis in 2.5% agarose gel in TBE buffer (pH 8.0) containing ethidium bromide (0.5 µg/ml) followed by visualization of the electrophoregram in the ultraviolet transilluminator (λ = 310 nm). The sizes of the amplified products are estimated in comparison with standard DNA markers.

Sequencing of the amplified products of the clb and cnf1 gene loci, as well as ribosomal DNA (16S rDNA) coding for 16S rRNA, was performed on the ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA). The sequenced sequences were aligned according to the nucleotide sequences of a number of reference strains of microorganisms published in GenBank, using the BLAST and CLUSTAL W programs (v. 1.83).

RESULTS AND DISCUSSION

Species of six microbial cultures were identified using bacteriological and molecular genetic analysis, in particular, by sequencing the ribosomal DNA locus

Table 1: The list of oligonucleotide primers used in the work

Gene locus	Oligonucleotide primers for PCR	PCR product (bp)
16S rRNA	ITS-F: 5/-GAT-TAG-ATA-CCC-TGG-TAG-3/ ITS-R: 5/-AGT-CAC-TTA-ACC-ATA-CAA-CCC-3/[16]	1215
papC	papC-F: 5/-cgt-tcg-ccg-ggt-atc-gtt-tct-cag-3/ papC-R: 5/-CCC-GTT-CCC-CAG-CGA-TTT-GTC-AC-3/[10]	724
papH	papH-F: 5/-TTA-AAG-ATA-ATC-GGG-TCA-T-3/ papH-R: 5/-GGA-ATC-AGA-GAA-AAG-GTT-3/[10]	858
afa	afa-f: 5/-GCT-GGG-CAG-CAA-ACT-GAT-AAC-TCT-C-3/ afa-r: 5/-CAT-CAA-GCT-GTT-TGT-TCG-TCC-GCC-G-3/[11]	750
eae	Eae-f: 5/-TCA-ATG-CAG-TTC-CGT-TAT-CAG-TT-3/ Eae-r: 5/-GTA-AAG-TCC-GTT-ACC-CCA-ACC-TG-3/[12]	482
bfpA	bfpA_114F: 5/-GTC-TGC-GTC-TGA-TTC-CAA-TA-3/ bfpA_521R: 5/-TCA-GCA-GGA-GTA-ATA-GC-3/[13]	408-414
hlyA	hlyA-F: 5/-GCA-TCA-TCA-AGC-GTA-CGT-TCC-3/ hlyA-R: 5/-AAT-GAG-CCA-AGC-TGG-TTA-AGC-T-3/[14]	534
lthB	LT-f: 5/-ACG-GCG-TTA-CTA-TCC-TCT-C-3/ LT-r: 5/-TGG-TCT-CGG-TCA-GAT-ATG-TG-3/[12]	273
sta	STa-1: 5/-GCT-AAT-GTT-GGC-AAT-TTT-TAT-TTC-TGT-A-3/ STa-2: 5/-AGG-ATT-ACA-ACA-AAG-TTC-ACA-GCA-GTA-A-3/[15]	190
stx	VTcom-f: 5/-GAG-CGA-AAT-AAT-TTA-TAT-GTG-3/ VTcom-r: 5/-TGA-TGA-TGG-CAA-TTC-AGT-AT-3/[12]	518
pks	pks-f: 5/-ATT-CGA-TAG-CGT-CAC-CCA-AC-3/ clbJ/K-up: 5/-CTG-TAT-GCC-TTT-ATC-ACT-GCC-3/[2,18]	1511
cnf1	CNF-1s: 5/-GGG-GGA-AGT-ACA-GAA-GAA-TTA-3/ CNF-1as: 5/-TTG-CCG-TCC-ACT-CTC-ACC-AGT-3/[2]	1112
cdtB-I	CDT-I-s: 5/-CAA-TAG-TCG-CCC-ACA-GGA-3/ CDT-I-as: 5/-ATA-ATC-AAG-AAC-ACC-ACC-AC-3/[2]	411
cdtB-IV	CDT-IV-s: 5/-CCT-GAT-GGT-TCA-GGA-GGC-TGG-TTC-3/ CDT-IV-as: 5/-TTG-CTC-CAG-AAT-CTA-TAC-CT-3/[2]	350
cif	cif-int-s: 5/-AAC-AGA-TGG-CAA-CAG-ACT-GG-3/ cif-int-as: 5/-AGT-CAA-TGC-TTT-ATG-CGT-CAT-3/[2]	383
ColE3	ColE3-F: 5/-TAA-GCA-GGC-TGC-ATT-TGA-TG-3/ ColE3-R: 5/-TCG-GAT-CTG-GAC-CTT-TCA-AC-3/[4]	413
ColE6	ColE6-F: 5/-ACC-GAA-CGT-CCA-GGT-GTT-3/ ColE6-R: 5/-TTT-AGC-CTG-TCG-CTC-CTG-AT-3/[4]	399
ColE7	ColE7-F: 5/-GCA-TTC-TGC-CAT-CTG-AAA-T-3/ ColE7-R: 5/-CTT-CTG-CCC-ACT-TTC-TTT-CG-3/[4]	431
ColE1	ColE1-F: 5/-TGT-GGC-ATC-GGG-CGA-GAA-TA-3/ ColE1-R: 5/-CTG-CTT-CCT-GAA-AAG-CCT-TTT-3/[4]	650

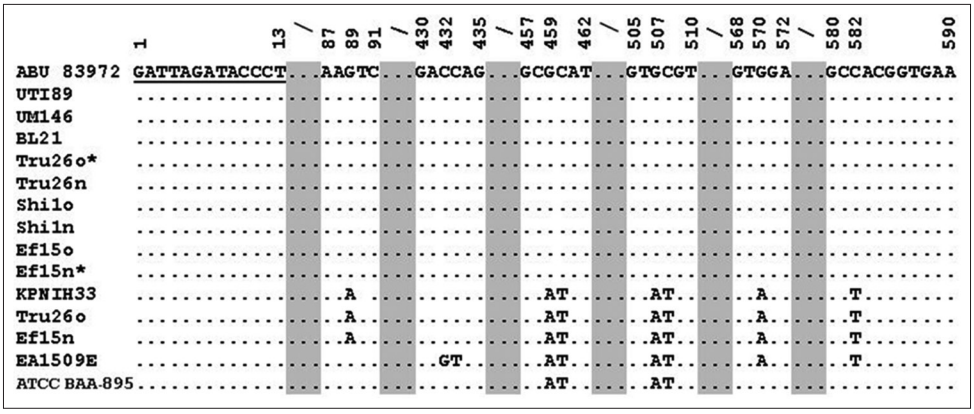

Figure 1: Single nucleotide polymorphism positions in analyzed locus of ribosomal DNA encoding the 16S rRNA of *Escherichia coli, Klebsiella pneumonia, Enterobacter aerogenes,* and *Citrobacter koseri.* Positions 1-13: ITS-F primer site: 5/-GATTAGATACCCTGGTAG-3/. Reference strains of *E. coli* (ABU 83972, UT189, UM146, and BL21), *K. pneumonia* (KPNIH33), *E. aerogenes* (EA1509E), and *C. koseri* (ATCC BAA-895)

(16S rDNA) amplified with the primers ITS-F and ITS-R.[16] Thus, Tru26n, Shi1o, Shi1n, and Ef15o were isolates from *E. coli* monocultures, the Tru26o and Ef15n samples were associated with *E. coli* and *K. pneumonia* microorganisms. Subsequently, a pure culture of *E. coli* (isolates Tru26o * and Ef15n *) was isolated from mixed bacterial cultures.

Single nucleotide polymorphism (SNP) positions in ribosomal DNA encoding the 16S rRNA of *E. coli, K. pneumonia, E. aerogenes, and C. koseri* [Figure 1] analyzed locus were shown to obtain some identification value in assessing the species, which belonging to bacterial taxa.

Presence of clb and CNF1 toxingens was detected using PCR and sequence analysis of microbial cultures by genetic determinants controlling adhesive, hlyA, and toxigenic activity.

Moreover, a positive amplification signal for the clb gene locus (clb) was obtained in Tru26o, Shi1o, Shi1n, and Ef15n samples, and cnf1 gene (cnfI) was only found out in Shi1o and Shi1n samples.

In addition to previously used primer set (pks-f + pks-r) for a specific PCR product of clb gene locus of 2119 bp in length[2,17] amplification, we also generated a new set of oligonucleotides (pks-f + clbJ/K-up)[2,18] initiating the less extended fragment of 1511 bp in length amplification.

The electrophoregram of PCR amplification of clb *E. coli* and *K. pneumonia* locus with primers pks-f + clbJ/K-up result is shown in Figure 2.

Moreover, corresponding identification SNP positions of *E. coli, K. pneumonia, Enterobacter aerogenes, and Citrobacter koseri colibactin* gene in analyzed locus are shown in Figure 3.

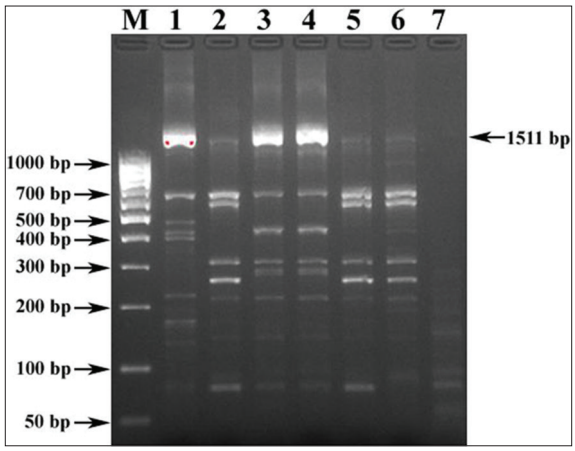

Figure 2: Electrophoregram of polymerase chain reaction amplification of colibactin *Escherichia coli* and *Klebsiella pneumonia,* with primers pks-f + clbJ/K-up locus. Notations: 1 - Tru26o; 2 - Tru26n; 3 - Shi1o; 4 - Shi1n; 5 - Ef15o; 6 - Ef15n *; 7 - OKO

The availability of nucleotide sequences of clb gene locus of the Shi1o and Shi1n monoculture samples of the E. coli and the mixed Tru26o and Ef15n cultures of the *K. pneumonia* genome was indicated by data from corresponding gene site sequence analysis directly, and indirectly by sequence analysis of ribosomal DNA fragment encoding 16S RRNA.

Pure E. coli strains (Tru26o * and Ef15n * isolates), isolated from the mixed bacterial cultures Tru26o and Ef15n, did not obtain a specific amplicon locus of clb gene, indicating clb gene to belonged to second association of mixed cultures, the *K. pneumonia* genome.

It should be noted that the absence of colicin-producing microorganisms in the analyzed samples was also confirmed by the sequencing of a non-specific amplified DNA fragment of approximately

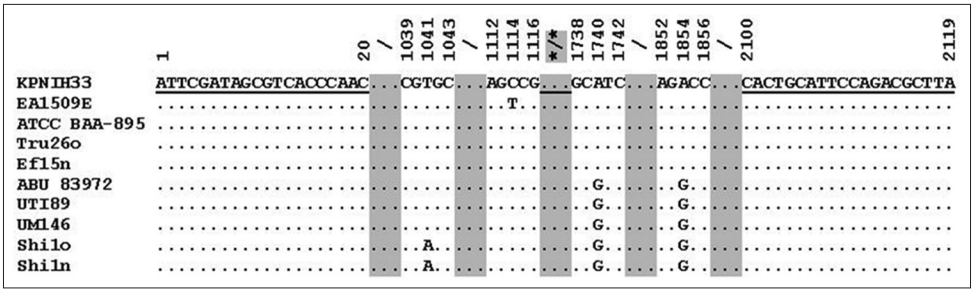

Figure 3: Single nucleotide polymorphism positions in the analyzed locus of the colibactin gene *E. coli, K. pneumonia, E. aerogenes,* and *Citrobacter koseri.* Positions 1–20: primer site pks-f: 5/-attcgatagcgtcacccaac-3/. Positions 2100-2119: primer site pks-r: 5/-taagcgtctggaatgcagtg-3/. */*. - positions 1492-1511: site clbJ/K-up: 5/-ctgtatgcctttatcactgcc-3/

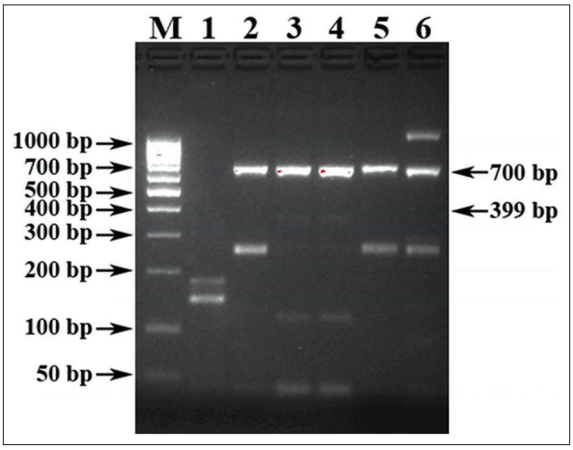

Figure 4: Electrophoregram of polymerase chain reaction result with primers ColE6-F and ColE6-R. Notations: 1 - Tru26o; 2 - Tru26n; 3 - Shi1o; 4 - Shi1n; 5 - Ef15o; 6 - Ef15n

700 bp [Figure 4] in length flanked by ColE6-F and ColE6-R cross-reactive primers capable of detecting in addition to colicin E6, also E2, E3, E5, E8, and E9.[4]

Summary

1. Species of microorganisms detected in patients with colorectal cancer were identified as a result of bacteriological and molecular genetic studies, the isolated isolates of which were monocultures of *E. coli* (Tru26n, Shi1o, Shi1n, and Ef15o) and mixed bacterial cultures of E. coli and *K. pneumonia* (Tru26o and Ef15n samples). The SNP positions of the 16S rRNA locus analyzed to have an identification value in assessing the species belonging to bacterial taxa.
2. A number of identified isolates were genotoxigenic in view of the ability to produce carcinogenic toxins - clb (Tru26o, Shi1o, and Shi1n samples) and cnfI (Shi1o and Shi1n samples). The genetic determinants controlling the adhesive (papC, papH, afa, eae, and bfpA), hlyA, and toxigenic (lthB, sta, stx, cdtB, cif, and colE) activity in the samples of microbial cultures were not detected.

CONCLUSION

The clb -producing microorganisms *E. coli* and *K. pneumonia*, as well as *E. coli* strains, isolated from the rectal mucosa of examined patients, producing another carcinogenic toxin (CNF1), were shown to be etiological agents of colorectal cancer development due to pathogenetic oncomarkers (clb and cnf1), which is also desirable to take into account when improving laboratory diagnostic and therapeutic prophylactic measures.

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