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Possível associação da presença de bactérias anaeróbias do trato gastrointestinal com a neoplasia retal: ocorrência e caracterização molecular bacteriana.

Tese apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

Área de concetração: Microbiologia

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Versão Corrigida

São Paulo
2020

Primeiramente gostaria de agradacer a Deus, foste Tu que me ensinaste que nada é impossível, que perante qualquer dificuldade quem acredita no teu amor encontrará o caminho da superação. Assim, meu Deus, a Ti dedico e agradeço por mais esta conquista.

Á minha mãe Zildete Gama que sacrificiou-se, dedicou-se e não mediu esforços para que eu tivesse a oportunidade de estudar e ter uma boa formação, hoje cheguei aonde estou graças a sua sementinha plantada. Serei eternamente grata.

Ao meu esposo Ricardo Wesley Alberca por ter me incentivado a fazer o doutorado e por sempre estar ao meu lado nos momentos de dificuldade e alegria. Agradeço também pela paciência, companherismo, e compreensão. Talvez não existam palavras suficientes e significativas que me permitam agradecer a você com justiça, com o devido merecimento. Mas é tudo que posso fazer, é te agradecer.

E ao meu filho Bernardo Alberca, por me dar forças para finalizar a produção dessa tese.

A ajuda de vocês e apoio foram muito importantes para mim, e nunca vou esquecer tudo que vocês fizeram por mim. Muito obrigada! Com todo o carinho e de coração eu agradeço, e para sempre minha gratidão será à vocês.

AGRADECIMENTOS

Ao Professor Dr. Mario Julio Avila-Campos (in memoriam), pela oportunidade, orientação, confiança, paciência e por transmitir todo o seu ensinamento e sabedoria. Para sempre será recordado com carinho, respeito e admiração pelo profissional e ser humano que era, fica aqui a minha eterna gratidão!

À minha co-orientadora Professora Dra. Viviane Nakano, pelos ensinamentos durante todos esses anos, pela orientação, parceria, amizade, dedicação e em especial por toda atenção disprendida para a produção dessa tese.

Ao Professor Dr. Nilton Lincopan pela aceitação da minha orientação na reta final do meu doutorado.

Ao Instituto de Câncer do Estado de São Paulo (ICESP), principalmente o setor de Endoscopia, ao Dr. Ulysses Ribeiro e em especial a Dra. Adriana Vaz Safatle Ribeiro, aos médicos residentes, as enfermeiras e técnicos de enfermagem por toda contribuição.

Aos pacientes e os familiares que permitiram que essa pesquisa fosse realizada.

À auxiliar de laboratório Márcia Harumi, pelo apoio técnico, companherismo e amizade, lembrarei com carinho das novas conversas.

Ao Dr. Santiago Lattar e a Dra. Viviane Arenas por toda contribuição, dedicação e conhecimentos compartilhados nesse trabalho.

Às amigas que ganhei no Laboratório de Anaeróbios, a Dra. Naiane Cardoso pela amizade, cumplicidade e todo apoio durante o meu doutorado. A MSc. Rosa Liliana Solis pela amizade, parceria e colaboração na pesquisa, lembrarei com carinho dos nossos momentos compartilhados. A Alejandra Guzmán e Luciane Cavalheiro, obrigada por tornarem meus dias mais fácieis no laboratório.

Aos colegas de laboratório que ali passaram, Claudia Freire, Érika Francisca, Filipe Menezes, Bruno Alves, Ana Paula Barbosa, Rita Rodrigues, Paula Betin e Dr. Luís Llanco e em especial à Dra. Miriam Fernandes pela confiaça a mim depositada, pelos ensinamentos compartilhados durante meu estágio.

À Gisele Santana e Renato Aguiar, secretaria de Pós-graduação, obrigada por todo suporte e proatividade.

Á Rosana Prisco (ICBIII) e ao Dr. Ricardo Wesley Alberca pela colaboração para o desenvolvimento desse trabalho.

Agradeço também aos funcionários do Departamento de Microbiologia.

À CAPES pelo apoio financeiro concedido durante a elaboração desta pesquisa.

APOIO FINANCEIRO

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

“Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo e nunca se arrependende.”

Leonardo da Vinci

RESUMO

ALBERCA, G. G. F**. Possível associação da presença de bactérias anaeróbias do trato gastrointestinal com a neoplasia retal: ocorrência e caracterização molecular bacteriana.** 2020. 123 f. Dissertação (Doutorado em Microbiologia) Instituto de Ciências Biomédicas, Universidade de São Paulo, 2020.

As interações da microbiota gastrintestinal com o câncer de reto (CR) vêm sendo muito estudadas, mas até o momento não há evidências científicas convincentes sobre essa relação. As bactérias anaeróbias são predominantes na microbiota residente do trato gastrintestinal. Bactérias como: *Bacteroides fragilis* e *Clostridium perfringens*, são encontradas na microbiota intestinal de humanos e animais, entrentanto são agentes oportunistas e sua patogenicidade está relacionada à produção de vários fatores de virulência como toxinas e enzimas hidrolíticas. Neste estudo, foram avaliados 40 pacientes de câncer de reto e 20 pacientes sem câncer. Sendo coletados dos pacientes com câncer: 25 amostras fecais, 38 amostras de sangue, 36 amostras de tecido tumoral e de tecido adjacente. Dos pacientes sem câncer foram coletados: 16 amostras fecais, 19 amostras de sangue e 14 amostras de tecido retal sadio. Foram realizados o isolamento para as bactérias anaeróbias do "grupo" *Bacteroide fragilis, Parabacteroides* spp. e *Clostridium* spp. em todas as amostras clínicas, bem como o estudo da diversidade genética e a detecção de bactérias intestinais e orais pela técnica quantitativa da PCR em tempo real. As espécies *B. ovatus* (*P* = 0,018) e *B. stercoris* (*P* = 0,018) apresentaram diferenças estatísticas no isolamento em relação aos pacientes com e sem câncer. Das 24 cepas de *B. fragilis* isoladas nenhuma abrigou o gene *bft* ou seus subtipos em ambos os grupos estudados. Na distribuição de espécies do gênero *Clostridium* não foi verificada diferenças entre os pacientes avaliados, mas a espécie *C. perfringens* foi a mais prevalente em ambos os grupos. Das cepas de *C. perfringens* isoladas de pacientes com câncer 80 perteceram ao toxinotipo A e 5 ao toxinotipo G. Dessas 85 cepas, 8 abrigaram o gene *tpeL,* 5 o gene *netB* e 6 *netE.* Nenhuma cepa abrigou essas três toxinas simultaneamente; e todas abrigaram os genes *nanI* e *nanJ.* Todas as 47 cepas dos pacientes controles, pertenceram ao toxinotipo A e apresentaram os genes *nanI* e *nanJ* e não abrigaram os genes *tpeL, becA, becB, netB, netE, netF e netG.* Sobre a diversidade genética de *B. fragilis* por AP-PCR foi verificada elevada diversidade e nenhuma cepa de paciente com câncer e sem câncer apresentaram 100% de homologia. Em *C. perfringens* foi verificada uma cepa de paciente sem câncer (S15.1) agrupo-se no mesmo cluster que outras três cepas de pacientes com câncer apresentando 100% de homologia. Foram isolados *B. fragillis, E. coli e E. faecalis* de 2 amostras de pacientes com câncer por hemocultura. Todas as demais amostras não apresentaram resultados positivos da hemocultura. Pelo ensaio de PCR em tempo real foi verificado diferenças estatísticas nas amostras fecais para o Filo *Bacteroidetes, E. coli, D. pneumosintes* e *F. nucleatum.* Nos tecidos avaliados, observou-se diferenças para o Filo *Bacteroidetes,* Filo *Firmicutes, Clostridium* Cluster I, *Lactobacillus* spp., *E. coli, P. gingivalis* e *A. actinomycetemcomitans.* Os resultados demonstraram que a microbiota fecal é bastante diversa e complexa, por isso mais estudos são necessários para analisar os possíveis efeitos da modulação da microbiota no desenvolvimento do câncer reto e possíveis terapias.

Palavras-chave: microbiota fecal, câncer retal, *Clostridium perfringens*, *Bacteroides fragilis*, toxinas.

ABSTRACT

ALBERCA, G. G. F**. Possible association of the presence of anaerobic bacteria from the gastrointestinal tract with rectal neoplasia: occurence and molecular characterization.** 2020. 123 p. Thesis (PhD in Science - Microbiology) Instituto de Ciências Biomédicas, Universidade de São Paulo, 2020.

The interactions of gastrointestinal microbiota with rectal cancer (RC) have been much studied, but there is no scientific evidence on this relationship. Anaerobic bacteria are predominant in the resident microbiota of the gastrointestinal tract. Bacteria such as: *Bacteroides fragilis* and *Clostridium perfringens*, are found in the intestinal microbiota of humans and animals, however they being opportunistic agents and their pathogenicity are related to the production of several virulence factors such as toxins and hydrolytic enzymes. In this study, 40 patients with rectal cancer and 20 patients without cancer were evaluated. From cancer patients: 25 fecal samples, 38 blood samples, 36 samples of tumor tissue and adjacent tissue were collected. From the patients without cancer were collected: 16 fecal samples, 19 blood samples and 14 samples of healthy rectal tissue. The isolation for anaerobic bacteria of the "group" *Bacteroide fragilis*, *Parabacteroides* spp. and *Clostridium* spp. was performed in all clinical samples, as well as the study of genetic diversity and the detection of intestinal and oral bacteria by the quantitative real-time PCR. The species *B. ovatus* (*P* = 0.018) and *B. stercoris* (P = 0.018) showed statistical differences in isolation in relation to patients with and without cancer. Of the 24 strains of *B. fragilis* isolated, none harbored the *bft* gene or its subtypes in both groups studied. In the distribution of species of the genus *Clostridium* there were no differences between the patients evaluated, but the species *C. perfringens* was the most prevalent in both groups. Of the strains of *C. perfringens* isolated from cancer patients 80 were to toxinotype A and 5 to toxinotype G. Of these 85 strains, 8 harbored the *tpeL* gene, 5 the *netB* gene and 6 the *netE* gene. No strain harbored these three toxins simultaneously; and they all harbored the *nanI* and *nanJ* genes. All 47 strains from the control patients belonged to the toxinotype A and had the *nanI* and *nanJ* genes and did not harbor the *tpeL, becA, becB, netB, netE, netF* and *netG* genes. About the genetic diversity of *B. fragilis* by AP-PCR was verified high diversity and no strain of cancer and control patient had 100% homology. In *C. perfringens* one control patient strain (S15.1) was found in the same cluster as three other cancer patient strains with 100% homology. *B. fragillis, E. coli and E. faecalis* were isolated from 2 samples of cancer patients per blood culture. All other samples showed no positive results from blood culture. The real-time PCR assay showed statistical differences for Philo *Bacteroidetes*, *E. coli*, *D. pneumosintes* and *F. nucleatum* in the fecal samples. In the evaluated tissues, differences were observed for Phylum *Bacteroidetes*, Phylum *Firmicutes*, *Clostridium* Cluster I, *Lactobacillus* spp., *E. coli*, *P. gingivalis* and *A. actinomycetemcomitans*. The results have shown that faecal microbiota is quite diverse and complex, so further studies are needed to analyse the possible effects of microbiota modulation on the development of rectal cancer and possible therapies.

Keywords: Fecal microbiota, rectal cancer, *Clostridium perfringens*, *Bacteroides fragilis*, toxins.

1. INTRODUÇÃO

O câncer de cólon e reto é considerado o terceiro tumor maligno mais prevalente nos países desenvolvidos, onde aproximadamente 1,8 milhões de novos pacientes/ano são observados, com 862.000 mortes anuais (WHO, 2018). Nos Estados Unidos da América a incidência de câncer colorretal tem diminuído entre os maiores de 50 anos e aumentado abaixo dessa faixa etária, essa mudança possivelmente se deve as alterações do estilo de vida (JAHANI-SHERAFAT *et al.*, 2018; RAWLA; SUNKARA; BARSOUK, 2019).

Segundo o Instituto Nacional de Câncer (INCA), em 2018, na população brasileira, o câncer de colorretal apresentou uma estimativa de 36.360 novos casos, sendo considerado o 3º tumor mais prevalente em homens com (16.830 casos) e o 2º mais comum em mulheres (17.900 casos). A estimativa para o ano de 2020 a 2022 é de 41 mil novos casos, possivelmente será considerado o 2° tumor mais prevalente em homens e mulheres (INSTITUTO NACIONAL DE CÂNCER, 2019; INSTITUTO NACIONAL DO CÂNCER, 2018).

Nas regiões Sul e Sudeste, em relação às regiões Norte e Nordeste, a população apresenta elevada incidência dessa neoplasia, provavelmente devido aos diferentes padrões alimentares e sócio-econômicos (INSTITUTO NACIONAL DE CÂNCER, 2019; PINHO; FRANÇA-JUNIOR, 2003).

As neoplasias que acometem o cólon e o reto devem ser tratadas de maneiras distintas, pois o cólon e o reto apresentam diferenças na anatomia, função e padrões metastáticos (TAMAS *et al.*, 2015). Dessa forma é possível que a microbiota intestinal bacteriana envolvida nesses processos seja distinta.

Estima-se que menos de 10% dos casos de desenvolvimento do câncer intestinal estejam relacionados a históricos familiares, como polipose adenomatosa familiar (FAP) e o câncer colorretal hereditário sem polipose (HNCCP); e 70% dos casos podem ser classificados como esporádicos (ou somáticos), ou seja, não estão relacionados a fatores hereditários (KRAVOCHUCK; CHURCH, 2017; PARREIRAS *et al.*, 2013; RONCUCCI; PEDRONI; MARIANI, 2017).

Os fatores envolvidos no desenvolvimento dos diversos tipos de câncer são: idade, gênero, fatores ambientais, doenças inflamatórias intestinais, dieta, tabagismo, sedentarismo, índice de massa corpórea (IMC), diabetes melitus, e

fatores genéticos (CARR *et al.*, 2018; ELINAV *et al.*, 2013; NUNEZ *et al.*, 2018). Interessantemente, DOUBENI et al., (2012) afirmam que os fatores de risco como: saneamento, educação escolar; e comportamento de risco como: dieta, falta de exercícios, e uso de medicamentos, entre outros, estão mais associados com o desenvolvimento de câncer de cólon do que ao câncer de reto.

A microbiota residente intestinal é constituída por várias espécies bacterianas comensais que fornecem ao hospedeiro uma proteção contra patógenos exógenos. Por outro lado, a microbiota residente pode causar inflamação ou tumorogênese devido à capacidade de apresentar atividades enzimáticas, como β-glicosidades, álcool desidrogenase, ácidos graxos de cadeia curta, nitroredutases, fitoquímicos, ácidos biliares secundários, entre outros (HUYCKE; GASKIN, 2004; LOUIS; HOLD; FLINT, 2014). Alterações na ecologia intestinal podem causar constipação e aumentar os riscos de desenvolver doenças, como: colite e câncer de cólon (DENIPOTE; TRINDADE; BURINI, 2010).

O papel da microbiota é de fundamental importância para a homeostase do intestino, podendo desempenhar um papel favorável ou desfavorável para o organismo. Alterações na homeostase acarretam uma série de desordens tanto na composição microbiana como no metabolismo do hospedeiro (HOLMES, A. J. *et al.*, 2017; HOLMES, E. *et al.*, 2012).

As bactérias anaeróbias são predominantes na microbiota residente da cavidade bucal, trato intestinal, e trato genital feminino, tendo como destaque espécies dos gêneros *Bacteroides, Bifidobacterium* e *Lactobacillus* (BEDANI *et al.*, 2010; SUMMANEN, 1993). Algumas dessas espécies bacterianas tais como os dos gêneros *Bifidobacterium* spp. e *Lactobacillus* spp., têm mostrado alguma relação simbiótica com a saúde do hospedeiro (GOMES; MALCATA, 1999; SHAH, N. P., 2007; SLAVIN, 2013).

Segundo GAO et al., (2015), o filo *Firmicutes* e os gêneros *Lactococcus, Bacteroides* e *Streptococcus* estariam presentes em maior abundância no tecido tumoral de câncer colorretal quando comparados com os tecidos adjacentes sem câncer. Espécies do gênero *Bacteroides* se destacam dentre a família *Bacteroidaceae*, por serem o gênero mais observado em infecções do trato gastrointestinal. A virulência dessas bactérias está associada à habilidade de aderir às superfícies celulares, devido à expressão de cápsulas, fímbrias e adesinas, produção de toxinas e enzimas hidrolíticas, e ácidos graxos de cadeia curta, que

inibem a resposta de células T e interfere na quimiotaxia celular, além de induzir a apoptose de macrófagos e polimorfonucleares, estimulando assim, a resposta inflamatória (OBISO, JR.; BEVAN; WILKINS, 1997).

Dentre as espécies do gênero *Bacteroides, B. fragilis* destaca-se por participar de infecções oportunistas de natureza endógena, assim como, de infecções extra-intestinais, urogenitais e bacteremias (SACK *et al.*, 1994). Sua patogenicidade está também relacionada a proteínas de membrana e produção de enzimas específicas, incluindo a enterotoxina ou fragilisina (SEARS, 2009; SEARS; GEIS; HOUSSEAU, 2014). A presença de *B. fragilis* enterotoxigênico (ETBF) em material fecal e em biópsia de cólon, e doença inflamatória intestinal tem sido relatada (BOLEIJ *et al.*, 2015; ULGER TOPRAK *et al.*, 2006).

Clostridium perfrigens pertence à microbiota residente intestinal de humanos. A virulência dessa bactéria é dependente da produção de toxinas, podendo produzir até 20 tipos diferentes de toxinas, tais como: alfa, beta, épsilon, iota, enterotoxina, NetB, TpeL, a mais recente, enterotoxina binária (BEC) e enzimas hidrolíticas (REVITT-MILLS; ROOD; ADAMS, 2015; YONOGI *et al.*, 2014). Antigamente, as cepas eram classificadas segundo a produção de quatro toxinas: toxina alfa (tipo A); toxina alfa e beta (tipo C), toxinas alfa, beta e épsilon (tipo B); toxinas alfa e épsilon (tipo D); e toxinas alfa e iota (tipo E) (UZAL, FRANCISCO *et al.*, 2014). Recentemente uma nova clasificação do toxinotipo foi proposta por Rood et al., (2018), acrescentando mais dois toxinotipos o F e o G: com a produção da toxina alfa e enterotoxina (tipo F) e toxina alfa e a NetB (tipo G), futuras alterações nessa classifcação podem vir a serem proposta com adição de outras toxinas. Essas toxinas apresentam ação letal ou tóxica contra as células epiteliais do hospedeiro; entretanto, a afinidade de cada toxina com as células, ainda não está totalmente definida (TWETEN, 2001).

Além disso, espécies toxigênicas de *C. perfringens*, podem expressar até três sialidases, sendo codificadas pelos genes *nanH*, *nanI* e *nanJ* (LI; MCCLANE, 2014; LLANCO; NAKANO; AVILA-CAMPOS, 2014). Essas neuraminidades ou sialidases são enzimas que auxiliam no processamento de oligossacarídeos contendo ácido siálico. A sialidase NanI é predominante em *C. perfringens* do tipo A quando comparadas com às outras duas sialidases (LI; MCCLANE, 2014).

A maioria das bactérias anaeróbias presente na microbiota intestinal produzem diversos fatores de virulência, principalmente toxinas que agridem os

tecidos do hospedeiro. Embora as ações tóxicas sejam conhecidas, até o momento não é observada na literatura, alguma relação com células tumorais, particularmente tumores de câncer de cólon e/ou reto.

A bactéria facultativa *Escherichia coli* é uma bactéria comensal que participa de processos homeostáticos no intestino, mas também pode causar danos ao hospedeiro através da sua patogenicidade provenientes da presença de genes de virulência (MAINIL, 2013; SAROWSKA *et al.*, 2019).

Apesar de alguns estudos recentes mostrarem que existe uma associação da presença da bactéria *Escherichia coli* em lesões tumorais (LIU *et al.*, 2019), evidências de causa e consequência ainda necessitam ser investigadas (WASSENAAR, 2018). Existem diversas hipóteses da ação pró-oncogênica de espécies de *E. coli*, como as que possuem a capacidade de produzir toxinas (ANTONIO HERNÁNDEZ-LUNA; LAGUNES-SERVIN; LOPEZ-BRIONES, 2016) e/ou servir como bactéria *driver* para bactérias *passenger* oncogênicas (TJALSMA *et al.*, 2012).

Dentre as toxinas produzidas pela *E. coli*, a colibactina demonstrou potente efeito carcinogênico em modelo murino de inflamação intestinal (BAKTHAVATCHALU *et al.*, 2018). A capacidade de induzir o processo oncogênico é dependente dos genes de *pks* presentes nas cepas de *E. coli* (ARTHUR *et al.*, 2012). O mecanismo pelo qual a colibactina influência no desenvolvimento do câncer está correlacionada com as propriedades de atuar no ciclo e morte de células eucarióticas (FAÏS *et al.*, 2018).

A cavidade oral é um ecossistema complexo, onde há a interação de diversos microrganismos, e observa-se um equilíbrio constante entre a atividade microbiana e a resposta imunológica do hospedeiro. Composta pelos filos *Firmicutes* (principalmente *Streptococcus*), *Bacteroidetes* (principalmente *Prevotella*) e *Proteobacteria* com *Fusobacteria, Actinobacteria e Haemophilus* segundo o projeto do microbioma humano desenvolvido pelo National Institute of Health (NIH) (HUSE *et al.*, 2012; KOLIARAKIS *et al.*, 2019; ZHOU *et al.*, 2013).

Bactérias anaeróbias orais como: *Porphyromonas gingivalis* e *Fusobacterium nucleatum* são consideradas oportunistas, pois em processo de disbiose oral corroboram com o desenvolvimento de doenças periodentais (AHN; SEGERS; HAYES, 2012). *P. gingivalis* e *F. nucleatum* possuem a capacidade de gerar alta

resposta pró-inflamatória (MCCOY *et al.*, 2013) e infecções crônicas (HAN; WANG, 2013).

Além disso, a presença de *P. gingivalis* em um processo inflamatório pode levar ao desenvolvimento de um microambiente pró-tumoral devido à sua capacidade de atuar na divisão celular e na apoptose das células do hospedeiro (MAO *et al.*, 2007).Também, estudos demonstram a associação de *F. nucleatum* com a neoplasia colorretal, entretanto, os mecanismos dessa relação ainda não estão totalmente esclarecidos (KOSTIC *et al.*, 2012). Ahn et al., (2012) detectaram o aumento de bactérias gram-negativas pertencentes as espécies dos gêneros *Fusobacterium, Porphyromonas* e *Prevotella* em amostras fecais de pacientes com câncer de cólon e reto. Esse link oral-cólon pode constituir uma outra rota para bactérias orais mediando respostas inflamatórias sistêmicas.

2. OBJETIVOS

2.1. Geral

Tendo em vista, a importância da participação das bactérias intestinais e orais como microbiotas residentes em processos clínicos, particularmente, em diversos tipos de câncer, este estudo visa obter dados microbiológicos sobre a possível associação dessas bactérias com a neoplasia retal.

2.2. Específicos

1. Análise qualitativa da presença de espécies dos gêneros *Bacteroides, Parabacteroides* e *Clostridium,* bem como de espécies toxigênicas de *B. fragilis* e *C. perfringens* em material fecal de pacientes com câncer e sem câncer de reto;
2. Análise qualitativa da presença de espécies dos gêneros *Bacteroides, Parabacteroides, Clostridium* e *Escherichia coli,* bem como de espécies toxigênicas de *B. fragilis* e *C. perfringens* em sangue de pacientes com câncer e sem câncer de reto;
3. Determinar a diversidade genética das cepas de *B. fragilis* e *C. perfringens* isoladas de pacientes com e sem câncer de reto e;
4. Análise quantitativa da presença de bactérias intestinais (*Bacteroides fragilis*, *Escherichia coli, Lactobacillus* spp.*, Bifidobacterium* spp., *Clostridium perfringens, Clostridioides difficile, Clostridium Cluster* I, e Filos *Bacteroidetes* e *Firmicutes*) e bactérias orais (*Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella nigrescens, Prevotella intermedia, Dialister pneumosintes,* e *Aggregatibacter actinomycetemcomitans*) em: fezes, sangue, tecido tumoral e tecido adjacente ao tumor de pacientes com câncer de reto; ou tecido retal sadio de pacientes sem câncer.

7. CONCLUSÃO

Com os resultados obtidos neste estudo permite-se concluir que:

- Pacientes com câncer de reto apresentaram maior idade média que os pacientes sem câncer de reto;
- Os isolados de *Clostridium perfringens* das amostras de fezes analisadas, apresentaram toxinotipo A e G; e a presença de cepas abergando genes das toxinas TpeL, NetB e NetE foi verificada apenas nos isolados de pacientes com câncer;
- Verificou-se a alta diversidade genética dos isolados de *Clostridium perfringens* e *Bacteroides fragilis*;
- A bacteremia foi observada em dois pacientes com câncer de reto;
- Na análise quantitativa utilizando o qPCR:
  - Nas amostras fecais foi possível verificar diferença estatística no Filo *Bacteroidetes*, *Escherichia coli*, *Dialister pneumosintes* e *Fusobacterium nucleatum*;
  - Na comparação dos tecidos saudáveis do paciente com câncer (adjacente) e do paciente sem câncer, houve diferença estatística no Filo *Bacteroidetes*; *Clostridium* Cluster I, *Lactobacillus* spp. e *Escherichia coli*;
  - Já em relação ao tecido tumoral comparado com o tecido sadio dos pacientes sem câncer, foi observado diferença estatística no Filo *Firmicutes* e *Porphyromonas gingivalis*;
  - No tecido tumoral com o tecido adjacente do paciente com câncer foi possível verifcar diferença estatística no Filo *Firmicutes*, *Clostridium* Cluster I, *Lactobacillus* spp., *Escherichia coli* e *A. actinomycetemcomitans*;
  - Em duas amostras de sangue de pacientes com câncer retal (os mesmos da hemocultura positiva) foi verificada a presença de bactérias do Grupo *B. fragilis* em baixa concentração. E nas demais amostras de ambos os grupos selecionados não foi detectado nenhum microrganismo avaliado nesse estudo.

Resumindo, o câncer de reto é uma patologia multifatorial e a influência das bactérias da microbiota fecal nesse quadro parece ser portador dependente. São necessários mais estudos para clarificar o real papel desses microrganismos no desenvolvimento, manutenção ou agravamento dessa doença.

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