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What genes in *Bacteroides fragilis* contributes to the tumoricidal effect in Colonrectal cancer?

Introduction

Excluding skin cancer, colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women in the US (National Cancer Institute 2016). CRC is associated with diets high in processed foods, red meat and chronic gastrointestinal inflammation (Hagland et al. 2015). CRC is expected to cause 49, 190 deaths this year (National Cancer Institute 2016). Twenty-five percent of patients diagnosed with CRC have a family history of this cancer (National Cancer Institute 2016b).

Fig. 1 The proportions of Bacteroides species identified in humans. Modified from Wexler et al. 2007.

The gut microbiome has been shown to be associated with the cell proliferation and apoptosis observed in cancer (Zackular et al. 2013). Specifically, when comparing healthy gut microbiomes to the gut microbiomes of CRC patients, the CRC patients tended to have altered gut microbiomes (Zackular et al. 2013). The main type of alteration noted is a drop in gut microbiota diversity. A drop in diversity leads to increased competition among the bacterial colonies for space and food, which causes dysbiosis (an imbalance) (Coyte et al. 2015). Figure 1 shows the *Bacteroides* species that most commonly found in the gut microbiome (Wexler 2007).

Understanding the role that the gut microbiota plays in the formation of tumors can lead to finding therapies to prevent colon cancer. So far, there have been several studies that have associated several bacteria species with the development or prevention of CRC. Zackular et al. 2013 have observed that changing the gut microbiome can change the rate of tumor growth and the size of the tumor. Of the many bacteria they found in the gut microbiome, three species *Bacteroides fragilis* (Bf), *Bacteroides thetaiotamicron* (Bt), and *Bacteroides uniformus* (Bu) showed an association with CRC. In an experiment to demonstrate the involvement of the gut microbiome in tumor progression Zackular et al. (2013) exposed germ-free mice to the feces and bedding from healthy mice (healthy community) or from tumor-bearing mice (dysbiotic community). Because normally mice engage in coprophagy, it is assumed the germ-free mice will acquire the gut microbiota from donor’s feces. To induce tumors, they treated all the mice with intraperitoneal injections of the chemical carcinogen azoxymethane (AOM). 73 days after AMO the colons were harvested and the number of tumors determined. Figure 2 summarizes the results of the experiment. The group of germ-free recipient mice that acquire their dysbiotic microbiome from a tumor-bearing donor (dysbiotic community) had twice as many tumors as the group that acquired their microbiome from the healthy donor (healthy community). The germ-free mice exposed to a dysbiotic community acquired a less diverse microbiome. Analysis of the composition of the gut microbiome using 16S rRNA PCR amplification showed a general increase in the populations of *Bacteroides and Erysipelotrichaceae* and a reduction in *Porphyromonadaceae*. The microbiome of germ-free mice exposed to a healthy community had an increase of *Odoribacter* and *Turicibacter,* which was not detected before and a decrease in *Prevotella* and *Porphyromonadaceae.* The authors observed that changes in the microbiome changes the rate of tumor growth and size of tumors.

Fig. 2 Number of tumors observed in the colon of mice with a healthy or dysbiotic microbiome community. Modified from Zackular et. al 2013.

Fig. 3 Tumors in the colon of mice with a healthy and dysbiotic microbiome communities. Modified by Zackular et. al 2013.

To test the effect of specific bacteria in tumor size, Vetizou et al. (2015) (Figure 4) feed germ-free mice a combination of broad-spectrum antibiotics (amphicillin, colistin and streptomycin) and a specific bacterium followed by an injection of tumor inducing chemicals. After 73 days the colons were harvested and and the size of the tumors determined. Figure 4 illustrates the results of the experiment and shows that tumors of mice with B. fragilis were significantly smaller.

Fig. 4 Effect of specific bacterium in the size of tumors. Modified by Vetizou et. al 2015.

Based on the finding that B. fragilis appears to reduce tumor size, I will design an experiment that aims to identify what is contained in Bf that is contributing to decreased tumor size. To answer this question, it is helpful to use a combination of bioinformatics searches and *in vitro* experiments to identify and isolate the genes the bacteria have and explore which genes lend to the tumoricidal effect. Now, this is a giant leap, nonetheless, it is a good place to start.

Experiment

Comparative genomic analyses of *B. vulgatus, B. distasonis, B. thetaiotamicron*, and *B. fragilis* identified a set of 1416 protein-coding genes shared by all these Bacteroides species (Xu et al. 2007). The Bf genome contains 4625 protein coding genes and 3209 of those are specific to Bf and are not present in the other Bacteroides. Because the genome is fully sequenced, I can use the genome to design ~3209 pairs of PCR primers flanking those 3209 genes. In order to do this, I will use a website called PRIMER and will do a random selection of 32 genes to test at a time. These primers will be used in PCR, amplifying each one of those genes ~32 PCR reactions at a time. The PCRs that produce a band of the expected size will be cloned into an expression vector. The plasmid construct will be transformed into E. coli and the culture with be administered by oral feeding.

Fig. 5 Davidson college pET vector

Cario (2013) discusses using different vectors to stimulate or suppress proteins that turn on/off the inflammation response that leads to tumorgenesis. They used Polymerase Chain Reaction (PCR) to amplify and isolate each target gene. PCR uses DNA polymerase to synthesize a new strand of DNA complimentary to the target strand to get a specific region of target sequence that we want to amplify. In order to do this, we must first denature the target DNA at a high temperature, around 94-95 degrees Celsius. Add DNA polymerase, which is an enzyme that makes new strands of DNA complimentary to our target sequence. According to NCBI, when doing PCR, Taq, *Thermis aquaticus*, is the most common DNA polymerase used because it is heat resistant (Anonymous 2016). Taq generates new DNA by using target DNA as a template and primers. Primers are short pieces of single stranded DNA that are complementary to the target sequence. Taq will begin synthesizing new DNA from the end of the primer onward. Add deoxynucleotide triphosphates (dNTPs), which are single units of bases A, T, G, and C. Taq will use these dNTPs as the building blocks for the new DNA strand.

Through PCR, we can exponentially amplify the target sequence and make a larger quantity of gene. In this experiment, we are amplifying an expression vector as a plasmid for several genes that Bf & Bd have in common. A plasmid is a chromosomal DNA molecule found in bacteria that usually carry gene(s) that are beneficial but not necessary for the cell (Linott 2015). An expression vector is a synthetic plasmid that is created to express genes and a myriad of other things (Linott 2015). We add it in the bacteria so when the bacteria undergo replication, therefore causing the plasmid to undergo transcription and translation and pop out a protein. We would put the target gene in the pLink part of the plasmid. Once the vector plasmid has been implanted in the *Escherichia coli* BL21(DE3), which had the T7 polymerase in it, we will send a signal to the culture to begin replication. This is done by adding Isopropyl beta-D-1-thiogalactopyranoside (IPTG), a molecule that mimics lactose and triggers the transcription of the lac operon. It is used to induce protein expression where the gene is under control of the lac operon. The Lac operon controls metabolism of lactose in the E. coli cell (Wikipedia 2016).

We do gel electrophoresis, which separate charged molecules according to size. We are doing it here to make sure that the primers we used for PCR amplify the correct target sequence. For example, if we wanted was 1520 base pairs, the DNA that appears after electrophoresis should be 1520 base pairs. In order to make sure the bands are the right size, the band generated is compared against a 1kb DNA ladder size marker. Smaller molecules migrate faster and farther than larger molecules. The gel is made of agarose powder and water. A comb is placed to make wells and then taken out when the gel is set. The gel is put in the electrophoresis tank and electrophoresis buffer is poured into the tank till the surface of the gel is covered. Dye is added to the DNA, then DNA is put into the top well. A DNA marker of known size is added to the first well to measure the experimental DNA against. The DNA is separated by passing an electrical current through the gel so that at the end of the gel has a positive charge at one end and a negative charge at the other end. Due to DNA’s negative charge, it will be attracted to the positive end of the gel. The electrical current is shut off when the DNA fragments have moved far enough along the gel to separate cleanly. To visualize the bands, put the gel in a UV transilluminator.

 We will have mice in two groups like Zackular et. al, group germ-free (GF) with AOM/DSS. We can break them up into groups (total of two groups), put tumors in two groups (group 1a is GF, Group 2a SPF) feed E. coli culture with vector via gavage, then group 1b (GF) and 2b (SPF) E. coli control (has plasmid, no insert). Harvest their intestine and count the number of tumors.

Results and Discussion

The results can tell us whether these bacteria have a gene or something that can be used to decrease tumorgenesis. It can also lead to more questions, like whether or not other bacteria have that gene, or is that gene the only one? Does it act alone or in tandem with others? Does a protein arise out of it that can be used? If there is no decrease in tumor growth, we would have to look at other factors or something else common to the bacteria. The main limitation of the experiment is that this is a very controlled setting so it would be very difficult to state what would work in a variable environment like the gut microbiome.

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