The role of fusobacterium nuleatum in colorectal cancer

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Abstract
Background: Colorectal cancer (CRC), the third leading cause of cancer-related deaths, is driven by host genetics and environmental factors. with one to two million new patients recorded each year, making it the 3rd most common malignancy and the 4th leading cause of cancer-related death. One of its most important risk factors is gut bacteria as it's involved in nutrition metabolism and absorption, drug metabolism, and xenobiotic clearance in healthy individuals. Furthermore, a healthy gut microbiota aids in the preservation of intestinal barrier integrity, defends against infections, and aids in immunomodulation. One of these microorganisms is Fusobacterium Nucleatum gram-negative anaerobic opportunistic bacteria that may affect the tumor microenvironment. The objective of this study: To investigate the role of Fusobacterium nucleatum as an associated factor with CRC. Patients and methods: This study was conducted from January 2022 to June 2022. Tissue biopsies were collected from a total of 35 colorectal patients, 35 of tumor-adjacent tissue and 35 biopsies of polyp tissue, from patients who had attended the Gastroenterology and Liver Diseases Hospital and 30 normal control tissue were collected from autopsies at the Forensics Medicine Department. All samples were homogenized and underwent DNA extraction and it was investigated for Fusobacterium nucleatum using nusG gene-specific primers by real-time PCR.Results: it's been found that Fusobacterium nucleatum is significantly higher in malignant tissue which shows positive results in 18 patients (51.4%), followed by resection tissue which shows positive results in 14 patients (40.0%), and benign tissue in 7 patients (20%), with significant P value (P =<0.001). Fusobacterium nucleatum is significantly higher in the rectum tumor tissue compared to other sites of tumors represented by 8 cases (80%) out of a total 10, with a significant P value of (P=0.048). And according to the staging system it was mostly expressed in patient with extensive tumor invasion (T4) with 15 patients (93.80%) out of 16 patients compared to patient with T1+T2+T3, with a significant P value (P =0.001). According to lymph node spreading of cancer it was mostly expressed in N2 with 11 patients (78.60%) out of 14 patients compared to patients with N0+N1, with significant P value (P =0.015). It was also mostly expressed in the metastases patient at M1 with 15 patients (75.00%) out of 20 patients, with significant P value (P =0.001). Conclusions
1. A higher abundance of F. nucleatum in CRC compered to normal and pre-malignant tissue which refers to the important role of the bacteria in cancer development.
2. F. nucleatum on the gastrointestinal mucosa has a significant impact on the onset and development of CRC. However, its contribution to the progression of cancer is still complex.

1. Introduction

A growing body of research points to the potential involvement of microbes in the development of colorectal cancer. Fusobacterium nucleatum appears to decrease antitumor immune response and enhance the growth of colonic neoplasia in animal models, among other microbial species (1). A high concentration of F. nucleatum in carcinoma tissue has been linked to the proximal tumor site, high-level microsatellite instability (MSI-high), and reduced density of T lymphocytes in tumor tissue (2,3). Additionally, enrichment of F. nucleatum has been seen in a subset of human colorectal neoplasms (4–7).
in clinical, pathological, and epidemiological investigations, the colorectum is often divided into the proximal colon (cecum to transverse colon), distal colon (splenic flexure to the sigmoid colon), and rectum because it is a lengthy organ (8). But numerous studies have shown that the percentages of colorectal malignancies with particular molecular characteristics, such as high MSI, high CpG island methylator phenotype (CIMP), and high BRAF and PIK3CA mutations, gradually rise throughout the bowel subsites from the rectum to ascending colon (9). These results support the colorectal continuum model rather than the dichotomy or trichotomy models and are in line with the theory that the microbiota, bacterial metabolites, and other contents of the large intestine change gradually (as opposed to suddenly) from the proximal to distal regions (10–13).
To investigate the role of Fusobacterium nucleatum as an associated factor with CRC the frequency and amount of Fusobacterium nucleatum were examined in the fresh tissue biopsy of colorectal cancer (CRC) patients, benign tissue and control using a real-time PCR technique.

2. Methods

Study population: Samples collection included

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tissue biopsies of the tumor from patients with colorectal cancer who are a week apart from the last antibiotic dose and never had chemotherapy or radiotherapy before and the adjacent tissue, also benign tissue biopsies from the endoscopic department and normal control tissue.

Tumor and adjacent tissue: A total of 35 colorectal cancer patients (18 men and 17 women) with ages ranging from 24 to 71 years old were included in this study. Direct interviews with the patients were conducted to obtain clinical data, along with investigations into their hospital records and past medical histories.

Control Group: 30 tissue biopsies from autopsies at the forensics medicine department and histopathologically proved free of malignancy which was taken as normal tissue (12 males and 18 females with a mean age of 45.11 years and a range between 21 and 70 years) and were used as controls for evaluation in real-time PCR.

Benign tumor: 35 endoscopic biopsies of polyp (22 female and 13 male with a mean age of 47.6 and a range between 29 and 72) were taken from the diagnostic endoscopy department at the Gastroenterology and Liver teaching hospital. All been histopathological proved to be benign polyposis.

Detection and quantification of *Fusobacterium nucleatum* bacteria using quantitative real-time PCR (q RT- PCR)

PrestoTM Mini gDNA Bacteria Kit Quick protocol (Geneaid Biotech Ltd, Taiwan) has been used to extract high purity genomic bacterial DNA from a fresh homogenization tissue sample, the amount of tissue F. nucleatum DNA were measured in all tissue biopsies, while blinded to data on tumor location and other clinical, pathological, and tumor molecular features. Custom TaqMan primer/probe sets for the nusG gene (Forward and Reveres) of F. nucleatum and for the reference human housekeeping gene (PGT)and the probe for each gene (PGT Probe (FAM-BHQ),Fusobacteria probe (FAM-BHQ) (14,15).

Reagent volumes were determined using the manufacturing Master Mix (Go Taq Prope qPCR Master Mix) protocol (Promega, USA) and based on the number of controls and samples plus one more reaction to insure a sufficient volume.

The template DNA (samples) and primers (provided by Alpha DNA Technologies, Canada) were added to PCR tubes in the concentrations mentioned. Nuclease-Free Water was added to PCR tubes to a total volume of 20 µl and then mixed by a brief vortex.

Assembling the GoTaq® Probe qPCR Master Mix reaction mix,the primers sequence and thermal programming of the reaction is showing in table 1,2,3.

Table (1): Assembling the GoTaq® Probe qPCR Master Mix reaction mix.

Component	Volume	Final Concentration
GoTaq® Probe qPCR Master Mix,2x	10µl	1x
Forward Primer (20x)	1µl	200nM-1µm
Reverse Primer (20x)	1µl	200nM-1µm
Hydrolysis Probe (20x)	1µl	100-300nM
Template DNA	2-5µl	250ng
Nuclease-Free Water	To a final volume of 20µl	

Table (2): Primer sequences used for amplification analysis of the *Fusobacterium nucleatum* bacteria, housekeeping gene, and their probes.

Gene		Primer sequence
nusG	Forward Primer	5′-CAACCATTACTTTAACTCTACCATGTTCA-3′
	Reverse Primer	5′-GTTGACTTTACAGAAGGAGATTATGTAAAAATC-3′
Fusobacteria probe (FAM-BHQ)		5′-TCAGCAACTTGTCCTTCTTGATCTTTAAATGAACC-3′
PGT	Forward Primer	5′-ATCCCCAAAGCACCTGGTTT-3′
	Reverse Primer	5′-AGAGGCCAAGATAGTCCTGGTAA-3′
PGT Probe (FAM-BHQ)		5′-CCATCCATGTCCTCATCTC-3′

Table (3): PCR program and thermal profile of Fusobacterium nucleatum bacteriaand the housekeeping gene.

Steps	Cycles	Temperature	Time
GoTaq® DNA Polymerase activation	1	95°C	10 Minutes
Denaturation	40	95°C	15 Seconds
Annealing and extension		58°C	30 Seconds

In the final step of the thermal protocol, the Real-time PCR (Mx3005P, Agilent Technologies, USA) instrument software automatically calculate the baseline cycles and threshold, and the amplification curve was given for each sample as the (Y) axis is the Ct – Threshold cycle, and the (X) axis the bacterial or the house-keeping DNA copy number.

Qualitative results were displayed on the (Report mode) screen. Samples that cross the threshold in the channel (FAM) are displayed as positive while those that did not cut the threshold were displayed as negative or (NO CT). According to the following table, the housekeeping gene was analyzed for those undetectable samples to prevent giving false

negative results.

3. Statistical Analysis

The statistical analysis of this case-controlled prospective study performed with the statistical package for social sciences (SPSS) 20.0 and Graph-pad prism Version 7. Numerical data were described as mean and standard deviation and analysis of variance test used for comparison among more than two groups. Categorical data were described as count and percentage. Chi-square test used to estimate the association between variables. The lower level of accepted statistically significant difference is bellow or equal to 0.05(16).

4. Results

Fusobacterium nucleatum in Real-time PCR Analysis

Using qPCR, the relative quantification of Fusobacterium nucleatum is significantly higher in malignant tissue which shows positive results in 18 patients (51.4%), followed by resection tissue which shows positive results in 14 patients (40.0%), and benign tissue in 7 patients (20%), with significant P value (P =<0.001) as it is shown in table 4.6.

Table (4): qPCR quantification results of *Fusobacterium.nucleatum*

		Group			
		Benign (n=35)	Control (n=30)	Malignant (n=35)	Resection (n=35)
Fusobacterium nucleatum	Positive	7	0	18	14
		20.0%	0.0%	51.4%	40.0%
	Negative	28	30	17	21
		80.0%	100.0%	48.6%	60.0%
P value		<0.001**			

**: High statistical significance (p≤0.001).

The relation between quantification of *Fusobacterium nucleatum* the site of the tumor and the cancer stages

Fusobacterium nucleatum is significantly higher in the rectum tumor tissue compared to other sites of tumors represented by 8 cases (80%) out of a total 10, with a significant P value of (P=0.048).

And according to the staging system it was mostly expressed in patient with extensive tumor invasion (T4) with 15 patients (93.80%) out of 16 patients compared to patient with T1+T2+T3, with a significant P value (P =0.001).

According to lymph node spreading of cancer it was mostly expressed in N2 with 11 patients (78.60%) out of 14 patients compared to patients with N0+N1, with significant P value (P =0.015).

It was also mostly expressed in the metastases patient at M1 with 15 patients (75.00%) out of 20 patients, with significant P value (P =0.001

Table (5): The relation between quantification of Fusobacterium nucleatum the site of the tumor and the cancer stages.

		Fusobacterium nucleatum			
		Positive		Negative	
		Count	%	Count	%
Site	appendix	2	100.00%	0	0.00%
	left colon	2	22.20%	7	77.80%
	Rectum	8	80.00%	2	20.00%
	right colon	1	20.00%	4	80.00%
	sigmoid	1	33.30%	2	66.70%
	upper colon	4	66.70%	2	33.30%
P value		0.048*			
Tumor	T1	0	0.00%	6	100.00%
	T2	0	0.00%	6	100.00%
	T3	3	42.90%	4	57.10%
	T4	15	93.80%	1	6.20%
P value		<0.001**			
Nodes	N0	N0	1	14.30%	6
	N1	N1	6	42.90%	8
	N2	N2	11	78.60%	3
P value		0.015*			
Metastases	M0	3	20.00%	12	80.00%
	M1	15	75.00%	5	25.00%

*: Statistical significance (p≤0.05).
**: High statistical significance (p≤0.001).

5. Discussion

Fusobacterium nucleatum abundance in CRC tissue

Large quantities of F. nucleatum have been correlated to unfavorable CRC results, according to some research. Also, according to some studies, the presence of F. nucleatum DNA in CRC tissue is associated with a greater mortality rate for people with colorectal cancer. And that the DNA of F. nucleatum may be used as a diagnostic for bad prognosis (17).

Relative quantification of Fusobacterium nucleatum was examined in this study and it showed significant higher results in malignant tissue which give a positive result in 18 patients (51.4%), followed by resection tissue which shows positive results in 14

patients (40.0%), and benign tissue in 7 patients (20%), with significant P value (P =<0.001).
This result agrees with Ito et al, (2015) (115) who found that F. nucleatum positivity was significantly higher in CRCs (56%) than in premalignant lesions of any histological type (p < 0.001).
agreed with another study by Suehiro et al (2017) (116) the levels of F. nucleatum were measured in fecal specimens from Japanese CRC patients by ddPCR, and F. nucleatum was found to be present in 54% (85/158) of the specimens.
agreed with Li Yo et al (2016) (18) who found F. nucleatum was over-represented in 88/101 (87.1%) CRC samples. The abundance of F. nucleatum determined by 2-ΔCT was significantly greater in tumor samples than in normal controls (P < 0.001).
Although there is a difference in percentage which comes from the difference in sample size.
In another study, the richness of F. nucleatum was evaluated by qPCR, and the samples were prepared from genomic DNA extracted from 149 primary CRC tissue samples; F. nucleatum was detected in 74% (111/149) of the CRC tissue samples (7).
Mima et al (2016) (19) found in Clinical and Translational Gastroenterology using a qPCR assay to detect F. nucleatum in FFPE tissue from CRC patients, that F. nucleatum was present in 2.5% (4/157) of rectal cancers and 11% (19/178) of cecum cancers, with a significant linear trend along all subsites. The percentage of Fusobacterium nucleatum-enriched CRC gradually increases from rectum to cecum, suggesting that the rate at which F. nucleatum is present may also differ among intestinal sites.
This viability of results might be due to the difference in sample size, geographical area and life habits of each cohort.
-F. nucleatum and colorectal cancer development:
High intra-tumoral Fusobacterium nucleatum load may be linked to negative prognostic consequences, according to prior research (20).
According to Li YY et al (2016), lymph node metastases are found in 52 out of 88 cases (59.1%) with a high abundance of F. nucleatum and in 0 out of 13 patients (0%), indicating that a high abundance of F. nucleatum is associated to the progression and metastasis of CRC (18).
In this study and according to the staging system Fusobacterium nucleatum was mostly expressed in patient with extensive tumor invasion (T4) which account 15 patients (93.80%) out of 16 patients compared to patient with T1+T2+T3, with a significant P value (P =0.001).
According to lymph node spreading of cancer it was mostly expressed in N2 with 11 patients (78.60%) out of 14 patients compared to patients with N0+N1, with significant P value (P =0.015).
It was also mostly expressed in the metastasis's patient at M1 with 15 patients (75.00%) out of 20 patients, with a significant P value (P =0.001).
This results agreed with de Carvalho et al (2019) (21) who found that the Fusobacterium nucleatum DNA in the tumor tissue was significantly associated with proximal tumors (p = 0.001), higher depth of invasion (p = 0.014), higher clinical stages (p = 0.033), poor differentiation (p = 0.011),and the presence of Fusobacterium nucleatum DNA in CRC tissue was also associated with a worse patient cancer-specific survival (69.9 vs. 82.2% in 5 years p = 0.028) and overall survival (63.5 vs. 76.5% p = 0.037).
Sun et al (2016) (22)found that F. nucleatum infection of tumor tissues was related with poorer tumor differentiation (P<0.001), deeper tumor invasion (P<0.001), lymph node metastasis (P=0.01), distant metastasis (P=0.001) and advanced TNM stage (P=0.034).
Based on these results some studies suggested that Fusobacterium nucleatum might consider a higher abundance of Fusobacterium nucleatum in CRC is associated with a shorter survival time. The Fusobacterium nucleatum status, peripheral nerve invasion, vascular thrombus, lymph node metastasis, and TNM staging were related factors affecting the prognosis of patients with CRC. Previous data indicate that high intra-tumoral F nucleatum load might be associated with poor prognostic effect(15,21).
How Fusobacterium, an oral bacteria, invades colorectal tissues is one frequently asked question, Some research has proposed a mechanism involving D-galactose-(1-3)-N-acetyl-D-galactosamine (GalGalNAc), which is over-represented in CRC cells, and the F. nucleatum adhere using Fap2 protein (23).
Conclusions:
1. A higher abundance of F. nucleatum in CRC compered to normal and pre-malignant tissue which refers to the important role of the bacteria in cancer development.
2. F. nucleatum on the gastrointestinal mucosa has a significant impact on the onset and development of CRC. However, its contribution to the progression of cancer is still complex.

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