Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 3660 - 3666
Received 25 April 2021; Accepted 08 May 2021.

Molecular Detection of *Fusobacterium* Persistence in Colorectal Cancer Patients

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Abstract

The presence of *Fusobacterium nucleatum* DNAwas detected in abundance relative total number of bacteria in 40 patient tissue samples with colorectal cancer and 20 normal tissue sample. *Fusobacterium* bacterium positivity was detected in 33 (82.51%) tissue samples with CRC. The results show that there was significant increase in copy number of *Fusobacterium nucleatum* isolates in patients when compared with control group.The results of the current study showed that there was no significant different between male and female in colorectal cancer patients.

Introduction

Fusobacterium was considered as part of the normal flora of the oropharynx formerly, but lately its pathogenic role is found especially as a driver of periodontitis and its association with intestinal diseases has been demonstrated (Griffen*et al*.,2012),Although it is still unclear whether *Fusobacterium* is the passenger or driver of Colorectal cancer (CRC), many studies have concluded that *Fusobacterium* is a novel risk factor for CRC development and progression, as well as a determinant affecting patient survival outcomes (Mehta *et al*.,2017) .

Fusobacterium is a genus of gram-negative anaerobic bacteria. It may act as a main anchor of biofilms that can induce periodontitis (Okuda *et al*.,2012), vaginitis (Machado *et al*.,2015) and other infections (Sanmillan*et al*., 2013).

In colorectal adenoma, an early event in CRC development, *Fusobacterium* is found to be enriched in comparison with surrounding normal tissue suggesting an essential role of *Fusobacterium* in the early onset of CRC. In recent years, a large number of studies have indicated that the intestinal flora is closely associated with the occurrence of CRC (Yamashiro *et al*.,2018).

Fusobacterium nucleatum adheres to and invades the intestinal mucosa through its surface adhesion factors and virulence proteins, and ultimately promotes the occurrence and development of CRC (Han *et al*.,2015). It has previously been identified that the absolute copy number of Fusobacterium*nucleatum* in CRC tissues may be used as an indicator to evaluate the prognosis of patients with CRC (Yamaoka *et al*.,2018).

Recent studies have demonstrated that *Fusobacterium nucleatum*is not only associated with the development of CRC, but also promotes chemotherapeutic resistance in colon cancer via TLR4/NF-κB pathway-induced autophagy (Zhang*et al*., 2019)

Fusobacterium nucleatum promotes the occurrence of CRC through several virulence mechanisms colonization, invasion, and modulation of host immune response (Bullman*et al*., 2017) . *Fusobacterium nucleatum* bacteria interact with each other by expressing a variety of different virulence factors, and can adhere to many different mammalian cell types, including epithelial and endothelial cells, polymorph nuclear neutrophils, monocytes, erythrocytes, fibroblasts, and natural killer (NK) cells (Liu *et al*., 2019).

Material and method

Patients

All patients in this study did not receive any dose of chemotherapy. The blood and tissue samples were also taken from the same patient.The samples collection and practical work of the present study extended through the period from February 2020 to the January 2021. Thirty Samples were colorectal cancer (CRC) patients were obtained formalin fixed paraffin embedded (FFPE) and fifteen fresh biopsies were obtained from patients who attended to gastrointestinal center in Imam Hussein medical city, Al-Kafeel Specialist Hospital, and Imam Zain Al-Abidin Hospital in Holy Karbala. Marjan Hospital and the Republican Hospital in BabilGovernorate. DNA was extracted from all samples to be used later in thestudy. Blood samples were obtained from all patients with confirmed CRC who visit Oncology Center of the Imam Hussein, medical city / Holy Karbala - Iraq, the patients were females and males with age range (20-80). All patients and control were from the same ethnic group (Arabic). Two ml of blood were obtained from each patient by vein puncture and put into Ethylenediaminetetraacetic acid (EDTA) tubes. DNA was extracted from EDTA tubes to be used later in thestudy.

Control group

Twenty of blood samples and the same number of colonic biopsieswere obtained from individuals who visit the gastrointestinal center in Imam Hussein medical city, and Al-Kafeel Specialist Hospitalin Holy Karbala. which complain of bleeding per rectum and colonoscopy were done for them as a diagnostic workup and their finding revealed negative endoscopy apart from bleeding hemorrhoid of the age ranging from (20-80) years, select this range of age to be similar to the

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ages of the patients under studyand also ensuring no have colorectal cancer they have .Apermissionwas taken from all individuals of control group after they were told about the aim and advantages of this study

2.3.The Commercial kits used in the present study

1. Genomic DNA extraction kit from blood uses (The ReliaPrep™ Blood gDNA Miniprep System) company Promega.
2. Genomic DNA extraction kit from (FFPE) specimens were carried out according to the manual of manufacturer of Promega company. Genomic DNA was extracted using Relia Prep™ FFPE gDNA Miniprep System. DNA isolation kit (GPSpin extraction/purification kits recommended.
3. The Isolation kit of Fusobacterium nucleatum company (GPS - Spain).
4. The DNA extraction from fresh biopsy specimens were carried out according to the manual of manufacturer of Promega company. Genomic DNA was extracted using ReliaPrep™ gDNA Tissue Miniprep System.

FusNuc MONODOSE dtec-qPCR Test Genetic detection of*Fusobacterium nucleatum*

Principle of the method

Polymerase chain reaction (PCR) allows the amplification of a target region from a DNA template by using specific oligonucleotides. In real-time PCR (qPCR), the accumulating amplified product can be detected at each cycle with fluorescent dyes. This increasing signal allows to achieve sensitive detection and quantification of pathogens.

Protocol & amplification regime

1.Add the desired volume of sample ranging from 5 µl up to a maximum qPCR volume of 20 µl and, when needed, complete this final volume by adding DNase/RNase free water (GREEN CAP) (i.e., 7 µl sample + 13 µl water). Vortex thoroughly and pulse-spin. To determine the sample volume, please consider the possible presence of inhibitors.

GPS™ reagents contains BSA and are compatible with all real-time PCR thermal cyclers, glass capillary or plate based. Plastic of the Generic tube is compatible with: StepOne™, StepOnePlus™, ABI 7500 Fast, LightCycler® 96, LightCycler® Nano, CFX96™, PikoReal™ 24-well, DNA Engine® systems, MiniOpticon™ 48-12 and Opticon®.

2. For other devices, please, transfer the content of the MONODOSE (20 µl) to appropriate tubes. Take into account that the fluorescent signal must be collected by using the FAM channel for the target. If the internal control is added use the HEX channel.Fluorogenic signal should be collected during this step by using the FAM channel for the target and by using the HEX channel for the internal control.

ReagentVolume	
Standard template dilution (i.e., 2 x 102 copies/µl) 1	5 µl
DNase/RNase free water (GREEN CAP)	10 µl
Sample	5 µl

volume reaction final 20 µl

Tube 4 of the curve dilutions series obtained from Standard Template (RED CAP). An optimal result should show a positive signal, equal or higher (same or lower Ct) than these found for the Positive Control alone (tube 4, 103 copies). Inhibition may be total (negative result) or partial, observing a considerable increase in the Ct when compared to this of the Standard.

Template dilution added. If inhibition is observed, a sample dilution to 1/10 may be recommended (if concentration is not close to detection limit). The matrix inhibition control is external, allowing to check the inhibition on the main target of interest.

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Result

Molecular Detection of *Fusobacterium nucleatum* by Quantitative Real time PCR

The presence of *Fusobacterium nucleatum* DNA was detacted in abundance relative total number of bacteria in 40 patient samples tissue with colorectal cancer and 20 normal tissue sample.*Fusobacterium* bacterium positivity was detected in 33(82.51%) of with CRC as shown in (table 3-1) and in 7 (17.5 %) of control sample.

Table (1): Comparison of Cq (Ct) and Copy number of *Fusobacterium nucleatum* between Control and Patient groups

Results	Control	Patient	P value
Cq	30.2369±1.6	27.8624±3	0.017*
Copy number	367.5985±179.09	3993.4909±1433	

The results show that there was significant increase in copy number of *Fusobacterium nucleatum* isolates in patients when compared with control group as shown in (table 3-1). and the range of CT value according to the copy number range from 30.23 ± 1.6 in control and 27.86 ± 3 in patient groups as shown in (table 3-2) and the result show that 7 (17.5 %) of tissue samples were negative for *Fusobacterium nucleatum* which due to non-detectible *Fusobacterium* DNA copies as shown in (table 3-2).

Table (2): Percentage distribution of *Fusobacterium nucleatum*detected from colorectal cancer (CRC) by qPCR assay

Results	All	Control	Patient
Positive	46 (76.7)	13 (65)	33 (82.5)
Negative	14 (23.3)	7 (35)	7 (17.5)
Total	60 (100)	20 (100)	40 (100)
P value	0<.0001*	0.180	0<.0001*

* represent a significant difference at p≤ 0.05.

Fusobacterium nucleatum play a causal role early in colorectal carcinogenesis, and may be involved in cancer initiation and progression stimulating the proliferation of CRC (Claudio *et al*., 2018).
(Kinsman*et al*., 2019) observed that *Fusobacterium nucleatum* was significantly more abundant in the CRC tumor tissue compared to the matched surrounding mucosa.
Human and functional studies provide evidence that *Fusobacterium nucleatum* mediated increased gut inflammation and chemo resistance, through immune signaling and autophagy activation, explains the poorer prognosis for CRC patients (TaChung et*al*., 2017). In different positions along the colon was determined using qPCR, *Fusobacterium nucleatum*was detected in samples from 60 % patient and 18% in control group.

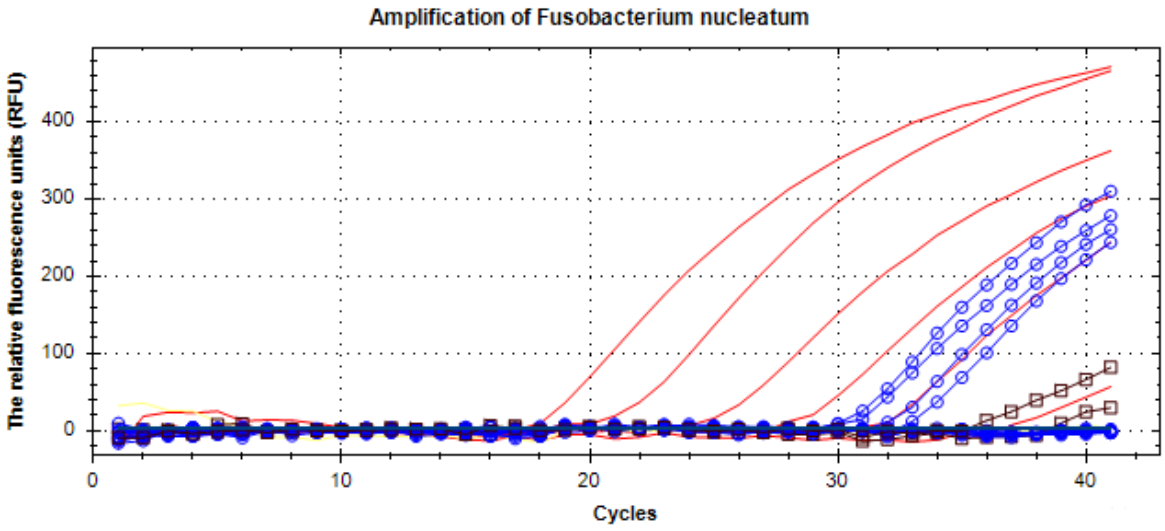

Figure (1): Identification and quantification of *Fusobacterium nucleatum* copy number by qPCR assay. This is the first run for 15 samples only, represent a standard curve amplification, represents amplification of

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patient samples. represents amplification of Control samples.

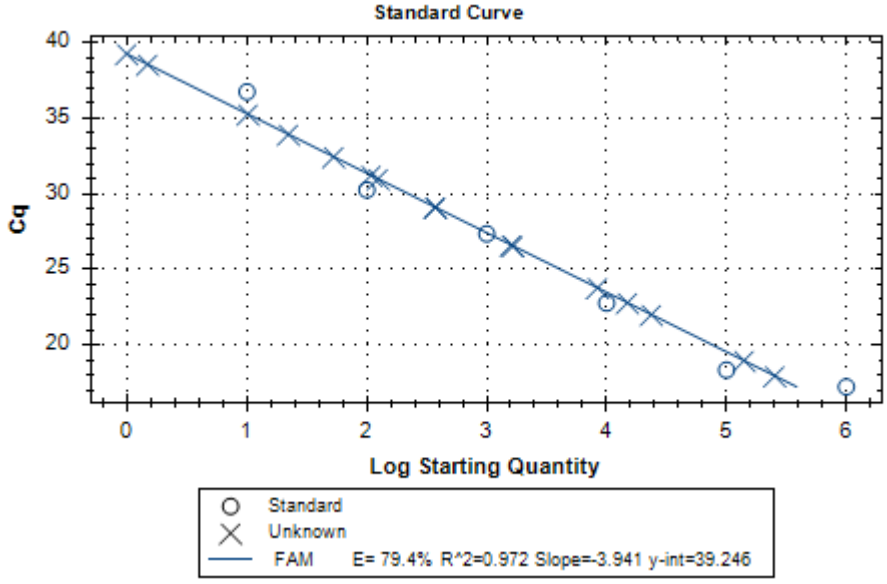

Figure (2): Amplification standard curve for quantification of *Fusobacterium nucleatum* copy number by qPCR assay

3.2 Demographic study

3.2. AGender of patients

Samples study were (60) samples and they were 22 male and 18 female for patients group, while for control group they were 10 male and10 female . The results of the current study showed that the male percentage value of colorectal cancer was no different significant between male and femaleTable (3-3).

Table (3): Comparison of Cq (Ct) and Copy number of Fusobacterium nucleatum between Control and Patient groups according to Gender

Results	Control		P value	Patient		P value
	Male (N=7)	Female (N=6)		Male (N=17)	Female (N=16)	
Cq	29.46±0.7	31.1433±0.58	0.06	27.7153±0.82	28.0188±0.67	0.777
Copynumber	580.6114±320	119.0833±15.2		5287.8147±2507	2618±1290	

* represent a significant difference at p≤ 0.05.

Murphy *et al.*,(2011) explained that men are more likely to have risk for CRC than women, due to hormonal and other risk factors.

The results of the present study disagreed with (White *et al.*, 2018) results who indicated that males have risk percentage more than female. This may explain due to that men are more likely to have a diet high in red and processed meat, be heavier consumers of alcohol, and more likely to smoke. Men also have a greater propensity to deposit visceral fat which is associated with increased risk of CRC.(Kim *et al.*, 2015), that CRC incidence in female is recorded more than in male. These results can be understood by understanding sex- and gender-related biological and sociocultural differences in colorectal cancer risk.(Yang *et al.*,2017) was demonstrated that female sex as a favorable prognostic factor in colorectal cancer, but the results have been very inconsistent.The results of the present study was disagreed with (Kang *et al* .,2017) recorded a percentage rate of women with CRC more than that of men**.** The results of the present study wasconsonant with (Hasan *et al*., 2021) in CRC cases no significant between the male and female.

3.2. B: Age of patients

The age range for patient involved in the work was (30-80)years. (Table 3-4).

Table(4): Comparison of Cq (Ct) and Copy number of *Fusobacteriumnucleatum*between Control and Patient groups according to Ag

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Age Groups	Control		Patient		P value
	Cq	Copy number	Cq	Copy number	
<45	30.71±0.25	149.87±22.96014	27.8±0.94	3096.9778±1886	0.017*
45-65	31.2050±0.59	116.4200±38.91	27.7±0.73	4981.3060±2205	0.012*
>65	29.8175±0.69	512.0413±285.427	28.4±1.27	1071.57±549.9	0.391
P value	0.522		0.920		

* represent a significant difference at p≤ 0.05.

From (Table 4) the results indicate that the risk of developing the disease begins at the age of 40 years and increases between 45-65 years for both sexes. Age can be considered an equally relevant risk factor for women and men, as this study consonantwithCancer. (Net Editorial Board.,2021) indicated the risk of colorectal cancer increases as people get older. Colorectal cancer can occur in young adults and teenagers, but most colorectal cancers occur in people older than 50. For colon cancer, the average age at the time of diagnosis for men is 68 and for women is 72. For rectal cancer, it is age 63 for both men and women. Older adults who are diagnosed with colorectal cancer face unique challenges, specifically regarding cancer treatment.

(Surveillance.,2016) recorded that the incidence and death rates for colorectal cancer increase with age. Overall deaths occur in people 50 and older. The median age at colon cancer diagnosis, 69 in men and 73 in women, is older than the median age at rectal cancer diagnosis, which is 63 in men and 65 in women.

Results of the current study similar to the results (Wong *et al.*, 2016) that showed age andgender-based risk stratification tool was found to be the highest progression and death with CRC with an average age of (50 – 70) years.

The results of the present study consonant with (Howlader*et al.*, 2016) indicated the risk of CRC increases with aging the median age at diagnosis for colon and rectal cancer is 60 year in both genders.

While,(Ankur *et al.*, 2019) showed in Hawaii that Between 2009 and 2013, patients had the highest incidence rates of colon cancer before the age of 50, which disagree with the results of current study due to the ethnicity and life style of patients.

The results of the present study were disagreed with (Vuik*et al.*, 2019) recorded for both sexes combined, in age group 30-39 years the CRC incidence increased.

The results of present study are in consonant with (Hassan*et al*. 2021) The risk of Colorectal cancer rises with age. Incidence rates increased in the over-45 age groups, with a significant 41.7% value being significantly higher than in the under 45 age groups also indicated by(Mosli *et al*.2012).

3.2. C: Location of patients

The results of the present study found a significant difference between urban and rural residents with colorectal cancer (table5).

Table (5): Comparison of Cq (Ct) and Copy number of *Fusobacterium nucleatum*between Control and Patient groups according to Location

Results	Control		P value	Patient		P value
	Urban (9)	Rural (4)		Urban (21)	Rural (12)	
Cq	30.43±0.37	29.8±1.33	0.677	28.0290±0.6	27.5708±0.94	0.692
Copy number	219.7467±70	700.2±1156		3838.0357±1935	4265.5375±2121	

* represent a significant difference at p≤ 0.05.

The results of the present study wasconsonant with(Denggui*et al* ., 2018) which indicated that the colorectal cancer in Shijiazhuang (urban) were considerably higher than in Shexian (rural) in both men (22.8 vs. 11.9/100,000) and women (15.0 vs. 9.3/100,000). The difference was like that between countries with high and medium human development indices according to GLOBOCAN 2012.The results of the present study were disagreed with (Jane Meza *et al*., 2018) This finding is no differences between the rural and urban populations with colorectal cancer.The results of the present study consonant with(Charles et*al*.,2020) their study consisted of the final group of 4,660 male CRC patients, 15.3% of them (n = 712) who lived in rural areas at the time of their cancer diagnosis. Compared to the rest of the patients who live in urban areas.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 3660 - 3666
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