



Fusobacterium nucleatum-Mediated Alteration in Expression of *VEGF* and *CCL3* Genes and *KRAS* Mutation in Colorectal Cancer Patients

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Abstract

Background: Colorectal cancer (CRC) is the third most common cancer worldwide, and its development is influenced by genetic and environmental factors, including the gut microbiota. Recent studies have reported an association between *Fusobacterium nucleatum* abundance and CRC.

Objectives: This study aimed to investigate the abundance of *F. nucleatum* in CRC and polyp patients and its association with the expression of chemokine ligand-3 (*CCL3*), vascular endothelial growth factor (*VEGF*), and nuclear factor-kappa B (*NF-KB1*) genes and the presence of deoxyribonucleic acid (DNA) mutations and polymorphisms in the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene.

Methods: A total of 80 biopsy samples were collected from CRC, polyp, and colitis patients. Moreover, *F. nucleatum* abundance was measured by quantitative polymerase chain reaction (qPCR). The expression of *CCL3*, *VEGF*, and *NF-KB1* genes was measured by reverse transcription polymerase chain reaction (RT-PCR). Additionally, *KRAS* gene mutations and polymorphisms were detected by the Mutation Surveyor software (V5.1.2).

Results: The results showed that *F. nucleatum* abundance was significantly higher in CRC and polyp patients than in colitis patients ($P < 0.05$). The expression of *CCL3* and *VEGF* genes was also significantly higher in *F. nucleatum*-positive samples ($P < 0.05$). However, *NF-KB1* gene expression was non-significant. *Fusobacterium nucleatum*-positive biopsy samples had a higher frequency of *KRAS* gene mutations and polymorphisms than *F. nucleatum*-negative CRC patients (odds ratio = 3). Most of the mutations observed in the positive samples were (6144A>AT,31E>E) at exon 2 of the *KRAS* gene.

Conclusions: The study findings suggest that *F. nucleatum* might play a role in CRC and polyp development and contribute to *KRAS* gene mutations. Therefore, targeting *F. nucleatum* in the gut microbiota could be a potential therapeutic strategy for preventing CRC and polyp development.

Keywords: *Fusobacterium nucleatum*, Colorectal Cancer, Polyps, *CCL3*, *VEGF*, *NF-KB1*, *KRAS* Gene Mutations

1. Background

Colorectal cancer (CRC) is a major cause of cancer-related mortalities worldwide, with a high incidence and mortality rate (1). Several factors have been identified to contribute to the development and progression of CRC, including genetic mutations, lifestyle factors, and the gut microbiome (2, 3). In recent years, there has been growing interest in the role of *Fusobacterium nucleatum* in various human diseases. *Fusobacterium nucleatum* is a gram-negative anaerobic bacterium commonly found in the oral cavity and gastrointestinal tract. It is known for its

ability to form biofilms and adhere to epithelial cells, facilitating its colonization and persistence in the gut environment (4). In particular, recent studies have shown that the abundance of *F. nucleatum* is increased in CRC patients compared to healthy controls (5, 6). *Fusobacterium nucleatum* has been suggested to promote tumorigenesis by interacting with host cells and promoting inflammation, inhibiting immune responses, and inducing deoxyribonucleic acid (DNA) damage (7, 8).

Previous studies have also reported that *F. nucleatum* is associated with the development of precancerous lesions, such as polyps (9, 10). However, the expression of specific

genes in *F. nucleatum*-positive samples and the association between *F. nucleatum* and DNA mutations in the *KRAS* gene, which are frequently found in CRC and associated with a poor prognosis (11), have not been fully explored. Emerging evidence has highlighted the significance of specific genes, such as *CCL3* and *VEGF*, in CRC. Chemokine ligand-3, also known as chemokine (C-C motif) ligand 3, is a pro-inflammatory chemokine involved in immune cell recruitment and activation (12). It has been implicated in tumor progression, angiogenesis, and metastasis in various cancers, including CRC (13). The dysregulation of *CCL3* expression in CRC has been associated with poor prognosis and advanced stages of the disease (14). Understanding the role of *CCL3* in CRC is essential for unraveling the complex interplay between inflammation, immune responses, and tumor development.

Vascular endothelial growth factor (*VEGF*) is a key regulator of angiogenesis, the formation of new blood vessels (15). In CRC, *VEGF* has been implicated in tumor vascularization and the promotion of tumor growth and metastasis (16). Increased *VEGF* expression has been correlated with poor prognosis and resistance to certain therapies in CRC patients (17). Therefore, investigating the role of *VEGF* in CRC is crucial for identifying potential therapeutic targets and developing effective treatment strategies. Despite the growing understanding of the involvement of *CCL3* and *VEGF* in CRC, the specific impact of *F. nucleatum* abundance on the expression of these genes still needs to be explored. By investigating the association between *F. nucleatum* and the expression of *CCL3* and *VEGF* in CRC patients, this study aimed to shed light on the potential molecular mechanisms underlying the tumorigenic properties of *F. nucleatum* in CRC and provide insights into new therapeutic approaches.

2. Objectives

This study aimed to investigate the abundance of *F. nucleatum* in CRC, polyp, and colitis patients and analyze the expression of *CCL3*, *VEGF*, and *NF-KB11* genes in *F. nucleatum*-positive samples. This study also aimed to compare the prevalence of DNA mutations and polymorphism in the *KRAS* gene between *F. nucleatum*-positive and -negative CRC patients. To achieve these aims, biopsy samples were collected from 100 patients diagnosed with CRC, polyps, or colitis and analyzed using quantitative polymerase chain reaction (qPCR), gene expression analysis, and DNA sequencing.

3. Methods

3.1. Study Design

This was a retrospective case-control study conducted between June 2021 and March 2022. The study was approved by the Ethics Committee of Erbil Health and Medical Technical College, Erbil, Iraq, and informed consent was obtained from all participants.

3.2. Study Participants

The study included a total of 100 patients who underwent colonoscopy and surgical operation during the study period. The participants were divided into 3 groups based on their diagnosis, namely the CRC group (n = 38), the precancerous group (n = 19) with polyps, and the colitis group (n = 18) with 1 control group (n = 25). The inclusion criteria for the study were patients aged 18 years and older who underwent colonoscopy and surgical operation for clinical reasons. All the studied participants were selected according to the colonoscopy results during the first diagnosis before starting chemotherapy or radiotherapy.

3.3. Sample Collection

During colonoscopy or surgical operation, biopsy samples were collected from the colon mucosa of all participants using standard biopsy tools. The biopsy samples were immediately placed in sterile tubes containing phosphate-buffered saline (PBS) and stored at -80°C until further analysis.

3.4. DNA Extraction

Genomic DNA was extracted from the biopsy samples using a test kit for DNA extraction (Favorgen Biotech Crop., Taiwan) according to the manufacturer's protocol. Briefly, the samples were initially prepared and subjected to cell lysis using a provided lysis buffer. Protein removal was performed to eliminate protein contaminants using Proteinase K, and DNA purification was achieved through column-based methods. Washing steps were carried out to remove residual impurities, followed by the elution of the purified DNA using an elution buffer. Deoxyribonucleic acid purity and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

3.5. Determination of the Abundance of *Fusobacterium nucleatum*

Real-time PCR was performed to determine the abundance of *F. nucleatum* in the biopsy samples. The 16S rRNA gene was used as a target gene for *F. nucleatum*, and a standard curve from standard *F. nucleatum* bacteria was prepared. Moreover, the log copies for the known samples were measured using the following formula:

$$\text{Number of copies} = (\text{DNA concentration in ng}/\mu\text{L}) \times (6.02 \times 10^{23}) / (\text{length of DNA template in bp} (3000000 \text{ bp}) \times 660)$$

The primers used for PCR amplification were as follows:

F. nucleatum (F: 5'-AGA GTT TGA TCC TGG CTC AG-3', R: 5'-GTC ATC GTG CAC ACA GAA TTG CTG-3')

The abundance of *F. nucleatum* was calculated based on the cycle threshold (Ct) values of the standard and samples. Then, the log number copies were calculated using Excel software. The PCR protocol for *F. nucleatum* was performed with an initial 5 minutes at 94°C; then, 30 cycles repeated; each cycle consisted of a denaturation process at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute, with 10 minutes at 72°C as a final extension.

3.6. Gene Expression Analysis

The relative expression levels of *CCL3*, *VEGF*, and *NF-KB1* genes were analyzed using qPCR. Total ribonucleic acid (RNA) was extracted from the biopsy samples using a special kit (Invitrogen, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Real-time PCR was performed using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA) on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The primers used for PCR amplification are shown in Table 1. The relative expression for the studied genes was calculated using the delta-delta Ct method.

Table 1. Forward and Reverse Sequences of Studied Primers

Primer Name	Forward Sequence	Reverse Sequence
<i>β-actin</i>	TCGAGCAATCTCAACTCGG	TGAAGGTAGTTTCGTTGGATG
<i>VEGF-A</i>	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
<i>NF-κB1</i>	CACAAGGCAGCAAATAGACGAG	TGGGGCATTTTGTTGAGAGTT
<i>CCL3</i>	TGTTGCCAAACAGCCACAC	CAGAGCAAACAATCACAACACAC

3.7. KRAS Mutation Analysis

The *KRAS* mutation analysis was performed using specific primers (F: 5'-TAGTCACATTTTCATTATTTTAT-3', R: 5'-AGATTACCTCTATTGTGGAT-3') in exon 2 of the *KRAS* gene, which contains the most common mutations associated with the presence or absence of *F. nucleatum*. The PCR products were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

3.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 9). The abundance of *F. nucleatum* and gene expression levels were compared between the groups using the one-way analysis of variance (ANOVA) test followed by Tukey's post-hoc test. The odds ratio (OR) for *KRAS* mutations in *F. nucleatum*-positive samples was calculated. Two-way ANOVA was applied to compare *F. nucleatum*-positive and -negative samples based on patient gender, and the Sidak test was used as a multiple comparison test. P-values less than 0.05 were considered statistically significant. Mutation Surveyor software (V5.1.2) manufactured by SoftGenetics (Pennsylvania, USA) was applied for *KRAS* mutation analysis. The Glutamic Acid Decarboxylase (GAD) and mutation reports were exported as the final results for the mutations.

4. Results

The abundance of *F. nucleatum* was determined using qPCR. The results showed that the mean \pm standard error (SE) of *F. nucleatum* abundance was 5.228 ± 0.057 and 5.291 ± 0.058 log copies/ng DNA in patients with CRC and precancerous polyps, respectively, which was significantly higher than in colitis patients (4.874 ± 0.182 log copies/ng DNA) ($P < 0.05$). However, the abundance of *F. nucleatum* was non-significant in both precancerous polyp and CRC patients (Figure 1). Regarding the normal cases, only one case was positive for *F. nucleatum*, which was not included in the figure and analysis due to a lack of replications.

The results for the relative expression of *CCL3*, *VEGF*, and *NF-KB1* genes in *F. nucleatum*-positive and *F. nucleatum*-negative samples in CRC patients using quantitative reverse transcription polymerase chain reaction (RT-qPCR) showed that the relative expression of *CCL3* and *VEGF* genes was significantly higher in *F. nucleatum*-positive samples than in *F. nucleatum*-negative samples ($P < 0.05$). However, the expression of *NF-KB1* was not significantly different between *F.*

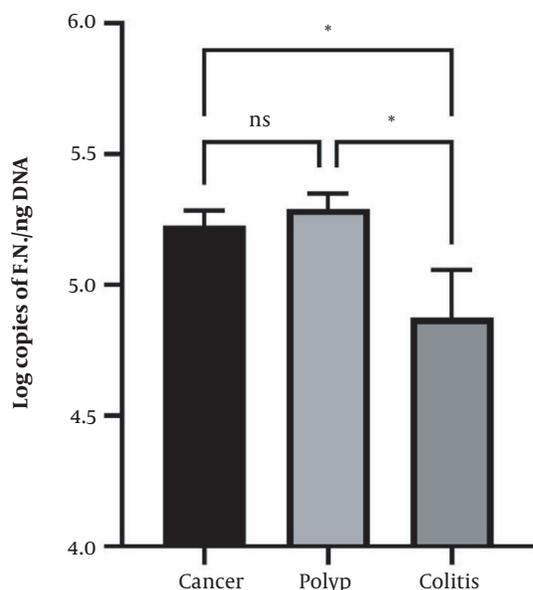


Figure 1. The abundance of *Fusobacterium nucleatum* in colorectal cancer (CRC), polyp, and colitis groups. Both the CRC and polyp groups showed a significantly higher abundance of *Fusobacterium nucleatum* ($P < 0.05$) than the colitis group. The data are expressed as mean and standard error (SE). * indicates the significant level at $P < 0.05$, and the one-way ANOVA test was applied with Tukey's post-hoc test for comparisons.

nucleatum-positive and *F. nucleatum*-negative samples (Figure 2). Gender-based data showed that *CCL3* expression was significantly increased in positive patients in both males and females. However, no significant change was observed in *VEGF* relative expression among *F. nucleatum*-negative and -positive samples in both male and female samples, which might be due to a reduction in the sample size of the groups after gender-based categorization (Figure 3).

The results of the association between *F. nucleatum* and *KRAS* gene mutations were assessed using Mutation Surveyor analysis in biopsy samples. The PCR products for *KRAS* primer were prepared and checked via an agarose gel (Figure 4). The results revealed a significantly higher frequency of *KRAS* gene mutations and polymorphism (OR = 3, $P < 0.05$) in *F. nucleatum*-positive biopsy samples than in *F. nucleatum*-negative CRC patients (Table 2). Most of the mutations observed in the positive samples were (6144A>AT,31E>E) at exon 2 of the *KRAS* gene (Figure 5).

5. Discussion

The current study examined the abundance of *F. nucleatum* in CRC, precancerous polyp, and colitis patients

Table 2. Frequency and Odds Ratio of *KRAS* Mutations in *Fusobacterium nucleatum*-Positive and -Negative Colorectal Cancer Samples

	<i>F. nucleatum</i> Positive	<i>F. nucleatum</i> Negative
No. of samples with mutation	6	4
No. of samples without mutation	8	16
Odd ratio (OR)	3	

and investigated its association with gene expression and DNA mutations. The results showed a significant increase in the abundance of *F. nucleatum* in both CRC and polyp patients, compared to colitis patients. This finding is consistent with the findings of previous studies that reported a positive correlation between *F. nucleatum* and CRC (5, 18, 19). One possible mechanism for this association is that *F. nucleatum* can adhere to the colorectal epithelium and induce chronic inflammation, leading to tumorigenesis (20). Furthermore, the present study demonstrated that *F. nucleatum*-positive biopsy samples had more DNA mutations and polymorphisms in the *KRAS* gene than *F. nucleatum*-negative CRC patients. This result suggests that *F. nucleatum* might promote CRC progression by increasing genetic instability and promoting the acquisition of oncogenic mutations. Previous studies have suggested that *F. nucleatum* can stimulate DNA damage and genomic instability in host cells by inducing chronic inflammation and inhibiting DNA repair mechanisms (20-22).

In addition, in this study, an increase in the expression of *CCL3* and *VEGF* genes was observed in *F. nucleatum*-positive samples than in *F. nucleatum*-negative samples. Both of these genes have been implicated in cancer progression and angiogenesis (23-25). Also known as macrophage inflammatory protein-1 alpha, *CCL3* is a chemokine that recruits immune cells to the site of inflammation and contributes to the progression of various cancers (26, 27). The *VEGF* gene is a key regulator of angiogenesis and has been shown to be upregulated in CRC (28). One possible mechanism for the increase in *CCL3* and *VEGF* expression in *F. nucleatum*-positive samples is that *F. nucleatum* can activate pro-inflammatory signaling pathways, such as nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) and signal transducer and activator of transcription 3 (STAT3), leading to the upregulation of these genes (18, 29). Overall, the present study's results provide further evidence of the association between *F. nucleatum* and CRC and suggest that

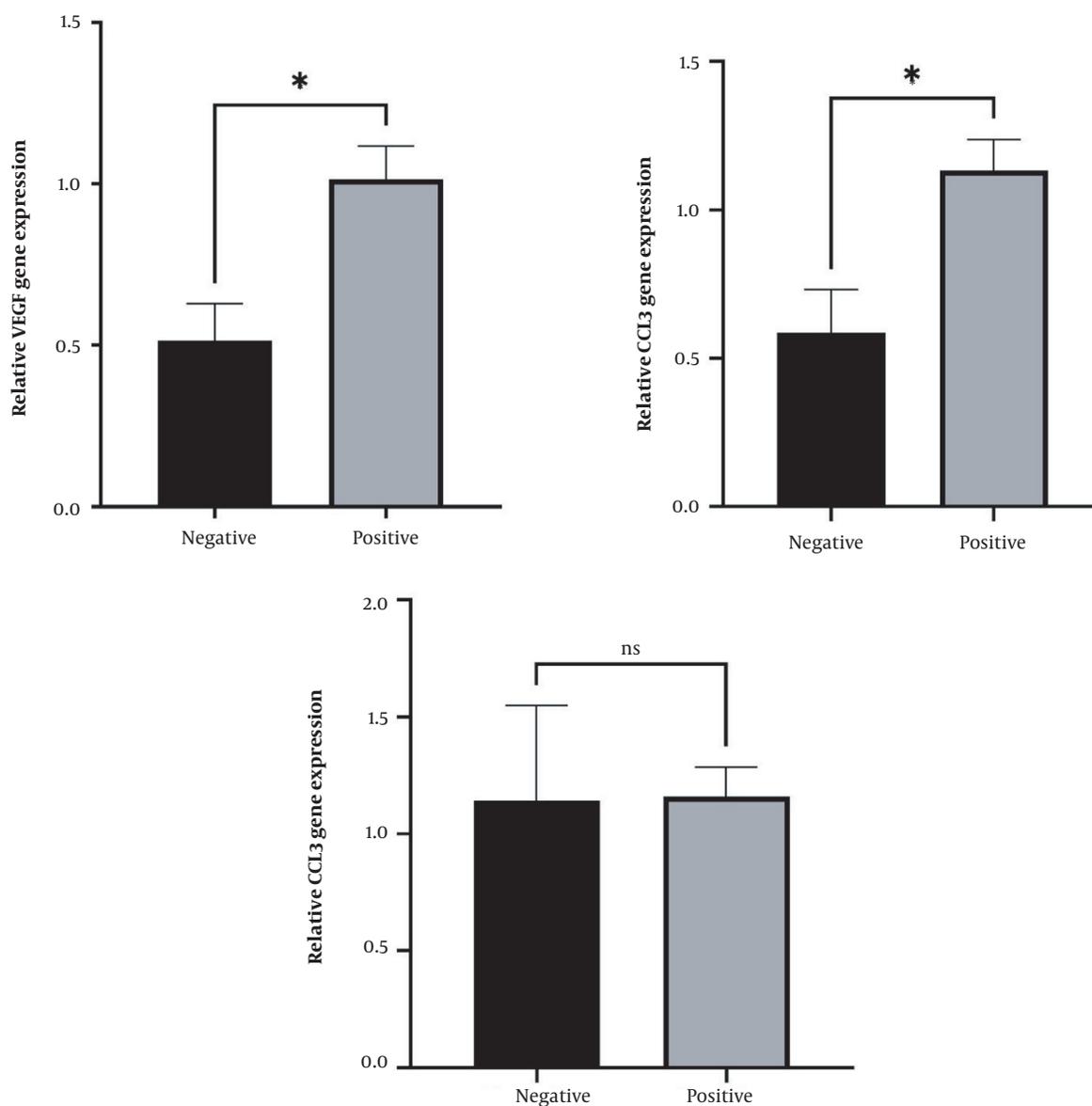


Figure 2. Relative expression of (A) VEGF, (B) CCL3, and (C) NF-KB11 in *Fusobacterium nucleatum*-positive and -negative patients. The expression of both VEGF and CCL3 genes was higher ($P < 0.05$) in *F. nucleatum*-positive samples than in *F. nucleatum*-negative samples. The data are expressed as mean and standard error (SE). * indicates the significant level at $P < 0.05$, and an unpaired Student's t-test was applied for comparisons.

F. nucleatum might promote tumorigenesis by inducing chronic inflammation, promoting genetic instability, and modulating gene expression. These findings might have important implications for the development of novel diagnostic and therapeutic strategies for CRC.

The present study has several limitations that should be acknowledged. Firstly, the sample size of the study was relatively small, and larger studies are needed to

confirm the obtained findings. Secondly, the present study was cross-sectional; therefore, it is impossible to establish causality between *F. nucleatum* and the development of CRC and polyps. Thirdly, this study did not investigate the functional role of *F. nucleatum* in CRC and polyps, and further studies are needed to explore the underlying mechanisms.

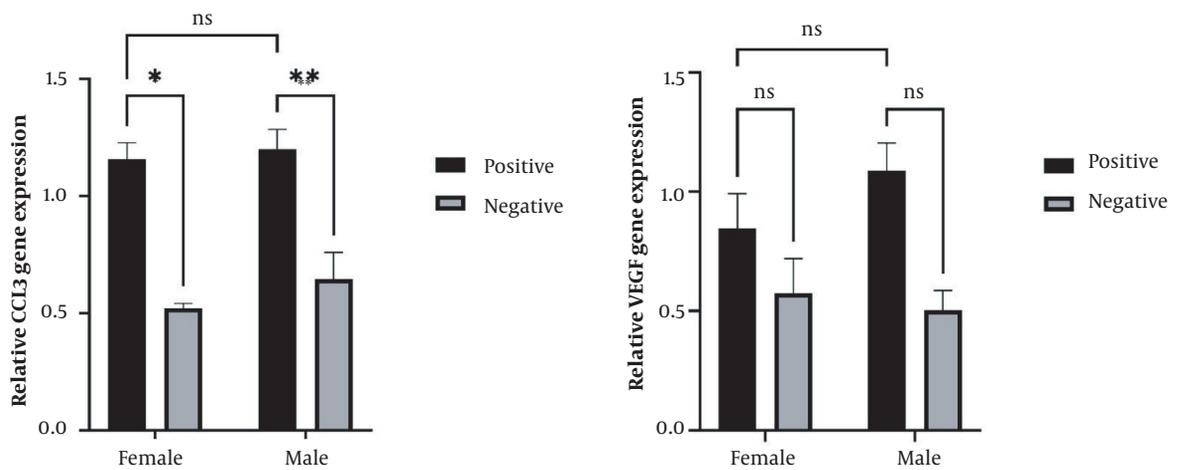


Figure 3. Relative expression of (A) VEGF and (B) CCL3 in *Fusobacterium nucleatum*-positive and -negative patients categorized according to gender (male and female). The expression of the CCL3 gene was higher ($P < 0.05$) in *F. nucleatum*-positive samples than in *F. nucleatum*-negative samples in both females and males. However, VEGF showed no significant differences between positive and negative infected patients in both males and females. The data are expressed as mean and standard error (SE). * indicates the significant level at $P < 0.05$, and the two-way ANOVA test was applied for comparisons with the Sidak test as post-hoc for multiple comparisons.

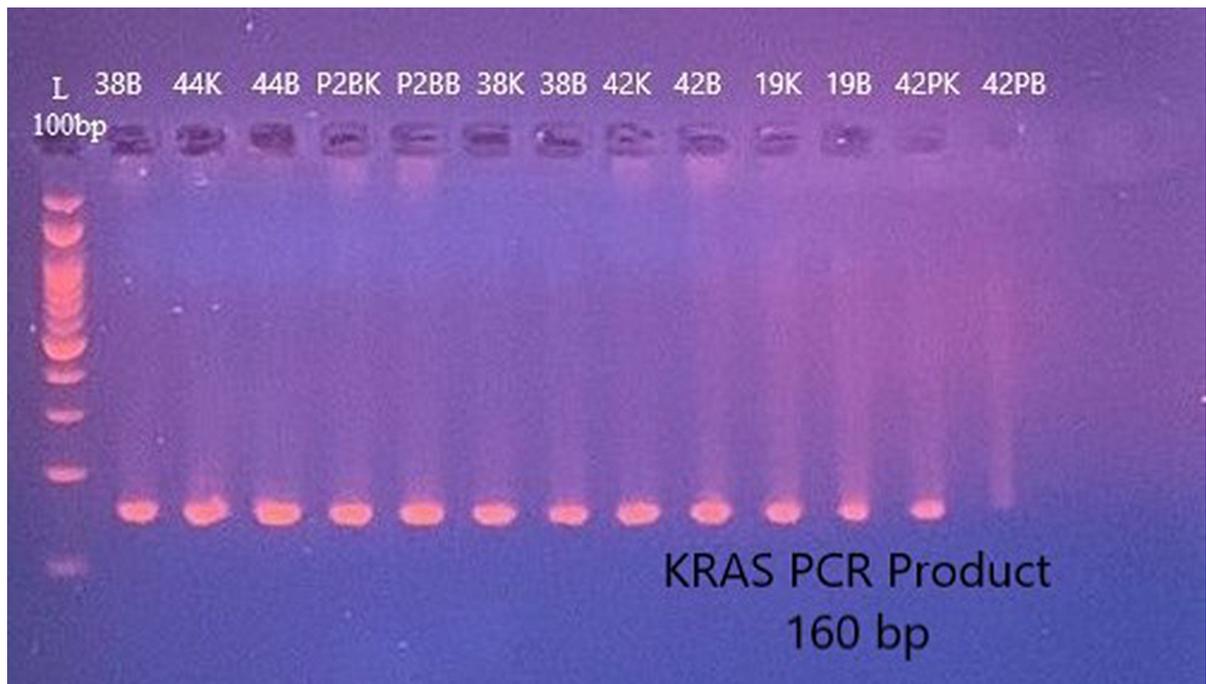


Figure 4. Gel image of polymerase chain reaction (PCR) products for KRAS mutations in *Fusobacterium nucleatum*-positive samples. The bands were obtained at 160 bp. The first lane (L) is the DNA ladder with 100 bp, and the other lanes are the studied samples.

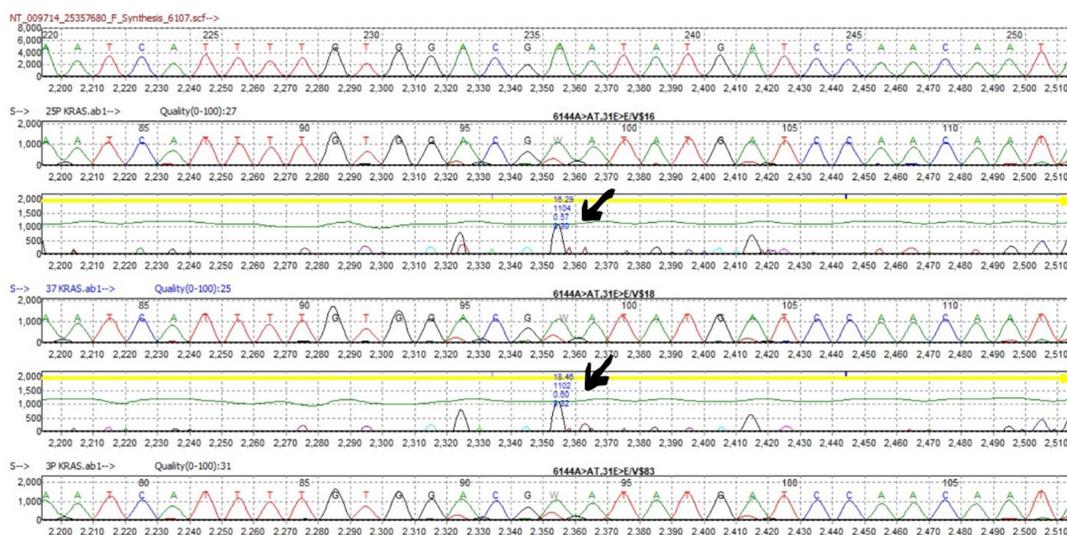


Figure 5. Glutamic acid decarboxylase (GAD) report of Mutation Surveyor Software (V 5.1.2) for *KRAS* mutations in *Fusobacterium nucleatum*-positive samples. The results showed a significantly higher frequency of *KRAS* gene mutations and polymorphism ($OR = 3, P < 0.05$) in *F. nucleatum*-positive biopsy samples than in *F. nucleatum*-negative colorectal cancer patients.

5.1. Conclusions

In conclusion, the current study provides evidence of the association between *F. nucleatum* and the development and progression of CRC and polyps. The findings of the increased abundance of *F. nucleatum*, increased expression of *CCL3* and *VEGF*, and increased DNA mutations and polymorphisms in the *KRAS* gene in *F. nucleatum*-positive samples suggest that *F. nucleatum* might play a role in the development and progression of CRC and polyps. Further studies are needed to explore the underlying mechanisms and potential therapeutic strategies targeting *F. nucleatum* in CRC and polyps.

Footnotes

Authors' Contribution: Study concept and design: F. K.; Acquisition of the data: H. T.; Analysis and interpretation of the data: H. T.; Drafting of the manuscript: H. T.; Critical revision of the manuscript for important intellectual content: H. T.; Statistical analysis: H. T.; Administrative, technical, and material support: H. T.; Study supervision: F. K.

Conflict of Interests: The study was self-funded by the author (Hataw J. Taher). This study is a Ph.D. project supervised by Prof. Dr. Fouad H. Kamel. The authors are not members of the editorial board or reviewers in this journal. There is no conflict of interest.

Data Reproducibility: The dataset presented in the study is available on request from the corresponding author during submission or after publication. The data are not publicly available due to privacy and ethics.

Ethical Approval: The current study was approved by the Human Ethics Committee of the Erbil Health and Medical Technical College, Erbil Polytechnic University (NO:0034; date: 5/6/2021). Standard procedures were followed throughout the research process.

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Informed Consent: Informed consent was obtained from all the participants.

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