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ORIGINAL ARTICLE

Basic Study

Relationship between *Fusobacterium nucleatum*, inflammatory mediators and microRNAs in colorectal carcinogenesis

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Abstract

AIM

To examine the effect of *Fusobacterium nucleatum* (*F. nucleatum*) on the microenvironment of colonic neoplasms and the expression of inflammatory mediators and microRNAs (miRNAs).

METHODS

Levels of *F. nucleatum* DNA, cytokine gene mRNA (*TLR2*, *TLR4*, *NFKB1*, *TNF*, *IL1B*, *IL6* and *IL8*), and potentially interacting miRNAs (miR-21-3p, miR-22-3p, miR-28-5p, miR-34a-5p, miR-135b-5p) were measured by quantitative polymerase chain reaction (qPCR) TaqMan® assays in DNA and/or RNA extracted from the disease and adjacent normal fresh tissues of 27 colorectal adenoma (CRA) and 43 colorectal cancer (CRC) patients. *KRAS* mutations were detected by direct sequencing and microsatellite instability (MSI) status by multiplex PCR. Cytoscape v3.1.1 was used to construct the postulated miRNA:mRNA interaction network.

RESULTS

Overabundance of *F. nucleatum* in neoplastic tissue compared to matched normal tissue was detected in CRA (51.8%) and more markedly in CRC (72.1%). We observed significantly greater expression of *TLR4*, *IL1B*, *IL8*, and miR-135b in CRA lesions and *TLR2*, *IL1B*, *IL6*, *IL8*, miR-34a and miR-135b in CRC tumours compared to their respective normal tissues. Only two transcripts for miR-22 and miR-28 were exclusively downregulated in CRC tumour samples. The mRNA expression of *IL1B*, *IL6*, *IL8* and miR-22 was positively correlated with *F. nucleatum* quantification in CRC tumours. The mRNA expression of miR-135b and *TNF* was inversely correlated. The miRNA:mRNA interaction network suggested that the upregulation of miR-34a in CRC proceeds *via* a *TLR2*/*TLR4*-dependent response to *F. nucleatum*. Finally, *KRAS* mutations were more frequently observed in CRC samples infected with *F. nucleatum* and were associated with greater expression of miR-21 in CRA, while *IL8* was upregulated in MSI-high CRC.

CONCLUSION

Our findings indicate that *F. nucleatum* is a risk factor for CRC by increasing the expression of inflammatory mediators through a possible miRNA-mediated activation of *TLR2*/*TLR4*.

Key words: Colorectal cancer; Colorectal adenoma; *Fusobacterium nucleatum*; Inflammation; Cytokines; MicroRNAs

Core tip: We examined the influence of *Fusobacterium nucleatum* (*F. nucleatum*) in colorectal adenoma (CRA) and colorectal cancer (CRC) on the mRNA expression of inflammatory mediators and the association with microRNA (miRNA) levels, *KRAS* mutation, and microsatellite instability (MSI). We suggest that *F. nucleatum* contributes to CRC development by increasing the expression of inflammatory mediators through a possible miRNA-mediated activation of *TLR2*/*TLR4*. The miRNA:mRNA interaction network suggests an upregulation of miR-34a in CRC *via* a *TLR2*/*TLR4*-dependent response to *F. nucleatum*. *KRAS* mutations were more frequent in *F. nucleatum*-infected CRC and were associated with a greater expression of miR-21 in CRA, while *IL8* was upregulated in MSI-high CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the three leading causes of cancer-related deaths and the third most frequently diagnosed cancer worldwide, with 1849518 new cases and 880792 deaths estimated in 2018[1]. In Brazil, CRC is the third most frequent cancer in men and the second most frequent cancer in women[2]. CRC is associated with chronic inflammation and oxidative processes that can induce malignant cell transformation and activate carcinogenic processes such as proliferation and angiogenesis[3].

The human intestinal microbiota is composed of many species that may play an important role in inflammatory gastrointestinal diseases, such as inflammatory bowel disease and CRC. Among the microbiota, *Fusobacterium nucleatum* (*F. nucleatum*) has emerged as a potential factor in CRC aetiology[4-7]. This bacterium is a gram-negative anaerobic commensal pathogen that is associated with several human diseases, especially those related to the oral and intestinal tracts[5,8]. Some studies suggest that *F. nucleatum* can cause a pro-inflammatory microenvironment in the intestine through deregulating inflammatory and immune responses, thereby promoting a microenvironment propitious for tumour initiation and CRC progression[5,6,9]. However, the mechanisms involved in this proposed tumourigenic process are still under discussion.

Studies have shown an abundance of *F. nucleatum* in tumour tissues and stool samples from CRC patients compared to adjacent normal tissues, colorectal adenomas (CRA) or even healthy subjects, and this observation was also correlated with shorter post-diagnosis overall survival[6,10-12]. These results reinforce the importance of *F. nucleatum* detection to assist in the identification of risk groups and early detection of CRC with implications for disease prognosis as well.

The interaction of microorganisms with the intestinal epithelium initially involves recognition by Toll-like receptors (TLRs)[13] and activation of the nuclear factor-kappa B (NF-κB) pathway, which is the main signalling pathway regulating inflammatory responses implicated in colorectal tumourigenesis[14]. NF-κB may facilitate tumour progression through the expression of pro-inflammatory cytokines[15], which have different roles in colorectal carcinogenesis. These pro-inflammatory cytokines include interleukin (IL) IL1B, which can induce tumour cell proliferation[16]; IL6 and IL8, which are related to tumour growth, angiogenesis and metastasis[17]; and tumour necrosis factor (TNF) A, which can decrease cell death[16,18].

F. nucleatum infection has also been associated with common CRC tumour genetic and epigenetic alterations, such as microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and mutations in the *BRAF* and *KRAS* genes[19-21]. These alterations are thought to be due to *F. nucleatum*-mediated inflammatory responses influencing the molecular pathways of colorectal carcinogenesis *via* generation of reactive oxygen species (ROS) and greater pro-inflammatory gene expression, resulting in aberrant DNA methylation and DNA damage.

MicroRNAs (miRNAs) have a well-established role in inflammatory processes and can serve as molecular markers for diagnosis, prognosis, and treatment response[22]. Several miRNAs have been associated with CRC development and progression[23-25], including investigations of *F. nucleatum*-induced inflammation and CRC[26-28].

However, the interaction and (dys)regulation between inflammatory genes and miRNAs in potential *F. nucleatum*-induced colorectal carcinogenesis has not previously been elucidated. Thus, we investigated the association of *F. nucleatum* abundance in CRA and CRC tissues with the expression of inflammatory genes (*TLR2*, *TLR4*, *NFKB1*, *TNF*, *IL1B*, *IL6* and *IL8*) and miRNAs (miR-21-3p, miR-22 -3p, miR-28-5p, miR-34a-5p and miR-135b-5p). These genes and miRNAs were selected due to their proposed involvement in the inflammatory process or colorectal carcinogenesis from the literature and public databases (TarBase v7.0 and miRBase 2.1)[29,30]. Our findings suggest that the host inflammatory response to *F. nucleatum* contributes to the neoplastic progression of CRA to CRC though *TLR2* and *TLR4* activation of the pro-inflammatory cytokines *IL1B*, *IL6* and *IL8* in a potentially miRNA-dependent process.

MATERIALS AND METHODS

Clinical samples

This study was approved by the Research Ethics Committee of IBILCE/UNESP, São José do Rio Preto (SP), Brazil (reference 1.452.373). Written informed consent was obtained from all individuals, and all samples were coded to protect patient anonymity.

A total of 43 fresh-frozen CRC tissue samples and the matched adjacent normal tissue (N-CRC) as well as 27 CRA tissue samples and the matched adjacent normal tissue (N-CRA) were collected from the Proctology Service of Hospital de Base and Endoscopy Center Rio Preto, both in SP (Brazil) during the period of 2010 to 2012.

All required information on demographic and clinical histopathological parameters was obtained from the patients' medical records. The inclusion criteria were patients with a confirmed diagnosis of pre-cancerous adenomas or sporadic CRC by standard clinical histopathological measures without previous chemotherapy and radiotherapy, and the exclusion criterion was patients with hereditary CRC.

Acid nucleic extraction and cDNA reverse transcription

Simultaneous extraction of total RNA and DNA from colorectal tissue samples was performed using the TRIzol reagent (Ambion, Carlsbad, CA, United States) and corresponding protocol from the manufacturer. A reverse transcriptase reaction was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) as described previously[31]. The synthesis of cDNA to the miRNAs was carried out with a TaqMan® MicroRNA Reverse Transcription Kit *(*Applied Biosystems, Foster City, CA, United States*)* according to the manufacturer's protocol.

Quantification of F. nucleatum

Quantification of *F. nucleatum* was performed in CRA, CRC and the respective adjacent normal DNA samples by quantitative real-time PCR (qPCR). TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, United States) with specific probes for the bacterial gene target *NusG* (5'-TCAGCAACTTGTCCTTCTTGATCTTTAAATGAACC-3' TAMRA probe FAM) and the human gene *PGT* (5'-CCATCCATGTCCTCATCTC-3' TAMRA probe FAM) as a reference were assayed by StepOnePlus Real-Time PCR (Applied Biosystems, Foster City, CA, United States). The reactions were performed separately for each gene at a 12 μL final volume using 20 ng of genomic DNA, 400 nmol/L primer (*NusG* sequences: 5'-CAACCATTACTTTAACTCTACCATGTTCA-3' and 3'-GTTGACTTTACAGAAGGAGATTATGTAAAAATC-5', *PGT*: 5'-ATCCCCAAAGCACCTGGTTT-3' and 3'-AGAGGCCAAGATAGTCCTGGTAA-5') (Invitrogen, Carlsbad, Califórnia, United States), 400 nmol/L probe and GoTaq probe 1 × qPCR Master Mix (Promega, Madison, Wisconsin, United States). The reaction was subjected to temperatures of 50 ℃ for 2 min, 95 ℃ for 10 min, then 60 cycles of 95 ℃ for 15 s and 57 ℃ for 1 min[10]. All reactions were performed in duplicate, and all experiments had a no-template control that was used to confirm no contamination in samples. Cq (cycle quantification) values were calculated after adjusting the threshold by StepOne software (v. 2.2.2) (Applied

Biosystems, Foster City, CA, United States), and all samples with a resulting Cq value were considered positive. Relative quantification (RQ) for the *F. nucleatum* gene (*NusG*) was calculated based on the $2^{-\Delta\Delta Ct}$ method[32] and was expressed for each group relative to the respective normal tissue samples both in an unpaired way, using the mean of adjacent normal samples as a calibrator for each group, and in a paired way, using the respective adjacent normal of each sample as its specific calibrator.

Relative quantification of inflammatory mediator genes and miRNAs

Relative quantifications for the expression of 7 inflammatory genes [*TLR2* (Hs00610101_m1), *TLR4* (Hs01060206_m1), *NFKB1* (Hs00765730_m1), *IL1B* (Hs01555410_m1), *IL6* (Hs00985639_m1), *IL8* (Hs00174103_m1) and *TNF* (Hs00174128_m1)] and 5 miRNAs [hsa-*miR-21*-3p (TM002438), hsa-*miR-22*-3p (TM000398), hsa-*miR-28*-5p (TM000411), hsa-*miR-34a*-5p (TM000426), and hsa-*miR-135b*-5p (TM002261)] were performed in 27 CRA and 43 CRC cDNA samples. Adjacent normal tissue samples of each lesion (CRA and CRC) were studied as a sample pool with the same amount of cDNA. The qPCRs were performed using the TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, United States) with specific probes for each gene according to the instructions from the manufacturer, in a final volume of 10 μL using 25 ng of cDNA for the cytokine genes and 0.66 ng for the miRNAs. All reactions were performed in duplicate, and all experiments had a no-template control. The reactions were subjected to the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). Cq values were calculated after adjusting the threshold by the StepOne software (v.2.2.2) (Applied Biosystems, Foster City, CA, United States). For the mRNA analyses, the *ACTB* and *GAPDH* genes were used as reference genes, as validated in a previous study[31]. For the miRNA analysis, the method of global normalization was employed[33]. RQ values were calculated using the $2^{-\Delta\Delta Ct}$ method[32], considering the pool of the respective adjacent normal samples as the calibrator and considering the Cq mean of CRA expression for the CRC samples.

miRNA-mRNA interaction networks

Prediction of targets regulated by miRNAs was performed using the miRNA Data Integration Portal bioinformatics tool (http://ophid.utoronto.ca/mirDIP/)[34]. A protein-protein interaction network was generated via the String database (version 9.1) using the target genes as an input[35]. The identified miRNAs and target genes were integrated into interaction networks by Cytoscape software (version 3.1.1)[36]. The biological function of the identified genes in the network was defined using the BiNGO tool in Cytoscape (version 3.0.2)[37]. Cytoscape software also provides a graphical visualization of the network with the nodes representing the genes, miRNAs, and/or proteins and the edges representing their interactions.

KRAS mutation

KRAS (codons 12 and 13) hotspot mutation regions were analysed by PCR, followed by direct sequencing, as previously described[38]. PCR was performed at a final volume of 15 μL under the following conditions: 1.5 μL of buffer (Qiagen, Venlo, The Netherlands), 2 mmol/L $MgCl_2$ (Qiagen, Venlo, The Netherlands), 100 mmol/L dNTPs (Invitrogen, Carlsbad, Califórnia, United States), forward and reverse primers at 0.2 mmol/L (Sigma Aldrich), 1 unit of HotStarTaq DNA polymerase (Qiagen, Venlo, The Netherlands) and 1 μL of DNA at 50 ng/μL. The *KRAS* primers used were 5'-GTGTGACATGTTCTAATATAGTCA-3' (forward) and 3'-GAATGGTCCTGCACCAGTAA-5' (reverse)[39].

PCR products were purified with ExoSAP (GE Technology, IL, United States) and then added to a sequencing reaction mix containing 1 μL of BigDye (Applied Biosystems, Foster City, CA, United States), 1.5 μL of sequencing buffer (Applied Biosystems, Foster City, CA, United States) and 1 μL of primer, followed by post-sequencing purification with a BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA, United States) according to the instructions from the manufacturer. Direct sequencing was performed on a 3500 xL Genetic Analyzer (Applied Biosystems, Foster City, CA, United States). All mutations were confirmed in a second, independent PCR experiment.

MSI status

MSI evaluation was performed using a multiplex PCR comprising six quasimonomorphic mononucleotide repeat markers (NR-27, NR-21, NR-24, BAT-25, BAT-26 and HSP110), as previously described[40,41]. Primer sequences, as described[41,42], were each reverse primer end-labelled with a fluorescent dye as follows: 6-carboxyfluorescein (6-FAM) for BAT-26 and NR-21; 20-chloro-70-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) for BAT-25, NR-27 and HSP110; and 2,7,8-benzo-5-fluoro-2,4,7-trichloro-5-carboxyfluorescein (NED) for NR-24. PCR was performed using a Qiagen Multiplex PCR Kit (Qiagen, Venlo, The Netherlands) with 0.5 μL of DNA at 50 ng/μL and the following thermocycling conditions: 15 min at 95 ℃; 40 cycles of 95 ℃ for 30 s, 55 ℃ for 90 s and 72 ℃ for 30 s; and a final extension at 72 ℃ for 60 min. Fragment analyses were performed on a 3500 xL Genetic Analyser (Applied Biosystems, Foster City, CA, United States) according to the instructions from the manufacturer, and the results were analysed using GeneMapper v4.1 (Applied Biosystems, Foster City, CA, United States). Cases exhibiting instability at two or more markers were considered to have high MSI (MSI-H), cases with instability at one marker were defined as having low MSI (MSI-L) and cases that showed no instability were defined as microsatellite

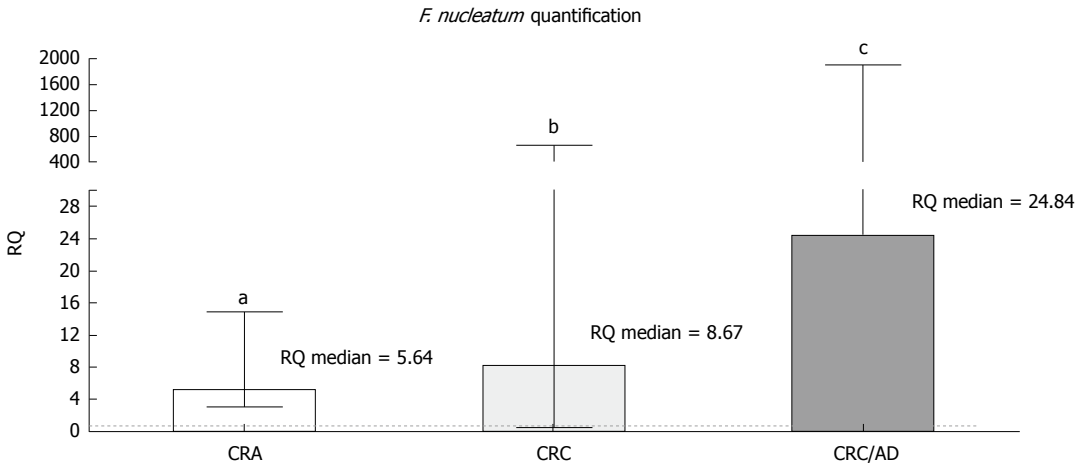

Figure 1 Relative quantification of the *Fusobacterium nucleatum NusG* gene in adenoma and colorectal cancer samples compared to adjacent normal tissues and colorectal cancer compared to adenoma tissue. Statistically significant differences, according to the Wilcoxon signed-rank test, were as follows: colorectal adenoma: $^{a}P = 0.0002$, colorectal cancer: $^{b}P = 0.0002$, colorectal cancer/adenoma: $^{c}P < 0.0001$. Median with interquartile range graph. CRA: Colorectal adenoma; CRC: Colorectal cancer; CRC/AD: Colorectal cancer/adenoma; RQ: Relative quantification; *F. nucleatum*: *Fusobacterium nucleatum*.

stable (MSS)[40].

Statistical analysis

KRAS mutation and MSI status were compared between groups using Fisher's exact test. The distribution of continuous data was evaluated using the D'Agostino-Pearson normality test. The Wilcoxon signed-rank test was used to test the significance of the RQ of the measured genes from the qPCR experiments. Spearman's rank correlation coefficient test was performed to compare the *F. nucleatum* quantification with the expression of the inflammatory genes and miRNAs. For all analyses, $P < 0.05$ was considered statistically significant. The analysis was performed by GraphPad Prism software (version 6.01).

RESULTS

Quantification of F. nucleatum in CRA and CRC tissues

Among 43 CRC and 27 CRA samples and their respective normal adjacent mucosa (N-CRC and N-CRA, respectively) quantified for *F. nucleatum*, 33 (76.7%) CRC samples, 31 (72.1%) N-CRC samples, 14 (51.8%) CRA samples, and 13 (48.1%) N-CRA samples were positive for bacterial DNA. Of these samples, the presence of the bacterium was observed in both the lesion and its matched normal mucosa for 6 CRA samples and 27 CRC samples. A significant increase in bacterial DNA was found for both CRA (RQ = 5.64) and CRC (RQ = 8.67) tissues compared to the respective normal adjacent tissues (Figure 1).

In addition, the analysis of *F. nucleatum* quantification for the CRC group was also performed in a paired way, using the respective adjacent normal tissue of each sample as its specific calibrator. This result was consistent with the unpaired analysis showing more *F. nucleatum* in tumour tissues (RQ = 17.71, $P = 0.0002$). For the CRA samples, this paired analysis was not performed due to the low potential of analysing the available 6 paired samples.

A comparison of CRC samples with CRA samples, using the Cq mean of CRA rather than the normal mucosa as a calibrator for the analysis, estimated the quantification of *F. nucleatum* as 24.84 times greater in CRC samples than in CRA samples ($P < 0.0001$) (Figure 1).

Gene expression of inflammatory mediators

Gene expression of inflammatory mediators in CRA samples and CRC samples relative to adjacent normal tissue samples showed significantly increased mRNA levels for *TLR4* (RQ = 2.27, P =0.0003), *IL1B* (RQ = 2.27, $P = 0.0047$) and *IL8* (RQ = 3.33, $P = 0.0006$) in CRA tissues (Table 1, Figure 2A) and for *TLR2* (RQ = 2.36, $P < 0.0001$), *IL1B* (RQ = 4.13, $P < 0.0001$), *IL6* (RQ = 6.67, $P < 0.0001$) and *IL8* (RQ = 6.36, $P < 0.0001$) in CRC tumours (Table 1, Figure 2B).

Additionally, elevated expression of *TLR2* (RQ = 1.68, $P < 0.0001$), *IL1B* (RQ = 4.79, $P < 0.0001$), *IL6* (RQ = 9.40, $P < 0.0001$) and *IL8* (RQ = 12.12, $P < 0.0001$) was observed in CRC tumours compared to CRA tissues (Table 1, Figure 2C).

miRNA gene expression

A similar estimation of miRNA relative gene expression was performed for CRA and CRC samples, in which miRNAs (miR-21, miR-22, miR-28, miR-34a, miR-135b) were quantified in each neoplastic tissue sample relative to a pool of the adjacent normal tissue. For the CRA group, only miR-135b was upregulated (RQ = 2.19, $P = 0.0074$) (Table 2, Figure 3A). However, for CRC samples, while miR-34a (RQ = 1.38, $P = 0.0029$) and miR-135b (RQ = 9.31, $P < 0.0001$) were upregulated, miR-22 (RQ = 0.27, $P < 0.0001$) and miR-28 (RQ = 0.65, $P = 0.0045$) were downregulated (Table 2, Figure 3B). Relative to the CRA group, CRC samples also presented a significant increase in the gene expression of miR-34a (RQ = 1.26, $P = 0.01$) and miR-135b (RQ =

Table 1 Relative quantification of mRNA expression of the inflammatory genes in adenoma and colorectal cancer samples compared with adjacent normal tissue samples and colorectal cancer samples relative to the adenoma group

	TLR2	*TLR4*	*NFKB1*	*IL1B*	*IL6*	*IL8*	*TNF*
CRA							
RQ median	0.87	2.27	1.02	2.27	1.12	3.33	0.75
RQ Range	0.36-10.80	0.32-11.25	0.45-3.38	0.14-76.76	0.09-80.41	0.10-874.50	0.14-2.60
P value	0.2940	0.0003[a]	0.5360	0.0047[a]	0.2473	0.0006[a]	0.0527
CRC							
RQ median	2.36	0.74	0.90	4.13	6.67	6.36	0.72
RQ Range	0.31-35.80	0.20-23.42	0.31-8.49	0.13-245.70	0.09-192.80	0.16-194.20	0.06-31.76
P value	< 0.0001[a]	0.4476	0.9855	< 0.0001[a]	< 0.0001[a]	< 0.0001[a]	0.2391
CRC/CRA							
RQ median	1.68	0.54	0.78	4.79	9.40	12.14	0.70
RQ Range	0.22-25.49	0.15-17.22	0.27-7.35	0.15-284.80	0.13-271.80	0.31-370.60	0.06-30.77
P value	< 0.0001[a]	0.0608	0.1237	< 0.0001[a]	< 0.0001[a]	< 0.0001[a]	0.1560

Wilcoxon signed rank test. [a]*P* value < 0.05 were considered statistically significant. CRA: Colorectal adenoma; CRC: Colorectal cancer; RQ: Relative quantification; TNF: Tumour necrosis factor; IL: Interleukin; NFKB: Nuclear factor kappa B; TLR: Toll-like receptor.

Table 2 Relative quantification of microRNAs in adenoma and colorectal cancer samples compared with adjacent normal tissue samples and colorectal cancer samples relative to the adenoma group

	miR-21	miR-22	miR-28	miR-34a	miR-135b
CRA					
RQ median	0.75	0.85	0.73	0.97	2.19
RQ Range	0.15-13.91	0.10-3.05	0.08-2.17	0.12-4.74	0.10-25.13
P value	0.6349	0.1747	0.1904	0.3306	0.0074[a]
CRC					
RQ median	0.53	0.27	0.65	1.38	9.31
RQ Range	0.08-12.53	0.026-2.78	0.11-7.27	0.20-30.0	0.45-74.00
P value	0.1725	< 0.0001[a]	0.0045[a]	0.0029[a]	< 0.0001[a]
CRC/CRA					
RQ median	1.01	0.59	0.77	1.26	2.64
RQ Range	0.15-23.91	0.06-6.07	0.14-8.79	0.18-27.51	0.13-20.96
P value	0.0969	0.5770	0.1271	0.0101[a]	< 0.0001[a]

Wilcoxon signed rank test. [a]*P* value < 0.05 were considered statistically significant. CRA: Colorectal adenoma; CRC: Colorectal cancer; RQ: Relative quantification.

2.64, *P* < 0.0001) (Table 2, Figure 3C).

Interactions between F. nucleatum abundance with the expression of inflammatory mediator genes and miRNAs

A correlation analysis was performed between the RQ values of inflammatory genes (*TLR2*, *TLR4*, *NFKB1*, *TNF*, *IL1B*, *IL6* and *IL8*) and *F. nucleatum* DNA levels in all neoplasms. For the CRA group, the only significant finding was a negative correlation between *TLR4* and *F. nucleatum* quantification (r = - 0.62, P = 0.0235). However, for CRC, significant positive correlations were observed for bacterial DNA levels with cytokines *IL1B* (r = 0.46, P = 0.0066), *IL6* (r = 0.47, P = 0.0059), and *IL8* (r = 0.54, P = 0.0013) (Table 3).

In the miRNA analysis, while no significant correlations was observed for the CRA group, there was a significant positive correlation between miR-22 expression and *F. nucleatum* (r = 0.38, P = 0.0331) in the CRC group (Table 3).

miRNA-mRNA correlation and interaction networks

Finally, independent of considerations of *F. nucleatum* levels, we also performed a correlation analysis between the RQ values of miRNAs and inflammatory genes in both CRA and CRC samples. For CRA samples, there was a positive correlation of *IL8* with miR-21 (r = 0.40, P = 0.0466), miR-34a (r = 0.44, P = 0.0296) and miR-135b (r = 0.46, P = 0.0200) (Table 4). Regarding CRC, several positive correlations were observed between miR-21, miR-22, and miR-28 and most of the inflammatory genes evaluated, and there was also a significant inverse correlation between miR-135b and *TNF* (r = - 0.32, P = 0.0411) (Table 4).

Additionally, we identified *in* silico a miRNA:mRNA interaction network that may be deregulated in CRC (Figure 4). This analysis demonstrated the interrelations of the inflammatory mediator genes alone and with their miRNA moderators (*e.g.*, miR-28 and miR-34a targeting *TLR4*, miR-22 targeting *TLR2* and miR-135b targeting *IL6*). The negative correlation of the expression of miR-135b and *TNF* found in this study (Table 4) was also demonstrated in this *in* silico a miRNA:mRNA interaction network, although not functionally validated in our study and not yet predicted by public databases[34] (Figure 4).

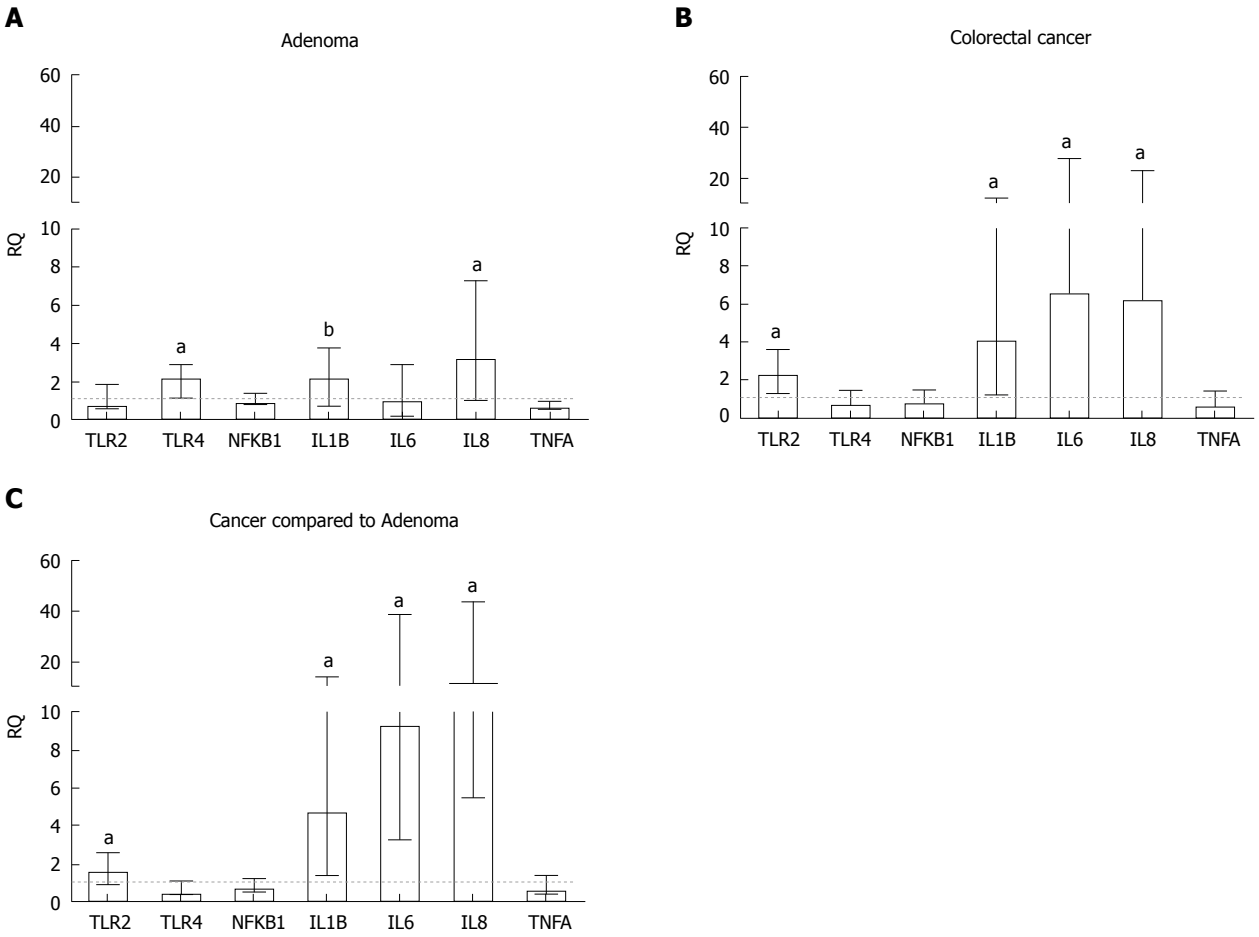

Figure 2 Relative quantification of inflammatory genes in (A) adenoma and (B) colorectal tumour tissue samples compared to a pool of respective adjacent normal tissue samples and (C) colorectal tumour tissue samples compared to adenoma samples. All samples were normalized to the reference genes *ACTB* and *GAPDH*. Statistically significant differences, according to the Wilcoxon signed-rank test, were $^{a}P \leq 0.001$ and $^{b}P \leq 0.01$. Median with interquartile range graph. RQ: Relative quantification.

KRAS mutation and MSI status in CRA and CRC tissues

Mutations in codons 12 and 13 of the *KRAS* gene were detected in 8/27 (30%) of CRA tissues and 11/43 (26%) of CRC tissues ($P = 0.7854$), of which p.Gly12Asp (G12D) and p.Gly13Asp (G13D) were the most frequent changes in both CRA and CRC tissues (Table 5). Regarding the MSI status, all the CRAs were microsatellite stable or MSI-low, while 7/43 (16%) of CRC samples were MSI-high ($P = 0.0382$; Table 5). The *KRAS* mutation was associated with *F. nucleatum* presence in CRC tumours ($P = 0.0432$) and had greater expression of miR-21 in CRA samples ($P = 0.0409$). The MSI-high tumour status was associated with increased expression of *IL8* ($P = 0.0171$). Other comparisons of the association between *KRAS* mutations and MSI status with *F. nucleatum* levels or cytokine gene and miRNA expressions showed no significant differences.

DISCUSSION

To better understand the possible mechanistic relationship between *F. nucleatum* presence and the immune response in CRC development, we evaluated the association between *F. nucleatum* quantification and the expression of cytokines and miRNAs involved in the inflammatory stress response in CRA and CRC tissues from a South American patient study. Our results corroborated previous studies showing that *F. nucleatum* is present in greater amounts in tumours compared to both CRA and matched normal tissues. Importantly, our work further suggests that the abundance of *F. nucleatum* can affect cytokine expression possibly via recognition by *TLR4* and *TLR2* with regulation by miRNAs such as miR-34a, miR-135b and miR-22.

Several studies in North American, European and Asian populations showed the overabundance of *F. nucleatum* when comparing CRC tissues with normal adjacent tissues, healthy subjects[5,10,11,37,40,43-45] or CRA tissues[10,43]. A recent meta-analysis concluded that intestinal *F. nucleatum* is a valuable diagnostic marker for CRC[46]. A different Brazilian study with a small sample size, also in the southeast region, previously showed greater levels of *F. nucleatum* in CRC (DNA levels in faecal samples of CRC patients were compared to those of healthy subjects)[47]. Our study of fresh tissue samples from Brazilian CRA and CRC patients

Table 3 Correlation analysis between the relative quantification of the inflammatory genes and microRNAs with the quantification of *Fusobacterium nucleatum* in adenoma and colorectal cancer samples

Spearman correlation coefficient (*r*)	CRA	CRC
TLR2	0.42	0.09
P value	0.1557	0.6335
TLR4	-0.62	-0.01
P value	0.0235[a]	0.9587
NFKB1	-0.40	-0.04
P value	0.1809	0.8045
IL1B	0.31	0.46
P value	0.3064	0.0066[a]
IL6	0.01	0.47
P value	0.9716	0.0059[a]
IL8	-0.07	0.54
P value	0.8166	0.0013[a]
TNF	0.30	0.23
P value	0.3156	0.2027
miR-21	-0.21	0.26
P value	0.4643	0.1467
miR-22	0.17	0.38
P value	0.5528	0.0331[a]
miR-28	0.04	0.01
P value	0.8872	0.9413
miR-34a	0.17	0.00
P value	0.5630	0.9905
miR-135b	-0.05	0.22
P value	0.8637	0.2163

Spearman correlation test. [a]*P* value < 0.05 were considered statistically significant. CRA: Colorectal adenoma; CRC: Colorectal cancer; TNF: Tumour necrosis factor; IL: Interleukin; NFKB: Nuclear factor kappa B; TLR: Toll-like receptor.

provided more evidence of the increasing abundance of *F. nucleatum* in the progression from adenoma to cancer.

The mechanism by which *F. nucleatum* functionally contributes to colorectal tumourigenesis has been investigated in several studies. It has been shown that this bacterium causes an inflammatory microenvironment more favourable to CRC development among other bacteria that colonize at the tumour site[6]. A carcinogenic mechanism proposed is that *F. nucleatum* promotes an oncogenic and inflammatory response *via* FadA, the main virulence factor of *F. nucleatum*, binding to E-cadherin and activating the B-catenin pathway[48]. In addition, the *F. nucleatum* lectin Fap2 binds to the Gal-GalNAc polysaccharide expressed by CRC cells, likely increasing immune-mediated inflammation[9]. Moreover, the presence of *F. nucleatum* in the gut affects tumour-related cytokines and activates the JAK/STAT and MAPK/ERK pathways involved in CRC tumour progression[49] (Figure 5).

In our study, in CRA disease tissues, we found that the mRNA expression of *TLR4*, *IL1B*, and *IL8* was increased, as was the expression of miR-135b. In CRC tumour tissues, the *TLR2* receptor and the IL genes *IL1B*, *IL6* and *IL8* were significantly upregulated when compared to adjacent normal tissues and to CRA tissues. miRNA levels of miR-34a and miR-135b were more highly expressed in CRC tumours, while miR-22 and miR-28 were downregulated (Figure 5). These findings indicate that several of these genes are already dysregulated in early CRA stages of colorectal neoplasia as well as in CRC; thus, these genes may contribute to inflammatory stresses that drive the progression from CRA to CRC. Greater *TLR2* expression appears to be a later event in colorectal carcinogenesis, as also indicated by our recent study showing increased mRNA and protein expression of *TLR2* in CRC tissues[31].

Recent studies have demonstrated that the TLR4/MYD88/NF-κB pathway is activated by *F. nucleatum* infection, which stimulates the overexpression of miR-21[28]. Moreover, the TLR4/MYD88 innate immune signalling and the miR-18a and miR-4802 expression in CRC patients with a high amount of *F. nucleatum* activate the autophagy pathway to control CRC chemoresistance[50]. In summary, these findings suggest the involvement of both *TLR2* and *TLR4* in *F. nucleatum* immune recognition.

Correlations of both inflammatory genes and miRNA expression with *F. nucleatum* levels showed positive associations with *IL1B*, *IL6*, and *IL8* as well as with miR-22 in CRC tissues. Increased expression of *IL1B*, *IL6* and *TNF* has also been reported by *in vitro* and animal model studies after *F. nucleatum* infection[51,52].

Together with existing data, our results are consistent with a scenario in which *F. nucleatum* triggers an increased expression of *IL1B*, *IL6* and *IL8*, which further adds to inflammatory pressures fuelling the progression of colorectal neoplasia. Our work suggests that this phenomenon may proceed by an alternative pathway involving the recognition by the *TLR2* receptor (Figure 5) to that pathway previously shown for the invasion of epithelial cells *via* FadA[48].

To date, several studies evaluated the abundance of *F. nucleatum* with miRNA expression in CRC[26-28]. In our study, we found a positive correlation between miR-22 and the abundance of *F. nucleatum* in CRC. miR-22 suppresses the expression of the *p38* gene, which can impair the production of dendritic cells in tumours[53]. As dendritic cells are important in the TLR-mediated recognition of microorganisms[54], patients with upregulated miR-22 may have a compromised immune system due to fewer dendritic cells, favouring the proliferation of microorganisms such as *F. nucleatum*. Therefore, this positive correlation observed between miR-22 and *F. nucleatum* levels may be related to its role in the immune response and should be further investigated.

In addition, we also observed associations between both the *KRAS* mutation and MSI status and the expression of inflammatory genes or miRNAs in CRA and CRC tissues. Interestingly, we observed a greater expression of miR-21 associated with the *KRAS* mutation in the CRA tissues, but not in CRC tissues. In non-small cell lung cancer (NSCLC), the overexpression of wild type *KRAS* or mutated *KRAS* (G12D) was

Table 4 Correlation analysis between the relative quantification of microRNAs with the inflammatory genes in adenoma and colorectal cancer samples

Spearman correlation coefficient (*r*)	miR-21	miR-22	miR-28	miR-34a	miR-135b
CRA					
TLR2	0.09	0.29	0.31	0.23	0.09
P value	0.6608	0.1655	0.1266	0.2606	0.6714
TLR4	0.18	0.06	0.18	0.41	0.38
P value	0.3892	0.7926	0.3955	0.0431	0.0598
NFKB1	-0.01	-0.17	-0.21	-0.19	-0.20
P value	0.9796	0.4252	0.3191	0.3650	0.3454
IL1B	0.29	0.15	0.09	0.45	0.38
P value	0.1667	0.4834	0.6555	0.0232	0.0610
IL6	0.30	0.08	0.05	0.26	0.24
P value	0.1430	0.7148	0.8237	0.2136	0.2417
IL8	0.40	0.18	0.17	0.44	0.46
P value	0.0466[a]	0.4017	0.4273	0.0296[a]	0.0200[a]
TNF	-0.21	-0.04	-0.03	-0.05	-0.11
P value	0.3083	0.8638	0.9012	0.8152	0.6084
CRC					
TLR2	0.29	0.53	0.18	0.19	-0.14
P value	0.0777	0.0005[a]	0.2790	0.2408	0.3923
TLR4	0.07	0.34	0.33	0.26	0.05
P value	0.668	0.0377[a]	0.0401[a]	0.1216	0.7586
NFKB1	0.18	0.31	0.02	-0.10	-0.30
P value	0.2779	0.0499[a]	0.8958	0.5462	0.0574
IL1B	0.53	0.43	0.13	0.22	-0.10
P value	0.0004[a]	0.0055[a]	0.4139	0.1668	0.5248
IL6	0.54	0.57	0.18	0.21	0.05
P value	0.0004[a]	0.0002[a]	0.2813	0.2095	0.7680
IL8	0.53	0.37	0.09	0.23	0.13
P value	0.0005[a]	0.0180[a]	0.5734	0.1506	0.4314
TNF	0.26	0.42	0.03	0.08	-0.32
P value	0.1094	0.0071[a]	0.8406	0.6264	0.0411[a]

Spearman correlation test. [a]*P* value < 0.05 were considered statistically significant. CRA: Colorectal adenoma; CRC: Colorectal cancer; TNF: Tumour necrosis factor; IL: Interleukin; NFKB: Nuclear factor kappa B; TLR: Toll-like receptor.

reported to modulate the expression of miRNAs, including miR-21 and miR-30c[55]. The authors showed that miR-30c and miR-21 were specifically activated by *KRAS* and played an important role in lung cancer development and chemoresistance by targeting crucial tumour suppressor genes. Moreover, we also observed a positive association between the expression of *IL8* mRNA and MSI-H colorectal carcinoma, independent of the presence and abundance of *F. nucleatum*. Recently, Hamada *et al*[20] reported an association of *F. nucleatum* levels with the immune response to colorectal carcinoma according to the tumour MSI status, suggesting an interplay between *F. nucleatum*, MSI status, and immune cells in the CRC tumour microenvironment. MSI-H colorectal carcinomas generate immunogenic peptides due to a mismatch repair deficiency, resulting in a strong anti-tumour immune response thought to underlie the reported favourable prognosis and better response to immunotherapies of this molecular subtype of CRC[20].

We also evaluated the correlation between the expression of miRNAs and inflammatory mediator genes, and we formulated a miRNA:mRNA interaction network based on predicted database targets[34]. The correlations were mainly positive, including miR-21, miR-34a and miR-135b with *IL8* in CRA tissues and between miR-21, miR-22 and miR-28 with most of the inflammatory mediator genes studied in CRC tissues. However, a negative correlation was observed between miR-135b and *TNF*.

miR-135b has been previously associated with an increased expression in CRC and CRA tissues[56-58] and has been proposed to be an oncomiR targeting several tumour suppressor genes[56,58]. Studies have proposed that the detection of miR-135b in stool samples can be used as a non-invasive biomarker for CRC and CRA[59], and silencing miR-135b may be considered a possible therapy for CRC[56,58]. Data showing that miR-135b indirectly inhibits the production of LPS (lipopolysaccharide)-induced *TNF* by suppressing the production of ROS and the activation of *NF-κB* in human macrophages[60] support the negative correlation found between miR-135b and *TNF* in this study. Although this proposed miRNA-mRNA relationship is not yet predicted by the major miRNA public databases[34], *TNF* may have an indirect immune regulation by miR-135b.

Regarding the role of miR-34a in CRC, studies demonstrated that it acts as both an oncomiR that is upregulated[61,62] and as a tumour suppressor that displays reduced expression[63-66]. According to our

Table 5 *KRAS* mutation and microsatellite instability status in adenoma and colorectal cancer groups

KRAS status/mutation type	CRA, *n* = 27 (%)	CRC, *n* = 43 (%)	*P* value
WT	19 (70.4)	32 (74.5)	0.7854
Mutation	8	11	
p.Gly12Ala (G12A)	1 (3.7)	0	
p.Gly12Ser (G12S)	1 (3.7)	1 (2.3)	
p.Gly12Val (G12V)	1 (3.7)	2 (4.6)	
p.Gly12Asp (G12D)	3 (11.1)	4 (9.3)	
p.Gly13Asp (G13D)	2 (7.4)	4 (9.3)	
MSI status			
MSS + MSI-L	25 + 2 (100)	34 + 2 (83.7)	0.0382[a]
MSI-H	0	7 (16.3)	

Fisher's exact test. [a]*P* value < 0.05 were considered statistically significant. WT: Wild; MSI: Microsatellite instability; MSS: Microsatellite stable; MSI-L: Microsatellite instability low; MSI-H: Microsatellite instability high; CRA: Colorectal adenoma; CRC: Colorectal cancer.

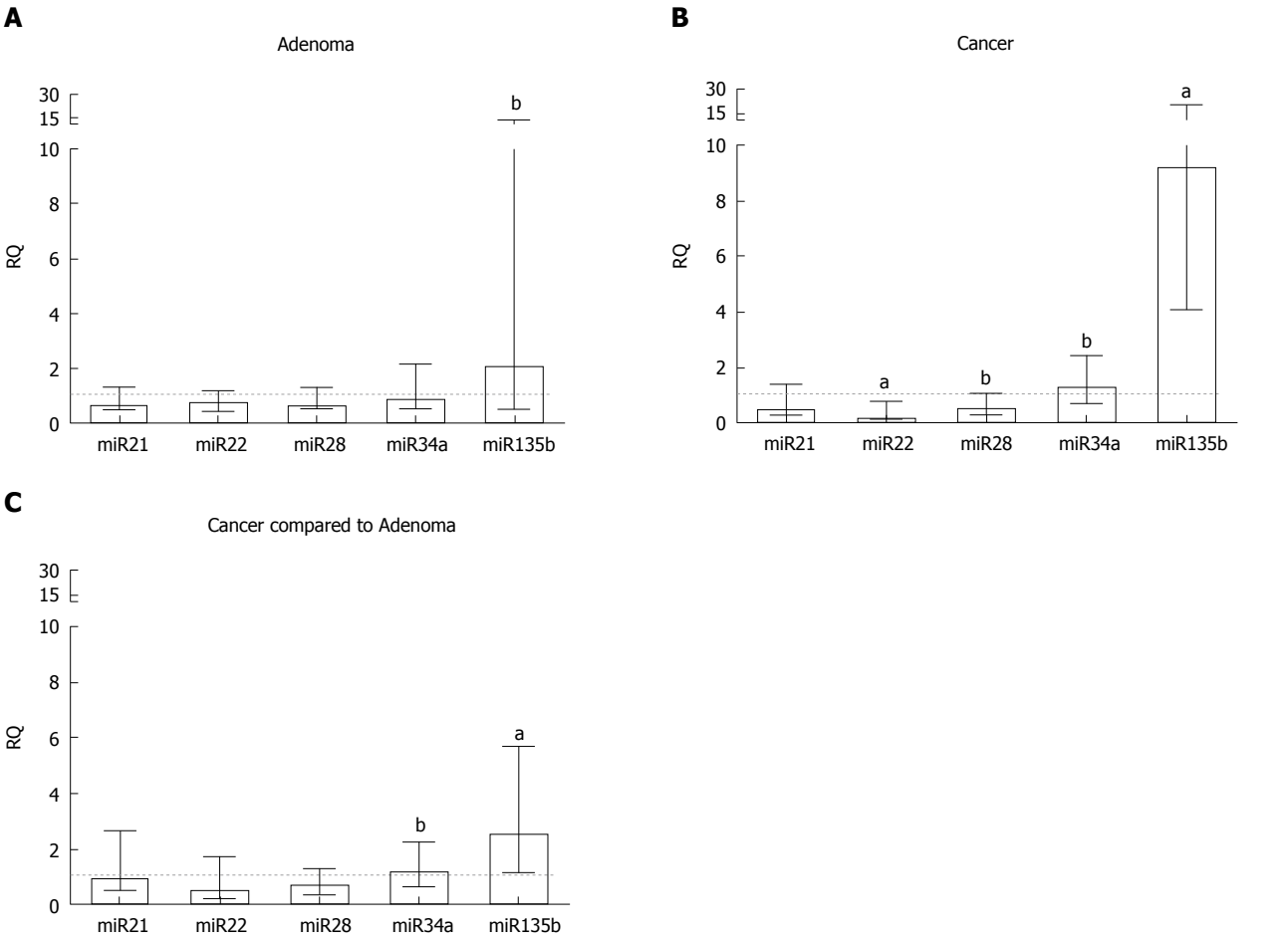

Figure 3 Relative quantification of microRNA genes in (A) adenoma and (B) colorectal tumour tissue samples compared to a pool of respective adjacent normal tissue samples and (C) colorectal tumour tissue samples compared to adenoma samples. Statistically significant differences, according to the Wilcoxon signed-rank test, were [a]$P \leq 0.001$ and [b]$P \leq 0.01$. Median with interquartile range graph. RQ: Relative quantification.

miRNA:mRNA interaction network analysis, miR-34a can target *TLR4* (Figure 4). This may explain the low expression of *TLR4* in CRC samples and overexpression in CRA samples, in which miR-34a had basal expression. Our data, together with that of previous studies, showed an increased expression of *TLR2* in CRC samples. Therefore, in CRC, a significant mechanism for the recognition of *F. nucleatum* may operate *via TLR2*, with consequent activation of ILs *IL1B*, *IL6* and *IL8 via* NF-κB. Moreover, *TLR2* is a predicted target of miR-22, which was downregulated in the CRC samples evaluated in our study (Figure 5).

Evidence suggests that miR-22 and miR-28 function as tumour suppressors. miR-22 can target key oncogenes for tumour invasion, metastasis and angiogenesis in CRC[67,68]. A recent study showed reduced expression of miR-28 in the tissues of CRC liver metastases[69]. However, the activity of this miRNA differs

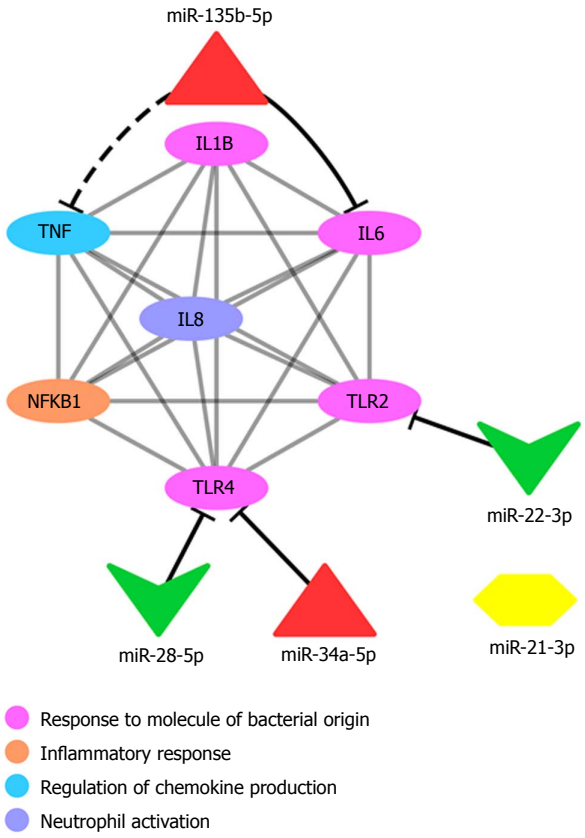

Figure 4 Protein interaction network showing microRNAs and their predicted gene targets. The protein interaction network (grey lines) shows the interaction between proteins encoded by target genes that are predicted to be regulated by microRNAs (miRNAs). Predicted interactions between miRNAs and target genes are shown by black lines. The dashed black line represents the possible interaction suggested in this study for *miR-135b-5p* and *TNF*. Ellipses represent target genes and/or proteins; red triangles represent upregulated miRNAs; green triangles represent downregulated miRNAs. TNF: Tumour necrosis factor; IL: Interleukin; NFKB: Nuclear factor kappa B; TLR: Toll-like receptor.

by 3p or 5p strand translation. miR-28-3p has been implicated in increased tumour migration and invasion, while miR-28-5p, analysed in our study, was reported to play a role in reducing tumour proliferation, migration and invasion in CRC[70].

Our results showed a greater level of *F. nucleatum* in CRA and CRC tissues, which was more striking for CRC samples, suggesting an expansion of *F. nucleatum* colonization during the progression from adenoma to adenocarcinoma. Our gene expression data suggested that this phenomenon may lead to increased inflammatory pressures during CRC development based on the high expression of pro-inflammatory ILs *IL1B*, *IL6* and *IL8* and the correlation with *F. nucleatum* levels. Immune recognition of *F. nucleatum* may be mainly mediated by TLR2 and/or TLR4 and dependent on interactions with differently regulated miRNAs. Together, these findings provide further potential mechanistic rationale for the immune-comprised pro-inflammatory role of *F. nucleatum* in colorectal carcinogenesis. Efforts to develop early detection strategies for CRC could include these biological interactors as potential functional biomarkers of *F. nucleatum*-mediated disease in addition to overall measures of *F. nucleatum* levels.

ARTICLE HIGHLIGHTS

Research background

Recently, *Fusobacterium nucleatum* (*F. nucleatum*), an anaerobic bacterial component of the oral and gut commensal flora, has emerged as a risk factor for colorectal cancer (CRC) development. Several studies have observed an association between overabundance of *F. nucleatum* in colonic tumor tissue compared to the normal matched mucosa. However, despite progress in this field the molecular mechanisms of how the bacterium etiologically contributes to carcinogenesis are still unclear.

Research motivation

We previously observed an association of the *TLR2*-196 to -174del genetic variant with increased CRC risk, together with an increased expression of *TLR2* mRNA and protein in tumor tissues[31]. The major postulated mechanism of *F. nucleatum*-mediated colorectal tumorigenesis involves immune related inflammatory responses. Therefore, we decided to extend our previous work by measuring the transcript levels of important mediators in the pathogen-activated immune and inflammatory response, including TLR2 / TLR4 receptor and cytokine genes, and then evaluating the association of their expression with *F. nucleatum* levels in colorectal tumors. As microRNAs have been shown to be epigenetic regulators of inflammatory responses, we further examined the involvement of miRNAs in modulating the bacterial - cytokine interaction.

Research objectives

The main objective of this study was to investigate the association between inflammatory genes and *F. nucleatum* in colorectal carcinogenesis, by examining tissues from the major colorectal neoplasms of adenoma and adenocarcinomas. A secondary objective was to examine the interaction of the bacterial mediated immune response with microRNA (miRNA) regulation. The elucidation of likely mechanisms whereby *F. nucleatum* may contribute to inflammatory mediated colorectal carcinogenesis will help to better understand the molecular pathways activated by this bacterium and where prevention and treatment strategies can be best targeted.

Research methods

Robust techniques were used for DNA quantification of *F. nucleatum* and RNA transcript measures of the inflammatory genes and miRNAs in normal and tumor tissues. For this purpose, we used TaqMan gene expression assays (Applied Biosystems, Foster City, CA, United States) with specific probes for each gene and miRNA for relative quantification. The reactions were analyzed using the StepOnePlus real-time PCR System (Applied Biosystems, Foster City, CA, United States). Mutation testing of the *KRAS* gene was performed by direct sequencing and microsatellite instability (MSI) evaluation was performed using a multiplex PCR. In addition, we also used a bioinformatic tool 'miRNA Data Integration Portal' (http://ophid.utoronto.ca/mirDIP/)[34] to build an miRNA: mRNA interaction network by using Cytoscape software (version 3.1.1)[36].

Research results

Ours results confirm the overabundance of *F. nucleatum* in adenoma and tumor neoplasms compared to their respective matched normal tissues, as previously found in several populations. We further suggest that this bacterial load increases the expression of TLR2 and TLR4 receptors and consequently of pro-inflammatory interleukins IL1B, IL6 and IL8. This immune-modulation of the inflammatory response to *F. nucleatum* colonic invasion also affects the expression of miRNA regulators of the inflammatory response. In particular, these miRNA:mRNA interactions network indicate a mechanism of colorectal carcinogenesis where altered expression of miR-34a, miR-135b, and miR-22, previously associated with CRC, occurs *via* a TLR2/TLR4 dependent response to *F. nucleatum*. In analyses stratified by tumor molecular characteristics, we observed that *KRAS* was more frequently mutated in tumors with *F. nucleatum*, and that an increased *IL-8* expression was associated with MSI-

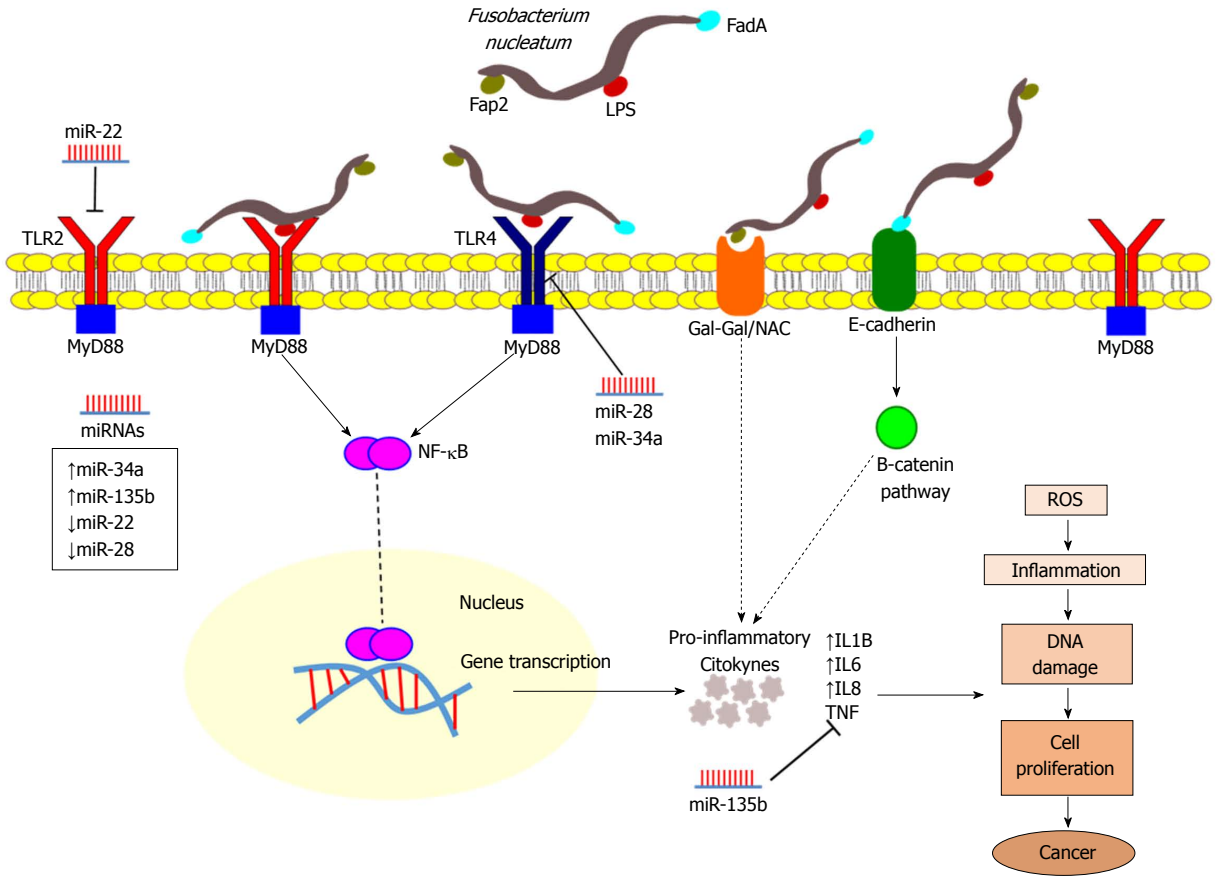

Figure 5 Representation illustrating the interactions of *Fusobacterium nucleatum* in colorectal cancer. *Fusobacterium nucleatum* (*F. nucleatum*) presents the virulence factors FadA and Fap2 and lipopolysaccharide (LPS), which are recognized by Toll-like receptors (TLR2 and TLR4) mediating *F. nucleatum* invasion and the promotion of colorectal cancer (CRC). FadA, a surface adhesion protein, can bind to E-cadherin on CRC cells, activating B-catenin signalling. Fap2, a galactose-sensitive haemagglutinin and adhesion protein binding to Gal-GalNAc, contributes to the invasive ability of *F. nucleatum*. The TLR2/TLR4/MYD88 pathway is activated in response to *F. nucleatum*, leading to the activation of *NF-κB*, a transcription factor that is involved in regulating the expression of many genes, leading to elevated expression levels of oncogenes and pro-inflammatory cytokines, mainly interleukin (IL)1B, IL6, IL8 and tumour necrosis factor, inducing the production of reactive oxygen species, which subsequently lead to inflammation and DNA damage that promotes tumour growth and progression. Furthermore, these pathways may be under the regulation of differentially expressed microRNAs. ROS: Reactive oxygen species; TNF: Tumour necrosis factor; IL: Interleukin; NF-κB: Nuclear factor kappa B; TLR: Toll-like receptor; miRNA: MicroRNA.

high status. Therefore, more studies of gene function and regulation within the inflammatory pathways impacted by *F. nucleatum* invasion are needed, along with consideration of tumor molecular subtypes.

Research conclusions

Our findings reinforce the increasing invasion of *F. nucleatum* during the colorectal adenoma to cancer development. This appears to increase expression of pro-inflammatory mediators and dysregulation of miRNA expression, leading to a more carcinogenic microenvironment alongside genetic alterations such as *KRAS* mutation and MSI-high. Therefore, together with other studies, our results suggest that *F. nucleatum* is involved in CRC development through immune responses to inflammatory stresses. Further work is needed to functionally demonstrate these postulated tumorigenic mechanisms, and also for early CRC detection and diagnosis strategies using biomarkers of *F. nucleatum* presence or the consequent immune response.

Research perspectives

The intestinal microbiota is very diverse and important for the maintenance of epitelial homeostasis. Disturbances of this microbiome balance appears to be a major factor in CRC etiology. *F. nucleatum* has been implicated in recent years, by *in vitro* and in mouse models, as a carcinogenic bacterium through generation of a microenvironment conducive to cancer development. Considering that *F. nucleatum* has been found to be highly abundant in both adenoma and CRC neoplasms, it may have uses as a tissue or non-invasive biomarker in faeces (or possibly mouth-rinse samples) for CRC and the early detection of adenomas (which may help define a higher risk group for CRC development due to the presences of the bacterium). However, further investigations are needed to understand the molecular mechanisms in the immuno-inflammatory response to the increased invasion of this bacterium into developing neoplasms, and if this can promote genetic and epigenetic alterations that may culminate in CRC development.

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