Elevated TNF-α level is correlated with NF-κB/p65 activation in patients with sporadic colorectal cancer

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Abstract. Colorectal cancer (CRC) is a major global health issue, and inflammation plays a crucial role in its development. Nuclear factor-κB (NF-κB) activation, typically mediated by tumor necrosis factor-α (TNF-α), is involved in the inflammatory response leading to cancer. The present study aimed to investigate the TNF-α/NF-κB signaling in sporadic CRC. NF-κB activation was evaluated by measuring the phosphorylation of the p65 subunit at specific residues (pS536 and pS529) through phospho-flow cytometry. TNF-α protein levels were measured using flow cytometry/ELISA and *TNF-α*, *NFKB1*, and *RELA* gene expression was assessed using reverse transcription-quantitative PCR. The results were then correlated with the Tumor-Node-Metastasis (TNM) classification. The findings of the present study revealed that NF-κB activation was higher in tumoral tissue than in normal adjacent mucosa. TNF-α gene expression and protein levels were also elevated in patients with CRC, particularly in advanced stages. The present study demonstrated a positive correlation between TNF-α and NF-κB/p65. The analysis of TNM groups suggested that the correlation between TNF-α and p65 (pS536) plays an essential role in CRC tumorigenesis. By contrast, TNF-α and p65 (pS529) signaling was revealed to be relevant for CRC progression. Evaluating *RELA* and *NFKB1* gene expression revealed molecular feedback during the inflammation response. The findings of the present study highlighted the TNF-α/NF-κB signaling pathway role in CRC development and may serve as a potential diagnostic and prognostic marker for the disease. Further research could provide valuable insights into the clinical implications of targeting this pathway in CRC diagnosis and treatment.

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Introduction

Colorectal cancer (CRC) is a major global health concern; it is the third most frequently diagnosed malignancy and a leading cause of cancer-related deaths worldwide (1). The 10-year survival rate for CRC is 58%, but this varies depending on race and ethnicity (2). In Mexico, the mortality rate due to CRC is increasing by 1.3% in women and 2.7% in men annually. However, public health insurance policies do not cover the cost of CRC screening despite its importance in diagnosis and treatment. This lack of programs focused on early detection is a cause of concern in Mexico (3). Certain lifestyle factors, such as smoking, unhealthy diet, obesity and high alcohol consumption, are associated with the incidence of sporadic CRC; these factors are essentially linked to chronic inflammation. The Tumor-Node-Metastasis (TNM) classification uses clinical parameters to illustrate the development of CRC, its clinical outcomes and how it responds to treatment (4).

Inflammation is extensively involved in several cancer hallmarks, including evasion of apoptosis, tumor growth, proliferation and metastasis, thereby playing a crucial role in promoting tumorigenesis (5). Nuclear factor-κB (NF-κB) is a family of inducible transcription factors that regulates the inflammatory response in various human malignant diseases by controlling the expression of pro-inflammatory genes (6,7). The NF-κB family comprises five hetero or homodimers: RelA/p65, RelB, NF-κB1 (p50/p105), NF-κB2 (p52/p100) and c-Rel. Multiple post-translational modifications, particularly phosphorylation, regulate NF-κB signaling (8,9). Several

studies have shown an association between increased activity of NF-κB and CRC development (8,10). The principal regulators of NF-κB activation are cytokines such as tumor necrosis factor-α (TNF-α), which trigger the IKK complex, leading to the phosphorylation of the p65 subunit and translocation of NF-κB dimers to the nucleus, regulating gene expression associated with tumorigenesis (8,11).

Previous studies indicate that TNF-α promotes phosphorylation of NF-κB/p65 on serine 536 and 529 in various inflammation-related pathologies, including cancer (12-15). TNF-α is a pro-inflammatory cytokine produced by macrophages and commonly found in the tumor microenvironment (12). In previous studies, and one by the present authors it was found that TNF-α gene expression was higher in advanced stages of patients with sporadic CRC compared with early stages. TNF-α activity has been proposed as a potential marker for CRC development (14-17). However, it remains uncertain what clinical impact TNF-α/NF-κB signaling has on patients with sporadic CRC.

The aim of the present study was to investigate the TNF-α/NF-κB signaling activity and its correlation with the development and progression of sporadic CRC. To assess the activation of NF-κB, the phosphorylation of p65 Ser536 and Ser529 was assessed through phospho-flow cytometry. Additionally, TNF-α gene expression was determined by reverse transcription-quantitative PCR (RT-qPCR) and the protein level was detected using flow cytometry and ELISA.

Materials and methods

Participants. In total, 122 patients >18 years old (adults) diagnosed with clinical and histological sporadic CRC who agreed to participate and signed the informed consent form were enrolled from December 2018 to June 2023 as experimental group in the present study. A calibration control group consisting of 30 tissue samples of individuals without a history of inflammatory bowel disease was also included. The collection of calibration control (non-CRC) tissues was conducted during the same time period. These individuals were included after undergoing routine preventive colonoscopy due to non-specific symptoms that ultimately resulted negative for neoplasia (such as chronic constipation, unexplained anemia or mild abdominal pain). The tissues were obtained as biopsies of non-cancerous colonic mucosa during these procedures. Therefore, no additional hospitalization was required specifically for sample collection. Additionally, blood samples were collected from 100 healthy individuals between December 2018 and May 2025 to establish a control group for evaluating serum TNF-α levels. All experimental and control samples were collected at the Civil Hospital of Guadalajara 'Dr. Juan I. Menchaca' (Guadalajara, Mexico). Patients who had taken drugs that could modify the natural activity of TNF-α, and NF-kB or those who voluntarily decided that the analysis of their samples should not be used in the project were excluded from this study. Further exclusions included those with unsuccessful laboratory procedures or with the impossibility of access to the new biological samples. The pathology service determined the CRC stage using the TNM classification according to the American Joint Committee on Cancer (AJCC) 8th edition (18). Only non-treated patients were included in the study. The present study was performed following the Declaration of Helsinki and was approved by the 'Hospital Civil de Guadalajara Ethics Committee' (approval no. 21711; Guadalajara, Mexico). The patients provided written and verbal informed consent for scientific purposes to publish any associated data from the present study.

Tissue samples. In each case, tumor tissue and adjacent normal mucosa from distant regions of the same patient's resected tumor were obtained. The sampling was performed according to the 'Cancer Care Quality Measures: Diagnosis and Treatment of Colorectal Cancer', issued by the 'Agency for Health Care Research and Quality' (19,20). Samples were collected using phosphate-buffered saline and transported to the laboratory for immediate flow cytometric experiments. For RNA isolation, RNAlater® Stabilization Solution (cat. no. AM7020; Thermo Fisher Scientific, Inc.) was used and further processed for qPCR analysis. Tissue samples from non-patients with CRC were collected during preventive colonoscopy using the same procedure. The remaining tissues were stored at -80˚C for any additional experiments.

Serum samples. Blood samples were collected preoperatively from 122 patients diagnosed with CRC constituting the experimental group, along with 100 healthy participants who served as the control group. The samples were centrifuged at 1,811 x g for 5 min at room temperature in the Eppendorf centrifuge 5810. Then, the serum was separated and stored at -80˚C until further analysis.

Flow cytometric assays. Tissues (5-10 mg) suspended in PBS were minced with a sharp blade. The procedure to obtain single-cell suspensions before flow cytometric experiments was carried out as proposed by Ali *et al* (21) and adapted to the samples as follows: The minced tissue was subjected to enzymatic digestion in 1 ml of 0.1% trypsin solution, followed by dissociation with 0.1% collagenase. The phospho-flow cytometric method was used to measure the phosphorylated NF-κB/p65 residues using the Perfix Phospho-Epitopes Exposure kit (cat. no. B26976; Beckman Coulter, Inc.), anti-NF-κB p65pS529-PE (cat. no. 130-120-252; Miltenyi Biotec GmbH) and anti-NF-κB p65pS536-Alexa Fluor (cat. no. A88939; Beckman Coulter, Inc.) at a working dilution of 1:50 (2 µl/test) incubated for 30 min at room temperature while protected from light, following the manufacturer's recommended protocol. To assess TNF-α activity, cell surface protein was detected. The cell suspensions were processed and stained with anti-human TNFα Alexa Fluor 488 antibody (cat. no. 53-7349-41 Affymetrix; Thermo Fisher Scientific, Inc.) using the 'Staining Cell Surface Antigens for Flow Cytometry Reagents' from Affymetrix eBioscience, which includes the following reagents and steps on its protocol: Pre-incubation of the cell suspension with 20 µl Human Fc Receptor Binding Inhibitor (cat. no. 14-9161) for 10-20 min at 2-25˚C. The TNFα Alexa Fluor 488 antibody at a working dilution of 1:200 (2 µl/test) was combined with an appropriate volume of Flow Cytometry Staining Buffer (cat. no. 00-4222) so that the final staining volume was 100 µl. Next, the cell suspensions were incubated for 40 min at 4˚C and protected from light. After incubation, the cells were washed by adding 2 ml/tube of Flow

Cytometry Staining Buffer and centrifuged at 400-600 x g for 5 min at room temperature. Data were acquired using a Gallios 10 Flow Cytometer (Beckman Coulter, Inc.). The Gallios software v.10 (Beckman Coulter, Inc.) was utilized to analyze 20,000 events. Gating was applied to exclude cell debris and autofluorescence.

ELISA. The TNF-α level was measured in serum samples of patients with CRC and control participants using the human TNF-α ELISA Kit (cat. no. KHC3011; Thermo Fisher Scientific, Inc.). Experiment validation was performed using standards provided by the kit manufacturers and analyzed with the MultiSkan FC automated microplate reader (Thermo Fisher Scientific, Inc.). The optical density at 450/550 nm was relied upon to detect the concentrations of each sample. The mean concentration and the standard deviation of the optical density of control patients were utilized to establish the ELISA test cut-off.

Gene expression. A total of 10-15 mg of tissue was cut into small pieces and then collected in 0.5 ml of TRI® reagent (cat. no. T3934; Sigma-Aldrich; Merck KGaA). Each sample was homogenized in Tissue Lyser LT (Qiagen Inc.) for 3 min at 25 Hz. The following steps of RNA isolation were performed according to the manufacturer's instructions (Qiagen Inc.). RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription was performed using 1 μg total RNA treated with DNase1 amplification grade and the High-Capacity cDNA Reverse Transcription kit (cat. no. 4387406; Applied Biosystems; Thermo Fisher Scientific, Inc.) in a GeneAmp PCR System 9700 thermal cycler. The reaction was carried out by following the given conditions: 25˚C for 25 min, 37˚C for 120 min, 85˚C for 5 min, and infinite hold at 4˚C.

qPCR was performed using 1 μl of cDNA per reaction, TaqMan® Gene Expression Master Mix (cat. no. 4369016; Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan®MGB Probes Gene Expression Assays (cat. no. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.) with FAM-NFQ detector for *NFKB1* (ID no: Hs00765730_m1), *RELA* (ID no: Hs00153294_m1) and *TNF* (ID no: Hs00174128_m1) primers. The reaction was performed in a 7900HT Fast Real-Time PCR System linked to SDS 2.4 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cycling conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, 95˚C for 15 sec, and 60˚C for 1 min (40 cycles). Relative quantification (RQ) analysis was performed using the Livak method ($2^{-\Delta\Delta Cq}$) with amplification efficiencies >95% (22). The *GUSB* gene (Hs99999908_m1) was used as a housekeeping reference and normal adjacent mucosa as a calibrator. The experimental samples were evaluated in triplicate.

Statistical analysis. The data were analyzed using the SPSS 20.0 software (IBM Corp.). The NF-κB/p65 activation was evaluated using Kruskal-Wallis test and Dunn's test for a post hoc analysis, and then differences in active cell percentage pairwise comparison among groups were assessed by the Mann-Whitney U test. The Wilcoxon matched-pairs test was used to examine differences in gene expression and anti-human TNF-α levels between tumor tissues and adjacent normal mucosa. TNF-α serum levels data were first evaluated for normality and homogeneity of variances, using the Shapiro-Wilk and Levene's tests, respectively. Therefore, a one-way analysis of variance (ANOVA) was conducted to compare the group means with Tukey's post hoc test. The correlation between molecular parameters and TNM groups was analyzed using the Pearson correlation coefficient test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Participants. A total of 122 patients with CRC were included, of which 83 were males and 39 were females, all unrelated to hereditary colorectal syndromes. The tissues were collected during surgery and in total, 92 tissues corresponded to colon and 30 to rectal cancer. The mean age was 56 years, ranging from 34 to 62 years; none of the participants had been previously treated with radiotherapy or chemotherapy. The TNM classification to establish tumor staging resulted in I=18, II=34, III=48 and IV=22 patients per stage. Participants were classified into early stages (E) and advanced stages (A) to analyze the results. The E group included 52 participants from stages I and II, while the A group included 70 participants from stages III and IV. This categorization was implemented to enable an in-depth analysis of the collected data and identify significant differences between the groups. A total of 30 individuals, of whom 8 were male and 22 were female, who were established with non-cancerous condition after colonoscopy and histopathology examination, were included as control calibrators for tissue experiments comparison. The mean age was 39 years, ranging from 26 to 48 years; none of the participants in the control group had an inflammatory bowel disease history (Table I). Additionally, blood samples were collected from 100 healthy individuals to establish a control group for evaluating serum TNF-α levels (Table II). This approach aimed to establish an appropriate cut-off threshold for the experimental groups, thus ensuring the validation of the assay and enabling the attainment of statistically significant results across all groups.

NF-κB/p65 activation in CRC development. Phospho-flow cytometric analysis of NF-κB revealed that both p65 (pS536) and (pS529) were active in patients with CRC. The activation levels were compared with those found in adjacent normal mucosa. Differences between early and advanced TNM groups were analyzed using non-neoplastic tissues as a control group; results are described below and reported in Table III. NF-κB/p65 (pS536) activation evaluated by TNM stages was reported as follows: I=45.1%, II=51.3%, III=50.8% and IV=53.4% (Fig. 1A). Compared with the control group, the activation was significantly higher in all stages ($P<0.05$). However, there was no significant increase between the early stages (48.2%) and advanced stages (52.1%; Fig. 1B). Furthermore, the analysis of NF-κB/p65 (pS529) activation by TNM stages revealed: I=27.3%, II=41.9%, III=60.8% and IV=75.3% (Fig. 1A). There was a significant increment between the early (34.6%) and advanced stages (68.05%; Fig. 1C). Therefore, NF-κB/p65 (pS529) activation exhibited a positive association with CRC progression ($P<0.05$).

Table I. Parameters of sporadic CRC and non-cancerous tissue control group.

A, Clinical parameters

Parameter	CRC group (n=122)	Non-cancerous tissue control group (n=30)
Age, years		
Mean ± SD)	56±7.31	39±6.57
≤40, N (%)	9 (7.4)	19 (63.3)
41-60, N (%)	54 (44.3)	11 (36.7)
>60, N (%)	59 (48.4)	0 (0.0)
Sex, N (%)		
Male	83 (68.0)	8 (26.7)
Female	39 (32.0)	22 (73.3)

B, Pathological parameters

Parameter	CRC group (n=122)	Control group (n=30)
TNM stage, N (%)		
I	18 (14.8)	N/A
II	34 (27.9)	N/A
III	48 (39.3)	N/A
IV	22 (18.0)	N/A
Tumor location, N (%)		
Colon	92 (75.4)	N/A
Rectum	30 (24.6)	N/A
Histopathology result, N (%)		
CRC	122 (100.0)	0 (0.0)
Non-cancerous condition	0 (0.0)	30 (100.0)
Disease history, N (%)		
IBD	97 (79.5)	0 (0.0)
No IBD	25 (20.5)	30 (100.0)

TNM, Tumor-Node-Metastasis; CRC, colorectal cancer; IBD, inflammatory bowel disease.

Table II. Parameters of healthy control group.

Clinical parameters

Parameter	Healthy control group (n=100)
Age, years	
Mean ± SD	46±6.39
≤40, N (%)	37 (37)
41-60, N (%)	63 (63)
>60, N (%)	0 (0)
Sex, N (%)	
Male	59 (68.0)
Female	41 (32.0)

Table III. Evaluation of NF-κB/p65 activation in Tumor-Node-Metastasis stages.

Stage	NF-κB/p65 (pS536) activation, %	NF-κB/p65 (pS529) activation, %
Early (I + II)	48.2	34.6
I	45.1	27.3
II	51.3	41.9
Advanced (III + IV)	52.1	68.05
III	50.8	60.8
IV	53.4	75.3

NF-κB, nuclear factor-κB; pS536/529, phosphorylated serine 536/529.

TNF-α cell surface protein is upregulated in CRC. It was found that both early and advanced CRC-stage groups exhibited significant upregulation of TNF-α in CRC cells compared with non-tumoral adjacent mucosa cells, as assessed through cell surface antigen evaluation. The comparison between tumor groups revealed a significant increase in TNF-α levels (E=18.8% and A=54.8%; P<0.05) as the disease progresses, with a difference of 36.0% (Fig. 2). The correlation between TNF-α cell surface levels and NF-κB activation of TNM groups showed a weak positive correlation with p65 (pS536; ρ=0.216; P<0.05), while the analysis with p65 (pS529) exhibited a strong positive correlation (ρ=0.801; P<0.05) as revealed in Table IV. The results closely matched the comparison between TNF-α serum levels and the phosphorylated NF-κB/p65 residues, suggesting that the behavior between TNF-α serum and cell surface antigen levels may be due to an effect associated with that producing NF-κB activation.

TNF-α serum levels provide early CRC diagnosis. The cut-off value was calculated using the formula: Cut-off=Mean concentration of controls + 3 times the standard deviation to validate the ELISA test. Additionally, the 95th percentile of the mean was considered, resulting in Cut-off=1.23+3x0.43)=2.52 pg/ml. This approach is a widely accepted and effective method for determining the presence of analytes in a given sample. By employing this method, the reliability and accuracy of the ELISA test is enhanced, enabling us to identify valid inferences from the results.

After measuring serum TNF-α levels, the mean values obtained for each group were as follows: Control group (C)=1.23±0.43 pg/ml; Early-stage group (E)=15.48±4.2 pg/ml; and Advanced-stage group (A)=47.42±7.1 pg/ml. The data met the assumptions of normality and homogeneity of variances, assessed using the Shapiro-Wilk and Levene's tests, respectively. Therefore, a one-way analysis of variance (ANOVA) was conducted to compare the group means. The ANOVA revealed statistically significant differences in TNF-α levels among the (P<0.0001). Tukey's post hoc

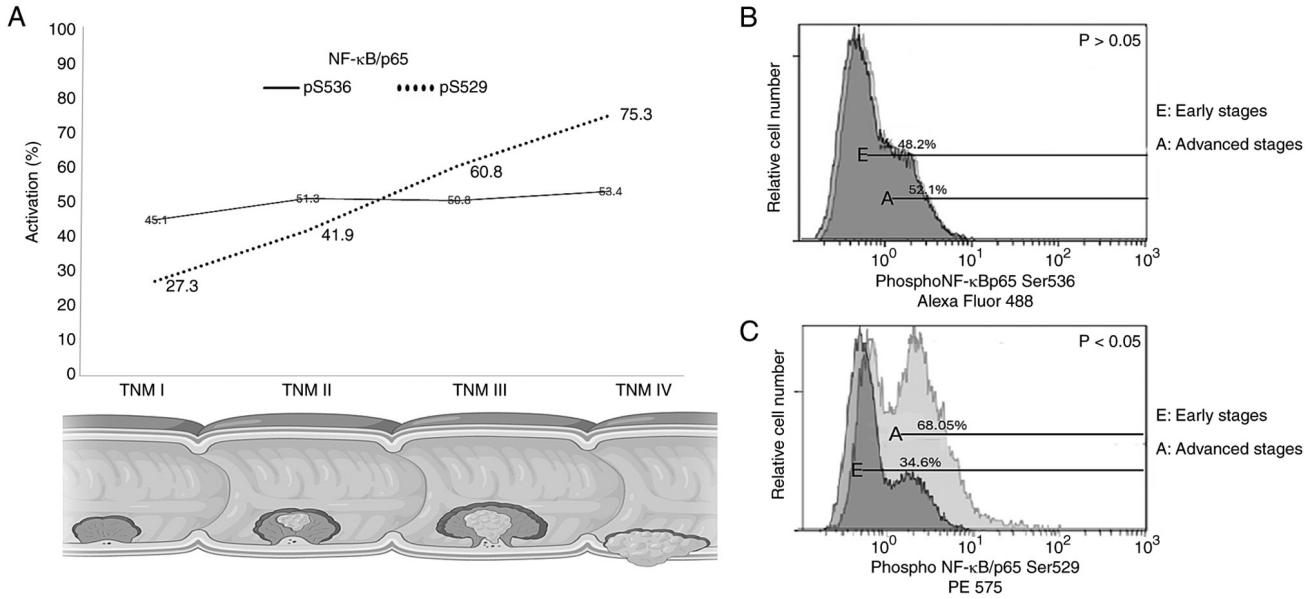

Figure 1. NF-κB/p65 activation in sporadic colorectal cancer. (A) NF-κB/p65 (pS536) and (pS529) activation evaluated by TNM classification. (B) Phospho-flow cytometric histograms of the mean NF-κB/p65 (pS536) activation reported no significant differences in early vs. advanced stages using Mann-Whitney U test (P>0.05). (C) Phospho-flow cytometric histograms of the mean NF-κB/p65 (pS529) activation reported significant differences in early vs. advanced stages using Mann-Whitney U test (P<0.05). NF-κB, nuclear factor-κB; pS536/529, phosphorylated serine 536/529; TNM, tumor-node-metastasis.

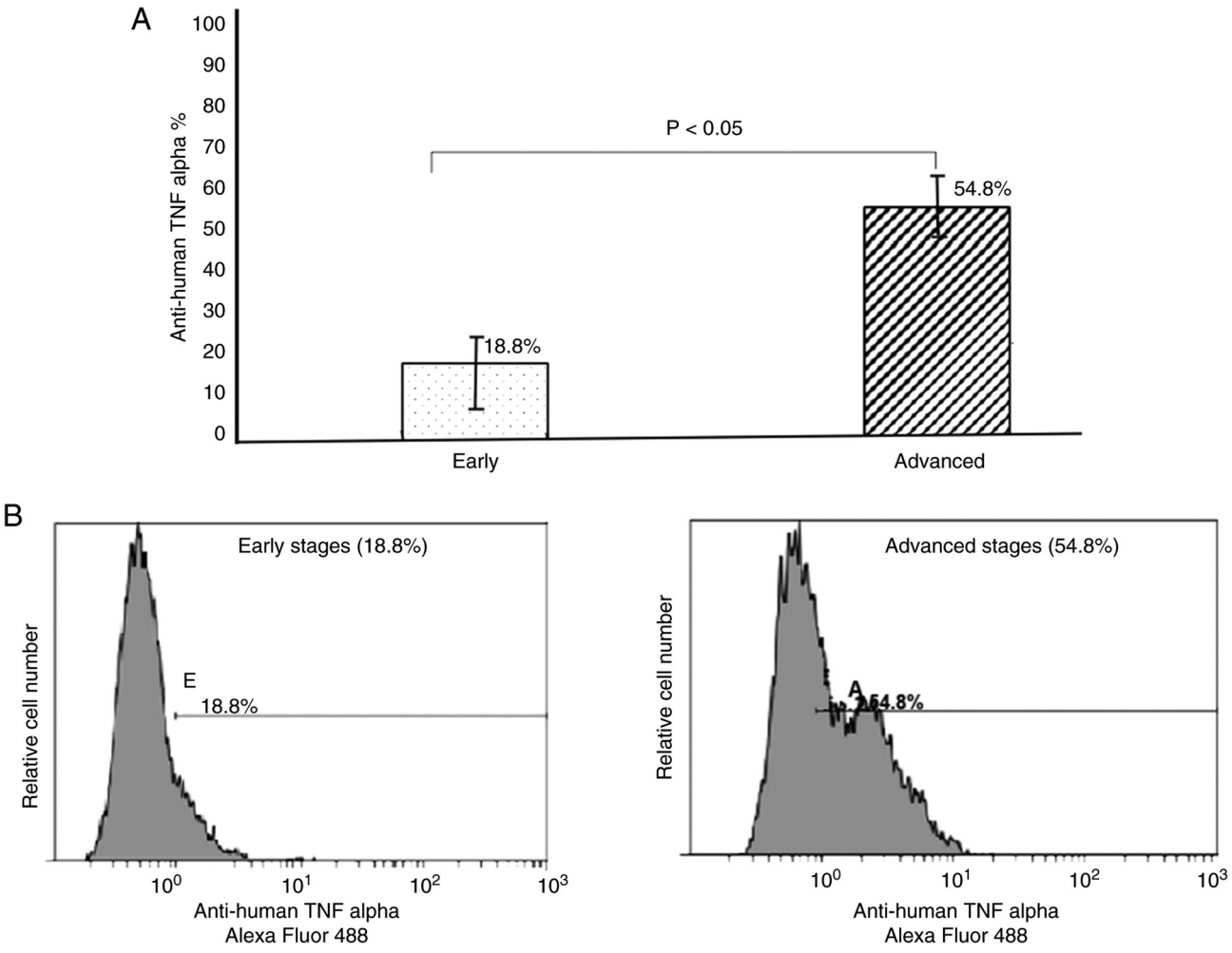

Figure 2. TNF-α cell surface levels in sporadic colorectal cancer. TNF-α cell surface activity in CRC group vs. normal adjacent mucosa as a calibrator was evaluated using Wilcoxon matched-pairs test (P<0.05). (A) TNF-α cell surface mean activity (%) comparative graph evaluating TNM groups and (B) Flow cytometric histograms of the mean TNF-α cell surface protein reported significant differences in early compared to advanced stages using unpaired Student' t-test (P<0.05). TNF-α, tumor necrosis factor α; TNM, tumor-node-metastasis.

Table IV. NF-κB/p65 correlation analysis with the evaluated parameters.

	Correlation with NF-κB/p65 (pS536) activation		Correlation with NF-κB/p65 (pS529) activation	
Parameter	ρ	Correlation	ρ	Correlation
TNF-α cell surface	0.216	Weak positive	0.801	Strong positive
TNF-α serum	0.469	Moderate positive	0.862	Strong positive
TNFA gene expression	0.616	Moderate positive	0.921	Strong positive
RELA gene expression	0.731	Moderate positive	0.891	Strong positive
NFKB1 gene expression	0.104	Weak positive	0.294	Weak positive

NF-κB, nuclear factor-κB; pS536/529, phosphorylated serine 536/529; TNM, Tumor-Node-Metastasis; TNF-α/*TNFA*, tumor necrosis factor-α; *RELA*, RELA p65 proto-oncogene; *NFKB1*, nuclear factor-κB subunit 1.

analysis showed that the E group exhibited significantly higher TNF-α levels compared with that in the C group (P<0.001), whereas the A group showed significantly higher levels compared with those in both the E and C groups (P<0.001 in both cases; Fig. 3).

The correlation between the serum level of TNF-α and NF-κB activation was assessed based on TNM groups; this analysis indicated a moderate positive correlation (ρ=0.469; P<0.05) with p65 (pS536), while a strong positive correlation (ρ=0.862; P<0.05) was observed with p65 (pS529; Table IV).

TNF gene expression interacts with the NF-κB heterodimer genes. Additionally, the impact on gene expression and the probable interaction of the TNF-α/NF-κB signaling pathway was determined. To identify the most stable housekeeping gene suitable for use as an internal control for the qPCR assays, three candidate genes were compared, namely *GUSB*, *ACTB* and *ABL*, in both experimental and calibrator samples. *GUSB* showed a mean Cq of 23.375±0.61 in experimental samples compared with 23.638±0.47 in calibrator samples (P=0.429). *ACTB* showed a median Cq of 29.785±1.02 in experimental samples compared with 28.914±1.24 in calibrator samples (P=0.686). *ABL* exhibited a median Cq of 28.726±1.57 in experimental samples and 28.278±1.34 in calibrator samples (P=0.739). These findings indicate that there were no significant differences in any case. However, *GUSB* exhibited minimal standard deviation among the three genes, making it the most suitable for the present experiment (data not shown). Therefore, it was selected as the internal control for qPCR assays.

The Livak method ($2^{-\Delta\Delta Cq}$) was utilized to quantify the relative expression of *TNFA*, *RELA* and *NFKB1* genes. All genes were upregulated in tumor tissue compared with that in the adjacent normal mucosa. A significant increase in *TNFA* and *RELA* expression in advanced stages compared with the early stages was found, with a fold change of A=37.8, E=13.2 and A=26.4 and E=11.9, respectively (P<0.05). However, no significant differences were observed in the expression of *NFKB1*, with a fold change of A=4.3 and E=3.8 (Fig. 4). These findings suggest that *TNFA* and *RELA* might play a role in the progression of the disease, whereas *NFKB1* might not be associated. The correlation test between gene expression profiles and NF-κB activation indicated that *TNFA* has a moderate

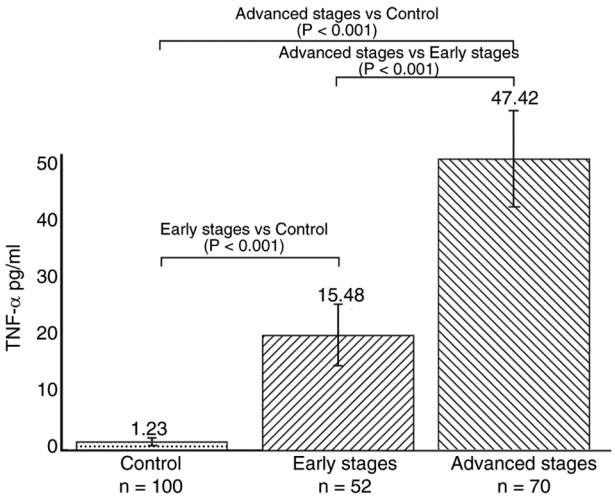

Figure 3. TNF-α serum levels in sporadic colorectal cancer. The advanced stages group level is statistically higher compared with that in the early stages group and the control group. The early stages group differed significantly from that in the control group. Statistically significant differences among groups were indicated based on Tukey's test.

positive correlation with p65 (pS536; ρ=0.616; P<0.05) and a strong positive correlation with p65 (pS529; ρ=0.921; P<0.05). Similarly, *RELA* presented a moderate positive correlation with p65 (pS536; ρ=0.731; P<0.05) and a strong positive correlation with p65 (pS529; ρ=0.891; P<0.05). Conversely, *NFKB1* revealed a weak positive correlation with both p65 (pS536) (ρ=0.104; P<0.05) and p65 (pS529; ρ=0.294; P<0.05). These findings are summarized in Table IV.

Discussion

CRC is characterized by dysregulated inflammation stimulus. An essential pro-inflammatory molecule involved is NF-κB, which promotes gene transcription in tumorigenesis (23). The NF-κB/p65 subunit phosphorylation is a critical post-translational activation mechanism triggered by TNF-α stimulation in cancer development (23-25). Recent studies demonstrated that dysregulated NF-κB activity contributes to tumor progression and confers drug resistance by inhibiting

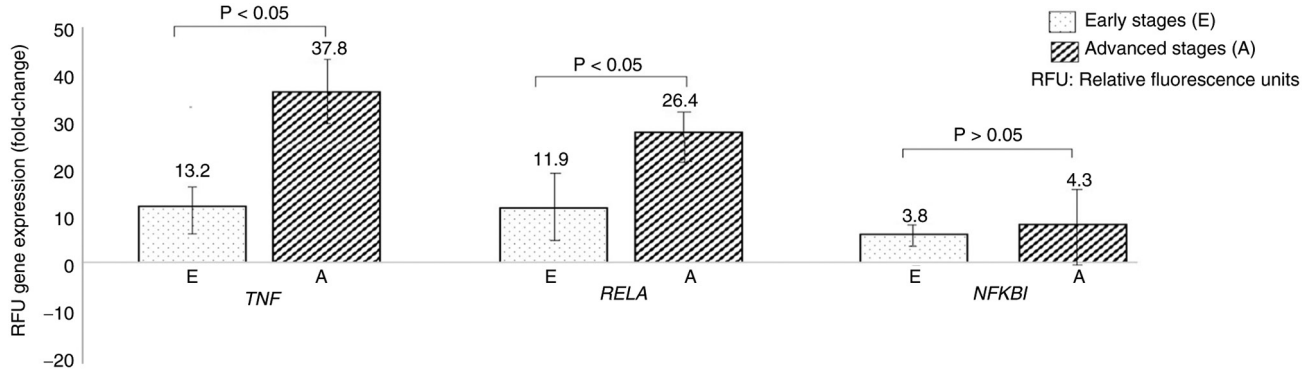

Figure 4. Relative gene expression levels in sporadic colorectal cancer. The Livak method was used to calculate the relative gene expression of *TNFA*, *RELA* and *NFKB1* in the CRC group compared with normal adjacent mucosa as a calibrator. *TNFA* and *RELA* exhibited significantly upregulated expression in advanced CRC stages compared with early stages using unpaired Student's t-test (P<0.05). No significant differences were observed in *NFKB1* between groups using unpaired Student's t-test (P>0.05). *TNFA*, tumor necrosis factor-α; *RELA*, RELA p65 proto-oncogene; *NFKB1*, nuclear factor-κB subunit 1.

apoptosis (26). The present study was designed to evaluate NF-κB activity in patients with sporadic CRC by measuring the phosphorylation of its subunit p65 residues (Ser529) and (Ser536) together with TNF-α cell surface protein, serum and gene expression levels. The goal was to determine whether there is a correlation between the NF-κB activation status and TNF-α levels in normal adjacent mucosa compared with tumor tissue. Patients were grouped according to TNM classification in early stages (I + II=E) and advanced stages (III + IV=A) to statistically analyze the disease progression. The validation of each experiment performed in this project was a fundamental part of reporting the obtained results.

NF-κB/p65 has been frequently reported to be upregulated in CRC. However, most studies have not compared normal adjacent mucosa with tumor tissue from the same patient or analyzed the results by comparison with the TNM classification (27-29). Berkovich *et al* (30) utilized immunohistochemistry to compare the expression of NF-κB in colonic adenocarcinoma specimens, colonic adenomas and inflammatory colonic tissues. The study observed a similar expression level between polypoid and inflammatory cases, but it was significantly higher in CRC than in both. The findings support the role of NF-κB activity early in the adenoma-to-carcinoma sequence, consistent with the results of the present study. Although the researchers did not evaluate the phosphorylated residues associated with the analysis, valuable findings were provided (30). Lewander *et al* (25,31) conducted two studies to assess the activation of NF-κB p65 (pS536) in Swedish patients with CRC using immunohistochemistry. NF-κB p65 (pS536) activation was higher in patients with CRC than in normal mucosa. Both studies concluded that NF-κB p65 (pS536) is an independent prognostic factor in patients with CRC, but it is not directly associated with the response of radiotherapy based on recurrence and survival (25). In accordance with these studies, the authors of the present study had previously published a study (15), in which upregulation of NF-κB/p65(pS536) in the tumor tissue was observed compared with the normal adjacent mucosa using immunohistochemistry in sporadic CRC. However, p65 (pS529) could not be statistically analyzed due to the limited quantity and quality of samples available during evaluation (15). According to previous studies, the nuclear immunopositivity of NF-κB/p65 in CRC cells has been widely reported and linked to their high transcriptional activity (25,31-33). However, conflicting findings have suggested that NF-κB is consistently present in the cytoplasm (15,31), which is contradictory to the predicted outcomes. The immunohistochemistry technique commonly used for diagnosis implies that the number of cells analyzed is a limiting factor in research experiments. Due to unexpected and controversial findings of some recent studies (15,16,25,31-33), an experimental design with larger sample groups to compare and use of phospho-flow cytometry to analyze a more significant and specific number of cells (20,000 events), are proposed.

Phospho-flow cytometry is a reliable method that consistently and reproducibly measures levels of phosphorylated proteins in single cells. This technique has recently been used for screening intracellular signaling events in different diseases (34-37). In 2023, Toney *et al* (34) applied the method in a CRC model, measuring the phosphorylation of STAT1, STAT3 and STAT5, induced by IL-6, IL-10 and IL-2, respectively. The experimental design of the present study involved significant challenges, as the authors were not able to find previous studies that used the technique to analyze NF-κB phosphorylated residues in CRC tissue. The fluorescence-labeled antibodies used in the present study recognized NF-κB only when phosphorylated on the specific amino acid residues (pS536) and (pS529), which strongly indicated the activation of the transcription factor. Previously, the authors of the present study have reported such evaluation in cell cultures of different pediatric leukemias with replicable results (38,39). Based on these precedents, a protocol for efficiently separating CRC cells was standardized for analysis by phospho-flow cytometry, obtaining the results reported in the present study. Furthermore, the present study revealed that both NF-κB/p65 (pS536) and (pS529) are significantly active in patients with sporadic CRC. The TNM analysis conducted on the results indicated that p65 (pS536) is strongly correlated to the onset of CRC, while p65 (pS529) is associated with CRC progression; these findings underscore the importance of how phospho-flow cytometry can integrate clinical and histopathological analysis with phospho-protein detection, which provides an advantage over conventional techniques in monitoring the activity of these biomarkers for early diagnosis and as indicators of progression. To the best of the authors' knowledge, this is

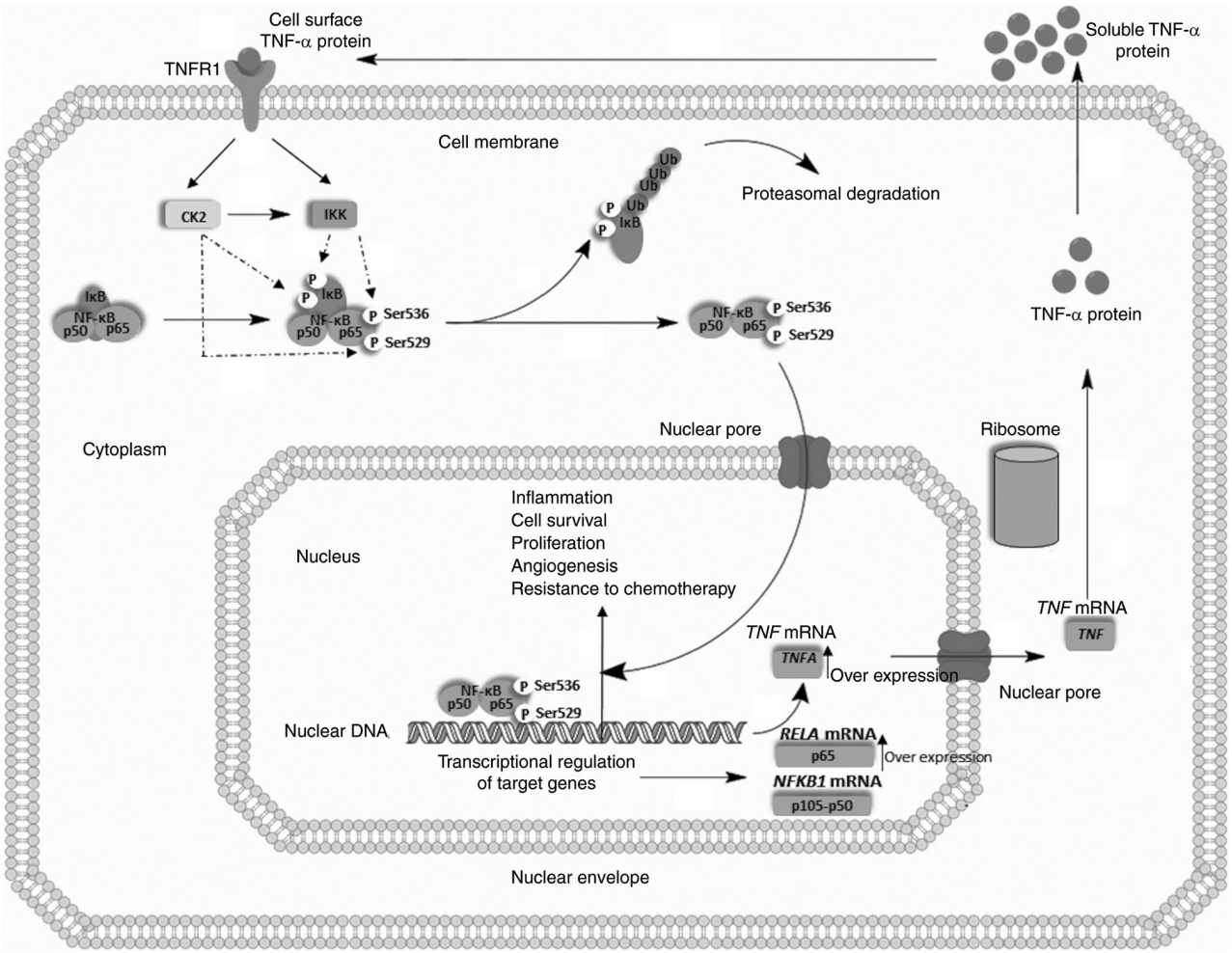

Figure 5. Molecular model of NF-κB activation in sporadic CRC.TNF-α/NF-κB signaling is regulated by IKK and CK2 activity, which in turn phosphorylates NF-κB/p65 (S536) and (S529) residues, respectively. The model indicates the feedback between NF-κB/p65 activation and TNF-α upregulation. CRC, colorectal cancer; *TNFA*/TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; pS536/529, phosphorylated serine 536/529; *RELA*, RELA p65 proto-oncogene; *NFKB1*, nuclear factor-κB subunit 1.

the first study that standardizes and proposes a protocol for evaluating NF-κB/p65 (pS536) and (pS529) in CRC tissue and adjacent normal mucosa cells.

A summary of the findings of the present study is described below along with a proposed model presented in Fig. 5. The role of TNF-α in the inflammatory stimulus leading to solid tumor development is well established (40-42). The present study provided substantial evidence of a gradual increase in cell surface TNF-α expression as CRC progresses. The results of the TNF-α cell surface analysis revealed a weak positive correlation with p65 (pS536), while a strong positive correlation was observed with p65 (pS529). These findings confirm an association between TNF-α and p65 (pS536), which is produced by IKK activity and is involved in the early stages of CRC. On the other hand, the signaling of TNF-α and p65 (pS529) mediated by the dysregulated activity of CK2 after TNF-α stimulus is critical to the progression of CRC; concurrently, CK2 potentiates IKK activity. The phosphorylation of IκB promotes its degradation, and consequently, NF-κB is released from the IκB inhibitory complex. NF-κB is translocated into the nucleus, resulting in the regulation of biological processes associated with tumorigenesis. NF-κB transcriptionally regulates the genes that codify to p65 (*RELA*) and p50 subunit (*NFKB1*). The *TNFA* gene is also regulated by NF-κB, and it is found to be overexpressedin the sporadic CRC model. TNF mRNA is exported to the cytoplasm and translated into the functional TNF-α protein in the ribosome. The tumoral cell identifies the increase in the TNF-α protein level and exports it out of the cell. The soluble TNF-α level is now increased, causing the proposed exponentially dysregulated TNF-α/NF-κB signaling in sporadic CRC. Cell surface proteins play an essential role in biomedical research due to their ability to serve as cellular markers and their extracellular accessibility for pharmacological intervention (Fig. 5). Despite their importance, information regarding individual cell surface protein repertoires, known as 'the surfaceome' remains limited (43). By evaluating the level of TNF-α on cell surfaces, the present study has revealed crucial insight into the molecular signaling of sporadic CRC. Therefore, the authors strongly recommend to include this assessment in any comprehensive analysis of sporadic CRC to better understand its molecular mechanisms.

Previous studies indicated that TNF-α gene expression levels are significantly higher in patients with CRC and

associated with advanced stages (44,45). Additionally, an increase of soluble TNF-α was observed in the serum of these patients. It has been reported that patients with low TNF-α serum levels have a significantly higher survival rate than those with high levels (44,46). The present study found that individuals with sporadic CRC had considerably higher levels of TNF-α in serum than healthy controls. Furthermore, within the tumoral group, there was a significant difference in TNF-α levels between patients in advanced stages and those in early stages. The study examined the association between TNF-α serum levels and NF-κB activation of TNM groups. The analysis showed a moderate positive correlation between TNF-α serum levels and p65 (pS536) activation and a strong positive correlation with p65 (pS529) activation. These findings suggest that TNF-α serum levels are associated with NF-κB activation, which is known to play a key role in tumor development and progression (Fig. 5). Overall, the results indicated that TNF-α serum levels could potentially serve as a predictive biomarker for CRC development, and the correlation with NF-κB activation further supports the involvement of TNF-α in CRC pathogenesis.

Similarly, increased levels of the *TNF-α* gene in CRC tissue and significant upregulation in advanced stages compared with early stages were observed. The correlation test between gene expression profiles and NF-κB activation indicated that *TNF-α* was strongly correlated with CRC progression. Upregulation of the *RELA* and *NFKB1* genes indicated a molecular feedback mechanism during the inflammatory response. These findings reinforced that the NF-κB pathway dysregulation impacts the transcriptional, translational and replicational levels (Fig. 5).

Despite its established relationship with various pathologies, there is a lack of clinically useful research on the TNF-α/NF-κB level profile. The results of the present study highlighted the importance of TNF-α/NF-κB interaction in CRC; therefore, its evaluation has potential as a biomarker in predicting CRC prognosis and development. Such predictive ability can allow for proactive measures to be taken in detecting and treating the disease earlier, which can positively impact patient outcomes. Identifying biomarkers in this context can aid in the early detection and timely intervention of CRC, a disease that globally poses significant health threats. With these considerations in mind, it is crucial to continue pursuing research into the potential of TNF-α assessment as a predictive biomarker for CRC. The implications of these findings are significant, as they could lead to the development of cost-effective and non-invasive screening methods for CRC in Mexico and in other developing nations.

In conclusion, the present study provides important insights into the relationship between TNF-α/NF-κB signaling and CRC development. It is demonstrated that assessing TNF-α/NF-κB activity is of utmost importance for patients diagnosed with sporadic CRC. The findings revealed that TNF-α and NF-κB/p65 have a positive correlation in the development of CRC. Moreover, the analysis of TNM groups suggests that the correlation between TNF-α and p65 (pS536) plays a crucial role in the early stages, contributing to the initiation and establishment of CRC. On the other hand, TNF-α and p65 (pS529) signaling are relevant to the advanced stages as well, which implies that they may play a role in the growth and spread of CRC. Overall, the present study highlighted the significance of TNF-α/NF-κB activity as a potential biomarker for monitoring and predicting CRC progression. It is essential to consider the potential limitations of this study. The methodological limitations are related to the relative quantification design of the data. It is important to note that while the control group patients did not have a diagnosis of adenocarcinoma of the colon or rectum, they might still have irregular levels of inflammatory markers. Despite these limitations, the invasiveness of the procedure for collecting the colorectal sample makes these individuals the ideal participants to be controls for the study. Additionally, the measurement of IKK and CK2 kinases, which are proposed as critical regulators of NF-kB activation, should be considered, as well as other residues phosphorylated by these kinases.

It was proposed that monitoring TNF-α/NF-κB activity could be a valuable parameter in managing CRC, thus increasing the percentage of success and improving the quality of life of the patient. Specifically, determining TNF-α serum levels could potentially be used as a diagnostic factor for early stages instead of relying on other, more invasive tests. This approach is particularly interesting due to the growing incidence of CRC worldwide. Indeed, detecting CRC in its early stages is critical for successful treatment outcomes. In light of these findings, the potential benefits of monitoring TNF-α/NF-κB activity and their molecular interactions in managing CRC must be further analyzed.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

UFSB participated in the study design, collection of the tissues, analysis and interpretation of the experimental data, as well as performed immunohistochemical experiments and RNA isolation, and was a significant contributor to the writing of the manuscript. DFS participated in the study design and collection of the tissues, analyzed and interpreted the experimental data, performed gene expression analysis and was a significant contributor to the writing of the manuscript. LBM participated in the study design, analysis and interpretation of the experimental data and was involved in drafting the manuscript and revising it critically for important intellectual content. ABJ

participated in the collection of the tissues, analyzed and interpreted the patient data and validated the gene expression assays. JAVP coordinated the clinicopathological diagnosis of the patients, participated in the surgeries of the patients, classified the samples according to the criteria of the study and revised the manuscript. JRCR made a substantial contribution to the conception and design of the study and was involved in drafting the manuscript and revising it critically for important intellectual content. ACR coordinated the present study, contributed substantially to its conception and design, as well as the analysis and interpretation of the experimental and clinicopathological data. ACR was also involved in drafting the manuscript and revising it critically for important intellectual content, and providing the final approval of the version to be published. UFSB, LBM and ACR confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was performed following the Declaration of Helsinki and was approved by the 'Hospital Civil de Guadalajara Ethics Committee' (approval no. 21711; Guadalajara, Mexico). The hospital Ethics Committee is affiliated to the 'Ethics Committee from the State of Jalisco' with investigation no. 77/UG-JAL/2011. The present study was registered at the National Bioethics Commission with the no. *CONBIOÉTICA* 14-CEI-008-20161212. The patients provided written and verbal informed consent for scientific purposes to publish any associated data from the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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