Expression profile of NF-κB regulated genes in sporadic colorectal cancer patients

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Abstract. Colorectal cancer (CRC) is the fourth leading worldwide cause of cancer-associated mortalities. Nuclear factor-κB (NF-κB) is a transcriptional regulator of multiple genes associated with CRC. Tumor tissue were compared with normal adjacent mucosa from 30 sporadic patients with CRC were investigated. A total of 8 non-CRC patients were analyzed as a control group. In the present study, the protein expression of NF-κB/p65 was detected by immunohistochemistry, and the gene expression profiles of cyclin D1 *(CCND1),* prostaglandin-endoperoxide synthase 2, vascular endothelial growth factor A, matrix metallopeptidase 9, BCL2 apoptosis regulator *(BCL2),* BCL2 like 1, nitric oxide synthase 2, tumor necrosis factor and arachidonate lipoxygenase were detected by reverse transcription-quantitative polymerase chain reaction. NF-κB/p65 and genes expression profiles were classified according to tumor-node-metastasis (TNM) clinicopathological parameters, followed by statistical analysis. Higher protein expression of NF-κB/p65 in the cytoplasm of tumor tissues compared with adjacent normal mucosa was reported; this increment was positively associated with all clinicopathological parameters, except for tumor localization site. The selected genes demonstrated a diverse associative pattern when analyzed with clinicopathological parameters. *CCND1* was positively associated with all TNM parameters and *BCL2* was negatively associated with all TNM parameters, thus indicating their importance as strong molecular biomarkers for CRC. According to these results, not all selected genes regulated by NF-κB/p65 show increased expression during CRC development, whereas the transcription factor did. The present study suggests that NF-κB/p65 overexpression is necessary for CRC establishment and progression, but its transcriptional activity is not sufficient to regulate all target genes in CRC. NF-κB/p65 and the gene expression profiles reported in the present study may be therapeutically useful. Considering the heterogeneity of the disease, the particular evaluation of these molecules may allow for the selection of proper diagnosis, treatment and follow-up for patients with sporadic CRC.

Introduction

Colorectal cancer (CRC) is a worldwide health problem being the fourth cause of death due to cancer (1). CRC tumorigenesis involves molecular deregulation of genes related to proliferation, tumor growth, antiapoptosis, invasiveness, metastasis and angiogenesis (2). Nuclear factor-κB (NF-κB) is a transcriptional factor that plays an important role in biological processes, comprises a family of five proteins grouped in homo or heterodimers (3). NF-κB is normally inactive, sequestered by IκBα inhibitor, but commonly has been reported active in cancer, and plays a key role in tumorigenesis by transcriptional regulation of multiple genes (4). Several reports have found higher NF-κB/p65 protein expression in CRC tissue compared to normal tissue (5-7). However, to our knowledge, the evaluation of NF-κB/p65 and genes expression profiles, in tumor tissue compared to adjacent normal mucosa from the same CRC patient, and its association with clinicopathological parameters has not been fully reported (8).

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Materials and methods

Patients. Thirty patients with sporadic CRC histopathological diagnosis who underwent to colonoscopy or surgery at Hospital Civil de Guadalajara 'Dr. Juan I. Menchaca', Jalisco, Mexico, were enrolled in this study after informed consent request, only non-treated patients were included. Eight patients classified as non-CRC were evaluated as comparative control group. The study was performed according to the declaration of Helsinki and was approved by the local Ethics Committee.

Tissues. Both, tumor tissue and its adjacent normal mucosa were obtained from respective areas in colonic or rectal resection specimens from the same patient according to the 'Cancer Care Quality Measures: Diagnosis and Treatment of Colorectal Cancer' from the 'Agency for Health Care Research and Quality' (9). CRC tissue samples for RNA isolation, were collected in RNAlater® Stabilization Solution (AM7020; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and in 10% neutral buffered formaldehyde (11-0705; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for immunohistochemistry analysis. Non-CRC patient's tissue samples were collected during colonoscopy, before pathological analysis according to the same procedure. CRC tissues collected for RNA isolation were transported to laboratory and processed immediately. Tissues collected in 10% of neutral buffered formaldehyde were examined microscopically to confirm the diagnosis and perform subsequent immunohistochemistry analysis. Remaining tissues were stored at -80˚C in case of extra analysis.

Immunohistochemistry. Tissue samples were fixed in 10% neutral buffered formaldehyde and embedded in paraffin wax. Each tissue sample was sectioned at a thickness of 4-5 μm, placed on slides, and deparaffinized by heat for 60 min at 65˚C. Slides were placed in xylene and serial alcohol solutions (100, 96, 80, and 50%). All procedure was performed using EnVision™ FLEX kit (Dako; Agilent Technologies, Inc. Santa Clara, CA, USA) as follows: Slides were washed for 5 min in wash buffer, treated in epitope retrieval solution for 20 min at 90˚C, and rewashed. To block endogenous peroxidase, slides were incubated in peroxidase-blocking reagent for 15 min at room temperature, washed for 5 min in wash buffer, and incubated for 1 h at room temperature with a mouse monoclonal primary antibody raised against the N-terminus of human NF-κB/p65 (dilution 1:50) (sc-8008; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Then, the slides were washed and incubated for 30 min at room temperature with secondary antibody coupled with peroxidase and rewashed. Visualization was made with 3,3'-diaminobenzidine and counterstaining with hematoxylin. Finally, slides were dehydrated with serial alcohol solutions (50, 80, 96 and 100%) and xylene. Positive and negative controls were included for each staining procedure, using a section of CRC tissue known as strongly NF-κB/p65-positive.

NF-κB/p65 staining evaluation. Evaluation of slides was made by two pathologists blinded to patient's characteristics. The slides were scored according to the method recommended by Abdullah *et al* (10), as follows: Intensity of staining was classified 'in crosses' from 0 to 3, as 0 (-) negative, 1 (+) weak, 2 (++) moderate, and 3 (+++) strong. The extent of staining referred as the percentage of positive epithelial cells in relation to the whole tumor area, was classified from 0 to 4, as 0 (0%), 1 (≤25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). The final staining score was calculated by the addition of staining intensity and the extent of staining. The scores have values between 0 and 7, and scores greater than or equal to 3 were classified as positive. For negative control, primary antibody was replaced by distilled water, as an internal control from each slide, staining of macrophages was considered.

Gene expression. A group of relevant genes in CRC was selected considering that NF-κB participates in their transcriptional regulation and its particular role in tumoral processes as described below: Proliferation, *CCND1* (11) and *PTGS2* (12); tumor growth, *TNF* (13), *ALOX* (14), and *NOS2* (15); anti-apoptosis, *BCL2* and *BCL2L1* (16); invasiveness and metastasis, *MMP9* (17); angiogenesis, *VEGFA* (18).

Tissues were collected in RNA*later*® RNA Stabilization Solution, transported to laboratory and processed immediately as follows: Tissue (10-20 mg) were cut into small pieces and collected in 0.5 ml of TRIzol® Reagent (cat. no. 15596; Thermo Fisher Scientific, Inc.). Then, each sample was homogenized in Tissue Lyser LT (cat. no. 85600; Qiagen GmbH, Hilden, Germany) for 3 min/25 Hz. Next steps of RNA isolation were made according to manufacturer's instructions (Life Technologies; Thermo Fisher Scientific, Inc.). Isolated RNA samples were stored at -80˚C. Reverse transcription (RT-PCR) was performed using 1 μg of total RNA treated with DNase1 amplification grade (cat. no. 18068; Life Technologies; Thermo Fisher Scientific, Inc.) and the High Capacity cDNA Reverse Transcription kit (cat. no. 4368813; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. The RT-PCR conditions were: 25˚C/25, 37˚C/120, 85˚C/5 min, and infinite hold at 4˚C. RT-Quantitative-PCR (RT-qPCR) was performed using TaqMan® Gene Expression Master Mix (cat. no. 4369016; Thermo Fisher Scientific, Inc.), and TaqMan® Gene Expression Assays with FAM-MGB fluorophore-quencher system. For each gene mRNA detection, the next assays were used (*PTGS2*, Hs00153133_m1; *BCL2*, Hs00608023_m1; *BCL2L1*, Hs00236329_m1; *CCND1*, Hs00765553_m1; *MMP9*, Hs00234579_m1; *VEGFA*, Hs00900055_m1; *TNF*, Hs99999043_m1; *NOS2*, Hs01075529_m1; *ALOX5*, Hs01095330_m1; *GUSB*, Hs00939627_m1; cat. no. 4331182, Thermo Fisher Scientific, Inc.). RT-qPCR was performed in a 7900 HT Fast Real-Time PCR System linked to SDS 2.4 software (Thermo Fisher Scientific, Inc.), PCR conditions were: 50˚C/2, 95˚C/10 min, 95˚C/15 sec and 60˚C/1 min (40 cycles). Gene expression assays were validated using β-glucoronidase (*GUS*), β-Actin (*ACTB*), and Abelson (*ABL*) genes as constitutive control (housekeeping genes). Gene-expression profiles were calculated by relative quantification using the $2^{-\Delta\Delta Cq}$ method (19).

Statistical analysis. Data was analyzed using the SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). The NF-κB/p65 staining scores differences of the 3 groups were evaluated using Kruskal-Wallis test, then differences of staining scores and positive cells percentage pairwise comparison among groups were evaluated by Mann-Whitney U test. Genes expression

Table I. Clinicopathological parameters of sporadic CRC patients (n=30).

Parameters	Value (%)
Clinical[a]	
Origin of tissue	
Colonoscopy	5 (16.7)
Surgery	25 (83.3)
Age (mean, 60 years)	
≤40	3 (10.0)
41-60	13 (43.3)
>60	14 (46.7)
Sex	
Male	22 (73.3)
Female	8 (26.7)
Tobacco consumption	
Yes	7 (23.3)
No	11 (36.7)
Occasional	12 (40.0)
Alcohol consumption	
Yes	11 (36.7)
No	2 (6.7)
Occasional	17 (56.6)
Pathological[a]	
TNM stage	
I	5 (16.7)
II	4 (13.3)
III	12 (40.0)
IV	9 (30.0)
Histopathological differentiation	
Well	0 (0.0)
Moderate	27 (90.0)
Poorly	3 (10.0)
Tumor site	
AC	3 (10.0)
TC	4 (13.3)
DC	7 (23.3)
SC	9 (30.0)
R	7 (23.3)
Tumor depth	
T1	3 (10.0)
T2	6 (20.0)
T3	11 (36.7)
T4	10 (33.3)
Lymph node status	
N0	9 (30.0)
N1	8 (26.7)
N2	13 (43.3)
Metastasis degree	
M0	21 (70.0)
M1	9 (30.0)
Laboratorial[b]	

Table I. Continued.

Parameters	Value
Tumor markers levels	
CEA, ng/ml	26.52 (1.35-59.42)
CA-19.9, U/ml	149.4 (53.6-206.9)
AFP, U/ml	207.1 (27.6-276.8)

[a]Data are presented as the number of patients (%). [b]Data are presented as the mean (range). AC, ascending colon; TC, transverse colon; DC, descending colon; SC, sigmoid colon; R, rectum; CEA, carcinoembryonic antigen; CA-19.9, carbohydrate antigen; AFP, α-fetoprotein antigen; CRC, colorectal cancer.

differences in tumor tissues vs. adjacent normal mucosa were performed by Wilcoxon signed-ranked test. To evaluate the association of NF-κB/p65 expression and gene-expression profiles with clinicopathological parameters, Pearson or Spearman rank correlation coefficient was used. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological parameters in patients. Histopathological diagnosis of CRC was confirmed by examination of hematoxylin and eosin staining before analysis. Twenty-three CRC patient's tissues corresponded to colon cancer and 7 to rectal cancer. Twenty-two men and 8 women were collected; none of them were treated with radiotherapy or chemotherapy. The average age was 60 years. Tumor staging was established by certified pathologists according to tumor-node-metastasis (TNM) classification. Tobacco and alcohol consumption was also registered; most of the patients were occasional consumers. CEA, CA 19.9 and AFP tumor markers levels were higher than normal values. Eight patients that attend 'Colon and rectum service', and were diagnosed CRC negative before histopathology studies, were evaluated as control group. CRC patients were classified according to TNM clinicopathological parameters for association evaluation. General features of patients are presented in Table I.

NF-κB/p65 immunostaining scoring. In tumor tissue group, all samples expressed cytoplasmic NF-κB/p65, the intensities of staining were majority 'moderate' (15/30) and 'strong' (11/30), the group showed a mean NF-κB/p65 extent of staining of 52.3% with a standard deviation of 18.2%. NF-κB/p65 intensities of staining in normal adjacent mucosa were mostly 'weak' (15/30) and 'non-staining' (12/30), the group showed a mean NF-κB/p65 extent of staining of 27.6% with a standard deviation of 11.8%. In non-CRC tissues, NF-κB/p65 intensity of staining was majority 'non-staining' (5/8), a mean NF-κB/p65 extent of staining of 18.8% with a standard deviation of 7.5% was reported for this group (Fig. 1; Table II).

An average staining score of 4.4 in tumor tissue group, 1.6 in normal adjacent mucosa and 0.8 in non-CRC tissues is reported. The analysis among 3 groups showed that they were statistically different ($X^2(1)=39.146$, $P<0.001$). Pairwise comparison of staining scores among groups showed that

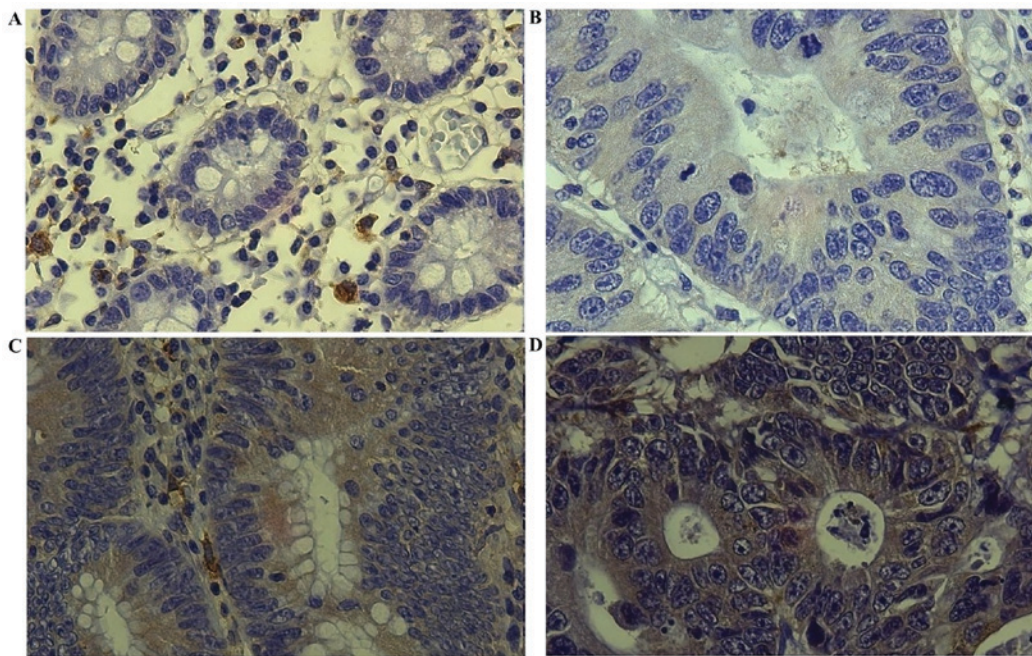

Figure 1. Representative staining patterns. Staining intensity of NF-κB/p65 is shown. (A) Normal mucosa with negative staining (-). (B) Tumor tissue with intensity of 1 (+). (C) Tumor tissue with intensity of 2 (++). (D) Tumor tissue with intensity of 3 (+++) (Magnification, x400). NF-κB, nuclear factor-κB.

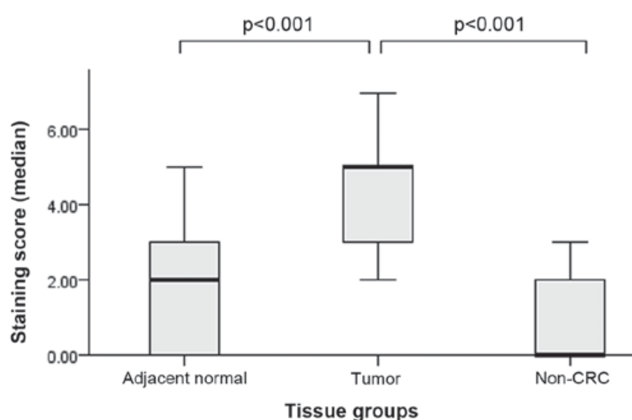

Figure 2. NF-κB/p65 staining scores analysis. Median staining score evaluation among normal adjacent mucosa, tumor tissue and non CRC tissue ($X^2(1)=39.146$, P<0.001). Pairwise comparison among groups is also shown (tumor tissue vs. normal adjacent mucosa, z=-5.707, P<0.001), (tumor tissue vs. non-CRC tissue (z=-4.126, P<0.001). NF-κB, nuclear factor-κB.

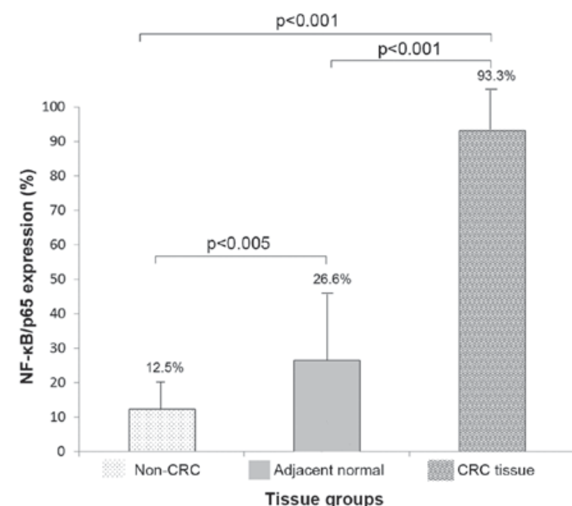

Figure 3. NF-κB/p65 positive expression. NF-κB/p65 (%) is significantly higher in tumor tissues vs. normal adjacent mucosa/non-CRC tissues (P<0.001).

tumor tissue was statistically higher than normal adjacent mucosa (z=-5.707, P<0.001), as well it was higher when compared to non-CRC tissue (z=-4.126, P<0.001). No difference was reported between normal adjacent mucosa and non-CRC tissue (Fig. 2).

Positive samples for NF-κB/p65 expression according to staining score (≥3), reported for each group were: 28/30 (93.3%) in tumor tissues, 8/30 (26.6%) in adjacent normal mucosa and 1/8 (12.5%) in non-CRC tissues. For statistical analysis, positive NF-κB/p65 samples were classified as '1' and negative samples as '0'. NF-κB/p65 positive samples were statistically higher than adjacent normal tissue and non-CRC tissue groups (P<0.001). Adjacent normal tissues also showed higher NF-κB/p65 positive samples when compared to non-CRC tissue group (P<0.05) (Fig. 3).

NF-κB/p65 expression and clinicopathological parameters. NF-κB/p65 expression was analyzed according to clinicopathological parameters; results are described below and reported in Table III.

NF-κB/p65 expression analyzed by tumor stages were reported as follows: I=27, II=36.6, III=63.5 and IV=70.1%. Stages II, III & IV are statistically higher than control groups (P<0.05), stage I was only statistically higher than non-CRC tissue group (P<0.05). As well, significantly increment of NF-κB/p65 expression in advanced stages compared to initial

Table II. NF-κB/p65 immunostaining in CRC and non-CRC patient's tissues.

Patients	Normal adjacent mucosa/Non-CRC			Tumor tissue		
	Intensity of staining	Extent of staining (%)	Staining score	Intensity of staining	Extent of staining (%)	Staining score
CRC						
1	0 (-)	0 (0)	0	1 (+)	2 (40)	3[a]
2	1 (+)	2 (35)	3[a]	1 (+)	2 (40)	3[a]
3	1 (+)	2 (35)	3[a]	1 (+)	2 (40)	3[a]
4	0 (-)	0 (0)	0	2 (++)	3 (60)	5[a]
5	1 (+)	1 (20)	2	2 (++)	2 (45)	4[a]
6	1 (+)	2 (40)	3[a]	3 (+++)	3 (75)	6[a]
7	0 (-)	0 (0)	0	1 (+)	3 (60)	4[a]
8	3 (+++)	2 (35)	5[a]	3 (+++)	2 (50)	5[a]
9	2 (++)	1 (20)	3[a]	3 (+++)	4 (90)	7[a]
10	1 (+)	1 (20)	2	2 (++)	3 (65)	5[a]
11	0 (-)	0 (0)	0	2 (++)	3 (60)	5[a]
12	1 (+)	1 (25)	2	2 (++)	2 (40)	4[a]
13	1 (+)	1 (20)	2	2 (++)	2 (40)	4[a]
14	0 (-)	0 (0)	0	1 (+)	2 (30)	3[a]
15	0 (-)	0 (0)	0	1 (+)	2 (35)	3[a]
16	0 (-)	0 (0)	0	3 (+++)	3 (75)	6[a]
17	0 (-)	0 (0)	0	2 (++)	3 (65)	5[a]
18	1 (+)	1 (25)	2	2 (++)	2 (45)	4[a]
19	0 (-)	0 (0)	0	1 (+)	2 (30)	3[a]
20	1 (+)	1 (20)	2	2 (++)	3 (60)	5[a]
21	0 (-)	0 (0)	0	2 (++)	2 (40)	4[a]
22	1 (+)	1 (20)	2	3 (+++)	2 (45)	5[a]
23	0 (-)	0 (0)	0	1 (+)	1 (20)	2
24	1 (+)	1 (10)	2	3 (+++)	2 (50)	5[a]
25	2 (++)	3 (60)	5[a]	2 (++)	4 (85)	6[a]
26	1 (+)	1 (20)	2	3 (+++)	3 (75)	6[a]
27	1 (+)	2 (40)	3[a]	2 (++)	3 (70)	5[a]
28	1 (+)	2 (0)	3[a]	3 (+++)	2 (50)	5[a]
29	1 (+)	1 (25)	2	2 (++)	3 (70)	5[a]
30	0 (-)	0 (0)	0	1 (+)	1 (20)	2
Non-CRC						
1	1 (+)	1 (15)	2	-	-	-
2	0 (-)	0 (0)	0	-	-	-
3	0 (-)	0 (0)	0	-	-	-
4	1 (+)	1 (15)	2	-	-	-
5	0 (-)	0 (0)	0	-	-	-
6	1 (+)	2 (30)	3[a]	-	-	-
7	0 (-)	0 (0)	0	-	-	-
8	0 (+)	0 (15)	0	-	-	-

[a]Samples positive to NF-κB/p65 (Staining score ≥3). Intensity of staining reported as score and num of crosses. 0 (-), no-staining; 1 (+), weak; 2 (++), moderate; and 3 (+++), strong. Extent of staining reported as score and percentage because of its variability. CRC, colorectal cancer; NF-κB, nuclear factor-κB.

stages was reported (III+IV=66.8±3.3% vs. I+II=31.75±4.8%; $P<0.05$), thus NF-κB/p65 expression is positively associated to CRC progression.

In histopathological differentiation groups, NF-κB/p65 expression was: 79.8% in poorly differentiated group and 46.8% in moderately differentiated group, both were statistically higher than control groups ($P<0.05$). No patients with well differentiated tumors were collected in this study. Analysis of poorly differentiated group vs. moderately differentiated group showed a statistical increment of 33% ($P<0.05$),

Table III. NF-κB/p65 expression (%) association with clinicopathological parameters in CRC patients (n=30).

Parameter	NF-κB/p65 expression (%)	P-value
CRC tissue		
Tumor stage		<0.05
I	27.00[a]	
II	36.62[b]	
III	63.51[b]	
IV	70.12[b]	
Histopathology differentiation		<0.05
Well	-	
Moderately	46.31[b]	
Poorly	79.81[b]	
Tumor localization		<0.05
Ascending colon	49.30[b]	
Transverse colon	45.63[b]	
Descending colon	40.74[b]	
Sigmoid colon	52.72[b]	
Rectum	31.33[a]	
Tumor depth		<0.05
T1	28.51[a]	
T2	34.33[a]	
T3	69.25[b]	
T4	74.84[b]	
Lymph node status		<0.05
N0	32.70[a]	
N1	49.12[b]	
N2	74.13[b]	
Metastasis degree		<0.001
M0	41.71[b]	
M1	73.57[b]	
Control group		
Non-CRC tissue	12.50	
Normal adjacent tissue	26.60[a]	

[a]Statistically different to non-CRC tissue group (P<0.05). [b]Statistically different to non CRC-tissue group and normal adjacent mucosa (P<0.05). NF-κB/p65 expression (%) in tumor tissue group is compared with control groups. CRC, colorectal cancer; NF-κB, nuclear factor-κB.

therefore NF-κB/p65 expression is positively associated to histopathological differentiation.

NF-κB/p65 expression analyzed by tumor localization sites is reported as follows: 49.3% in ascending colon (AC); 45.6% in transverse colon (TC); 40.7% in descending colon (DC); 52.7% in sigmoid colon (SC), and 31.3% in rectum (R). AC, TC, DC, and SC groups were statistically higher than control groups (P<0.001), while R group was only statistically higher than non-CRC tissue group. DC group vs. SC group NF-κB/p65 expression was statistically different (P<0.005). No other significative difference was reported between groups.

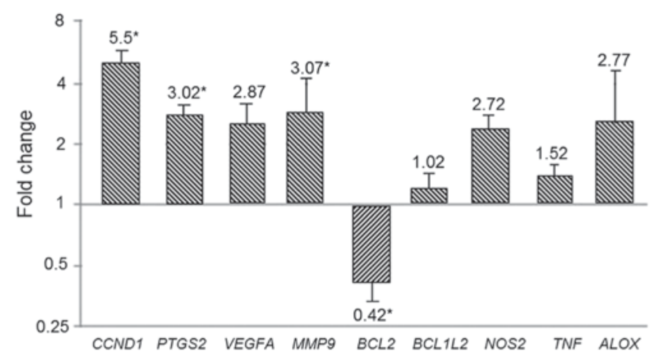

Figure 4. Gene expression profiles. Relative expression (folds) of selected genes comparing tumor tissue relative to normal adjacent mucosa. Statistical differences are shown (*P<0.001).

Certainly NF-κB/p65 showed higher expression in CRC tissues, but according to previous data, there is no association with tumor localization.

NF-κB/p65 expression reported for tumor depth groups were: 28.5% in T1; 34.3% in T2; 69.2% in T3, and 74.8% in T4. T3 and T4 groups were statistically higher than control groups (P<0.05); T1 and T2 groups did not showed significative differences when compared to normal tissue group but they were statistically higher than non CRC group. T3 and T4 groups were also statistically higher than T1 and T2 groups (P<0.05). NF-κB/p65 expression is positively associated to tumor depth.

NF-κB/p65 expression analysis in lymph node status showed the next data: 32.7% in N0; 49.1% in N1, and 74.1% in N2. N1 and N2 were statistically higher than control groups (P<0.05); N0 group did not showed significative differences when compared to normal tissue group, but it was statistically higher than non CRC group. N1 and N2 groups were statistically higher than N0 (P<0.05). As well, N2 was statistically higher than N1 (P<0.05). According to these results, NF-κB/p65 expression is positively associated to lymph node status in our patients.

NF-κB/p65 expression in metastasis groups was statistically higher than control groups: M0: 41.7 and M1: 73.5%, (P<0.001). Likewise, M1 was statistically higher than M0 (P<0.001). NF-κB/p65 expression positively increments according to metastasis degree.

Housekeeping genes evaluation. Ct median of selected endogenous genes in tumor tissue compared to adjacent normal mucosa were the following: *GUSB*, 30.375 vs. 29.638 (P=0.489); *ACTB*, 29.785 vs. 28.914 (P=0.686), and *ABL*, 28.726 vs. 28.278 (P=0.739). There were not significant differences in any case. *GUSB* constitutive gene, which exhibited the minimal standard deviation, was selected as internal control for the RT-qPCR assays.

Relative quantification of gene expression. Cq data was analyzed using the $2^{-\Delta\Delta Cq}$ method to obtain the relative quantification of genes. *CCND1*, *PTGS2,* and *MMP9* were overexpressed in tumor tissue compared to adjacent normal mucosa (5.5; 3.02; 3.07-folds, respectively (P<0.05). While *BCL2* decreased its expression (0.42-folds, P<0.05). *VEGFA, BCL2L1, NOS2, TNF,* and *ALOX* did not show significant differences (Fig. 4).

Table IV. Gene expression association with clinicopathological parameters in CRC patients (n=30).

Parameter	Relative quantification of gene expression (mean fold difference)								
	CCND1	PTGS2	VEGFA	MMP9	BCL2	BCL2L1	NOS2	TNF	ALOX
Tumor stage									
I	2.01±1.31	1.70±1.12	1.11±0.98	1.90±1.80	0.57±0.54	0.51±2.17	0.11±0.09	1.35±1.23	0.96±0.23
II	2.90±1.83	1.40±1.23	2.31±1.33	1.52±0.89	0.54±0.33	0.46±0.42	0.39±0.51	1.28±1.09	1.97±1.61
III	7.10±2.52	3.22±3.12	3.12±1.84	3.10±1.22	0.38±0.27	1.01±0.81	4.10±1.42	1.57±1.21	3.17±1.42
IV	10.00±2.21	5.81±5.03	5.01±1.61	5.83±4.21	0.21±0.19	1.21±1.13	6.29±1.93	1.91±1.73	4.99±1.71
r_s, (P-value)	0.97 (0.02)[a]	0.90 (0.94)	0.98 (0.01)[a]	0.88 (0.07)	-0.96 (0.03)[a]	0.92 (0.07)	0.95 (0.04)[a]	0.89 (0.10)	0.95 (0.01)[a]
Histological differentiation									
Well	-	-	-	-	-	-	-	-	-
Moderately	4.71±3.73	2.16±2.01	2.66±1.91	1.96±1.21	0.55±0.72	0.59±0.36	2.46±1.12	1.22±1.12	2.13±1.22
Poorly	6.32±1.21	3.92±1.10	3.13±0.96	4.21±1.53	0.29±0.11	0.99±0.51	3.3±2.71	1.82±0.96	3.41±1.73
r_s (P-value)	0.85 (0.05)[a]	0.90 (0.11)	0.78 (0.01)[a]	0.79 (0.03)	-0.91 (0.01)[a]	-0.83 (0.12)	-0.54 (0.23)	-0.69 (0.27)	-0.89 (0.34)
Tumor localization									
Ascending colon	6.31±2.34	2.71±1.71	5.54±2.81	1.79±1.11	0.45±0.21	0.80±0.31	2.39±1.23	2.47±1.31	1.9±0.35
Transverse colon	3.61±1.82	3.55±1.23	1.62±1.32	3.42±2.03	0.38±0.12	0.65±0.34	3.05±1.72	0.99±0.34	3.62±1.36
Descending colon	6.65±4.26	1.63±1.12	3.35±2.90	4.66±4.23	0.49±0.26	0.91±0.61	1.17±0.96	1.32±1.02	2.48±1.81
Sigmoid colon	6.98±3.81	5.32±4.20	1.83±1.12	4.48±3.11	0.49±0.24	0.96±0.67	4.41±1.23	1.22±0.67	1.05±0.43
Rectum	3.95±1.52	2.05±1.71	2.06±1.21	1.05±0.76	0.39±0.29	0.63±0.31	3.38±1.71	1.67±0.97	4.80±1.51
rs, (P-value)	-0.40 (0.50)	-0.10 (0.87)	-0.21 (0.47)	0.11 (0.37)	-0.10 (0.61)	-0.21 (0.87)	-0.28 (0.58)	-0.19 (0.59)	0.23 (0.71)
Tumor depth									
T1	1.98±1.42	1.56±1.11	1.45±1.21	2.21±1.81	0.54±0.22	0.86±0.64	1.21±0.78	1.29±0.37	1.21±0.56
T2	3.92±1.80	2.31±1.33	2.88±1.80	1.94±1.22	0.49±0.18	0.62±0.47	2.24±1.35	1.87±0.46	1.69±0.79
T3	6.38±2.01	2.99±1.94	3.34±1.23	3.62±1.73	0.34±0.28	0.73±0.38	3.62±1.92	1.51±0.28	3.87±1.23
T4	9.72±4.33	5.26±1.72	3.85±1.71	4.55±2.01	0.31±0.171	0.95±0.71	4.45±2.41	1.41±0.41	4.31±1.54
r_s, (P-value)	0.94 (0.01)[a]	0.78 (0.03)[a]	0.64 (0.07)	0.81 (0.06)	-0.89 (0.01)[a]	0.54 (0.08)	0.91 (0.01)[a]	0.24 (0.35)	0.82 (0.01)[a]
Lymph node status									
N0	1.91±1.22	1.22±0.95	1.23±0.67	1.49±1.35	0.74±2.73	0.83±0.28	1.29±1.32	1.09±0.97	1.96±1.13
N1	4.62±1.85	3.21±1.41	3.01±1.44	3.73±1.82	0.32±1.73	0.76±0.64	2.37±1.93	1.11±0.92	2.59±1.51
N2	9.97±4.21	4.66±2.74	4.43±2.91	4.02±2.11	0.20±2.12	0.78±0.10	4.98±2.61	2.36±1.21	3.76±1.44
r_s, (P-value)	0.84 (0.03)[a]	0.79 (0.04)[a]	0.90 (0.03)[a]	0.71 (0.08)	-0.92 (0.01)[a]	0.41 (0.26)	0.78 (0.02)[a]	0.62 (0.26)	0.61 (0.14)

Table IV. Continued.

Parameter	Relative quantification of gene expression (mean fold difference)								
	CCND1	PTGS2	VEGFA	MMP9	BCL2	BCL2L1	NOS2	TNF	ALOX
Metastasis degree									
M0	2.94±1.13	2.33±1.33	1.24±1.13	1.70±0.87	0.56±0.48	0.78±0.19	1.38±1.22	1.17±1.02	1.36±1.23
M1	8.06±3.71	3.73±1.72	4.52±1.71	4.46±2.82	0.28±0.15	0.80±0.45	4.38±2.93	1.87±1.13	4.18±3.11
r_s, (P-value)	0.91 (0.04)[a]	0.51 (0.12)	0.79 (0.03)[a]	0.31 (0.31)	-0.95 (0.03)[a]	0.11 (0.58)	0.81 (0.03)[a]	0.21 (0.48)	0.89 (0.06)

Fold change normalized with GUSB endogenous gene. [a]Statistically significant correlation (P<0.05). CRC, colorectal cancer; CCND1, cyclin D1; PTGS2, prostaglandin-endoperoxide synthase 2; VEGFA, vascular endothelial growth factor A; MMP9, matrix metallopeptidase 9; BCL2, BCL2, apoptosis regulator; BCL2L1, BCL2 like 1; NOS2, nitric oxide synthase 2; TNF, tumor necrosis factor; ALOX, arachidonate lipoxygenase; T, tumor; N, node; M, metastasis.

Gene-expression profiles and clinicopathological parameters. Gene's expression data was analyzed according to clinicopathological parameters; results are described below and reported in Table IV.

Positive association of *CCND1, VEGFA, NOS2,* and *ALOX* as well as a negative association of *BCL2* with tumor stage progression was reported (P<0.05). Significant overexpression in the advanced stages group compared to initial stages in *CCND1* (III+IV=8.55±1.45 vs. I+II=2.45±0.45; P<0.05), *VEGFA* (I+II=1.2±0.3 vs. III+IV=4.55±1.3; P<0.05), *NOS2* (I+II=0.25±0.14 vs. III+IV=5.2±2.1; P<0.05) and *ALOX* (I+II=1.46±0.5 vs. III+IV=4.08±0.19; P<0.05) corroborate the positive association. No significant difference was found in gene expression of *PTGS2, MMP9, BCL2L1,* and *TNF* during tumor progression.

Gene expression association with histopathological differentiation groups, was statistically positive in the case of *CCND1, MMP9* and *VEGFA*, while *BCL2* expression was negatively associated (P<0.05). No association of histopathological differentiation with *PTGS2, MMP9, BCL2L1, NOS2, ALOX* and *TNF* gene expression was reported.

No association between tumor localization site and expression of any evaluated gene was reported.

In the case of tumor depth, positive association was observed with *CCND1, PTGS2, NOS2* and *ALOX* expression, negative association with *BCL2* expression was also reported (P<0.05). *VEGFA, MMP9, BCL2L1,* and *TNF* expression did not showed any association.

Lymph node status and gene expression was positively associated in the case of *CCND1, PTGS2, VEGFA,* and *NOS2* (P<0.05). Negative association with *BCL2* was also reported (P<0.05). No association with *MMP9, BCL2L1, TNF,* and *ALOX* was found.

Metastasis degree and gene expression was positively associated in the case of *CCND1, VEGFA* and *NOS2*, and negative association was reported with *BCL2* (P<0.05). No association was observed in *PTGS2, MMP9, BCL2L1, TNF,* and *ALOX*.

Discussion

In the present study, NF-κB/p65 and genes expression association with clinicopathological parameters of CRC patients was investigated. We reported higher NF-κB/p65 cytoplasmic expression in tumor tissue compared to normal adjacent mucosa; our findings are consistent with previous studies (20). NF-κB/p65 expression in CRC showed discrepancy in stained protein localization, nevertheless nuclear staining has been mainly reported (20,21). NF-κB/p65 detected in previous reports, similar as our study, indicates released IκBα, but we were not able to confirm the transcriptional activity. We hypothesize that NF-κB/IκBα binding alterations could masked the nuclear localization signal in p65 as other studies suggest (22).

NF-κB/p65 expression by CRC stages has been commonly evaluated. Higher levels in advanced stages compared to initial stages, evaluated by immunohistochemistry are reported in this study. According to our results, NF-κB/p65 increment was positively associated with tumor stage progression. We suggest that NF-κB/p65 cytoplasmic expression may play a

key role in CRC progression probably by molecular changes in its downstream pathway. In addition, positive association between NF-κB/p65 expression with histopathology differentiation, tumor depth, lymph node status and metastasis degree was reported, but no association with tumor localization was observed in any case. NF-κB/p65 association with clinicopathological parameters has not been entirely described. A meta-analysis in solid tumors, reported a positive association with lymph node status and metastasis degree (23), but the conclusions are still in contradiction. Our results suggest that NF-κB/p65 is involved in CRC establishment and progression by promoting clinicopathological parameters development.

Gene expression analyzed in tumor tissue vs. normal adjacent mucosa, showed overexpression in *CCND1, PTGS2* and *MMP9*, decreased expression of *BCL2*, while no significant differences in expression of *BCL2L1, VEGFA, TNF, ALOX* and *NOS2* was reported. Overexpression of *CCND1* in tumor tissue was observed in this study as in others (24), considering that *CCND1* promotes the transition of G1- to S-phase of cell cycle it may play a key role in tumor cells proliferation in the evaluated tissues. *PTGS2* was similarly reported overexpressed in tumor tissue as others studies did using different methodologies (25-27), but no alteration of *PTGS2* expression has been also observed, these contradictory reports suggest that *PTGS2* activity in CRC is still unclear. According to our results, we hypothesize that proliferation and survival processes due to *PTGS2* overexpression plays an important role in CRC. *MMP9* overexpression observed in this study agreed with previous reports that relate its expression with invasiveness and metastasis (28-30). In this study we just report decreased expression of *BCL2* in CRC, higher expression in tumor tissue than in normal mucosa had been reported (31). *BCL2* promotes tumorigenesis by inhibition of apoptosis, according to our results; we suggest that once CRC is established it began to decrease its activity and consecutively its gene expression. Previous studies report higher expression of *BCL2L1, VEGFA, TNF, ALOX* and *NOS2* in tumor tissue than in normal mucosa, in this study we did not observed difference in expression of these genes. *BCL2L1* is associated with apoptosis and opposite of our results its overexpression has been previously reported (32). *VEGFA* overexpression has been observed and associated with advanced tumor stages and poor clinical outcome (33), but one study reported no difference at protein level similar as our results (34). *TNF* is involved in tumor promotion and progression; previous reports have found expression of *TNF* in 94% of tumors (35) in disagreement to our results. *ALOX* activity is related with tumor growth and invasiveness of solid tumors, opposite to our results *ALOX* overexpression in CRC has been reported (36). Similarly, *NOS2* overexpression in CRC has been observed and related to angiogenesis (37), while according to our results, decreased expression of *NOS2* in CRC tumor was previously reported (38).

Even though we did not found significant differences in expression of all selected genes between tumor and normal adjacent mucosa, we analyze the association of these genes with clinicopathological parameters due to their importance in tumorigenesis. The results of these analyses are discussed below: The strong positive association of *CCND1* with all TNM parameters, excluding tumor localization, confirmed its role during CRC tumorigenesis; as well its association with tumor progression suggests its potential as tumor marker in early stages. The activity of *MMP9* during invasiveness processes in CRC was confirmed in this study due to its positive association with tumor stages, but we can found any association with metastasis as other groups did (28-30). Antiapoptosis activity of *BCL2* has been associated to different cancer types, as well as CRC, in this study *BCL2* was negatively associated with all TNM parameters, supporting the highest expression level in normal adjacent mucosa of CRC patients we found; this data is completely disagreeing with previous reports (31), we suggest that others antiapoptotic factors play more important role than *BCL2* does in our CRC patients, even though we considered that is necessary to evaluate *BCL2* protein expression to reinforce our results. The role of *VEGFA* in solid tumors is confirmed with the positive association in lymph node status and metastasis degree CRC groups, according to others reports the poor clinical outcome is related to *VEGFA* expression (33), we confirmed this data in our CRC advanced group when *VEGFA* is overexpressed, so we also suggest its role as a possible tumor marker in advanced disease. Surprisingly, *TNF* and *BCL1L2* genes that has been several times reported associated with different types of solid tumors (32,35), did not showed association with any clinicopathological parameter, differences in their expression between tumor vs. normal adjacent mucosa was reported neither. Further studies should analyze the *TNF* and *BCL1L2* proteins vs. NF-κB/p65 activity in human CRC samples. *ALOX* overexpression influences in CRC development, and it is observed according to the positive association with tumor stages and tumor depth reported in this study. Tumor growth mediated by inflammation, supposed to be one of the most important processes related to *ALOX* overexpression (36), this data is supported by our results. *NOS2* showed a positive association with TNM parameters, but it was not significantly associated to histopathological differentiation and tumor localization. *NOS2* expression was not statistical different in tumor vs. normal adjacent mucosa, nevertheless we suggest that *NOS2* activity is related to tumorigenesis, absolute quantification analyses could help in future studies to confirm the *NOS2* role in CRC establishment and development (38).

According to results discussed above, not all selected genes regulated by NF-κB/p65 increment their expression as the transcription factor did; probably these genes are partially regulated by others transcription factors in CRC. These results suggest that NF-κB activity is necessary but not sufficient in CRC establishment. To our knowledge the present study reports for the first time, the selected genes expression and NF-κB/p65 association with clinicopathological parameters in sporadic CRC. Due to NF-κB/p65 nucleus translocation is required for its transcriptional activity; we suggest that others quantitative techniques are necessary to statistically associate NF-κB activity with gene expression profiles in CRC; conditions of our experiments did not permit us to work more deeply on it because some of our patients are already under treatment (non-inclusion criterion) or because we are not able to take more tissue samples (the patient did not continue attending hospital or because they passed away). Meta-analysis association studies are indispensable to completely understand the behavior of these molecules in CRC.

Higher NF-κB/p65 expression in CRC tissue compared to normal adjacent mucosa from the same patient is reported,

and this increment was positively associated with clinicopathological parameters, except for tumor localization. The monitoring and regulation of this transcription factor may be therapeutically useful in CRC patients.

NF-κB regulated genes showed an irregular expression pattern when compared CRC tissue vs. normal adjacent mucosa; *CCND1, PTGS2* and *MMP9* were overexpressed. *VEGFA, BCL2L1, NOS2, TNF* and *ALOX* did not changed, while *BCL2* decreases its expression level. Results of genes expression association with clinicopathological parameters are summarized below; *CCND1* was positively associated with all TNM parameters. *PTGS2* was associated with tumor depth and lymph node status. *VEGFA* showed a positively association with lymph node status and metastasis degree. *MMP9* was positively associated with tumor stages. *NOS2* showed a positive association with tumor stages, tumor depth, lymph node status and metastasis degree. *ALOX* was positively associated with tumor stages and tumor depth. *BCL1L2* and *TNF* did not showed association with any clinicopathological parameter, while *BCL2* was negatively associated with all TNM parameters. Tumor localization site was not related with any of the evaluated genes. According to the association of these genes with different clinicopathological parameters, they may be considered for the selection of proper diagnosis, treatment and follow-up for CRC patients.

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