Increased Nuclear Factor- κ B/RelA Expression Levels in Human Colorectal Carcinoma in North Indian Patients

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Abstract Colorectal cancer is a major cause of cancer-related death in many countries. Inflammatory pathway is considered to play a major role in colorectal carcinogenesis. Nuclear factor kappa B (NF- κ B) pathway is a link between inflammation and cancer. NF- κ B is a transcription factor which belongs to the Rel family. Activation of NF- κ B has been shown to play a role in cell proliferation, apoptosis, cytokine production, and oncogenesis. The aim of the present study was to evaluate the expression levels of NF- κ B/RelA in colorectal carcinoma using Real-time PCR. For this study, tumor tissue was taken from general surgery

OT of PGIMER, Chandigarh from twenty-seven patients of colorectal cancer treated by surgery. Adjacent colonic mucosa specimens were also collected from all patients as normal control tissue. Real-time PCR was performed to determine the nuclear factor- κ B/RelA expression levels in twenty-seven pairs of colorectal adenocarcinoma and adjacent normal colonic tissues. Out of 27 CRC patients, 18 were males and 9 females. Mean age of patients was 51.1 ± 14.8 years. Most of the cases were males (67%). Seventy percent of the cases were early (I–II) and 30% were advanced (III–IV) tumor stage. The quantitative relative expression of NF- κ B mRNA was found to be significantly higher ($p < 0.05$) in CRC tissues as compared with that in adjacent normal colon tissues. From this study, we can conclude that RelA/NF- κ B pathway is expressed constitutively in colorectal adenoma and adenocarcinomas. Thus, RelA/NF- κ B might play an important role in colorectal tumorigenesis.

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Introduction

Colorectal cancer (CRC) is a worldwide disease. It is the third most common cause of cancer in both women and men and is also the third most common cause of cancer death [1]. Inflammation plays a major role in CRC development, including tumor initiation, progression, and invasion [2]. Numerous factors have been regarded as potential diagnostic and prognostic markers in CRC [3].

Inflammatory pathway is considered to play a major role in colorectal carcinogenesis. Inflammation and cancer have been considered to be closely linked for many years. NF-

κB is the most crucial factor in the development of neoplasms. Although the molecular mechanisms are not well understood, studies nominate NF-κB as a central molecule responsible for the transition from inflammation to cancer [4].

Nuclear factor kappa B (NF-κB) is a transcription factor, which belongs to the Rel family [5]. NF-κB is considered to have an impact on many cellular phenomena such as inflammation, immune responses, proliferation, apoptosis, tumor progression and differentiation [6]. The stimuli that activate members of the Rel family are extensive and growing and it emphasizes their central role in transcriptional responses [7]. NF-κB exists in the cytoplasm in the majority of cell types as homodimers or heterodimers of a family of structurally related proteins [8]. NF-κB activation is associated with CRC. Colon cancer cell lines and human tumor samples as well as nuclei of stromal macrophages in sporadic adenomatous polyps have been found to have increased NF-κB activity [4, 9].

NF-κB activation has also been observed in many solid tumors, but so far no oncogenic mutations responsible for NFκB activation in carcinomas have been identified. However, through its ability to up-regulate the expression of tumor promoting cytokines, such as interleukin 6 or tumor necrosis factor α and survival genes, such as Bcl-XL, NFκB provides a critical link between inflammation and cancer [10]. The status of RelA/NF-κB and its role in apoptosis have been well studied in colorectal cancer cell lines [11–13], but information about the expression of RelA/NF-κB in colorectal tumors using Real-time PCR and its relation to the clinicopathological parameters is still lacking. Moreover, the role of NF-κB in colorectal carcinogenesis has not been thoroughly studied in the Indian population.

Thus the present study was carried out to elucidate NFκB/RelA expression levels in human colorectal carcinoma using Real-time PCR and its correlation to the histopathological grade and pathological stage of the tumor.

Materials and Methods

Tissue Specimen

Tumor tissue was taken from twenty-seven patients of colorectal cancer after surgical intervention at general surgery Operation theatre (OT) at PGIMER, Chandigarh. Unaffected colonic mucosal specimens were also collected in parallel with the tumor specimens from all patients as normal control tissue. Immediately after resection, tissue samples were transported on ice to the laboratory and tissue was washed with ice cold phosphate buffered saline (PBS)

and snap frozen in liquid nitrogen and stored at −80 °C until use. Each patient was explained about the nature of the study and a written informed consent was obtained. The present study was approved by institutional ethics committee.

Total RNA Extraction and Reverse Transcription

Total RNA was isolated from tumor and normal adjacent colonic mucosa using TRIzol reagent according to the manufacturer's instructions. (Invitrogen, USA). A total of 1 μg RNA was treated with 1 U DNase 1 (Fermentas) for 30 min at 37 °C and then heat inactivated at 65 °C for 10 min before reverse transcription to eliminate genomic DNA contamination.

cDNA was synthesized from 1 μg RNA by reverse transcription using Thermo Scientific's RevertAid First Strand cDNA Synthesis Kit (Invitrogen) following manufacturer's instructions in a total volume of 20 μl.

Quantitative Real Time PCR

Real-time PCR analysis was performed on 96 Real-Time PCR system (Roche, Indianapolis, IN) using the SYBR Green PCR Master Mix (Roche Indianapolis, IN). The PCR reaction mixture consisted of 1 × SYBR green reagent (Roche), 10 pmol of each forward and reverse primers including 1 μl cDNA in a final volume of 25 μl reaction. Cycling conditions were 50 °C for 10 s and 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. β-actin was used as an internal control. The primers used in the experiment were NF-κB: Forward, 5'-CCCACGAGCTTGTAGGAAAGG-3'; NF-κB: Reverse; 5'-CTGGATGCGCTGACTGATAG-3' and β-actin: Forward, 5'-TCTACAATGAGCTGCGTG-3'; β-actin: Reverse, 5'-CCTTAATGTCACGCACGA-3'. Specificity of the PCR products of NF-κB and β-actin was confirmed by melting curve analysis and agarose gel electrophoresis. All real time qPCR analyses were performed in duplicate. Relative quantification of the expression of each gene was calculated by the $2^{-\Delta(\Delta Ct)}$ method [14].

Statistical Analysis

SPSS 20.0 software was used to perform statistical analysis. Statistical significance in NF-κB mRNA expression between two groups was evaluated using one sample *t* test and Mann–Whitney test. Whereas, significance among more than two groups was evaluated using Kruskal–Wallis test. *p* values of < 0.05 were considered statistically significant. All results are represented as mean ± standard error (S.E.).

Results

Patients Characteristics

Out of 27 patients of colorectal cancer, 18 (67%) were males and 9 (33%) females. The patients were in the age group of 19–75 years with mean age of 51.1 ± 14.8 years. Most of the cases were males (67%). Seventy percent of the cases were early (I–II) and 30% were advanced (III–IV) tumor stage. The detailed clinical characteristics of patients are summarized in Table 1.

Correlation of NF- κ B mRNA Expression with Clinicopathological Features of Colorectal Cancer Patients

The association between NF- κ B expression and clinicopathological features are presented in Table 2. The quantitative relative expression of NF- κ B mRNA was found to be significantly higher in CRC tissues as compared with that in adjacent normal colon tissues ($p = 0.0001$; Fig. 1). However, no significant change in NF- κ B mRNA expression was observed between early stages and advanced stages of CRC ($p = 0.872$; Fig. 2). Also, there was no significant correlation between NF- κ B expression and tumor location ($p = 0.171$; Fig. 3). Similarly, there was no

significant correlation between NF- κ B expression and age, gender, TNM stage, lymph node status, histological type and histological grade (Table 2).

Discussion

Colorectal cancer is a leading cause of morbidity and mortality worldwide. It is believed to inflict for more than half a million deaths annually. CRC is a multistep process that involves multiple genetic and epigenetic aberrations, which lead to simultaneous failure of protective mechanisms and also activation of tumorigenic pathways. Nuclear factor- κ B (NF- κ B) is a transcription factor that activates several genes responsible for innate and adaptive immune responses and also regulates cell-cycle progression. Thus, NF- κ B plays a major role in the onset of molecular events leading to tumorigenesis. However, the precise role of NF- κ B activation in CRC is still unclear [15].

In the present study, an attempt was made to quantify the RelA/NF- κ B mRNA levels in 27 paired samples of colorectal tumor tissue and corresponding normal colorectal mucosa using Real-time PCR. We observed a significantly higher expression of NF- κ B mRNA in CRC tissues as compared with that in adjacent normal colon tissues and the same trend was seen in earlier studies also. Moorchung et al. [16] observed a constitutive activation of NF- κ B in CRC, after performing Immunohistochemistry that revealed nuclei of the epithelial cells in the malignant areas had a mean NF- κ B staining of 46.72%, whereas, nuclei of the lymphocytes had a mean NF- κ B staining of 32.79%. Further, Kojima et al. [5] demonstrated by using EMSA and Immunohistochemistry that NF- κ B gets constitutively activated as compared to non neoplastic tissues in human colorectal carcinoma tissues. In another study, results of Immunohistochemical staining showed that the increased expression of NF- κ B in colorectal tumorigenesis plays an important role in the pathogenesis of colon cancer in humans by mediating the transition from colorectal adenoma with low-grade dysplasia to adenocarcinoma [17]. Charalambous et al. [18] using immunohistochemistry, observed that NF- κ B-p65 and to a lesser extent NF- κ B-p50 are not only upregulated in human malignant epithelial cells of colorectal cancer patients, but they are also significantly activated. The advantage of the present study is that we used Real-time PCR to quantify the NF- κ B/RelA mRNA levels in the tissue samples as compared to the Immunohistochemistry techniques used by other authors. Real-time qPCR (quantitative polymerase chain reaction qPCR) is one of the most powerful and sensitive gene analysis techniques available and is used for a broad

Table 1 Clinical characteristic of the patients with colorectal cancer

Clinical characteristics	N (%)
Patients (n)	27
Age	
≤ 50 years	13 (48)
> 50 years	14 (52)
Sex	
Male	18 (67)
Female	9 (33)
Tumor location	
Proximal	16 (59)
Distal	11 (41)
Clinical stage (TNM)	
Early (I–II)	19 (70)
Advanced (III–IV)	8 (30)
Histopathological type	
Adenocarcinoma	22 (81)
Other types	5 (19)
Histopathological grade	
Well differentiated	2 (7.5)
Moderately differentiated	23 (85)
Poorly differentiated	2 (7.5)

Table 2 Correlation between NF- κ B mRNA expression and clinicopathological features of colorectal cancer patients

Clinicopathological parameters	Number of cases (%)	RQ (mean $\pm$ SE)	<i>p</i> value
Patients (n)	27 (100)		
Age group			
$\leq$ 50 years	13 (48)	8.11 $\pm$ 1.75	0.189
$>$ 50 years	14 (52)	5.21 $\pm$ 1.15	
Sex			
Male	18 (67)	6.67 $\pm$ 1.35	0.932
Female	9 (33)	6.87 $\pm$ 1.85	
Tumor location			
Proximal	16 (59)	5.53 $\pm$ 1.27	0.171
Distal	11 (41)	8.58 $\pm$ 1.87	
TNM stage			
I + II	19 (70)	6.86 $\pm$ 1.40	0.872
III	8 (30)	6.46 $\pm$ 1.81	
Lymph node metastasis			
Yes	8 (30)	6.46 $\pm$ 1.82	0.851
No	19 (70)	6.93 $\pm$ 1.41	
Histopathological type			
Adenocarcinoma	22 (81)	7.50 $\pm$ 1.37	0.467
Other types	5 (19)	5.27 $\pm$ 1.78	
Histopathological grade			
Good + moderate	25 (93)	6.88 $\pm$ 1.16	0.668
Poor	2 (7)	5.04 $\pm$ 3.53	

Colorectal cancer, RQ relative quantification * $p < 0.05$, statistically significant difference **Fig. 1** NF- κ B gene expression in CRC and adjacent normal colon tissues. NF- κ B mRNA levels in CRC as well as adjacent normal colon tissues were determined by real time PCR. β -actin mRNA levels wereused to normalize NF- κ B mRNA expression. Statistical analysis was done by means of the one sample *t* test. *** $p < 0.001$

Fig. 2 NF- κ B gene expression in different grades of CRC. NF- κ B mRNA levels in different grades of CRC tissue were determined by real time PCR. β -actin mRNA levels were used to normalize NF- κ B

mRNA expression. Statistical analysis was done by means of the Mann–Whitney test ($p = 0.872$)

Fig. 3 NF- κ B gene expression in different sites of CRC. NF- κ B mRNA levels in different sites of CRC tissue were determined by real time PCR. β -actin mRNA levels were used to normalize NF- κ B

mRNA expression. Statistical analysis was done by means of the Mann–Whitney test ($p = 0.171$)

range of applications including quantitative gene expression analysis.

Further in the present study, we also analyzed the association of NF- κ B expression with the histopathological grade and pathological stage of the tumor. However, we observed no significant change in NF- κ B mRNA

expression between early stages and advanced stages of CRC. Also, there was no significant relationship of NF- κ B expression with age, gender, tumor site, TNM stage, lymph node status histological type and histological grade. Similarly, in another study also, no association between NF- κ B staining and the clinic-pathological parameters were

observed. Moorchung et al. [16] found a significant correlation between the grade of NF- κ B staining in the malignant epithelial cells with the tumor and nodal status. Likewise, another study [5] showed significantly increased NF- κ B activation in the more progressed subgroups, such as the T3 + T4 subgroup and Stage II < subgroup. In the present study, may be due to less number of patients, the significant correlation could not be obtained between the NF- κ B expression and histopathological grade and pathological stage of the tumor. On further sub grouping, the patient number in each group further decreased. Hence we could not find any relation between the expression and other parameters.

In conclusion, we could effectively demonstrate the increased expression of constitutive NF- κ B/RelA in human colorectal carcinomas and established its correlation with tumor progression using Real-time PCR. However, further investigations with large sample size are warranted to understand the mechanism of NF- κ B activation so as to provide new regimen for colorectal cancer patients.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest in this study.

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