Nuclear factor-κB p65 (RelA) transcription factor is constitutively activated in human colorectal carcinoma tissue

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AIM: Activation of transcription factor nuclear factor-κB (NF-κB) has been shown to play a role in cell proliferation, apoptosis, cytokine production, and oncogenesis. The purpose of this study was to determine whether NF-κB was constitutively activated in human colorectal tumor tissues and, if so, to determine the role of NF-κB in colorectal tumor progression.

METHODS: Paraffin sections of normal epithelial, adenomatous and adenocarcinoma tissues were analyzed immunohistochemically for expression of RelA, Bcl-2 and Bcl-\(\chi\), proteins. Electrophoretic mobility shift assay (EMSA) was used to confirm the increased nuclear translocation of RelA in colorectal tumor tissues. The mRNA expressions of Bcl-2 and Bcl-\(\chi\) were determined by reverse transcription polymerase chain reaction (RT-PCR) analysis. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method.

RESULTS: The activity of NF-κB was significantly higher in adenocarcinoma tissue in comparison with that in adenomatous and normal epithelial tissues. The apoptotic index (AI) significantly decreased in the transition from adenoma to adenocarcinoma. Meanwhile, the expressions of Bcl-2 and Bcl-\(\chi\), protein and their mRNAs were significantly higher in adenocarcinoma tissues than in adenomatous and normal epithelial tissues.

CONCLUSION: NF-κB may inhibit apoptosis via enhancing the expression of the apoptosis genes Bcl-2 and Bcl-\(\chi\). And the increased expression of RelA/nuclear factor-κB plays an important role in the pathogenesis of colorectal carcinoma.

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immunohistochemistry. Tissue specimens were snap-frozen immediately in liquid N₂ and stored at -80 °C for EMSA assays and RT-PCR analysis. The study was approved by the Institutional Board of the Ethics Committee of Wuhan University under full consideration of the declaration on human rights of Helsinki.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue blocks were cut into 5 μm thick and mounted onto glass slides. After that, they were kept in an oven at 4 °C overnight. Immunostaining was performed as previously described with a slight modification. Sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. To improve the quality of staining, microwave oven-based antigen retrieval was performed. Slides were probed with either anti-RelA (1:50, mouse monoclonal, Santa Cruz Biotechnology), anti-Bcl-2 (1:100, mouse monoclonal, Santa Cruz Biotechnology) or anti-Bcl-xL (1:100, mouse monoclonal, Santa Cruz Biotechnology). Sections were washed three times with PBS for 10 min each and incubated with biotin-labeled anti-mouse IgG for 1 h at room temperature. After three washes with PBS for 10 min each, sections were stained with a streptavidin-peroxidase detection system. Incubation with PBS instead of the primary antibody served as a negative control. In specimens containing positive cells, the positive cells were counted in ten randomly selected fields under high power microscope (200-fold or 400-fold magnification) for each sample, and the average was expressed as the density of positive cells.

**Determination of apoptosis**

The TUNEL assay, originally described by Gavrieli et al., was used with minor modifications. Briefly, tissue sections of 5 μm were mounted onto glass slides, deparaffinized, hydrated, and treated for 15-30 min at 37 °C with proteinase-K (Roche Co., 20 μg/mL in 10 mmol/L Tris-HCl buffer, pH 7.4). Slides were rinsed twice with PBS. Then, 50 μL of TUNEL reaction mixture (450 μL nucleotide mixture containing fluoresceinated dUTP in reaction buffer plus 50 μL enzyme TdT from calf thymus, Roche Co.) were added to the samples. To ensure homogeneous distribution of the TUNEL reaction mixture on tissue sections and to avoid evaporative loss, slides were covered with coverslips during incubation. Slides were incubated in a humidified chamber for 60 min at 37 °C. After rinsed, slides were incubated with anti-fluorescein antibody, with Fab fragment from sheep, conjugated with horse-radish peroxidase for 30 min at 37 °C. Slides were rinsed twice with PBS. Then, 50-100 μL of DAB substrate was added and incubated for 10 min at room temperature. Samples can be counterstained prior to analysis by light microscope. Positive signals were defined as presence of a distinct brown color nuclear staining of the neoplastic cells or morphologically defined apoptotic bodies. The apoptotic index (AI) was determined by counting a total of at least 1 000 neoplastic nuclei in 10 randomly chosen fields at 400-fold magnification. Apoptotic cells were identified using a TUNEL assay in conjunction with characteristic morphological changes such as cell shrinkage, membrane blebbing, and chromatin condensation, to distinguish apoptotic cells and apoptotic bodies from necrotic cells.

**EMSA**

Nuclear extracts were harvested according to protocols described previously. In brief, fresh samples were minced and homogenized in 400 μL of hypotonic lysis buffer A (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF). Homogenized tissues were incubated on ice for 5 min, NP-40 was added to a final concentration of 5 g/L, and samples were vigorously mixed and centrifuged. The cytoplasmic proteins were removed and the pellet nuclei were resuspended in 50 μL buffer C (20 mmol/L HEPES pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF). After 30 min agitation at 4 °C, the samples were centrifuged and supernatants, containing nuclear proteins, were transferred to a fresh vial. The protein concentrations of nuclear extracts were determined by Bio-Rad protein assay. The nuclear extracts were stored at -80 °C until use. Nuclear protein extracts of carcinomas, adenomas, and normal tissues were analyzed by EMSA for NF-xB nuclear translocation as previously described. EMSA binding reaction mixture contained 8 μg protein of nuclear extracts, 2 μg of poly (deoxyinosinic- deoxyctydilic acid) (Sigma Co.), and [32P]-labeled double-stranded oligonucleotide containing the binding motif of NF-xB probe (4 000 cpm) in binding buffer (10 mmol/L HEPES pH 7.9, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 100 mL/L glycerol, and 0.2 g/L albumin). The sequence of the double-stranded oligomer used for EMSA was 5'-AGTTGAGGAGCTTCCCCAGGC-3'. The reaction was incubated for 30 min at room temperature before separation on a 50 g/L acrylamide gel, followed by autoradiography. For supershift experiments, 2 μg of mouse monoclonal antibodies against the p65 subunit (Santa Cruz Biotechnology) of NF-xB was incubated with the nuclear extracts 10 min before the addition of the [32P]-labeled probe and then analyzed as described.

**RT-PCR**

The mRNA expressions of Bcl-2 and Bcl-xL were assessed using RT-PCR standardized by coamplifying housekeeping gene β-actin, which served as an internal control. Total RNA was isolated from the normal epithelial, adenomatous and adenocarcinoma tissues by the single-step method. Total RNA was reversely transcribed into cDNA and used for PCR with human specific primers for Bcl-2, Bcl-xL and β-actin. Sequences of Bcl-2 primers were 5'-CAAGTGGACCTCCTGACCCCTT-3‘ (forward primer) and 5’-GCTTCGTATCTGATGACCC-3‘ (reverse primer), generating a 240 bp PCR product; for Bcl-xL, the forward primer was 5’-AGTTGAGGAGCTTCCCCAGGC-3‘ and the reverse primer was 5’-ATCAATGGCAAACCATCTTCG-3‘, generating a 216 bp PCR product; for β-actin, the forward primer was 5’-ACGGGGAGAT CGTCCGGTGC-3‘ and the reverse primer was 5’-CTACTTGCTTGCTGATCCAC-3‘, producing a 102 bp PCR product. Briefly, the PCR was amplified by 32 repeat denaturation cycles at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. During the first cycle, the denaturation was extended to 2 min, and in the final cycle the extension step was extended to 5 min. PCR products were separated on 15 g/L agarose gels containing 0.5 g/L of ethidium bromide and visualized by UV transillumination.

**Statistical analysis**

All statistical analyses were performed with SPSS10.0 statistical package for Microsoft Windows. Student’s t test and one-way analysis of variance (ANOVA) were used to compare continuous variables among groups. Correlation coefficients between continuous variables were calculated by the method of Pearson’s correlation coefficient. The χ² test was used to compare binominal proportions. A P value of <0.05 was considered significant.

**RESULTS**

**RelA/NF-xB expression in colorectal carcinoma tissues**

To investigate whether RelA/NF-xB-DNA binding activities were altered in human colorectal carcinoma tissues, we first carried out immunohistochemical analyses. The monoclonal antibodies used in this study detected only activated RelA
proteins[17]. RelA staining was shown as brown color and detected in normal colorectal mucosa, colorectal adenoma and colorectal adenocarcinoma specimens. In colorectal adenoma and adenocarcinoma, positive staining of RelA was mainly observed in the cytoplasm, and nuclear staining for RelA was also detected (Figure 1). Tissues of colorectal adenocarcinoma showed more cells with nuclear staining for RelA than those in colorectal adenoma tissues. No nuclear staining for RelA was found in normal colorectal mucosa. As shown in Table 1, the density of RelA-positive cells was significantly increased (P<0.01) in the transition from normal mucosa to adenoma and adenocarcinoma.

**EMSA**

To confirm the finding that RelA/NF-κB-DNA binding activities were activated in human colorectal carcinoma tissues, we carried out EMSA analyses. Figure 2 shows increased NF-κB DNA binding activity in adenocarcinoma tissues compared with that in adenoma and normal tissues. HPIAS-1000 SOFTWARE ANALYSIS took the image of electrophoresis. The absorbance of EMSA bands showed that the RelA/NF-κB complexes were not presented in normal colorectal epithelium, 0.6587±0.0021 in adenocarcinoma, and 0.2153±0.0013 in adenoma. The RelA expressions were significantly increased (P<0.05) in the transition from normal colorectal epithelium to colon tumor tissues. To confirm the specificity of NF-κB DNA binding, we performed supershift analysis with antibodies specific for RelA (p65) and a competitive study with a 50-fold excess of unlabeled oligonucleotide. An antibody specific for RelA which recognizes RelA/NF-κB heterodimer, unlabeled oligonucleotide diminished the intensity of RelA/NF-κB complexes, indicating that complex was the NF-κB binding-specific band. Our results showed that RelA was frequently activated in human colorectal tumor tissues but not in normal colon tissue.

**Bcl-2 and Bcl-xL protein expression in colorectal carcinoma tissues**

In the present study, the expressions of Bcl-2 and Bcl-xL were also investigated using immunohistochemistry. Immunostaining specific for Bcl-2 and Bcl-xL was cytoplasmic and shown as brown color (Figures 3, 4). The expressions of Bcl-2 and Bcl-xL were both significantly associated (r = 0.95,0.88; P<0.05) with RelA expression in adenoma and adenocarcinoma.

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>RelA</th>
<th>Bcl-2</th>
<th>Bcl-xL</th>
<th>AI</th>
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<tr>
<td>Normal</td>
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<td>15.62±0.75</td>
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<tr>
<td>Adenocarcinoma</td>
<td>30</td>
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<td>78.23±8.33</td>
<td>77.32±6.51</td>
<td>31.53±3.71</td>
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</tbody>
</table>

Figure 2 Electrophoretic mobility shift assay demonstrating increased nuclear translocation and DNA binding of NF-κB. A: lane 1, positive control (using Hela nuclear extract); lane 2, normal; lanes 3-6, adenocarcinoma; lanes 7-10, adenoma. B: lane 1, positive control (using Hela nuclear extract); lanes 2-3, specific competitor (using excess of unlabeled oligonucleotide); lanes 4-5, adenoma; lanes 6-7, adenocarcinoma; lane 4 and 6, supershift (addition of p65 antibodies to the nuclear extracts).

Figure 3 Immunohistochemical staining of Bcl-2 in tissue sections of colorectal adenoma (A) and adenocarcinoma (B). Bcl-2 expression is restricted to the cytoplasm of cancer cells. x200.
**Figure 4** Immunohistochemical staining of Bcl-xL in tissue sections of colorectal adenoma (A) and adenocarcinoma (B). Bcl-xL expression is restricted to the cytoplasm of cancer cells. ×200.

**Figure 5** The mRNA expressions of Bcl-2 and Bcl-xL were assessed using RT-PCR standardized by co-amplifying the housekeeping gene β-actin. A: the mRNA expression of Bcl-2. Lanes 1-3, adenocarcinoma; lanes 4-6, adenoma; lane 7, normal; lane 8, marker. B: the mRNA expression of Bcl-2. Lane 1, marker; lane 2, normal; lanes 3-5, adenoma; lanes 6-8, adenocarcinoma.

**Figure 6** TUNEL staining in tissue sections of colorectal adenoma (A) and adenocarcinoma (B). TUNEL staining is restricted to the nucleus of apoptotic cells. ×200.

**Bcl-2 and Bcl-xL mRNA expression in colorectal carcinoma tissues**

RT-PCR analysis of mRNA expressions of Bcl-2 and Bcl-xL was standardized by co-amplifying these genes with the housekeeping gene β-actin. HPIAS-1000 SOFTWARE ANALYSIS took the image of electrophoresis with β-action as internal standard. The relative absorbance of mRNA expression for Bcl-2: 2.43±0.27% in normal tissues, 17.96±1.51% in adenoma, and 36.71±2.17% in adenocarcinoma; for Bcl-xL: 3.54±0.33% in normal tissues, 23.02±2.11% in adenoma, and 39.71±2.49% in adenocarcinoma (Figure 5). Our results showed that colon tumor tissue constitutively expressed Bcl-2 and Bcl-xL. The mRNA expressions of Bcl-2 and Bcl-xL were significantly increased (P<0.05) in the transition from normal colorectal epithelium to colon tumor tissue.

**Cell apoptosis**

In this study, TUNEL staining was restricted to the nucleus of apoptotic cells. TUNEL-positive staining cells were detected in normal colorectal mucosa, adenoma, and adenocarcinoma. The AI was significantly increased (P<0.01) in the transition from normal colorectal mucosa to adenoma, but decreased from adenoma to adenocarcinoma (Figure 6). There was no association between the AI and the histological classification of adenoma and adenocarcinoma. The density of RelA-positive cells inversely correlated with the AI in the transition from adenoma to adenocarcinoma (r = -0.89; P<0.001).

**DISCUSSION**

We have demonstrated that RelA-DNA binding activity was constitutively activated in the majority of human colorectal carcinomas. Whereas the role for RelA/NF-κB in tumorigenesis has not firmly established, recent work has suggested that it may play a role in this process. RelA/NF-κB activation has been shown to be necessary for tumor formation in Hodgkin lymphoma cells[26,27]. More recently, the inhibition of RelA/NF-κB activity through the use of specific NF-κB inhibitors (gliotoxin and MG132) resulted in spontaneous caspase-independent apoptosis in Hodgkin and Reed-Sternberg cells[28]. Also, an increase in RelA/NF-κB levels was identified in breast cancer
cell lines, primary human breast cancer, hepatocellular carcinoma, pancreatic adenocarcinoma, and gastric carcinoma when compared with nontransformed controls or normal tissues.\(^{29-32}\) In addition, NF-κB transcriptional activity was required for oncogenic Ras-induced cellular transformation, which likely occurred through the inhibition of transformation-associated apoptosis.\(^{33}\) However, the role of NF-κB in colorectal tumorigenesis is unknown and currently under investigation.

We primarily used immunohistochemistry to detect NF-κB activation in human colorectal carcinoma tissues. Its expression was significantly increased in the transition from normal colorectal mucosa to adenoma and adenocarcinoma. In our immunohistochemical analyses, we used monoclonal antibodies to detect RelA/NF-κB-DNA binding activities, and their sensitivity and specificity have been characterized previously. They were useful in differentiating between activated and inactivated forms of RelA and facilitated the detection of the activated RelA proteins. In the current investigation, only 10-20% of RelA/NF-κB protein was detectable in the nucleus, which was consistent with previous reports.\(^{34,35}\) And 80-90% of RelA still remained in the cytoplasm when RelA proteins were activated. It is unclear why the majority of RelA/NF-κB proteins that were freed from IκB remained in the cytoplasm. Possible explanations for this are: (1) IκB was mutated and therefore could not bind to RelA and masked the transcriptional signal in RelA; (2) mutations in RelA inhibited IκB binding to RelA, and (3) the NF-κB upstream signal transduction cascades were constitutively activated.

The Bcl-2 proto-oncogene is an apoptosis inhibitor originally described in association with the t(14;18) (q32;q21) translocation in follicular B cell lymphoma, which places the Bcl-2 gene under the stimulatory control of the IgH promoter-enhancer at 14q32. Resulting in increased Bcl-2 mRNA and protein and inhibition of apoptosis. The Bcl-xL, proto-oncogene, a member of Bcl-2 family, is a homologue of Bcl-2 and is an apoptosis inhibitor. The Bcl-2 and Bcl-xL oncoproteins have been described in normal colonic mucosa, where these were restricted to the epithelial regenerative compartment and the intestinal crypt bases. In our study, the expression of Bcl-2 and Bcl-xL was increased in the transition from normal mucosa to adenoma and adenocarcinoma. These results also show that the increased RelA/NF-κB expression occurred concomitantly with an increased expression of Bcl-2 and Bcl-xL. To date, a number of gene products that inhibit apoptosis have been identified. These include Bcl-2 and Bcl-xL. Indeed, Bcl-2 and Bcl-xL have been identified as NF-κB target gene, but the exact role NF-κB plays in its regulation remains controversial.

In recent years, increasing evidence indicates that activation of NF-κB plays an important role in coordinating the control of apoptotic cell death. NF-κB has been shown to prevent Fas-induced death in B cells through the upregulation of Bcl-2 and Bcl-xL expression, but has also been demonstrated to promote apoptosis in thymocytes by downregulating Bcl-2 and Bcl-xL gene expression.\(^{36}\) However, the exact mechanism of NF-κB in the regulation of apoptosis is not entirely clear. There are at least two distinct mechanisms by which NF-κB blocks apoptosis: (1) induction of antiapoptosis factors including IEX-1L, TRAF1, TRAF2, c-IAP-1, c-IAP-2 etc; (2) interference of apoptotic pathway by protein-protein interaction. However, these two distinct mechanisms are not mutually exclusive since either mechanism alone cannot fully explain the antiapoptotic action of NF-κB. In our study, apoptosis was significantly decreased in the transition from adenoma to adenocarcinoma, which was in contrast to the expressions of RelA, Bcl-2, and Bcl-xL. We also observed an inverse relation between AI and the expression of RelA in the transition from adenoma to adenocarcinoma, implying that increased RelA protein expression to a certain level might be anti-apoptotic and thus promote tumorigenic cell behavior. The anti-apoptotic role of NF-κB has been well characterized and various down-stream targets of NF-κB, including Bcl-2 and Bcl-xL, have been identified. In the present study, the statistical correlation between the increased expression of RelA, elevated Bcl-2 and Bcl-xL expression implies that in colorectal tissue, activation of RelA might exhibit anti-apoptotic effects at least in part through upregulation of Bcl-2 and Bcl-xL expression. This has been shown to decrease mitochondrial permeability changes and cytochrome C release and thus to block apoptosis.\(^{39,40}\)

To date, NF-κB has been believed to play an important role in coordinating the control of apoptotic cell death. However, the mechanism by which NF-κB blocks apoptosis is still controversial. Some laboratories have reported that activation of NF-κB is able to either promote or prevent apoptosis, depending on different stimuli and different cell types.\(^{41-44}\) For example, Grimm et al.\(^{45}\) reported that serum starvation activated NF-κB and induced human embryonic kidney cells into apoptosis. Qin et al.\(^{46}\) found that NF-κB activation contributed to the excitotoxin-induced death of striatal neurons. However, somewhat inconsistent results have also been presented by Beg and Baltimore\(^{47}\) that NF-κB activation generally inhibited apoptosis in embryonic fibroblasts. A question arises: who is right on earth? We think the answer is expected by further studies.

In conclusion, our results demonstrate that the RelA/NF-κB pathway is activated constitutively in colorectal carcinoma tissues, suggesting that activation of RelA/NF-κB might play an important role in colorectal tumorigenesis. Further studies are required to elucidate the mechanisms of NF-κB activation and to determine whether NF-κB might serve as a therapeutic target in the anti-neoplastic treatment of colorectal cancer.

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