NF- κ B and Bcl-3 Activation Are Prognostic in Metastatic Colorectal Cancer

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Key Words

NF- κ B • P65 • P50 • Colorectal carcinoma

Abstract

Purpose: NF- κ B is an antiapoptotic transcription factor that has been shown to be a mediator of treatment resistance. Bcl-3 is a regulator of NF- κ B that may play a role in oncogenesis. The goal of this study was to correlate the activation status of NF- κ B and Bcl-3 with clinical outcome in a group of patients with metastatic colorectal cancer (CRC). **Methods:** A retrospective study of 23 patients who underwent surgical resection of CRC at the University of North Carolina (UNC). Activation of NF- κ B was defined by nuclear expression of select components of NF- κ B (p50, p52, p65) and Bcl-3. Tissue microarrays were created from cores of normal mucosa, primary tumor, lymph node metastases and liver metastases in triplicate from disparate areas of the blocks, and an intensity score was generated by multiplying intensity (0–3+) by percent of positive tumor cells. Generalized estimating equations were used to note differences in intensity scores among normal mucosa and nonnormal tissues. Cox regression models were fit to see if scores were significantly associated with overall survival. **Results:** p65 NE was significantly higher in primary tumor and liver metastases than normal mucosa (both $p < 0.01$). p50 nuclear expression was significantly

higher for all tumor sites than for normal mucosa (primary tumor and lymph node metastases $p < 0.0001$, liver metastases $p < 0.01$). Bcl-3 nuclear expression did not differ significantly between normal mucosa and tumor; however, nuclear expression in primary tumor for each of these components was strongly associated with survival: the increase in hazard for each 50-point increase in nuclear expression was 91% for Bcl-3, 66% for p65, and 52% for p50 (all $p < 0.05$). **Conclusions:** Activation of canonical NF- κ B subunits p50 and p65 as measured by nuclear expression is strongly associated with survival suggesting NF- κ B as a prognostic factor in this disease. Primary tumor nuclear expression appears to be as good as, or better than, metastatic sites at predicting prognosis. Bcl-3 nuclear expression is also negatively associated with survival and deserves further study in CRC.

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Introduction

The NF- κ B family of transcription factors is comprised of homo- or heterodimers of the subunits p50, p52, p65 (RelA), RelB, and c-Rel [1]. The canonical NF- κ B p50/p65 is the best-studied of the dimers. p50/p65 exists in the cytoplasm, and is activated by translocation to the nucleus after proteasomal degradation of a natural se-

questering protein I κ B. Upon entering the nucleus, NF- κ B acts as a transcription factor for a large number of proteins, a substantial fraction of which are well-described antiapoptotic proteins. As such, nuclear localization has been used as a surrogate for activation of NF- κ B for study in archived human tissue samples where the use of more accurate measures, such as electromobility gel shift assays, are not feasible. NF- κ B is important to study in solid tumors because of its potential to act downstream of a number of oncogenic pathways making it a desirable therapeutic target with potential for activity across a broad range of cancers.

The mechanisms by which NF- κ B is constitutively active in solid malignancies remain an area of active study. In rare cases, mutations in NF- κ B subunits or translocations of the atypical I κ B protein Bcl-3 result in abnormal activation [2]. Aside from this, NF- κ B can be activated in a number of ways including cellular stress, DNA damage, exposure to TNF, and by activation of various oncogenic pathways [3]. Perhaps the most relevant oncogenic pathways of NF- κ B interactions from a standpoint of colorectal cancer (CRC) are those between the NF- κ B and the RAF/MEK/ERK and the PI3 kinase (PI3K)/AKT pathways. Importantly, both of these pathways are activated by KRAS mutation in CRC [4] and both are potentially targetable by drug therapies. AKT is a central mediator of cellular survival that is activated by a number of upstream signals including growth factor signals such as ERBB signaling [5] via PI3K and negatively regulated by the tumor suppressor PTEN. AKT has been shown to activate I κ B kinase (IKK) and hence NF- κ B in several settings [6, 7]. Similarly, both HER2 signaling (which occurs through RAS) and oncogenic HRAS expression can activate NF- κ B [5, 8]. It would therefore be useful to know whether the correlation between NF- κ B and the MAPK pathway or the AKT pathway is stronger in CRC.

Recently it has been suggested that NF- κ B activation is associated with resistance to therapy in gastrointestinal malignancies, most notably in predicting resistance to chemoradiation in esophageal cancer [9] and predicting resistance to a combination of irinotecan and cetuximab in CRC [10]. Neither of these studies, however, addressed the possibility that NF- κ B is a prognostic rather than predictive marker. Furthermore, the literature on the role of NF- κ B in disease progression in colorectal neoplasms is sparse.

We therefore embarked on a study with several aims: (1) to determine whether p50 and/or p65 nuclear expression is prognostic in patients with metastatic CRC who were treated surgically, (2) to determine which NF- κ B

Table 1. Patient characteristics

Age		
<50 years	3/23	13%
50–70 years	13/23	57%
>70 years	7/23	30%
Sex		
Male	10/23	43%
Race		
Black	5/23	22%
White	14/23	61%
Other	4/23	17%
Presentation		
Synchronous	20/23	87%
Differentiation		
Moderate	20/23	87%
Poor	3/23	13%
Primary tumor site		
Rectum	2/23	8.6%
Proximal colon	7/23	30.4%
Distal colon	14/23	60.8%
Resection type		
R0	13/23	56.5%
R1	3/23	13.0%
R2	1/23	4.3%
Palliative/not resected	5/23	21.7%

subunit appears to be more predictive of survival, (3) to determine whether there are differences in NF- κ B activation in primary tumors, lymph node metastases and liver metastases, and (4) to assess whether NF- κ B activation correlates more strongly with RAF/MEK/ERK pathway activation or AKT activation using antibodies against phosphorylated ERK and AKT.

Methods

Tissue Microarray Creation

We identified 23 patients with metastatic CRC who had undergone surgical resection for metastatic disease between 1993 and 2005, and who had at least normal mucosa, primary tumor and one metastatic site biopsied or resected. Results of surgery are noted in table 1. Tissue specimens from normal colorectal mucosa, primary tumor site in colon, lymph node and hepatic metastases were stored in archived paraffin-embedded blocks. A tissue microarray (TMA) was created using fixed and paraffin-embedded tumors and adjacent nonneoplastic colorectal mucosa which were cored with a 1-mm needle and inserted into a recipient paraffin block (Beecher Instruments, Sun Prairie, Wisc., USA). Samples from the same tumor were staggered in the array and a map was created for later identification of the identity of individual cores.

Table 2. Antibodies and dilutions used for staining of TMAs

Target	Antibody	Manufacturer	Cat. No.	Dilution	Positive control
p50	p50NLS	Santa Cruz	sc-114NLS	1:100	breast carcinoma, Panc-1 cell block
p65	F-6	Santa Cruz	sc-8008	1:1,000	tonsil, Panc-1 cell block
Bcl-3	H-146	Santa Cruz	sc-13038	1:50	tonsil
AKT	Ser-473	Cell Signaling Systems	736E11	1:25	breast carcinoma and prostate carcinoma
ERK	Thr-202/Tyr-204	Cell Signaling Systems	D13.14.4E	1:200	breast carcinoma and NSCLC

Clinical data including survival were abstracted from University of North Carolina (UNC) medical records under an Institutional Review Board-approved protocol. All survival data were censored as of the last time of survival data collection in December 2007.

Immunohistochemistry Staining

Antibodies utilized for immunohistochemistry (IHC) are detailed in table 2. Briefly, unstained 5- μ m-thick sections were baked at 60°C for 15 min to 1 h. Baked sections were soaked twice in fresh xylene for 5 min each, then soaked in 100% ethanol for 3 min and blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 min. Slides were soaked in 95% ethanol for 3 min, 70% ethanol for 3 min, rinsed in distilled water, and soaked in Dako wash buffer (Dako Cat. No. S3006; Dako, Glostrup, Denmark) for 5 min. Slides were then steamed in a Black & Decker steamer for 25 min using antigen retrieval buffers (Dako) for each primary antibody to be studied (table 2) and then allowed to cool for at least 20 min. Sections were transferred to Dako wash buffer for 5 min. Endogenous biotin was neutralized by incubating the slides in a biotin blocking system (Dako Cat. No. X0590) for 10 min at room temperature in each of the two solutions. Sections were then exposed to the primary antibodies at the titers specified in table 2 for 30 min at room temperature. After rinsing in Dako wash buffer, slides were incubated with the Dako LSAB2 biotinylated link for 10 min at room temperature, rinsed in Dako wash buffer, and then incubated with the Dako LSAB2 streptavidin-horseradish peroxidase for 10 min at room temperature. After additional rinsing in Dako wash buffer, detection of the antibody/antigen complex was visualized using 3,3'-diaminobenzidine for 5 min. Slides were then rinsed in water, lightly counterstained in filtered Mayer's hematoxylin, rinsed, dehydrated, cleared, and mounted. The cells of interest in each core were scored for percentage reactivity and signal strength in both the cytoplasm and the nuclei. Simultaneously stained normal colorectal mucosa and no primary antibody-stained normal mucosa served as negative controls in each experiment.

Scoring of TMAs

One surgical pathologist (W.K.F.) blinded to clinical information scored all TMA cores by multiplying the average staining intensity seen in a given core on a scale of 0–3+ by the percentage of cells that were positive to any degree, creating a range of possible scores of 0–300. Triplicate cores were staggered in the TMA to reduce the chance of staining or interpretive bias, and results were averaged among informative cores.

Statistical Analysis

Generalized estimating equations, which account for correlated data, were used to estimate the differences in protein scores among normal and nonnormal tissues, and the differences among nonnormal tissues. Spearman's rank correlation coefficient was used to describe the strength of association between marker scores from the three nonnormal tissues. Bootstrap percentile methods were used to compute the p values for these coefficients. The Kaplan-Meier method was used to construct the survival curves with the scores categorized into four groups: (0, 50), (50, 100), (100, 200) and (200, 300). The log-rank test was used to compare differences among these survival curves. Cox regression models were fit to see if the score for each protein and location combination was significantly associated with overall survival. Statistical analyses were performed with SAS statistical software (SAS Institute Inc, Cary, N.C., USA) and R software (<http://www.r-project.org/>).

Results

Primary tumors, lymph node metastases (if positive for cancer), liver metastases and normal colonic mucosa from 23 patients were examined. Patient characteristics are described in table 1. Most patients had a synchronous disease presentation and the majority had moderately differentiated tumors. Nine of 23 patients received oxaliplatin and 20/23 had documented therapy with 5-fluorouracil. Overall median survival for the entire group was 24 months (95% CI: 18, 60).

As has been reported in other studies, we found that p50 and p65 are more frequently localized to the nucleus, and hence considered activated in primary and metastatic CRC when compared with normal mucosa (fig. 1). p65 nuclear staining was positive (100 or greater) in 7 of 23 (30%) primary tumors versus 3 of 22 (14%) normal mucosae. The mean staining intensity of p65 was also significantly higher for tumor than for normal tissue ($p = 0.001$). p50 primary tumor nuclear staining was positive at a greater frequency with 18 of 22 (82%) patients positive, but nuclear reactivity for p50 was also more frequent than observed for p65 in normal mucosa with 7 of 22

Fig. 1. Histograms of nuclear staining score of p65, p50 and Bcl-3 in mucosa, primary tumor, liver and lymph node tissue demonstrating differences only between tumor (of any site) and normal mucosal tissue.

(32%) cases positive. The difference in mean staining intensity between primary tumor and normal mucosa staining for p50 was also significantly different ($p < 0.0001$). Representative IHC is depicted in figure 2.

There were no significant differences in nuclear p50 or p65 expression between primary and metastatic tumors (either nodal or liver metastases, fig. 1), suggesting that activation of NF- κ B may commonly occur prior to the development of distant disease. p52 subunit nuclear staining, a finding that has been reported in breast [11] and hepatocellular cancers [12], was not seen in any of the cases in this study.

Another interesting finding was that p50 and p65 nuclear positivity were only weakly correlated ($\rho = 0.27$, $p = 0.025$) suggesting one of several possibilities:

(1) there are false negative results using the p65 antibody, (2) there are false positives using the p50 antibody, or (3) some tumors preferentially express nuclear p50 homodimers or non-p65 containing heterodimers of p50. These questions could not be tested directly given the lack of frozen tissue for electrophoretic mobility shift assays.

Importantly, nuclear expression of both p65 and p50 was a strong predictor of survival (fig. 3). Cox regression modeling revealed that each 50-point increase in tumor staining score for P65 was associated with a 66% increase in the hazard of death ($p = 0.0008$), while each 50-point increase in p50 nuclear staining was associated with a 52% increase in the hazard of death ($p = 0.024$).

Bcl-3 is a potential activator of p50 and p52 homodimers [13, 14] but is not known to interact with p50/p65

Fig. 2. Representative stain for p53 representing zero nuclear staining (a), 2+ nuclear reactivity in 50% of cells (score = 100) (b), and 3+ nuclear reactivity in 60% of cells (c).

heterodimers. In our study, Bcl-3 nuclear expression was frequently observed both in normal mucosa and in tumor tissue (all *p* values for differences in expression were not significant). Interestingly, however, there was a strong association between tumor Bcl-3 nuclear expression and survival (log-rank *p* = 0.007) (fig. 4a). For each 50-point increase in Bcl-3 staining score, a patient's hazard of death nearly doubled (Cox regression *p* = 0.002). Increasing Bcl-3 expression in normal mucosa was not associated with survival (Cox regression *p* = 0.7) (fig. 4b). We also assessed correlation between Bcl-3 and the p50 subunit staining; the Spearman's ρ for correlation was 0.29 (*p* = 0.014) suggestive of a modest correlation. Our interpretation of this finding is that a subset of CRC may utilize Bcl-3 overexpression with

coactivation of p50 homodimers as a mechanism of NF- κ B activation.

To further elucidate possible mechanistic pathways to account for the observed constitutive p50 and/or p65 activation we performed IHC for activated (phosphorylated) forms of AKT and ERK, both of which have been described as potential upstream activators of NF- κ B in the literature. Neither marker was associated with survival in our dataset. There was strong correlation between positivity for phospho-ERK staining and positivity for p65 and p50 nuclear localization (ρ for p50 = 0.43, *p* = 0.0009; ρ for p65 = 0.35, *p* = 0.009). There was no apparent correlation between phospho-AKT positivity and p50 and p65 nuclear localization.

Fig. 3. Kaplan-Meier plot of overall survival based on p65 (**a**) and p50 (**b**) IHC score by quartiles in primary tumor tissue.

Discussion

The results of this study demonstrate in a cohort of patients with metastatic CRC that NF- κ B activation has prognostic importance. Frequency of nuclear localization of NF- κ B was similar in primary and metastatic tumors, suggesting that NF- κ B activation generally occurs prior to metastatic spread in colon cancer. Recently, it has been suggested that NF- κ B nuclear expression predicted resistance to a combination of irinotecan and cetuximab in metastatic CRC [10] based on examination of p65 (alone) in a cohort of patients who had all been treated with the drug combination. Our study suggests the possibility that NF- κ B was simply prognostic rather than predictive of cetuximab response.

Our findings are consistent with those observed in several other solid tumor settings. Zhou et al. [15] demonstrated that NF- κ B activation, particularly DNA binding by the p50 subunit of NF- κ B, could identify a high-risk subset of ER-positive, primary breast cancers destined for early relapse despite adjuvant endocrine therapy with tamoxifen. NF- κ B activation has been suggested as a predictor of resistance to chemoradiation in esophageal cancer [9] based on a sample of patients who were all treated similarly.

Additional findings of our study may speak to the methods by which NF- κ B activation is assessed in clinical samples. Both p50 and p65 are measurable by IHC, and in spite of the fact that they are presumed to be part

of a heterodimer, the two appear to be expressed in colorectal tumor nuclei at different frequencies. This may be secondary to particular performance characteristics of the two antibodies in paraffin-embedded tissues, or to differences in subunit composition in different tumors. Utilization of antibodies to both subunits may be ideal until such issues are better elucidated. Additionally, our results suggest that primary tumor tissue should be equivalent to metastatic tissue in terms of assessing NF- κ B positivity.

The mechanisms by which NF- κ B is constitutively active in solid malignancies remain an area of active study. NF- κ B can be activated in a number of ways including cellular stress, DNA damage, exposure to TNF, and by activation of certain oncogenic pathways [3]. Perhaps the most relevant oncogenic pathway/NF- κ B interactions in CRC are those between the NF- κ B and the RAF/MEK/ERK and the PI3K/AKT pathways. This is because both pathways are frequently active in CRC via mutation of pathway elements including KRAS, BRAF, and PI3KCA [16].

AKT has been shown to activate IKK and hence NF- κ B in several settings [6, 17]. Both HER2 signaling and oncogenic HRAS expression can activate NF- κ B [5, 6, 8, 17]. Madrid et al. [18] have also shown that oncogenic HRAS requires PI3K and AKT to stimulate the transcriptional activity of NF- κ B. Activated PI3K and AKT stimulate NF- κ B-dependent transcription by stimulating transactivation domain 1 of the p65 subunit rather than

Fig. 4. a Kaplan-Meier plot of overall survival based on Bcl-3 IHC score by quartiles in primary tumor tissue. **b** Kaplan-Meier plot for Bcl-3 staining by quartiles in normal mucosa.

inducing NF- κ B nuclear translocation via I κ B degradation. In our patient sample and within the confines of assay by IHC we were not able to detect an association between positivity for AKT activation and nuclear positivity for either p65 or p50.

On the other hand, we did identify a correlation between phospho-ERK staining and both p65 and p50 nuclear positivity suggesting that ERK activation may be an important determinant of NF- κ B activation status in CRC. It has been shown that RAS enhances NF- κ B transcriptional activity through an RAF-dependent signaling pathway that utilizes p38 MAP kinase [19]. It is likely that oncogenic RAF enhances NF- κ B transcriptional activity through an autocrine feedback loop using the MEK/ERK pathway. In a previous work by Norris and Baldwin [19], conditioned medium from RAF-transformed cells activated NF- κ B transcriptional activity when added to parental cells. Also recent work has shown that oncogenic RAF activates NF- κ B in HEK293 cells through an autocrine loop that activates SAPK [20]. Our results support that the RAF/MEK/ERK pathway could be a principal driver of NF- κ B activation in CRC, a hypothesis that will be testable in studies of RAF or MEK inhibitors in BRAF mutant CRC.

Lastly, Bcl-3 is an oncogenic regulator of NF- κ B activity that has not been studied in human CRC samples to our knowledge. Our study suggests that Bcl-3 may be important in CRC and should be studied further. Bcl-3 is an atypical I κ B family member that acts in the nucleus rather

than the cytoplasm as a co-activator of NF- κ B. Bcl-3 has been demonstrated to act as an activator of p50 and p52 homodimers [12] and appears frequently in breast and hepatocellular carcinomas. The presence of Bcl-3 and p50 together in the nucleus suggests that this mechanism is active in a fraction of CRC, and could potentially help to explain the discordance between p50 and p65 positivity that was noted in our study.

The main limitations of our study are sample size and heterogeneity of metastatic presentation. Because one of the main goals of the study was to examine several tumor sites and several markers all in triplicate, we limited the number of patients examined to keep the number of evaluation points to a manageable number for one pathologist. The need to find patients with multiple tumor sites available to assay also limited the number of patients eligible for analysis. The magnitude of survival difference given this small sample size was large; this, however, suggests it would now be important to evaluate NF- κ B expression in a dataset where patients did or did not receive an anti-EGFR agent to clarify whether there are both prognostic and predictive, or just prognostic implications of NF- κ B activation. We would propose the use of an antibody against p50 for such a study based on our results. In the meantime, we also recently validated the prognostic implications of NF- κ B in 70 samples from patients with nonmetastatic primary rectal carcinoma in a separate study [21].

In summary, this study provides evidence that NF- κ B could be an important prognostic marker in CRC and should be studied further in a larger sample of patients. The continued study of mechanisms of NF- κ B activation in colon cancer and the study of therapeutic targeting of NF- κ B should remain a priority. Lastly, Bcl-3's role in CRC and other solid malignancies needs to be further defined both in the laboratory and in the clinic.

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