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Clinicopathological significance of nuclear factor-kappa B, HIF-1 alpha, and vascular endothelial growth factor expression in stage III colorectal cancer

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Nuclear factor-κB (NF-κB), hypoxia-inducible factor 1α (HIF-1α), and vascular endothelial growth factor (VEGF) are involved in cell proliferation, invasion, angiogenesis, and metastases. The principal objective of this study was to assess the prognostic significance of NF-κB, HIF-1α, and VEGF expression in stage III colorectal cancer. Tumor tissues from 148 patients with stage III colorectal carcinoma, all of whom underwent potentially curative resection, were immunohistochemically evaluated using monoclonal antibodies against NF-κB, HIF-1α, and VEGF. Positivity rates of NF-κB, HIF-1α, and VEGF were 47.3%, 42.6%, and 61.5%, respectively. NF-κB expression in tumor tissues was correlated significantly with HIF-1α expression ($P < 0.001$), VEGF expression ($P = 0.044$), and the presence of vascular invasion ($P = 0.013$). Univariate analysis demonstrated that NF-κB expression was associated with poor 5-year overall survival (55.8 months *vs* 76.9 months, $P = 0.012$). Multivariate analysis verified that NF-κB was independently associated with adverse outcomes (relative risk: 1.92, $P = 0.049$). However, HIF-1α and VEGF did not appear to be related to clinical outcomes. NF-κB expression in tumor tissue is associated with angiogenesis and poor 5-year overall survival in stage III colorectal cancer patients. (*Cancer Sci* 2010; 101: 1557–1561)

Colorectal cancer (CRC) is the second most common cause of cancer-related death in the USA,[1] and the third most frequent cancer in Korea.[2] There is relatively little information available about the clinical significance of prognostic markers in stage III CRC.

In this regard, many studies have focused on the important role of certain biomarkers in tumor angiogenesis in CRCs.[3–6] As cancer cell proliferation may outpace the rate of angiogenesis, thus inducing hypoxia,[7] hypoxia results in the expression of a number of gene products. The most important of these proteins is thought to be hypoxia-inducible factor-1 (HIF-1). Hypoxia-inducible factor-1 (HIF-1), a heterodimer composed of the oxygen-regulated subunits HIF-1α and HIF-1β, mediates the transcription of the gene for vascular endothelial growth factor (VEGF).[8] Hypoxia-inducible factor-1α (HIF-1α) overexpression is associated with tumor angiogenesis and tumor cell proliferation and invasion in CRCs.[4,5]

Vascular endothelial growth factor (VEGF) is the best-known pro-angiogenic growth factor, and its stimulation under hypoxic conditions plays a critical role in promoting the survival of malignant cells, in local tumor growth and invasion, and in the development of metastases.[9] Hypoxia-inducible factor-1α (HIF-1α) expression has been correlated not only with VEGF, but also with the level of angiogenesis in tumors, which is measured as microvessel density.[10] Clinical studies have correlated VEGF expression with poor prognosis in CRCs.[3,4]

The transcription factor nuclear factor-κB (NF-κB) performs a gatekeeper function in certain critical biologic functions, including cell survival, proliferation, and migration.[11] The constitutive activation of NF-κB has been observed and related positively to angiogenesis and tumor growth in CRCs.[12] In a previous report, NF-κB was identified as a direct modulator of HIF-1α expression, and the HIF-1α promoter was shown to be responsive to selective NF-κB subunits.[13] Additionally, increased NF-κB expression is accompanied by increased VEGF expression.[14]

The principal aims of this study were to evaluate the expression of the NF-κB, HIF-1α, and VEGF proteins in surgically excised stage III CRC tissues on tissue array slides, and to elucidate its clinical significance and prognostic value for patients with CRCs.

Materials and Methods

Patients. A total of 148 patients were included in this study, which spanned the period from January 2002 to January 2006. All patients had histologically confirmed stage III adenocarcinoma of the colon or rectum, and had undergone a potentially curative resection, with neither gross nor microscopic evidence of residual disease. Tumor samples were acquired from these patients and each sample was fixed in formalin and embedded in paraffin wax. Clinical outcomes were followed from the date of surgery to either the date of death or August 2009. This study was approved by the institutional review board of the Dong-A University Medical Center.

Construction of tissue microarray. One-milimeter cores were removed from the CRC samples that had previously been formalin-fixed and paraffin-embedded. For all of the arrays, three cores of different areas of the tumor were removed from each case and were placed into a new blank recipient paraffin block in a manner previously described,[15] and 4-μm thick sections were taken for all of the immunohistochemical stainings. Full cross-sections from the paraffin blocks were used for five of the CRCs, along with the adjacent normal colorectal tissue, to confirm the staining patterns observed on the tissue microarrays.

Immunohistochemistry. Immunohistochemical stainings for NF-κB, HIF-1α, and VEGF were performed on the tissue microarray slides via the avidin–biotin–peroxidase complex method. All of the sections were deparaffinized through a series of xylene baths, and rehydration was conducted using a series of graded alcohol solutions. In order to enhance immunoreactivity, microwave antigen retrieval was conducted for 30 min at

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750 W in citrate buffer (pH 6.0). After 10 min of blocking of the endogenous peroxidase activity with 5% hydrogen peroxidase, the samples were incubated with the primary antibody for 1 h at room temperature. The primary antibodies were a mouse monoclonal antibody directed against NF-κB p65 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a monoclonal antibody directed against HIF-1α (1:50; Novus Biologicals, Littleton, CO, USA) and a rabbit polyclonal antibody directed against VEGF (1:100; Santa Cruz Biotechnology). An EnvisionChem Detection Kit (DakoCytomation, Carpinteria, CA, USA) was utilized for the secondary antibody at room temperature for 30 min. After the tissue samples were washed in Tris-buffered saline for 10 min, 3, 3′-diaminobenzidine was administered as a chromogen, and Mayer's hematoxylin counterstain was applied.

Immunohistochemical assessment. The percentage and intensity of the immunoreactive tumor cells in each core was recorded and the final values of the positive tumor cells were assessed as the mean of the immunoreactivity in three cores. The presence of tumor tissue in at least two interpretable cores was required for the inclusion of a case in the statistical analysis. All of the slides were independently evaluated by an experienced pathologist (M.S.R.) who had no knowledge of any of the clinicopathologic data. Nuclear factor-kappa B (NF-κB) and VEGF immunoreactivities were defined as those evidencing a cytoplasmic staining pattern, and HIF-1α immunoreactivity was defined as a nuclear with/without cytoplasmic staining pattern of the lesional tissue, with a minimal background. The percentage scoring of the immunoreactive tumor cells was as follows: 0 (0%), 1 (1–10%), 2 (11–50%) and 3 (>50%). The staining intensity was scored visually and stratified as follows: 0 (negative), 1 (weak, if it was a blush), and 2 (strong, if it was obviously positive at ×20 magnification). A final score was obtained for each case by multiplying the percentage and the intensity score. Therefore, tumors with multiplied scores exceeding 4 (i.e. tumors with a strong intensity of >10% of the tumor cells) were regarded as having positive immunoreactivity to NF-κB, HIF-1α, and VEGF, respectively; all of the other scores were considered negative.

Statistical analysis. The associations between NF-κB, HIF-1α, and VEGF expression and the clinicopathologic parameters (sex, age, carcinoembryonic antigen [CEA], tumor size, histological grade, depth of bowel wall invasion, number of positive lymph node, vascular invasion) were assessed using an χ^2-test or Fisher's exact test. Disease-free survival was defined as the length of time from surgery to initial disease recurrence. Overall survival was defined as the length of time from surgery to death. The Kaplan–Meier method was utilized to construct curves for disease-free survival and overall survival. Data on patients who died without any evidence of disease recurrence were censored at the time of death for disease-free survival calculations. The log-rank test was employed to compare distributions. In order to identify the independent factors that were related significantly to patient prognosis, we utilized Cox's proportional hazard analysis via a stepwise procedure. All tests were two-sided, and *P*-values of <0.05 were considered statistically significant. Analyzes were conducted using SPSS version 14.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics. Of the 148 patients with stage III colorectal cancer, 78 (52.7%) were men, and the median age was 60 ± 11 years (range, 22–82 years). Seventy-five patients were diagnosed with colon cancer, and 73 were diagnosed with rectal cancer. All patients had adenocarcinoma, largely of histological grades 1 (53.4%) and 2 (40.5%). Only 10 patients harbored T2 lesions, and the number of positive lymph nodes was greater than four in 62 of the patients. All patients underwent surgical resection and 120 (81%) patients received 5-fluorouracil-based postoperative adjuvant chemotherapy or chemoradiation. The median follow-up duration was 53.2 months.

Expressions of NF-κB, HIF-1α, and VEGF. Among the 148 archival specimens, 70 (47.3%), 63 (42.6%), and 91 (61.5%) were positive for NF-κB, HIF-1α, and VEGF expression, respectively. The immunostaining pattern for NF-κB and VEGF was cytoplasmic, whereas the HIF-1α expression patterns in the tumor cells evidenced mixed nuclear/cytoplasmic staining (Fig. 1). Expression of VEGF was frequently detected at the invading edge of the tumor margin and at the periphery of necrotic regions with the tumor mass.

Correlations between NF-κB, HIF-1α, VEGF expressions, and clinicopathological parameters. Details of these relations are listed in Table 1. NF-κB expression was correlated significantly with vascular invasion ($P = 0.011$). No significant correlations were noted between NF-κB expression and sex, age, CEA, tumor size, histological grade, depth of bowel wall invasion, or number of positive lymph nodes. However, HIF-1α and VEGF expressions were not significantly associated with conventional clinicopathologic variables. As shown in Table 2, increased NF-κB protein expression was correlated with increased HIF-1α ($P < 0.001$) or VEGF expression ($P = 0.044$). Additionally, increased HIF-1α expression was also associated with high VEGF expression ($P = 0.005$).

Expressions of NF-κB, HIF-1α, and VEGF and clinical outcomes. Univariate analysis demonstrated that CEA ($P = 0.001$), number of positive lymph nodes ($P = 0.004$), and vascular invasion ($P = 0.005$) were all associated significantly

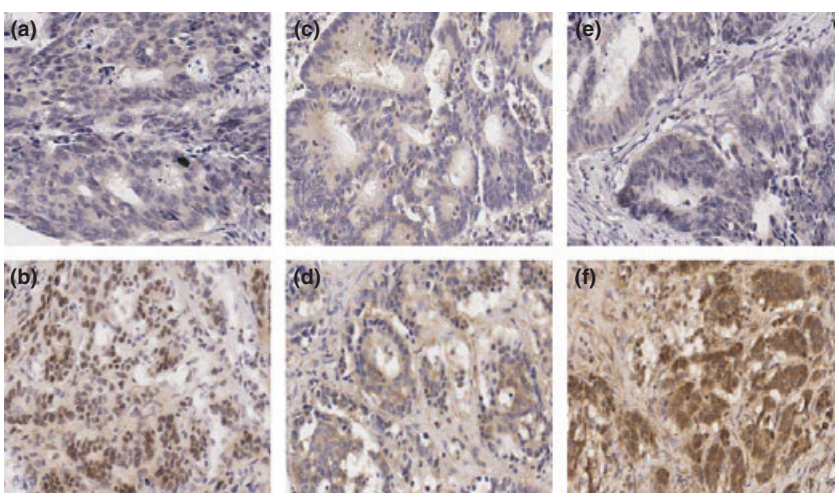

Fig. 1. Representative examples of immunohistochemical staining in colorectal cancer. Negative control of hypoxia-inducible factor-1α (HIF-1α) (a), nuclear staining of HIF-1α (b), negative control of nuclear factor-kappa B (NF-κB) (c), cytoplasmic staining of NF-κB (d), negative control of vascular endothelial growth factor (VEGF) (e), and cytoplasmic staining of VEGF (f). Original magnification: ×200.

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Table 1. Correlation between the expression of NF-κB, HIF-1α, VEGF, and clinicopathological parameters

	Total 148	NF-κB		*P*-value	HIF-1α		*P*-value	VEGF		*P*-value
		−	+		−	+		−	+	
T stage										
2	10	4	6	0.405	8	2	0.135	1	9	0.055
3	138	74	64		77	61		56	82	
Positive LN										
1–3	86	42	44	0.267	48	38	0.267	30	56	0.285
≥4	62	36	26		37	25		27	35	
Tumor size										
<5 cm	50	28	22	0.566	29	21	0.921	16	34	0.245
≥5 cm	98	50	48		56	42		41	57	
Vascular invasion										
+	47	32	15	0.011	28	19	0.719	21	26	0.293
−	101	46	55		57	44		36	65	
Grade										
1	79	42	37	0.969	49	30	0.309	28	51	0.605
2	60	31	29		30	30		26	34	
3	9	5	4		6	3		3	6	
CEA (ng/mL)										
<5	89	44	45	0.442	53	36	0.510	35	54	0.588
≥5	59	32	27		31	28		20	39	

CEA, carcinoembryonic antigen; HIF-1α, hypoxia-inducible factor-1α; LN, lymph node; NF-κB, nuclear factor-kappa B; VEGF, vascular endothelial growth factor.

Table 2. Correlation of NF-κB, HIF-1α, and VEGF expression

	NF-κB		*P*-value	HIF1-α		*P*-value
	(–) (*n* = 78)	(+) (*n* = 70)		(–) (*n* = 85)	(+) (*n* = 63)	
HIF-1α						
(–) (*n* = 85)	59	26	<0.001			
(+) (*n* = 63)	19	44				
VEGF						
(–) (*n* = 57)	36	21	0.044	41	16	0.005
(+) (*n* = 91)	42	49		44	47	

HIF-1α, hypoxia-inducible factor-1α; NF-κB, nuclear factor-kappa B; VEGF, vascular endothelial growth factor.

with 5-year disease-free survival. However, NF-κB, HIF-1α, or VEGF expressions were not associated with 5-year disease-free survival in any patients. When we examined 120 patients who received adjuvant chemotherapy, there was no difference of 5-year disease free survival according to NF-κB, HIF-1α, or VEGF expressions. Carcinoembryonic antigen (CEA) ($P = 0.003$), number of positive lymph nodes ($P = 0.040$), and vascular invasion ($P = 0.027$) were also significantly associated with 5-year overall survival. Patients with an NF-κB-expressing cancer had significantly worse 5-year overall survival rates than those without (55.8% *vs* 76.9%, $P = 0.012$; Fig. 2). On the other hand, HIF-1α and VEGF expression were not correlated with 5-year overall survival ($P = 0.264$, $P = 0.697$, respectively). During the follow-up period, 56 patients relapsed and received palliative chemotherapy. The response rate was significantly high in patients with NF-κB-negative expression (62.5% *vs* 18.8%, $P = 0.001$). Overall survival after palliative chemotherapy was much longer in patients with negative NF-kB expression, though this was statistically insignificant (19.5 *vs* 10.6 months, $P = 0.15$). To examine the independent prognostic value of NF-κB expression, we utilized a multivariate Cox

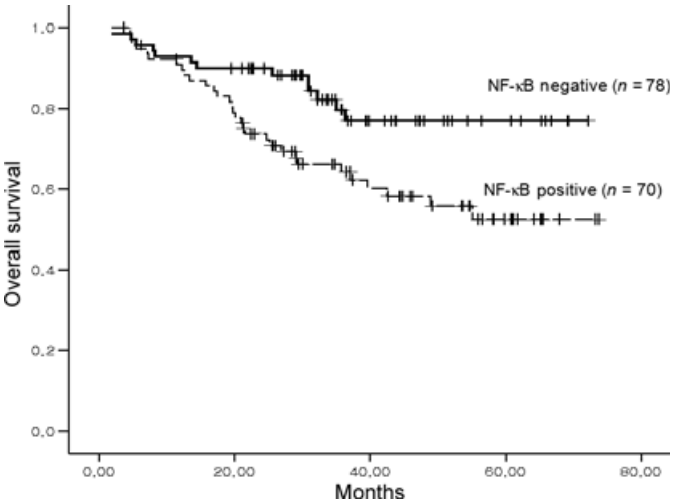

Fig. 2. Overall survival curve of patients with stage III colorectal cancer according to nuclear factor-kappa B (NF-κB) expression ($P = 0.012$).

proportional hazard analysis to control for other prognostic factors. Accordingly, CEA, vascular invasion, and number of positive lymph nodes were identified as independent prognostic factors of disease-free survival. Carcinoembryonic antigen (CEA) (hazard ratio = 2.503; 95% confidence interval [CI], 1.36–4.59; $P = 0.013$) and NF-κB expression (hazard ratio = 1.93; 95% CI, 1.00–4.71; $P = 0.049$) were identified as significant predictors of overall survival, after controlling for the other clinicopathological parameters (Table 3).

Discussion

The TNM staging systems are standard tools that are used to predict the likelihood of long-term survival in colorectal cancers (CRCs). However, conventional staging procedures may not be able to predict prognosis precisely, as some patients appear to exhibit a more aggressive progress than others at the identical stage. Therefore, many studies have been undertaken thus far to clarify the prognostic and predictive markers in patients with stage III CRCs.[16,17]

Neoangiogenesis is essential for tumor growth and metastases, as well as for the progression of invasive cancer. This is because cancer cell proliferation may outpace the rate of angiogenesis, thus inducing hypoxia.[7] Hypoxia-inducible factor-1α (HIF-1α) performs a critical function in the adaptation to hypoxia, and regulates the transcription of many genes relevant to key aspects of cancer pathogenesis.[18] Hypoxia-inducible factor-1α (HIF-1α) was overexpressed in multiple types of human cancer, including lung, prostate, breast, and colon carcinomas, even in preneoplastic lesions.[19] Hypoxia-inducible factor-1 (HIF-1) overexpression has been associated with radiation therapy and chemotherapy resistance, increased risk of invasion and metastasis, and poor clinical outcomes in cancers.[20]

There have been a few reports regarding HIF-1α protein expression in CRCs, but the results with regard to its association with tumor clinicopathological features or impact on patient prognosis have been inconclusive.[4–6] In this study, no significant correlations were detected between HIF-1α expression and clinicopathological parameters except for vascular invasion, and HIF-1α expression was not related to disease-free survival or overall survival. The lack of prognostic significance of HIF-1α may be attributable to an intricate relationship between HIF-1α and the mutation of tumor suppressor genes. In ovarian carcinoma, HIF-1α overexpression alone was of no prognostic value.

Table 3. Multivariate analysis of clinicopathological parameters

	Disease-free survival		Overall survival	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
NF-κB				
Negative *versus* positive	1.134 (0.659–1.956)	0.651	1.929 (1.004–4.706)	0.049
Vascular invasion				
Negative *versus* positive	1.754 (1.003–3.069)	0.049	1.681 (0.900–3.139)	0.103
CEA				
<5 ng/mL *versus* ≥5 ng/mL	2.365 (1.371–4.080)	0.002	2.503 (1.364–4.592)	0.003
Positive lymph node				
1–3 *versus* ≥4	2.007 (1.139–3.535)	0.016	1.590 (0.855–2.959)	0.143

CEA, carcinoembryonic antigen; CI, confidence interval; HR, hazard ratio; NF-κB, nuclear factor-kappa B.

However, the combination of HIF-1α protein overexpression with nonfunctional p53 is indicative of poor prognosis.[21] Further studies will be necessary to clarify this issue in CRCs.

Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis, both *in vitro* and *in vivo*, and VEGF expression is mediated by HIF-1α during hypoxia.[9] In our study, HIF-1α and VEGF expression were positively related. Tumor VEGF expression was shown to be a significant marker for tumor recurrence or reduced survival independent of conventional clinicopathological variables in several cancers.[22] However, the prognostic value of VEGF expression in CRCs remains unclear; some studies have suggested that tumor VEGF expression may be an independent prognostic factor for both disease-free[3] and overall survival in patients with CRCs,[3,4] whereas others have reported no such association.[23] In our observations, VEGF overexpression is not correlated with clinicopathological variables, and our findings do not appear to confirm the significant association between VEGF expression and unfavorable prognosis in CRCs. Our data may be influenced by the type of antibody, staining technique, and the scoring system of immunostaining. Additionally, our study population differs from those of previous studies, in that we observed only stage III CRC patients. These factors may contribute to the conflicting data regarding the prognostic value of VEGF expression in this study.

Nuclear factor-kappa B (NF-κB) is a transcription factor that has emerged as important to the development and progression of malignant cancers. Nuclear factor-kappa B (NF-κB) regulates genes that promote tumor cell proliferation, survival, metastasis, inflammation, invasion, and angiogenesis.[11] It has been previously reported that constitutive NF-κB activation is observed in CRCs, and it plays a key role in angiogenesis.[12] Additionally, a previous report asserted that increased NF-κB expression is correlated with angiogenesis in CRCs, and a significant association was noted to exist between NF-κB and VEGF.[14] Nuclear factor-kappa B (NF-κB) has also been reported to perform a pivotal role in hypoxia-induced HIF-1α mRNA expression.[24]

The results of our immunohistochemical studies have demonstrated that 47.3% of resected tumors demonstrated NF-κB positivity. A significant correlation was noted to exist between NF-κB, VEGF, and HIF-1α expression. However, we found no association between NF-κB staining and the clinicopathological parameters, with the exception of vascular invasion. The levels of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) in supernatant of NF-κB knockdown CRC cells showed a marked reduction relative to the wild type.[12] This suggests that other angiogenic chemokines were related to NF-κB. Patients with an NF-κB-expressing cancer had a significantly shorter 5-year overall survival time, but NF-κB was not related to disease-free survival. Constitutive NF-κB activation is associated with strong resistance to chemotherapy and radiotherapy.[25] Therefore, our results indicate that the activation of NF-κB in response to chemotherapy may constitute a principal mechanism of chemoresistance following tumor relapse. In order to determine whether or not NF-κB constituted an independent prognostic factor, we conducted a multivariate analysis. Cox proportional hazard models indicated that nuclear NF-κB staining was an independent predictor and portended a 1.9-fold increase in the 5-year risk of death.

In conclusion, we have demonstrated that NF-κB expression is associated with HIF-1α and VEGF expression in colorectal carcinoma. Our results show that NF-κB overexpression is significantly correlated with vascular invasion and poor overall survival.

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