NF-κB activity is downregulated by *KRAS* knockdown in SW620 cells via the RAS-ERK-IκBα pathway

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Abstract. The relationship between *KRAS* and NF-κB in colorectal cancer is not clear. Western blotting was used to determine whether *KRAS* knockdown in SW620 cells altered the levels of NF-κB-p65 and other molecules. Furthermore, we investigated the association between the *KRAS* status and NF-κB expression in 167 colorectal cancers tumor tissues and their correlation with overall survival (OS) of patients with *KRAS* mutations and activated NF-κB. RAS, p-ERK, p-IκBα and p65 expression was decreased in SW620 cells with *KRAS* knockdown. The MEK inhibitor U0126 downregulated p-ERK, p-IκBα and p65 levels in SW620 cells. p65 activation in tumors with *KRAS* mutations was higher (50.8%) than in tumors with the wild-type *KRAS* gene (30.6%) (P=0.012). Compared to patients with other types of tumors, OS was lower (median 28.4 months) in patients with *KRAS* mutations and NF-κB activation, vs. a median of 46.3 months in patients with other types of tumors (P=0.005). NF-κB activation was reduced in SW620 cells with KRAS knockdown, possibly via the RAS-ERK-IκBα pathway. The presence of both *KRAS* mutations and the active form of NF-κB in CRC tumors indicates poor patient prognosis.

Introduction

The oncogene *KRAS* is a commonly mutated gene in colorectal cancer (CRC), which occurs in approximately 35-40% of the tumors (1). It has been reported that CRC tumors with *KRAS* mutations show tolerance to chemotherapy and EGFR monoclonal antibodies (2-5) and treatment of *KRAS* mutation-containing CRC tumors require new strategies to replace or complement current therapies.

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Nuclear factor-κB (NF-κB) is a transcription factor that has emerged as an important player in the development and progression of malignant cancers. NF-κB targets genes that promote tumor cell proliferation, survival, metastasis, inflammation, invasion, angiogenesis and resistance to chemo- and radiotherapy (6). Constitutive or aberrant activation of NF-κB is frequently encountered in CRC (7-9). Therefore, the inhibition of NF-κB activation and its signaling pathway offers a potential strategy for cancer therapy (6,10,11).

Accumulating evidence has shown a strong relationship between *KRAS* mutation and the NF-κB signaling pathway. It is well established that NF-κB transcriptional activity can be activated through the ras-raf signaling pathway (12). Recently, it was shown that NF-κB activity was required to suppress apoptosis, promote abnormal cell proliferation and tumorigenesis which is induced by the ras oncogene (13-17). Barbie *et al* (18) used systematic RNA interference to detect synthetic lethal partners of oncogenic *KRAS* and found that the non-canonical IκB kinase TBK1 was essential in lung cancer cells that contain mutant *KRAS*. These results suggested that the NF-κB signaling pathway could be a promising therapy target for tumors with *KRAS* mutations.

In CRC, the study of this phenomenon is very limited and results are inconsistent. A study by Cadoret *et al* (19) suggested that Caco-2 cells, which had an inserted, functional Ha-ras oncogene, showed reduced constitutive NF-κB activity and p65 expression. In the present study, we assessed the possible relationship between *KRAS* and NF-κB in CRC. We show that *KRAS* knockdown led to reduced NF-κB transcriptional activity in colon cancer cells, which was possibly mediated through the RAS-ERK-IκBα-NF-κB signaling pathways. Moreover, we found that NF-κB activation was linked with *KRAS* mutations in primary colorectal cancers and patients whose tumors contained a *KRAS* mutation and in which NF-κB signaling was activated had a poor prognosis.

Materials and methods

Cell lines. The human colorectal carcinoma cell lines (SW620, HCT8, HCT116, HT29, Caco-2 and HCA-7) were maintained as instructed by the American Type Culture Collection. DLD-1 and LoVo were purchased from Institute of Cell Bank in China and were maintained according to their instructions.

According to the literature, *KRAS* mutations are present in the DLD-1, SW620, HCT8, HCT116, and LoVo cell lines and the wild-type *KRAS* gene is present in the Caco-2, HT29 and HCA-7 cell lines.

Reagents. JHS-23 (20), a selective inhibitor of nuclear translocation of NF-κB p65, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U0126 (21), an ERK1/2 inhibitor, was purchased from Cell Signaling Technology. Antibodies were obtained from Cell Signaling Technology;anti-NF-κB p65 #5970, anti-IκBα #4792, anti-phospho-IκBα (Ser32) #4792, anti-phospho-IκBβ (Thr19/Ser23) #4793, anti-IKKβ #3551, anti-phospho-IKKα/β (Ser176/180) #1147, anti-PARP #1442, anti-p38 MAPK #9212, anti-phospho-p38 MAPK (Thr180/Thr182) #4511, anti-P44/42 MAPK #4695, anti-phospho-p44/42 MAPK (Thr202/Thr204) #4370, anti-Akt #4685, anti-phospho-Akt (Ser473) #4060, anti-PI3K #4292, anti-STAT5 #9363, anti-β-actin #4967, anti-Ras #3915), anti-IκBβ (sc-74451), and anti-IKKα (sc-130153) were from Santa Cruz Biotechnology.

KRAS siRNA. The KRAS siRNA kit, negative control siRNA and positive MAPK control siRNA were purchased from Qiagen (no. 301799). DLD-1, SW620, LoVo, HCT116 and HCT8 cells were transfected with siRNAs according to the manufacturer's protocol. Transfection efficiency was checked using a MAPK siRNA control.

Establishment of stable KRAS knockdown SW620 colon cancer cells. KRAS shRNA lentiviral particles (sc-35731-v) and control shRNA lentiviral particles (sc-108080) were purchased from Santa Cruz Biotechnology. To establish stable knockdown cell lines, we transfected shRNA lentiviral particles into SW620 colon cancer cells, and cultured the cells in the presence of 2.5 μg/ml puromycin. Cells were characterized for KRAS knockdown by western blot analysis.

Cell viability assay. Cells (SW620, HCT8, HCT116, HT29, Caco-2, DLD-1, LoVo and HCA-7) were seeded at a density of 5,000 cells/cm^2 in 96-well plates and incubated in 100 μl fresh medium per well containing various concentrations of JSH-23. Forty-eight hours later, the number of viable cells in each well was then determined after treatment using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation assay (Promega, Madison WI). Briefly, 20 μl of Cell Titer 96 AQ_{ueous} One Solution were added to each well, including three wells containing only medium for background subtraction. The cells were then incubated at 37˚C for 30 min. The absorbance at 490 nm was then determined using a SpectraMax 340 plate reader/spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). Each combination of cell line and drug concentration was set up in three replicate wells, and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of untreated controls. Results are presented as mean ± standard deviation (SD).

Cells (SW620, HCT8, HCT116, DLD-1 and LoVo) were transfected with KRAS siRNAs after 72 h, and the number of viable cells in each well was determined. Each cell line was set up in three replicate wells, and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of untransfected controls. Results are presented as mean ± SD. Cell lines that do or do not require K-Ras to maintain viability were called '*KRAS*-dependent' and '*KRAS*-independent', respectively.

Total cellular protein extraction. Cells in 10-cm dishes were washed with cold PBS and harvested by scraping following addition of lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 mM dithiothreitol, and a protease inhibitor cocktail from Roche) for 15 min on ice. After centrifugation (13,000 rpm, 4˚C, 10 min), the supernatants were collected and frozen (-70˚C) until use.

Nuclear-cytoplasmic fractionation. Cells in 10-cm dishes were washed with cold PBS and harvested by scraping following addition of lysis buffer A (20 mM HEPES pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, and a proteinase inhibitor cocktail). Lysis was completed on ice for 10 min. Supernatants containing the cytoplasmic fractions were collected after centrifugation (2,000 rpm, 4˚C, 5 min). The pellets were washed three times in lysis buffer A and then lysed in buffer B (20 mM HEPES pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, and a proteinase inhibitor cocktail from Roche) for 30 min on ice. After centrifugation (13,000 rpm, 4˚C, 15 min), the recovered supernatants containing the nuclear lysates were collected and frozen (-70˚C) until use.

Western blotting. For western blot analysis, 25 μg of proteins were analyzed for each sample on an 8% or 10% of SDS-PAGE, gel transferred onto 0.2 μm nitrocellulose membranes, blocked with 3% bovine serum albumin and incubated with primary antibodies overnight at 4˚C followed by appropriate species-specific secondary antibodies. Following rigorous washes with Tris-buffered saline and Tween, and the membranes were developed by chemiluminescence.

Patient selection. The study retrospectively enrolled patients with histologically proven colorectal cancer and confirmed *KRAS* status from January 2006 to December 2008. Key inclusion criteria were the availability of a follow-up for at least 24 months from the diagnosis of primary tumors. Exclusion criteria were the lack of sufficient tissue available for IHC. The study protocol was approved by the Ethics Committee of the Fujian Provincial Cancer Hospital. Informed written consent was obtained from the patients according to the Declaration of Helsinki.

NF-κB immunohistochemistry. NF-κB was evaluated on 3-5 mm thick tissue sections obtained from paraffin-embedded specimens fixed in 10% formalin. The sections were deparafinized, rehydrated and treated with 0.3% H_2O_2 in methanol at room temperature for 30 min. Sections were incubated with anti-NF-κB p65 monoclonal antibody for 1 h at room temperature. Incubation with the secondary antibody was performed for 30 min, followed by application of diaminobenzidine chromogen for 5 min. Subsequently, the slides were counterstained with Meyer's hematoxylin for 1 min, dehydrated in a

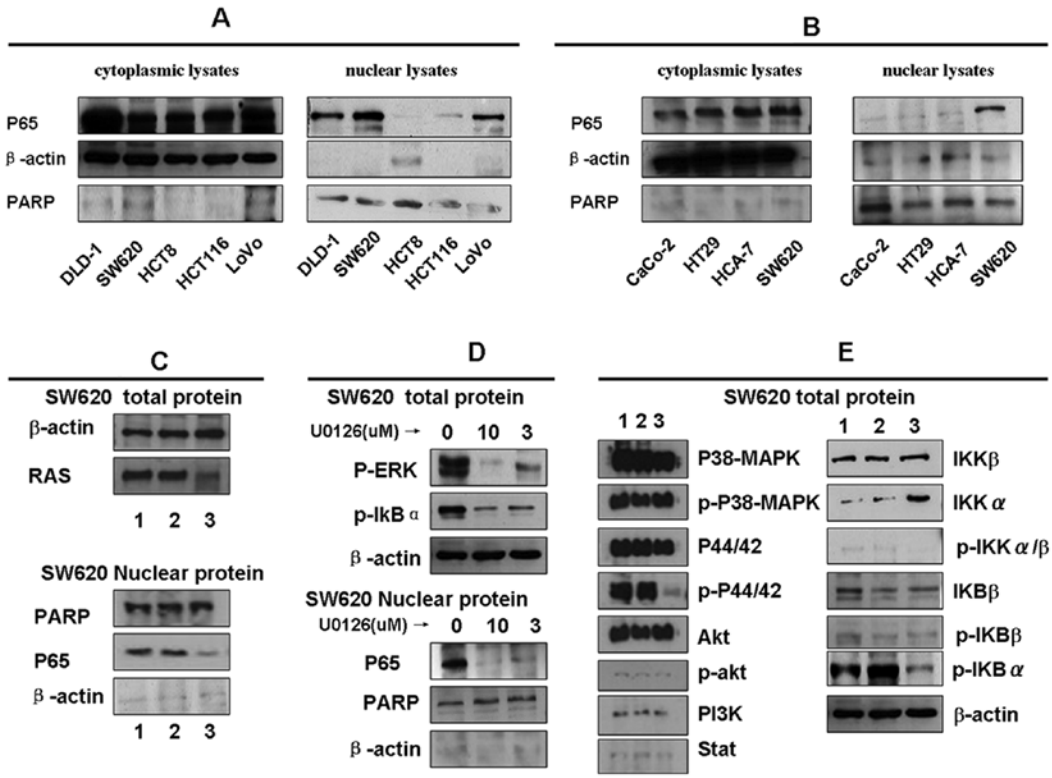

Figure 1. (A and B) Constitutive activation of NF-κB in colon cancer cells. Cytoplasmic and nuclear lysates were analyzed by western blot analysis. β-actin (cytoplasmic) and PARP (nuclear) were used to determine purity. (C) Decreased NF-κB activation in KRAS knockdown SW620 cells. SW620 cells were transfected by *KRAS* shRNA or control shRNA lentiviral particles. RAS (total cellular lysates) and p65 (nuclear lysates) were analyzed by western blot analysis. β-actin (cytoplasmic) and PARP (nuclear) were used as a loading control. Line 1: negative control, Line 2: control shRNA, Line 3: KRAS shRNA. (D) U0126 downregulated nuclear p65 and phosphorylation of ERK, and IκB in SW620 cells. Cells were treated with 3 or 10 μM U0126 and collected after 30 min, followed by immunoblotting using anti-p65, anti-phospho-ERK or -IκB antibodies. β-actin (cytoplasmic) and PARP (nuclear) were used as a loading control. (E) Decreased NF-κB activation in *KRAS* knock-down SW620 cells probably through the RAS-ERK-IκBα pathway. SW620 cells were transfected by KRAS shRNA or control shRNA lentiviral particles. Cell extracts were immunoblotted to detect the indicated protein species. Line 1: negative control, Line 2: control shRNA, Line 3: KRAS shRNA.

graded series of alcohols, treated with xylene, and covered with a coverslip. NF-κB detected expression was detected as nuclear and cytoplasmic brown staining of neoplastic cells. Positive tumor expression of NF-κB as only distinct nuclear immunostaining, was considered as activated NF-κB. The lymphocytes within the tissue sections which showed positive nuclear staining were used as positive internal controls. As a negative control, non-immune buffer was substituted for the primary antibody.

Evaluation of KRAS mutation. The *KRAS* mutation status was analyzed by PCR and by sequencing analysis as described in a previous report (22).

Statistical analysis. The association between NF-κB expression and *KRAS* mutation status or other clinical and pathological features was estimated by the Fisher's exact test or one-way ANOVA. The overall survival (OS) time was calculated as the period from the date of diagnosis until death from any cause or until the last follow-up. Univariate analysis of overall survival was estimated according to the Kaplan-Meier method and analyzed by the log-rank test. The Cox proportional hazards model was applied to the multivariate survival analysis in a step-down fashion, adjusted for those variables with a P-value cut-off of 0.10 at univariate analysis. Hazard ratios and 95% confidence intervals (CIs) were estimated by use of a stratified Cox regression analysis. Statistical analysis was performed with SPSS software version 13.0 for Windows. A two-sided P-value <0.05 was considered statistically significant.

Results

Constitutive activation of NF-κB was found in colon cancer cells with KRAS mutation and KRAS dependence. We examined by western blot analysis whether NF-κB was constitutively active in colorectal carcinoma cells with different *KRAS* status; *KRAS* mutations were observed in the DLD-1, SW620, HCT8, HCT116, and LoVo cell lines and the wild-type *KRAS* gene was found in the Caco-2, HT29 and HCA-7 cell lines. Constitutive NF-κB activation was observed in 3/8 cell lines (DLD-1, LoVo and SW620) all of which had mutated *KRAS* genes; among them SW620 showed relatively strong NF-κB activity (Fig. 1A and B). We then used *KRAS* siRNA to deplete *KRAS* in colorectal cancer cell lines harboring *KRAS* mutations to identify which cancer cell lines required *KRAS* to maintain viability. It was found that cell proliferation were significantly inhibited in SW620, DLD-1 and LoVo cells, whereas the suppression of *KRAS* had no effect on the proliferation of HCT116 or HCT8 cells (Fig. 2). These observations indicated that colon cancer cell lines with constitutive NF-κB

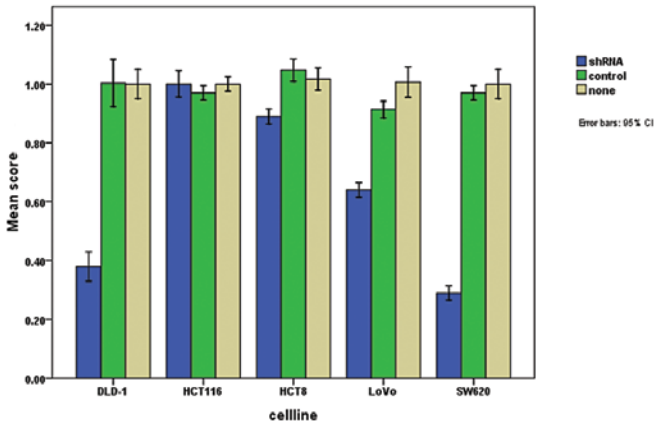

Figure 2. Constitutive activation of NF-κB in colon cancer cells with *KRAS* mutation and *KRAS* dependence. Viability of *KRAS* mutant cells by *KRAS* siRNA and control siRNA transfected cells. Percentage of cell growth is shown relative to untransfected cells.

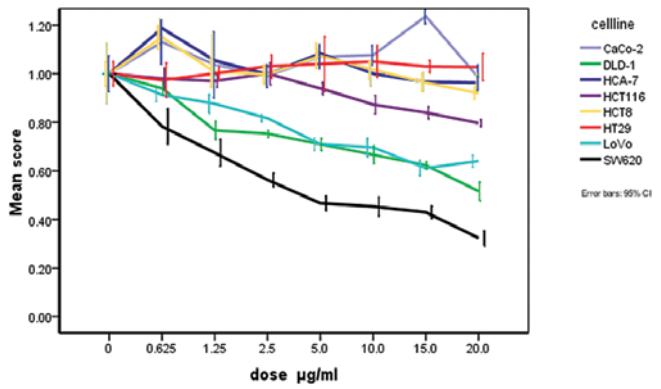

Figure 3. Colon cancer cells with constitutive NF-κB activation were sensitive to JSH-23. Cells were treated with JSH-23 at the indicated concentrations and viable cells were measured after 48 h of treatment. Percentage of cell growth is shown relative to untreated controls.

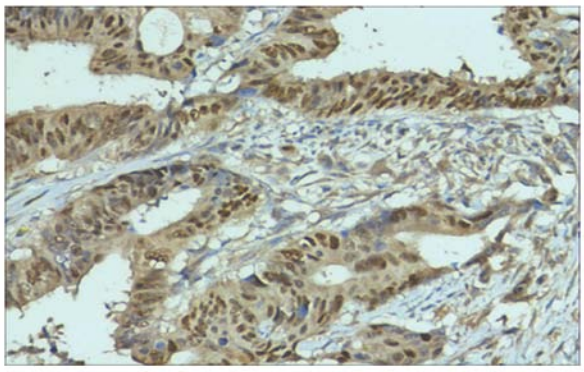

Figure 4. Immunohistochemical positive staining for the nuclear factor-κB subunit p65 in primary colorectal cancer. Positivity of the tumor for NF-κB expression was defined as distinct nuclear immunostaining.

Table I. Correlation between nuclear NF-κB immunohistochemical expression and *KRAS* status in colorectal cancer.

		NF-κB expression		
		Negative	Positive	
	Total	N %	N (%)	P-value
KRAS				
Wild	108 (64.7)	75 (69.4)	33 (30.6)	0.012[a]
Mutation	59 (35.3)	29 (49.2)	30 (50.8)	
G12D	28 (16.7)	9 (32.1)	19 (67.9)	0.029[a]
G12V	9 (5.4)	4 (44.4)	5 (55.6)	
G13D	19 (11.4)	14 (73.7)	5 (26.3)	
Others	3 (1.8)	2 (66.7)	1 (33.3)	

KRAS mutation, PCR and sequencing analysis. NF-κB p65, immunohistochemistry analysis.

activation may require *KRAS*. We then treated the cells with the NF-κB inhibitor JSH-23 to test whether JSH-23 had an effect on the proliferation of colorectal carcinoma cells. As shown in Fig. 3, JSH-23 significantly decreased the proliferation of SW620, DLD-1 and LoVo cell lines, but had no effect on other cell lines.

Decreased NF-κB activation in KRAS knockdown cells probably through the RAS-ERK-IκBα pathway. To determine the effect of *KRAS* knockdown on NF-κB activity, SW620 cells, that show relatively strong NF-κB activity, were transfected with *KRAS* shRNA lentiviral particles. To monitor *KRAS* gene knockdown, RAS was detected by western blotting. As shown in Fig. 1C, markedly decreased RAS was observed following shRNA mediated knockdown of *KRAS*. NF-κB p65 nuclear localization was also decreased in SW620 cells transfected with *KRAS* shRNA lentiviral particles.

Since RAS can activate the kinase RAF, the mitogen-activated ERK1 and ERK2, PI3K, and many other proteins to promote cell proliferation, we performed western blot analysis to investigate the possible signaling pathways associated with reduced NF-κB activity. As shown in Fig. 1E, western blot analysis using antibodies specific for ERK1/2 (p44/42), p38 and their phosphorylated forms showed that phosphorylated ERK1/2 was nearly diminished in *KRAS* knockdown cells. However, p38 was shown to be constitutively phosphorylated in this cell line and slightly affected by *KRAS* knockdown. In addition, no significant changes in the expression and phosphorylation of Akt were found in SW620/KRAS shRNA cells. The expression of other molecules, such as PI3K and Stat, were very weak in SW620 cells.

In our study, phosphorylated IκBα (at serine 32), which is an excellent marker of NF-κB activation, was decreased in SW620 cells after *KRAS* knockdown. Other key signaling molecules in the NF-κB signaling pathways including IKKα, IKKβ, IκBβ and their phosphorylated forms were not affected by *KRAS* knockdown. These findings indicate that reducing NF-κB activation by *KRAS* knockdown probably involved RAS, ERK and IκBα, which are independent of IKK (Fig. 1E).

U0126, which inhibits ERK1/2, was used to investigate the role of the ERK1/2 pathway in constitutive NF-κB activation in SW620 cells. As shown in Fig. 1D, U0126 at a 3 or 10 μM

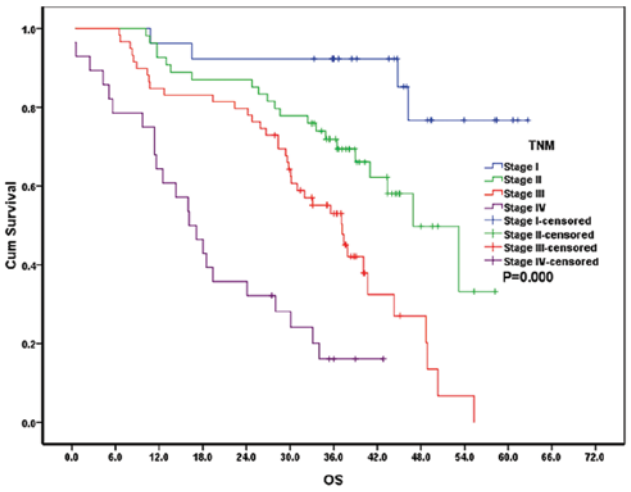

Figure 5. TNM stage influences on oversll survival.

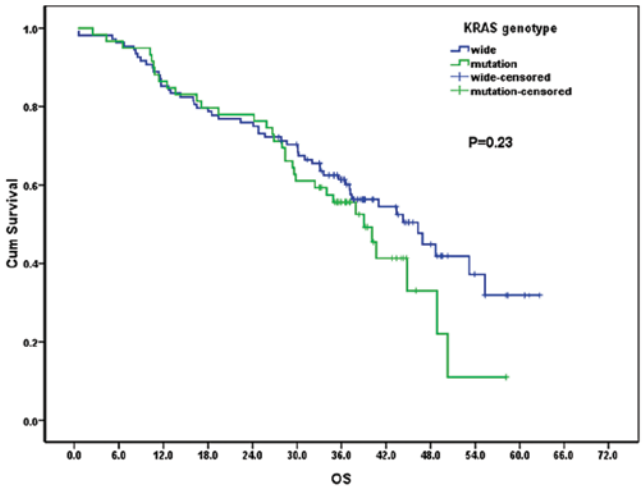

Figure 6. The *KRAS* genotype does not influence overall survival (OS). No significant differences were observed in overall survival among patients with different *KRAS* status. The median OS was 39.0 months (95% CI, 33.3-44.7) in patients with *KRAS* mutation, 46.3 months (95% CI, 36.8-55.8) in patients with wild-type *KRAS*, P=0.23.

concentration almost completely blocked ERK1/2 phosphorylation and nuclear expression of p65; implying that constitutive NF-κB activation in SW620 cells was also dependent on ERK1/2. U0126 also inhibited the phosphorylation of IκBα, suggesting that ERK1/2 also exerted an effect on IκBα or its upstream signaling components.

NF-κB p65 immunohistochemical expression was linked with KRAS status in colorectal cancer. KRAS status was determined in 224 CRC patients seen in the hospital from January 2006 to December 2008 and 167 patients were eligible for our analysis. Immunohistochemistry for NF-κB and *KRAS* mutation analysis was performed in primary tumors in all patients. NF-κB nuclear expression was positive in tumor samples from 63 patients (37.7%) and negative in the remaining 104 patients (62.3%). *KRAS* mutations were detected in tumor samples from 59 patients (35.3%). The remaining 108 patients (64.7%) were negative for a *KRAS* mutation. NF-κB p65 expression in

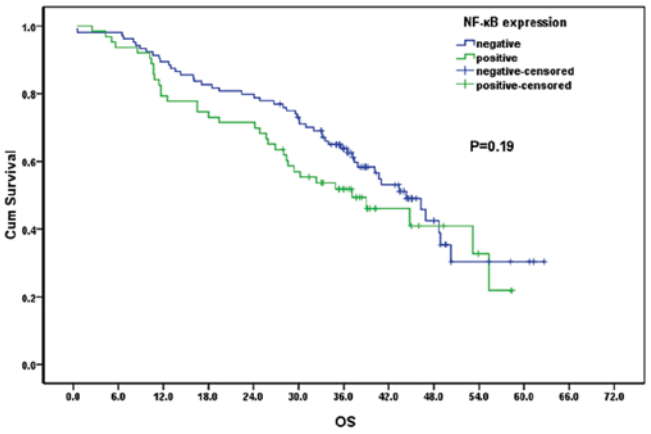

Figure 7. NF-κB expression does not influence overall survival (OS). No significant differences in OS among patients with different NF-κB expression levels were observed. Median OS was 37.1 months (95% CI, 24.1-50.0) in patients with positive NF-κB expression, 44.3 months (95% CI, 38.9-49.7) in patients with negative NF-κB expression (P=0.19).

Table II. TNM stage and $KRAS^{+}NF\kappa B^{+}$ type are independent prognostic factors for OS.

	RR	95% CI	P-value
TNM stage			<0.001
IV	1		
I	0.033	0.011-0.102	
II	0.134	0.071-0.256	
III	0.332	0.193-0.572	
KRAS/NF-κB			<0.001
$KRAS^{+}NF\kappa B^{+}$	1		
Others	0.259	0.125-0.538	

CI; confidential interval; RR, relative risk.

KRAS mutated cancers were much higher than in tumors with the *KRAS* wild-type gene, 50.8 vs. 30.6% (P=0.012). Fig. 4 and Table I show that a significantly greater percentage of tumors with G12D and G12V *KRAS* mutations were positive for NF-κB expression, 67.9 and 55.6%, respectively, compared to 26.3% tumors with the G13D mutation (P=0.029). One of our aims was to explore clinical and pathological parameters of colorectal cancer that were related with *KRAS* mutations and NF-κB activation. Thirty patients (18.0%) showed both NF-κB expression and *KRAS* mutation, and these patients are referred to as $KRAS^{+}NF\text{-}\kappa B^{+}$. However, there was no difference in the clinical or pathological features between the $KRAS^{+}NF\text{-}\kappa B^{+}$ type and other types of tumors (data not shown).

KRAS mutation and NF-κB expression indicate a poor prognosis in colorectal cancer. There were 86 deaths by September 1, 2011. Median follow-up time was 35.4 months. In univariate analysis, survival parameters were influenced by the TNM stage, while other tested variables such as gender, age at diagnosis, smoking history, tumor location, histology, *KRAS* status and NF-κB expression did not reach a statistically significant

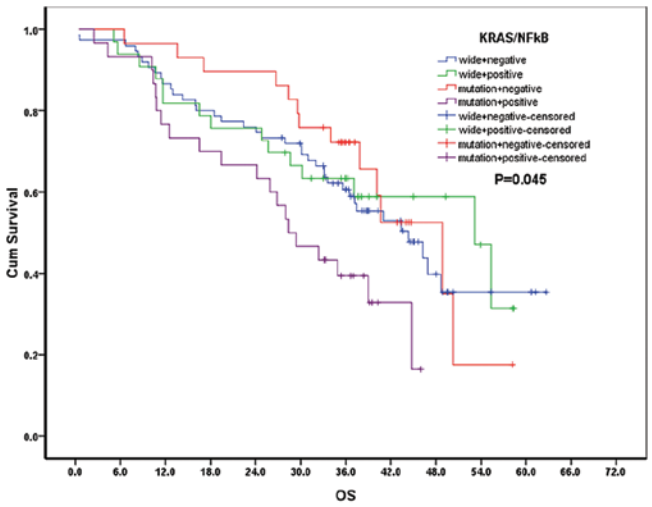

Figure 8. The influence of different combinations of *KRAS* status and NF-κB IHC expression on overall survival (OS). Patients were divided into four groups depending on the combination of *KRAS* status and NF-κB expression $KRAS^+NF\text{-}\kappa B^+$, $KRAS^+NF\text{-}\kappa B^-$, $KRAS^-NF\text{-}\kappa B^+$, and $KRAS^-NF\text{-}\kappa B^-$. The median OS and 95% CI were: 28.4 months (21.0-35.8), 48.9 months (37.3-60.5), 53.2 months (36.7-69.7) and 44.3 months (34.4-54.2), respectively (P=0.045). There was no significant difference in overall survival among patients with $KRAS^+NF\text{-}\kappa B^-$, $KRAS^-NF\text{-}\kappa B^+$, and $KRAS^-NF\text{-}\kappa B^-$.

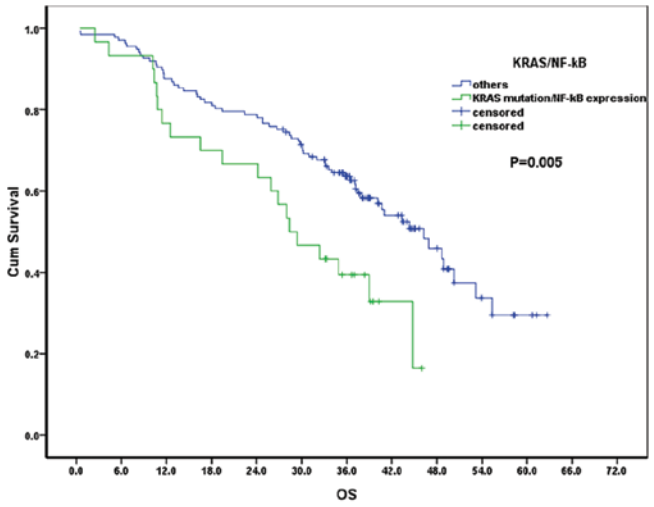

Figure 9. The influence of different combinations of *KRAS* status and NF-κB IHC expression on overall survival (OS). Cancer patients with $KRAS^+NF\text{-}\kappa B^-$, $KRAS^-NF\text{-}\kappa B^+$, $KRAS^-NF\text{-}\kappa B^-$ were grouped together and are indicated as 'others' vs. $KRAS^+NF\text{-}\kappa B^+$ patients. Comparing the two groups we found a statistically significant longer median OS of 46.3 months (95% CI, 39.4-53.2) in the 'others' group vs. 28.4 months (95% CI, 21.0-35.8) in the $KRAS^+NF\text{-}\kappa B^+$ patients (P=0.005).

level by univariate analysis (Figs. 5-7). However, different combinations of *KRAS* status and NF-κB expression showed significant effects on overall survival. In fact, compared with survival in patients with $KRAS^+NF\text{-}\kappa B^+$ tumors, in which the OS was 28.4 months (95% CI, 21.0-35.8), the OS rate was significantly higher (P<0.0001) in patients with other types of tumors (46.3 months; 95% CI, 39.4-53.2) (Figs. 8 and 9). Multivariate analysis included variables such as TNM stage and combinations of *KRAS* and the NF-κB type. In the Cox regression model, TNM stage and $KRAS^+NF\text{-}\kappa B^+$ tumors were confirmed to be an independent prognostic factors. Compared with $KRAS^+NF\text{-}\kappa B^+$ tumors, other types of tumors were associated with longer OS; relative risk (RR) 0.259; 95% CI, 0.125-0.538 P<0.0001. (Table II).

Discussion

This study led to three major conclusions. The first major conclusion of our study is that NF-κB activation is linked to the *KRAS* status in colorectal cancer and NF-κB activation was decreased in *KRAS* knockdown SW620 cells. So far, only one study has addressed *KRAS* mutations that showed an effect on NF-κB activation in colorectal cancer (19). This report demonstrated that p65 expression and NF-κB activity was downregulated in Caco-2 cells after an activated Val-12 human Ha-ras gene transfection. Conversely, we found that constitutive NF-κB activation was downregulated in *KRAS* knockdown SW620 cells. Moreover, in this study, we showed that the frequency of active NF-κB expression in patients with *KRAS* mutations were significantly higher than in patients with wild-type *KRAS*, 50.8 vs. 30.6% (P=.012), which is consistent

Table III. *KRAS* genotype, p53 genotype and NF-κB activation in 8 colon cancer cell lines.

	KRAS genotype	*KRAS* dependence	NF-κB activity	p53 genotype
HCT116	M	-	-	W
HCT8	M	-	-	W
SW620	M	+	+	M
LoVo	M	+	+	W
DLD-1	M	+	+	M
Caco-2	W	N	-	W
HT29	W	N	-	M
HCA-7	W	N	-	M

M, mutation; W, wild; N, not done.

with a previous report that demonstrated a trend between nuclear NF-κB expression and *KRAS* mutations in patients with colorectal cancer (23).

Some possible explanations responsible for these contrary findings are discussed. First, our results showed that constitutive NF-κB activation was not found in all *KRAS* mutation-containing cells, but only in cells with *KRAS* mutations in which cell viability was *KRAS*-dependent. Cancer cell lines harboring *KRAS* mutations can be broadly classified into the *KRAS*-dependent and the *KRAS*-independent groups. Comparing these two classes of cancer cells, *KRAS*-dependent cells were associated with a well-differentiated epithelial phenotype, and there are gene expression signature differences between *KRAS*-dependent and *KRAS*-independent cancer cells (24).

Recently, it was shown that suppression of TBK1 induc-esapoptosis in human lung cancer cell lines that depends on oncogenic *KRAS* expression (18). In addition, Myelan *et al* reported that NF-κB was activated with both loss of wild-type p53 and gain of *KRAS* mutations in lung cancer cells (17). Based on these reports, we searched the literature for the p53 gene status of the eight colon cancer cell lines (Table III). To our surprise, it seemed that constitutive NF-κB activation was found in cell lines with both *KRAS* and p53 mutations. Taken together, these findings indicate that the differences in NF-κB activation could be attributed to its interaction with *KRAS* under some conditions, which should be further examined.

The second major conclusion of this study is that reduced NF-κB activation by *KRAS* knockdown was dependent on ERK and IκBα, which is possibly IKK-independent. The phosphorylation of a specific serine residue, Ser32/36, in IκBα by the upstream regulators such as IKK, results in polyubiquitination and subsequent degradation by 26S proteasomes, causing release of the NF-κB dimer and promoting its translocation to the nucleus, activating various κB-responsive genes (25). In our assays, phosphorylated IκBα (at serine 32) was decreased in *KRAS* knockdown SW620 cells. We also found that *KRAS* knockdown led to reduced NF-κB activation without an effect on activating IKKα/β. We cannot completely rule out the possibility that IKKα and IKKβ activation was too weak to be detected in our assays. Our results suggested that a kinase other than IKKα or IKKβ may phosphorylate IκBα and thereby activate NF-κB. Recently, evidence showed that NF-κB activation was involved with phosphorylated ERK (26-30). The data are consistent with a recent study which suggested that ERK had a role in NF-κB activation. The exact role for ERK in the NF-κB pathway still remains to be fully clarified.

The third major conclusion of this study is that colorectal cancer patients with *KRAS* mutation and NF-κB expression had a poor prognosis. Evidence shows that colorectal cancer with *KRAS* mutations or NF-κB expression is tolerance to chemotherapy and EGFR monoclonal antibodies (2-5,31). Therefore, it suggests that patients with both NF-κB expression and *KRAS* mutations would be highly resistant to current therapeutic strategies. This subtype of patients should receive more attention. Until now, there is no complete agreement on the prognostic role of either *KRAS* mutations or NF-κB expression (32,33). In addition, previous studies have not examined the outcome of patients with CRC who harbor both *KRAS* mutations and NF-κB expression. In our study, neither *KRAS* mutation nor NF-κB activation was validated as an independent poor prognostic factor. However, different combinations of *KRAS* status and NF-κB expression showed an effect on overall survival. Patients with $KRAS^{+}NF\text{-}\kappa B^{+}$ type tumors showed a statistically significant decrease in survival compared with patients having other types of tumors. This analysis was limited by the retrospective design of the study and by the small sample size of patients in the $KRAS^{+}NF\text{-}\kappa B^{+}$ cohorts. Based on the above observations, it is hypothesized that $KRAS^{+}NF\text{-}\kappa B^{+}$ may represent a unique subtype of CRC.

In summary, this study demonstrated that NF-κB activation was reduced in SW620 cells with *KRAS* knockdown and that this effect may involve the RAS-ERK-IκBα pathway. The presence of both a *KRAS* mutation and the activation of NF-κB in a colorectal cancer tissue specimen indicates a poor prognosis for the patient.

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