IκBKβ and *NFκB1*, NSAID use and risk of colorectal cancer in the Colon Cancer Family Registry

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The NFκB-signaling pathway regulates cell proliferation and inflammation. Activation of the pathway is implicated in the etiology of colorectal cancer (CRC). NSAIDs may reduce CRC risk partially through a nuclear factor-kappa B (NFκB)-dependent pathway. In this study, we investigated associations between 34 *NFκB1* and 8 *IκBKβ* tagSNPs and CRC risk and examined interactions with non-steroidal anti-inflammatory drug (NSAID) use. Using conditional logistic regression, we investigated these associations among 1584 incident CRC cases and 2516 sibling controls from the Colon Cancer Family Registry. Three *IκBKβ* SNPs were associated with a statistically significant lower colorectal or colon cancer risk: rs9694958 (A>G intron 5) (colorectal: OR_{hzv} = 0.26(0.07–0.99), P_{trend} = 0.048, P_{adj} = 0.25), rs10958713 (A>C intron 19) (colon: OR_{hzv} = 0.62(0.42–0.92), P_{trend} = 0.005, P_{adj} = 0.03) and rs5029748 (C>A intron 2) (colon: OR_{het} = 0.72(0.56–0.91), P_{trend} = 0.01, P_{adj} = 0.08). We replicated trends associated with *NFκB1* and *IκBKβ* variants identified in a previous study (rs4648110 (T>A intron 22), rs13117745 (G>A intron 5) and rs3747811 (T>A intron 1)). *IκBKβ*'s rs6474387 (C>T intron 20) and rs11986055 (A>C intron 2) showed substantially lower colon cancer risk among current NSAID users ($P_{interaction}$ = 0.01 and $P_{interaction}$ = 0.045, respectively), whereas *NFκB1*'s rs230490 (G>A 5′ (outside UTR)) and rs997476 (C>A 3′ (outside UTR)) showed higher CRC risk among current NSAID users ($P_{interaction}$ = 0.01 and $P_{interaction}$ = 0.03, respectively). These findings suggest that variants in *NFκB1* and *IκBKβ* are associated with CRC risk and NSAIDs may function partially through an NFκB-dependent pathway. The SNPs identified here should be considered for future functional studies and may be useful in designing a pharmacogenetic approach to preventive NSAID use.

Introduction

Results from epidemiologic and experimental studies have pointed to chronic inflammation as an important cause of colorectal cancer (CRC) (1–3). The exact mechanism(s) through which inflammation contributes to CRC etiology and the extent to which inflammation differentially impacts colon and rectal cancer etiology are still being investigated (4); however, growing evidence suggests that one relevant mechanism is the nuclear factor-kappa B (NFκB)-signaling pathway (5). The NFκB signaling pathway regulates cell proliferation, apoptosis and inflammatory responses, through activation of an ubiquitous transcription factor, NFκB (5). Constitutive activation of NFκB has been observed in 40% of CRC tissues and in 67% of CRC cell lines (6).

NFκB is found as a homo- or heterodimeric complex (7). The most commonly studied NFκB complex is the RelA:p50 heterodimer (8). In the cytoplasm, RelA:p50 is normally bound to an inhibitory protein, IκBα, rendering the transcription factor inactive. In response to a wide array of stimuli, including proinflammatory cytokines and chemokinase, the IκB kinase (IKK) complex phosphorylates IκBα, causing degradation of IκBα and activation of NFκB. Once activated, NFκB translocates to the nucleus where it binds to the promoter region of its target genes (5,7). The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, with IKKβ required for activation of the RelA:p50 dimer (5). The *NFκB1* gene encodes p50 and the *IκBKβ* gene encodes IKKβ. Initial studies have suggested that genetic variability in *NFκB1* and *IκBKβ* is associated with CRC risk (9,10) and that genetic variability in *IκBKβ* is associated with CRC survival (11).

Several studies, including randomized trials, have shown that the regular use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with decreased CRC incidence and mortality (12–14). Reduction in inflammation by NSAIDs is generally attributed to inhibition of prostaglandin synthesis (3,15), but there is evidence that several NSAIDs, including aspirin, also inhibit NFκB activation (16,17) and induce CRC cell apoptosis through an NFκB-dependent mechanism (18–20). Thus, it is biologically plausible that variation in NFκB signaling pathway genes, including *NFκB1* and *IκBKβ*, modify the inverse association between NSAID use and CRC risk.

We have shown previously that genetic variability in prostaglandin synthesis and inflammation can influence risk of CRC (21–26). The purpose of this study was to investigate the association between genetic variability in *NFκB1* and *IκBKβ* and risk of CRC and to investigate the interaction between genetic variability in *NFκB1* and *IκBKβ* and NSAIDs on the risk of CRC using a case–unaffected sibling control design in the Colon Cancer Family Registry (CCFR).

Materials and methods

Study population and questionnaire data

The study population has been described previously (27). Briefly, CRC cases were recruited for the CCFR from six registry centers. The CCFR cases were probands and affected relatives diagnosed with primary invasive CRC from 1998 to 2002 who were interviewed within 5 years of diagnosis. Controls were siblings without a CRC diagnosis at the time of ascertainment. Although eligibility requirements varied slightly, registry centers typically required participants to be between the ages of 20 and 74 (27).

Standard questionnaires were used to collect epidemiologic data from CCFR study participants regarding demographic characteristics, medical history, NSAID use, family history of cancer, smoking history, diet, physical activity, height and weight. Regular NSAID use was defined as use two or more times per week for at least 1 month. Current NSAID use was defined as regular use in the 2 years prior to study enrollment. Blood and tissue samples were collected according to standardized procedures. Individuals were excluded from this study if the case did not have at least one matched unaffected sibling as a control and if an individual's sex determined by genotyping did not match reported sex on the questionnaire. Only self-reported Caucasian study participants, ascertained through population-based recruitment, were included in this analyses (sample sizes among other racial/ethnic groups and clinic-based populations were too small to assess associations).

Informed consent was obtained from all participants. The Institutional Review Boards at each CCFR site approved the study.

Abbreviations: BMI, body mass index; CCFR, Colon Cancer Family Registry; CI, confidence interval; CRC, colorectal cancer; FDR, false discovery rate; IKK, IκB kinase; LD, linkage disequilibrium; MAF, minor allele frequency; NFκB, nuclear factor-kappa B; NSAID, non-steroidal anti-inflammatory drugs; OR, odds ratio; UTR, untranslated region.

SNP selection

A list of SNPs examined in this study can be found in Supplementary Table 1, available at *Carcinogenesis* Online. The procedure for tagSNP selection for the parent study has been published previously (28). Briefly, tagSNPs for *NFκB1* ($n = 42$) and *IκBKβ* ($n = 10$) were selected using Haploview Tagger (29) with the following criteria: minor allele frequency (MAF) >5%, pairwise r^2 of >0.95 and distance from closest SNP of >60 base pairs (bp). The 5′ and 3′ untranslated regions (UTR) for each gene were extended to include the most up- or downstream SNP within the linkage disequilibrium (LD) block (~10 kb upstream and 5kb downstream). In regions of no or low LD, SNPs with a MAF >5% at a density of ~ 1 per kb were selected from either HapMap (29) or dbSNP (30). The tag SNPs included two non-synonymous SNPs each from *NFκB1* and *IκBKβ*.

Genotyping and quality control

DNA was extracted from peripheral blood leukocytes, and genotyping was performed at Translational Genomics Institute (TGen, Phoenix, AZ). SNPs were genotyped on the Illumina 1536 GoldenGate platform (31). Quality control checks were based on Illumina metrics, as described previously (32). Three SNPs (rs4648099, rs6813322 and rs6474387) that were not successfully genotyped on the Illumina platform were genotyped using Sequenom's iPLEX Gold (Sequenom, Inc). Five percent of the samples were re-genotyped to assess reliability. Concordance among duplicates was >99.8% for all SNPs. SNPs were excluded if the Hardy–Weinberg equilibrium P value among controls was <0.0001 or the call rate was <85%. The two non-synonymous SNPs from *IκBKβ*, rs2272763 and rs17875749, were excluded from analysis because they were not encountered in our population. From *NFκB1*, eight SNPs were excluded: two SNPs were not in Hardy–Weinberg equilibrium (including one non-synonymous SNP, rs4648099), and six SNPs were in LD ($r^2 > 0.90$) with another SNP with nearly identical genotype frequencies. After these exclusions, eight *IκBKβ* SNPs and 34 *NFκB1* remained for analysis (Supplementary Table 1, available at *Carcinogenesis* Online).

Statistical analysis

Single SNP main effects. Conditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI). A variable that indicated unique sibling kinships was used as the matching variable, and models were adjusted for age and sex. For each SNP, the risk of CRC was estimated using a codominant model, unless any cell had fewer than five individuals, in which case a dominant model was used. A global P value (two degrees of freedom for codominant models), determined using the likelihood ratio statistic, was used to test for differences between carriers of the variant allele and homozygous carriers of the wild-type allele. A trend test was performed using the log-additive model to determine if there was a dose-dependent association with the variant allele.

Interaction analyses. Conditional logistic regression was used to investigate the interaction between SNPs and current NSAID use, as well as between SNPs of *NFκB1* and *IκBKβ*. OR estimates and 95% CIs were calculated for each combination of genotype(s) and/or NSAID use status (current or former/never). In this study population, current NSAID use was more strongly inversely associated with CRC risk than other aspects of NSAID use and thus we chose it as the primary variable for interaction analysis. The reference groups were composed of individuals who were homozygous for the wild-type allele(s) and never/former users of NSAIDs. Because use of NSAIDs may be associated with other known risk factors for CRC, NSAID interactions were adjusted for smoking (continuous pack-years), body mass index (BMI) (continuous) and physical activity (four-level ordinal variable based on average MET hours) in addition to age (continuous) and sex. For SNP-SNP interactions, only SNPs independently associated with CRC risk (global $P < 0.05$) were combined with the other SNPs in this study to create composite genotypes. To avoid small cell counts, only the dominant model and composite genotypes that represented at least 5% of the population were examined. The likelihood ratio test compared models with and without the multiplicative interaction term(s) to determine if an interaction was statistically significant ($P_{interaction}$). All tests of statistical significance used a two-sided P value and $\alpha = 0.05$. Analyses were carried out using SAS v.9.3 (SAS Institute Inc, Cary, NC).

Multiple testing corrections. The correction method for multiple correlated tests developed by Conneely and Boehnke was used for main effects analyses (33). P values adjusted for correlated tests are referred to as P_{act}, with $P_{act} < 0.05$ considered statistically significant. All P value adjustments were conducted in R (version 2.14.1). The more conservative family wise error rate methods of multiple testing corrections often prevent the detection of true associations. Thus, the false discovery rate (FDR) control method developed by Benjamini and Hochberg (11,34,35), was used to identify SNPs noteworthy at the 25% FDR levels for both main effect and NSAID interaction analyses. Tables II andIII include only SNPs that had a significant global P, P_{trend} or $P_{interaction}$ value or were noteworthy by FDR.

Replication of previous study results

We used this study to attempt replication of the results of Curtin *et al.* (9). (the only other study to examine the association between genetic variation in these genes and colorectal cancer using a tagSNP method). Curtin *et al*. examined the association between individual or composite genotypes of *NFκB1* and *IκBKβ* SNPs and colon or rectal cancer adjusted for age, sex, center and race under the additive, dominant and recessive models. In the replication analysis, only ORs and 95% CIs are presented for individual genotypes or composite genotypes, as these were the parameters reported by Curtin *et al*. (9).

Results

Characteristics of the study sample are presented in Table I. There were no obvious differences between cases and unaffected sibling controls by age, regular NSAID use, pack-years of cigarette smoking and physical activity or BMI. Cases were more likely than controls to be male.

SNP risk estimates

Among the tagSNPs in *IκBKβ*, one SNP was associated with lower CRC risk, before corrections for multiple testing (Table II). Individuals with the homozygous variant genotype of rs9694958 (A>G intron 5) had an almost 4-fold lower CRC risk than individuals homozygous for the wild-type allele ($OR_{hzv} = 0.26$, 95% CI 0.07–0.99, global $P = 0.048$, $P_{act} = 0.25$).

After stratifying by tumor subsite, two SNPs in *IκBKβ* were statistically significantly associated with lower colon cancer risk (Table II). Carriers of rs10958713 (A>C intron 19) had 1.4- to 1.6-fold lower colon cancer risk ($OR_{het} = 0.73$, 95% CI 0.57–0.92; $OR_{hzv} = 0.62$, 95% CI 0.42–0.92, global $P = 0.02$, $P_{act} = 0.09$). The variant allele showed a dose-dependent association, which remained statistically significant after correction for multiple testing ($P_{trend} = 0.005$, $P_{act} = 0.03$). Carriers of rs5029748 (C>A intron 2) had a 1.4-fold lower colon cancer risk

Table I. Selected characteristics of colorectal cancer cases and unaffected sibling controls, the Colon Cancer Family Registry, 1998–2002

Characteristic	Cases ($N = 1584$)[a]	Unaffected sibling controls ($N = 2516$)[a]
	n (%) or mean ± SD	n (%) or mean ± SD
Age (years) ± SD	53.5 ± 10.8	54.0 ± 11.7
Male	810 (51.1)	1126 (44.8)
Center site		
Ontario	296 (18.7)	491 (19.5)
Los Angeles	319 (20.1)	444 (17.7)
Australia	317 (20.0)	554 (22.0)
Hawaii	6 (0.4)	7 (0.3)
Mayo	266 (16.8)	502 (20.0)
Seattle	380 (24.0)	518 (20.6)
Tumor site		
Proximal	526 (33.2)	—
Distal	460 (29.0)	—
Rectal	523 (33.0)	—
Regular NSAID use[b]		
Never/former	1234 (78.3)	1916 (76.9)
Current	343 (21.8)	577 (23.1)
Physical activity		
Inactive	375 (23.7)	585 (23.3)
Less active	424 (26.8)	679 (27.0)
Active	374 (23.6)	564 (22.4)
Very active	338 (21.3)	548 (21.8)
BMI ± SD	27.4 ± 6.0	26.8 ± 5.5
Cigarette smoking (pack-years) ± SD	12.9 ± 19.5	11.7 ± 19.3

[a]Numbers may not add to total because of missing data.
[b]Regular use defined as at least two pills per week for at least one month. This variable was chosen as the most predictive NSAID variable in the CCFR.

Table II. Association between selected SNPs in *IκBKβ* and *NFκB1* and colorectal, colon or rectal cancer risk adjusted for age and sex[a]

	Colorectal Cancer					Colon Cancer					Rectal Cancer				
	Cases/controls[b]	OR	95% CI	Global P (P_{act})[c]	P_{trend}	Cases/controls[b]	OR	95% CI	Global P (P_{act})[c]	P_{trend}	Cases/controls[b]	OR	95% CI	Global P (P_{act})[c]	P_{trend}
IκBKβ															
rs5029748 (C>A intron 2)															
C/C	908/1411	1.00	Ref.			580/873	1.00	Ref.			267/457	1.00	Ref.		
C/A	587/961	0.86	(0.71–1.03)			325/573	0.72	(0.56–0.91)			208/334	1.08	(0.78–1.49)		
A/A	89/144	0.83	(0.56–1.22)	0.25	0.11	54/89	0.72	(0.44–1.18)	0.02*,[d] (0.11)	0.01** (0.08)	30/48	1.17	(0.60–2.28)	0.86	0.59
rs9694958 (A>G intron 5)															
A/A	1344/2122	1.00	Ref.			809/1286	1.00	Ref.			431/721	1.00	Ref.		
A/G	232/366	1.01	(0.79–1.29)			146/234	1.06	(0.78–1.45)			71/107	1.06	(0.68–1.64)		
G/G	8/27	0.26	(0.07–0.99)	0.048* (0.25)	0.48	4/14	0.32	(0.04–2.42)			3/11	0.26	(0.04–1.54)		
A/G or G/G	240/393	0.99	(0.77–1.26)			150/248	1.04	(0.76–1.42)	0.82	—	74/118	1.04	(0.67–1.61)	0.88	—
rs10958713 (A>C intron 19)															
G/G	693/1097	1.00	Ref.			451/671	1.00	Ref.			197/362	1.00	Ref.		
G/A	712/1122	0.91	(0.75–1.09)			410/698	0.73	(0.57–0.92)			246/365	1.23	(0.89–1.69)		
A/A	178/295	0.81	(0.59–1.09)	0.36	0.15	97/166	0.62	(0.42–0.92)	0.02*,[d] (0.09)	0.005** (0.03*)	62/110	1.13	(0.66–1.93)	0.47	0.40
NFκB1															
rs4648072 (M507V)															
A/A	1565/2475	1.00	Ref.			949/1512	1.00	Ref.			499/826	1.00			
A/G[e]	19/41	0.62	(0.24–1.60)	0.32	—	10/23	0.63	(0.20–1.97)	0.43	—	6/13	0.64	(0.10–4.12)	0.64	—
rs10489113 (A>G 3′ outside UTR)															
A/A	1054/1639	1.00	Ref.			643/996	1.00	Ref.			332/549	1.00	Ref.		
A/G	465/782	0.87	(0.72–1.05)			289/480	0.85	(0.66–1.08)			145/260	0.89	(0.63–1.26)		
G/G	64/94	0.95	(0.62–1.47)	0.36	0.28	27/59	0.53	(0.29–0.97)	0.07	0.04* (0.48)	28/29	2.13	(1.02–4.46)	0.06	0.49

[a]Limited to SNPs with a significant global P or P_{trend} (<0.05), noteworthy by FDR, or a non-synonymous SNP.
[b]Unaffected sibling controls.
[c]P_{act} is the adjusted P value for multiple correlated tests. The adjusted P value is only presented for $P < 0.05$.
[d]Noteworthy at 25% FDR level.
[e]No individuals with the G/G genotype.
*$P \leq 0.05$, **$P \leq 0.01$.

Table III. Association between NSAID use and colorectal, colon or rectal cancer risk stratified by selected *IκBKβ* and *NFκB1* SNP genotypes[a,b]

Genotype	Colorectal Cancer				Colon Cancer				Rectal Cancer			
	Never or former NSAID use		Current NSAID use		Never or former NSAID use		Current NSAID use		Never or former NSAID use		Current NSAID use	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
IκBKβ												
rs6474387 (C>T intron 20)												
C/C	1.00	Ref.	0.91	(0.75–1.08)	1.00	Ref.	0.93	(0.72–1.18)	1.00	Ref.	0.80	(0.54–1.19)
C/T or T/T[c]	1.00	(0.72–1.40)	0.56	(0.31–1.02)	1.11	(0.71–1.72)	0.38	(0.16–0.86)	1.10	(0.65–1.86)	0.84	(0.35–2.04)
			$P_{interaction}$ = 0.10				$P_{interaction}$ = 0.01**,[c]				$P_{interaction}$ = 0.93	
rs11986055 (A>C intron 2)												
A/A	1.00	Ref.	0.90	(0.74–1.09)	1.00	Ref.	0.89	(0.70–1.14)	1.00	Ref.	0.81	(0.56–1.18)
A/C or C/C[d]	1.10	(0.72–1.67)	0.48	(0.23–1.01)	1.25	(0.73–2.16)	0.44	(0.17–1.13)	1.24	(0.72–2.53)	0.67	(0.20–2.28)
			$P_{interaction}$ = 0.06				$P_{interaction}$ = 0.045*,[c]				$P_{interaction}$ = 0.56	
NFκB1												
rs4648072 (M507V)												
A/A	1.00	Ref.	0.87	(0.72–1.05)	1.00	Ref.	0.85	(0.67–1.08)	1.00	Ref.	0.80	(0.55–1.15)
A/G[e]	0.71	(0.27–1.87)	0.25	(0.02–2.74)	0.60	(0.17–2.06)	0.56	(0.05–6.01)	0.60	(0.11–3.25)	—[f]	—
			$P_{interaction}$ = 0.39				$P_{interaction}$ = 0.95				$P_{interaction}$ = 0.32	
rs230490 (G>A 5′ outside UTR)												
G/G	1.00	Ref.	0.61	(0.44–0.84)	1.00	Ref.	0.69	(0.46–1.04)	1.00	Ref.	0.42	(0.22–0.82)
G/A	0.92	(0.73–1.15)	0.84	(0.62–1.13)	0.99	(0.74–1.33)	0.85	(0.58–1.24)	0.79	(0.55–1.15)	0.70	(0.40–1.23)
A/A	0.93	(0.67–1.28)	1.29	(0.82–2.03)	1.14	(0.76–1.73)	1.38	(0.78–2.44)	0.63	(0.35–1.14)	1.21	(0.52–2.82)
			$P_{interaction}$ = 0.008**,[c]				$P_{interaction}$ = 0.26				$P_{interaction}$ = 0.02*	
rs997476 (C>A 3′ outside UTR)												
C/C	1.00	Ref.	0.80	(0.65–0.98)	1.00	Ref.	0.81	(0.62–1.04)	1.00	Ref.	0.67	(0.45–1.01)
C/A or A/A[d]	1.08	(0.77–1.5)	1.57	(0.99–2.48)	0.82	(0.53–1.25)	1.09	(0.60–2.00)	1.58	(0.88–2.84)	2.38	(1.10–5.18)
			$P_{interaction}$ = 0.03*				$P_{interaction}$ = 0.16				$P_{interaction}$ = 0.08	

[a]Limited to SNPs with a significant interaction *P* (<0.05) or noteworthy by FDR.
[b]Adjusted for age, sex, BMI, pack-years and physical activity.
[c]Noteworthy at 25% FDR level.
[d]Dominant model, cases <5.
[e]No individuals with the G/G genotype.
[f]No cases and one control with A/G genotype.
*$P \le 0.05$, **$P \le 0.01$.

(OR_{het} = 0.72, 95% CI 0.56–0.91; OR_{hzv} = 0.72, 95% CI 0.44–1.18, global P = 0.02, P_{act}=0.11). The variant allele showed a dose-dependent association (P_{trend} = 0.01, P_{act} = 0.08). The rs5029748 and rs10958713 variants were noteworthy at the 25% FDR level for associations with colon cancer risk; thus the chance that these SNPs are truly associated with risk is ~75%.

No tagSNP in *IκBKβ* had a statistically significant association with rectal cancer risk. The complete analysis of the associations between SNPs in *IκBKβ* and colorectal cancer is presented in Supplementary Table 2, available at *Carcinogenesis* Online.

No SNP in *NFκB1* was statistically significantly associated with risk of colorectal, colon or rectal cancer and no SNP was noteworthy by FDR. Individuals carrying one copy of the single non-synonymous SNP in *NFκB1*, M507V (rs4648072), showed a reduced, but statistically non-significant, risk of CRC (OR_{het} = 0.62, 95% CI 0.24–1.60, global P = 0.50). The rs10489113 variant allele (A>G 3′ outside UTR) had a dose-dependent association when the analysis was restricted to colon cancer, prior to correction for multiple correlated tests (P_{trend} = 0.04, P_{act} = 0.48) (Table II). Individuals homozygous for this variant allele had an ~2-fold lower risk of colon cancer (OR_{hzv} = 0.53, 95% CI 0.29–0.97). The complete analysis of the associations between SNPs in *NFκB1* and colorectal cancer risk is presented in Supplementary Table 3, available at *Carcinogenesis* Online.

SNP-SNP interactions

We did not observe any statistically significant interactions between SNPs (data not shown). Of the three SNPs independently associated with CRC and, therefore, potentially eligible for this analysis, rs9694958 was not included because individuals with the risk-associated genotype represented <5% of the population.

NSAID interactions

Current NSAID use was associated with a statistically non-significant reduced CRC risk in the study population (OR = 0.87, P = 0.13). Two SNPs in *IκBKβ*, rs6474387 (C>T intron 20) and rs11986055 (A>C intron 2), altered the association between NSAID use and colon cancer risk (Table III). Current NSAID users who carried the rs6474387 variant allele had statistically significant lower risk of colon cancer (OR = 0.38, 95% CI 0.16–0.86), which was not observed in current NSAID users who did not carry the variant allele ($P_{interaction}$ = 0.01). The interaction between rs11986055 and NSAID use was similar (LD r^2 between rs6474387 and rs11986055 was 0.50); only current NSAID users who carried the variant allele had lower colon cancer risk (OR = 0.47, 95% CI 0.17–1.13, $P_{interaction}$ = 0.045). FDR analysis identified the interactions for both rs6474387 and rs11986055 as noteworthy at the 25% level when the analysis was restricted to colon cancer. There were no statistically significant or noteworthy interactions between *IκBKβ* SNPs and NSAID use for rectal cancer.

For the non-synonymous SNP in *NFκB1*, M507V, there was no statistically significant interaction with NSAID use. Two other tagSNPs in *NFκB1*, rs230490 (G>A 5′ outside UTR) and rs997476 (C>A 3′ outside UTR), were associated with a statistically significantly altered association between NSAID use and CRC risk (Table III). Among individuals with the homozygous wild-type and heterozygous genotypes of rs230490 current NSAID use was associated with lower CRC risk; however, among individuals with the homozygous variant genotype current NSAID use resulted in a trend toward higher CRC risk (OR = 1.29, 95% CI 0.82–2.03, $P_{interaction}$ = 0.008). The interaction between rs230490 and NSAID use remained statistically significant when the analysis was restricted to rectal cancer risk, but not for colon cancer risk. There was also a nominally significant SNP-NSAID interaction for rs997476, with higher CRC risk

Table IV. Comparison between OR for *IκBKβ* and *NFκB1* genotypes and composite genotypes identified by Curtin *et al*. (9). and results from the CCFR population

Cancer site	Gene(s)	SNP(s)	Comparison(s)	Model(s)	Curtin *et al*. (9)		CCFR	
					OR[d]	95% CI	OR[e]	95% CI
Colon	*IκBKβ*	rs2272733	TT versus CC	Additive[f]	0.56	(0.34–0.93)	0.90	(0.54–1.52)
	NFκB1	rs4648110	AA versus TT	Additive	0.65	(0.44–0.94)	0.68	(0.45–1.04)
			AA versus TT/TA	Recessive	0.66	(0.45–0.96)	0.65	(0.37–1.13)
	NFκB1	rs13117745	TT versus CC	Additive	0.61	(0.37–1.00)	0.80	(0.50–1.28)
			TT versus CC/CT	Recessive	0.64	(0.39–1.04)	0.89	(0.45–1.76)
	IκBKβ/NFκB1	rs3747811/rs4648110	(TT / TT) versus (TA or AA / AA)	Dominant/recessive	0.54	(0.34–0.87)	0.56	(0.28–1.12)
Rectal	*NFκB1*	rs230510[a]	TT versus AA	Additive	0.65	(0.49–0.87)	1.13	(0.73–1.79)
			TT versus AA/AT	Dominant	0.79	(0.51–0.94)	1.17	(0.84–1.63)
	NFκB1	rs3821958[b] or rs230521	GG versus AA	Additive	1.32	(1.00–1.75)	0.80	(0.50–1.29)
	NFκB1	rs11722146[c] or rs12509517	GA/AA versus GG	Dominant	1.24	(1.03–1.51)	0.99	(0.71–1.37)
	NFκB1	rs13117745	TT versus CC	Additive	1.69	(0.93–3.07)	1.22	(0.65–2.26)
	IκBKβ/NFκB1	rs3747811/(rs11722146[c] or rs12509517)	(TT/CC) versus (TA or AA/CG or GG)	Dominant/dominant	1.11	(0.83–1.50)	1.02	(0.63–1.65)

Odds ratios are for the specified comparison and model of inheritance.
[a]The MAF of rs230510 is close to 50% and the polymorphism results in a complementary base change (A to T), therefore it is possible the studies are investigating opposing associations.
[b]rs3821958 was not genotyped in CCFR, rs3821958 and rs230521 have a LD r^2 of 0.966 and the same MAF according to the Genome Variation Server.
[c]rs11722146 was not genotyped in CCFR, rs11722146 and rs12509517 have a LD r^2 of 1.000 and the same MAF according to the Genome Variation Server.
[d]Adjusted for age, sex, center and race.
[e]Restricted to Caucasians and adjusted for age and sex.
[f]The OR is two times the â from the log-additive model.

among carriers of the variant allele who were current NSAID users (OR = 1.57, 95% CI 0.99–2.48) ($P_{interaction}$ = 0.03).

Replication of previous findings

Table IV compares the findings of this study with the previous study by Curtin *et al.* of genetic variability in *IκBKβ* and *NFκB1* and the risk of colon and rectal cancer (9). Curtin *et al.* identified three individual genotypes and one composite genotype of *IκBKβ* and *NFκB1* that were each inversely associated with colon cancer risk. We also observed trends toward a lower risk of colon cancer for the same genotypes. The >30% reduction in risk associated with homozygous carriers of *NFκB1*'s rs4648110 variant allele (T>A intron 22) was nearly identical between the two studies under both the additive and recessive models of inheritance (recessive model: OR_{Curtin} = 0.66, 95% CI 0.45–0.96 versus OR_{CFR} = 0.65, 95% CI 0.37–1.13). The composite genotype composed of *IκBKβ*'s rs3747811 (T>A intron 1) and *NFκB1*'s rs4648110 was also associated with nearly identical lower risk estimates (OR_{Curtin} = 0.54, 95% CI 0.34–0.87 versus OR_{CFR} = 0.56, 95% CI 0.28–1.12).

Curtin *et al*. also identified four individual genotypes of *NFκB1* and one composite genotype of *IκBKβ* and *NFκB1* that were associated with rectal cancer risk. Carriage of the rs13117745 (G>A intron 5) variant resulted in a trend toward higher rectal cancer risk in both studies under the additive model (OR_{Curtin} = 1.69, 95% CI 0.93–3.07 versus OR_{CFR} = 1.22, 95% CI 0.65–2.26). The risk estimates for the remaining SNPs were not replicated.

Discussion

We identified three SNPs in *IκBKβ* that were statistically significantly associated with lower colorectal or colon cancer risk. In addition, we report similar trends in risk estimates for many of the *IκBKβ* and *NFκB1* SNPs identified by Curtin *et al*. (9). These findings contribute to the evidence that variation in *IκBKβ* and *NFκB1* is associated with CRC risk. The activation of NFκB is a key survival mechanism of premalignant cells because it can block apoptosis by regulating anti-apoptosis proteins (36) and inhibit accumulation of reactive oxygen species (37). Furthermore, the NFκB transcription factor induces expression of cytokines and chemokines, which help sustain an inflammatory state known to contribute to colorectal carcinogenesis (1,5,38). Although the functions of many of the tagSNPs identified here are not established, if these polymorphisms directly or indirectly modify the activation of this key transcription factor, this may explain their observed associations with CRC risk.

Evidence from this study and previous studies suggests that variants in *IκBKβ* are associated with a lower risk of CRC, particularly colon cancer. All statistically significant or noteworthy genetic variation in *IκBKβ* identified here and by Curtin *et al.* trended toward a lower risk of colon cancer (9). The two intronic tagSNPs that had statistically significant associations with lower colon cancer risk in this study, rs5029748 and rs10958713, have no known functional impact, but have been previously associated with risk of colorectal neoplasia (9,39): in a case–control study of metachronous colorectal adenoma risk, rs10958713 was independently associated with a 36% (95% CI 13–54%) lower risk (39) and carriers of the rs5029748 variant allele who also carried polymorphisms in *NFκB1* and *IL6* had an 80% (95% CI 33–94%) lower risk of colon cancer (9). It seems plausible that variation in *IκBKβ* could negatively affect the function or expression of the cognate protein, IKKβ, thereby impairing NFκB activation and the resulting inflammation. Reduced levels of inflammation reduce risk of CRC (1,2) and this particular mechanism may have a stronger impact on colon than rectal cancer. A similar mechanism may also operate with regard to survival, as genetic variation in *IκBKβ* has also been associated with increased CRC survival among CCFR cases (11).

Contrary to previous studies (9,10) we did not observe statistically significant associations between *NFκB1* SNPs and CRC risk. The non-synonymous SNP, M507V, was associated with a 40% reduced risk in heterozygous individuals, but this was not statistically significant (M507V has no demonstrated functional impact and is predicted to be functionally tolerable by SIFT (Sorting Tolerant From Intolerant) software (40)). In the Curtin *et al.* study, individual SNPs in *NFκB1* were mainly associated with the risk of rectal cancer; the only SNP associated with both colon and rectal cancer risk modified the risks in opposing directions (9). The associations in this study may not have reached statistical significance because family based study designs have lower power to identify main effects (41) and because of the limited number of rectal cancer cases in our study population.

Additionally, if variation in *NFκB1* modifies the risk of colon and rectal cancer in opposing directions, the associations could have counterbalanced each other in analyses of CRC.

This study replicated several of the colon and rectal cancer risk estimates associated with variation in *NFκB1* and *IκBKβ* identified by Curtin *et al*. (9). In both studies, rs4648110 of *NFκB1* was associated with a ~35% decreased risk of colon cancer on its own and, when combined with rs3747811 of *IκBKβ*, a ~45% decreased risk. Using the UCSC Genome Browser (http://genome.ucsc.edu/), we found that rs4648110 is located in a potential enhancer site (42). Another *NFκB1* SNP, rs13117745, which is in moderately high LD with rs4648110 ($r^2 = 0.71$), also had replicated trends across the two studies; carriers of the variant allele had similar lower colon cancer risk and higher rectal cancer risk in the two studies. Opposing risk effects for colon and rectal cancer were also observed with rs10489113 of *NFκB1* in this study. *NFκB1* is not the first gene to be identified to have opposite associations with colon and rectal cancer (43–45). Colon and rectal cancers probably have distinct (as well as overlapping) etiologies (46,47) and these findings suggest that NFκB, and the inflammation that results from its activation, may impact colon and rectal cancer carcinogenesis differently.

The findings here are a preliminary step toward a pharmacogenomic approach to NSAID use. Although NSAID use is known to decrease risk of CRC, NSAID use brings potentially serious gastrointestinal toxicity and bleeding (3,14,15,48). Therefore, it would be valuable to identify individuals with altered benefit from NSAID use to ensure that any potential harm is well outweighed by the benefits. In this regard, polymorphisms such as rs6474387 and rs11986055 in *IκBKβ* may be of particular importance because they interact with NSAIDs such that carriers of the minor allele have a statistically significantly lower risk of colon cancer, whereas carriers of the wild-type allele did not have decreased risk with NSAID use. We also observed a very strong pharmacogenetic interaction for *NFκB1* rs230940 ($P = 0.008$). This SNP definitely should be followed up in other studies. Recent findings have indicated that non-aspirin NSAIDs may be less protective than aspirin itself against rectal cancer (49); therefore, individuals with genotypes known only to only increase rectal cancer risk should not be prescribed non-aspirin NSAIDs as preventative treatment.

Most studies point to the inhibition of the cyclooxygenases (COX-1 and COX-2) in the prostaglandin synthesis pathway as the main mechanism by which NSAIDs reduce inflammation and risk of CRC (3,15,50). However, current research also points to inhibition of the NFκB-signaling pathway as a mechanism through which NSAIDs may reduce CRC risk by reducing inflammation and promotion of apoptosis (18–20). Indeed, we observed statistically significant interactions between genetic variation in *IκBKβ* and *NFκB1* and NSAID use, which is further evidence of a link between NSAIDs and the NFκB-signaling pathway. There are indications that some NSAIDs, including aspirin, directly impact the function of IKKβ *in vitro* (the protein encoded by *IκBKβ*) by competitively interfering with ATP binding (17). It is possible that variation in *IκBKβ* could affect the binding of NSAIDs to IKKβ, providing an explanation for the interaction between variation in *IκBKβ* and NSAID use. The fact that variation in both *IκBKβ* and *NFκB1* interacted with NSAID use to modify CRC risk serves as further evidence for including the NFκB-signaling pathway in future studies of the chemopreventive effects of NSAIDs.

There are several strengths to this study. We were able to replicate many of the results reported by Curtin *et al.* (9). The large sample size in this study made it possible to examine colon and rectal cancer separately; given the potential differences in etiology, it is important for studies to be able to make distinctions between the tumor sites. The case–unaffected sibling control study design also helps avoid false positives that can result from population stratification, and increases the power to detect gene–NSAID interactions (51). There are several weaknesses to this study as well. Associations with rare variants that were below our MAF cut-off (5%) and genetic variants that were not well represented in the relevant database may have been missed. Further, the family based study design may have reduced the power of the main effect analyses (41).

Identifying common genetic variants associated with CRC can help predict risk and contribute to our understanding of mechanisms of colorectal carcinogenesis. Additionally, examining pharmacogenetic interactions between common genetic variants and NSAID use may allow for more targeted recommendations of NSAIDs as chemopreventive agents. Ultimately, our findings, which partially replicated the results of a previous study, suggest that variants in *IκBKβ* and *NFκB1* are probably associated with CRC risk. Genetic variants in *IκBKβ* appear to alter the risk of colon cancer, whereas variants in *NFκB1* appear to result in risk modifications in opposite directions for colon and rectal cancer. The present results also begins to shed light on links between variation in key NFκB-signaling pathway genes and NSAID use, suggesting that the prostaglandin synthesis pathway is not the only pathway that should be considered when investigating NSAID effects on CRC risk. The common variants identified here should be considered for future research, not only in epidemiologic studies of CRC, but also in functional studies of these genes.

Supplementary material

Supplementary Tables 1–3 can be found at http://carcin.oxfordjournals.org/

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References

1. Fantini,M.C. *et al.* (2008) Cytokines: from gut inflammation to colorectal cancer. *Curr. Drug Targets*, **9**, 375–380.
2. Rizzo,A. *et al.* (2011) Intestinal inflammation and colorectal cancer: a double-edged sword? *World J. Gastroenterol.*, **17**, 3092–3100.
3. Ulrich,C.M. *et al.* (2006) Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. *Nat. Rev. Cancer*, **6**, 130–140.
4. Rhodes,J.M. *et al.* (2002) Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. *Trends Mol. Med.*, **8**, 10–16.
5. Wang,S. *et al.* (2009) NF-kappaB signaling pathway, inflammation and colorectal cancer. *Cell. Mol. Immunol.*, **6**, 327–334.
6. Sakamoto,K. *et al.* (2009) Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. *Clin. Cancer Res.*, **15**, 2248–2258.
7. Baud,V. *et al.* (2009) Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat. Rev. Drug Discov.*, **8**, 33–40.
8. Pomerantz,J.L. *et al.* (2002) Two pathways to NF-kappaB. *Mol. Cell*, **10**, 693–695.
9. Curtin,K. *et al.* (2010) Exploring multilocus associations of inflammation genes and colorectal cancer risk using hapConstructor. *BMC Med. Genet.*, **11**, 170.
10. Andersen,V. *et al.* (2010) Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. *BMC Cancer*, **10**, 484.

11. Coghill,A.E. *et al.* (2011) Genetic variation in inflammatory pathways is related to colorectal cancer survival. *Clin. Cancer Res.*, **17**, 7139–7147.
12. Algra,A.M. *et al.* (2012) Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol.*, **13**, 518–527.
13. Flossmann,E. *et al.* (2007) Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet*, **369**, 1603–1613.
14. Dube,C. *et al.* (2007) The use of aspirin for primary prevention of colorectal cancer: a systematic review prepared for the U.S. Preventive Services Task Force. *Ann. Intern. Med.*, **146**, 365–375.
15. Cross,J.T. *et al.* (2008) A review of gene-drug interactions for non-steroidal anti-inflammatory drug use in preventing colorectal neoplasia. *Pharmacogenomics J.*, **8**, 237–247.
16. Karin,M. *et al.* (2004) The IKK NF-kappa B system: a treasure trove for drug development. *Nat. Rev. Drug Discov.*, **3**, 17–26.
17. Yin,M.J. *et al.* (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature*, **396**, 77–80.
18. Din,F.V. *et al.* (2004) Evidence for colorectal cancer cell specificity of aspirin effects on NF kappa B signalling and apoptosis. *Br. J. Cancer*, **91**, 381–388.
19. Yamamoto,Y. *et al.* (1999) Sulindac inhibits activation of the NF-kappaB pathway. *J. Biol. Chem.*, **274**, 27307–27314.
20. Stark,L.A. *et al.* (2001) Aspirin-induced activation of the NF-kappaB signaling pathway: a novel mechanism for aspirin-mediated apoptosis in colon cancer cells. *FASEB J.*, **15**, 1273–1275.
21. Poole,E.M. *et al.* (2007) Genetic variability in prostaglandin synthesis, fish intake and risk of colorectal polyps. *Carcinogenesis*, **28**, 1259–1263.
22. Poole,E.M. *et al.* (2012) Genetic variability in IL23R and risk of colorectal adenoma and colorectal cancer. *Cancer Epidemiol.*, **36**, e104–e110.
23. Poole,E.M. *et al.* (2010) Genetic variation in prostaglandin E2 synthesis and signaling, prostaglandin dehydrogenase, and the risk of colorectal adenoma. *Cancer Epidemiol. Biomarkers Prev.*, **19**, 547–557.
24. Ulrich,C.M. *et al.* (2004) Polymorphisms in PTGS1 (=COX-1) and risk of colorectal polyps. *Cancer Epidemiol. Biomarkers Prev.*, **13**, 889–893.
25. Ulrich,C.M. *et al.* (2005) PTGS2 (COX-2) -765G > C promoter variant reduces risk of colorectal adenoma among nonusers of nonsteroidal anti-inflammatory drugs. *Cancer Epidemiol. Biomarkers Prev.*, **14**, 616–619.
26. Poole,E.M. *et al.* (2006) Prostacyclin synthase and arachidonate 5-lipoxygenase polymorphisms and risk of colorectal polyps. *Cancer Epidemiol. Biomarkers Prev.*, **15**, 502–508.
27. Newcomb,P.A. *et al.* (2007) Colon Cancer Family Registry: an international resource for studies of the genetic epidemiology of colon cancer. *Cancer Epidemiol. Biomarkers Prev.*, **16**, 2331–2343.
28. Levine,A.J. *et al.* (2010) A candidate gene study of folate-associated one carbon metabolism genes and colorectal cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, **19**, 1812–1821.
29. Thorisson,G.A. *et al.* (2005) The International HapMap Project Web site. *Genome Res.*, **15**, 1592–1593.
30. Sherry,S.T. *et al.* (2001) dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res.*, **29**, 308–311.
31. Shen,R. *et al.* (2005) High-throughput SNP genotyping on universal bead arrays. *Mutat. Res.*, **573**, 70–82.
32. Levine,A.J. *et al.* (2010) Genetic variability in the MTHFR gene and colorectal cancer risk using the colorectal cancer family registry. *Cancer Epidemiol. Biomarkers Prev.*, **19**, 89–100.
33. Conneely,K.N. *et al.* (2007) So many correlated tests, so little time! Rapid adjustment of P values for multiple correlated tests. *Am. J. Hum. Genet.*, **81**, 1158–1168.
34. Benjamini,Y. *et al.* (2001) Controlling the false discovery rate in behavior genetics research. *Behav. Brain Res.*, **125**, 279–284.
35. Benjamini,Y. *et al.* (1995) Controlling the false discovery rate—a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B-Methodological*, **57**, 289–300.
36. Kucharczak,J. *et al.* (2003) To be, or not to be: NF-kappaB is the answer—role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene*, **22**, 8961–8982.
37. Luo,J.L. *et al.* (2005) IKK/NF-kappaB signaling: balancing life and death—a new approach to cancer therapy. *J. Clin. Invest.*, **115**, 2625–2632.
38. Barnes,P.J. *et al.* (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.*, **336**, 1066–1071.
39. LeRoy,E.C. *et al.* (2011) Genes in the insulin and insulin-like growth factor pathway and odds of metachronous colorectal neoplasia. *Hum. Genet.*, **129**, 503–512.
40. Kumar,P. *et al.* (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat. Protoc.*, **4**, 1073–1081.
41. Witte,J.S. *et al.* (1999) Asymptotic bias and efficiency in case-control studies of candidate genes and gene-environment interactions: basic family designs. *Am. J. Epidemiol.*, **149**, 693–705.
42. Kent,W.J. *et al.* (2002) The human genome browser at UCSC. *Genome Res.*, **12**, 996–1006.
43. Slattery,M.L. *et al.* (2007) CDX2 VDR polymorphism and colorectal cancer. *Cancer Epidemiol. Biomarkers Prev.*, **16**, 2752–2755.
44. Slattery,M.L. *et al.* (2005) Energy balance, insulin-related genes and risk of colon and rectal cancer. *Int. J. Cancer*, **115**, 148–154.
45. Slattery,M.L. *et al.* (2004) Dietary calcium, vitamin D, VDR genotypes and colorectal cancer. *Int. J. Cancer*, **111**, 750–756.
46. Kapiteijn,E. *et al.* (2001) Mechanisms of oncogenesis in colon versus rectal cancer. *J. Pathol.*, **195**, 171–178.
47. Frattini,M. *et al.* (2004) Different genetic features associated with colon and rectal carcinogenesis. *Clin. Cancer Res.*, **10**(12 Pt 1), 4015–4021.
48. Süleyman,H. *et al.* (2007) Anti-inflammatory and side effects of cyclooxygenase inhibitors. *Pharmacol. Rep.*, **59**, 247–258.
49. Ruder,E.H. *et al.* (2011) Non-steroidal anti-inflammatory drugs and colorectal cancer risk in a large, prospective cohort. *Am. J. Gastroenterol.*, **106**, 1340–1350.
50. Langman,M.J. *et al.* (1999) Adverse upper gastrointestinal effects of rofecoxib compared with NSAIDs. *JAMA*, **282**, 1929–1933.
51. Gauderman,W.J. (2002) Sample size requirements for matched case-control studies of gene-environment interaction. *Stat. Med.*, **21**, 35–50.

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