NFκB1 and NFκBIA Polymorphisms Are Associated with Increased Risk for Sporadic Colorectal Cancer in a Southern Chinese Population

Shunxin Song1,2,*, Dianke Chen1,*, Jiachun Lu4, Jiawei Liao1, Yanxin Luo1,2, Zuli Yang1,2, Xinhui Fu1, Xinjuan Fan1, Yisheng Wei3, Lei Yang4, Lei Wang1,2, Jianping Wang1,2

1 Gastrointestinal Institute of Sun Yat-Sen University, The Sixth Affiliated Hospital of Sun Yat-Sen University, Guangzhou, People’s Republic of China, 2 Department of Colorectal Surgery, The Sixth Affiliated Hospital of Sun Yat-Sen University, Guangzhou, People’s Republic of China, 3 Department of Gastrointestinal Surgery, The Second Affiliated Hospital of Guangzhou Medical College, Guangzhou, People’s Republic of China, 4 The Institute for Chemical Carcinogenesis, The State Key Laboratory of Respiratory Disease, Guangzhou Medical College, Guangzhou, People’s Republic of China

Abstract

Background: Nuclear factor κB (NFκB) plays a key role in the regulation of apoptosis. The function of NFκB is inhibited by binding to NFκB inhibitor (IκB), and disruption of the balance of NFκB and IκB is related to the development of many diseases, including tumors. Therefore, we hypothesized that the NFκB1 (-94del/insATTG) and NFκBIA (2758 A->G) polymorphisms were associated with colorectal cancer (CRC) susceptibility.

Methods: In a hospital-based case–control study of 1001 CRC patients and 1005 cancer-free controls frequency matched by age and sex, we genotyped polymorphisms using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and performed luciferase assays and Western blotting analysis to identify whether genetic variants in NFκBIA alter its gene expressions and functions and thus cancer risk.

Results: We found that both NFκB1 -94 ins/delATTG and NFκBIA 2758 A>G polymorphisms were correlated with CRC risk (OR = 1.47, 95% CI = 1.14–1.86, and OR = 1.38, 95% CI = 1.14–1.66, respectively). Furthermore, when evaluated these two polymorphisms together, the combined genotypes with 2 variant (risk) alleles (2758GG and -94ins/ins-del/ins) were associated with an increased risk of CRC (OR = 1.71; 95% CI = 1.23–2.38) compared to 0 variant, and the significant trend for 2 variant (risk) alleles were more pronounced among subgroups of aged <60 years, women, never drinkers, never smokers, persons with a normal BMI and those with a family history of cancer. The AA+AG variant carriers were significantly higher in peritumoral tissues than those of the 2758GG genotype.

Conclusion: NFκB1 and NFκBIA polymorphisms appear to jointly contribute to risk of CRC. These two variants may be a genetic modifier for CRC susceptibility in this southern Chinese population.

Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women around the globe, and it is estimated that there were approximately 1.2 million newly diagnosed CRC cases and 605,700 related deaths in 2005 [1]. Records from the municipal death registry of the city of Guangzhou, Guangdong, China, indicate that CRC is the fifth most common cancer. The mortality rate was dramatically increased from 4.33/105 persons in 1970’s to 12.13/105 persons in 2000’s [2]. The majority of CRC cases (approximately 80%) are sporadic [3], but a hereditary predisposition is present in 20–35% of patients, suggesting that both genetic and environmental factors contribute to CRC development [4]. Alcohol drinking and tobacco use [5,6], dietary and lifestyle factors [7], and inflammatory bowel disease such as ulcerative colitis [9,9] have shown to be
associated with CRC risk. Although many people are exposed to these risk factors, only some of the exposed individuals develop CRC, indicating that genetic variation partly determines individual susceptibility to colorectal tumorigenesis.

Apoptosis, a highly regulated cellular process, participates in development, tissue homeostasis maintenance and elimination of unwanted cells [10]. Dysregulation in this process is likely to contribute to tumorigenesis [11]. The biochemical pathways of apoptosis are complicated and depend on not only the cells but also the initiators of apoptosis. Substantial evidence suggests that the occurrence and development of cancer is associated with both extended cell survival and suspended apoptosis in precancerous lesions and, consequently, aberrant apoptosis may allow for unchecked cell growth [12].

Nuclear factor kappa B (NFκB) is a major transcription regulator of the immune response, cell adhesion, differentiation, proliferation, and apoptosis [13]. Five members, p50/p105, p65/RelA, c-Rel, RelB, and p100/p52 in the NFκB family have been identified, and the dimeric form of NFκB p50/RelA is the major form [14]. In the resting cell, NFκB is inactivated in the cytoplasm through association with a sequestering inhibitory protein, IkBα, β or γ, and the most common protein of this family is the NFκB inhibitor α (NFκBIA) [15]. In the classical activation pathway, the phosphorylation and degradation of the inhibitory proteins lead to NFκB dissociation from the NFκB complex and translocation to the nucleus, where it can activate the transcription of a large number of genes [16]. As an important transcription factor, NFκB mediates the survival response by inhibiting p33-dependent apoptosis and up-regulating anti-apoptotic members of the Bcl-2 family and caspase inhibitors [17,18]. In contrast, NFκB is also activated by both the extrinsic and intrinsic apoptotic stimuli and mediates upregulation of pro-apoptotic genes such as TRAIL R2/DR5, Fas, and Fas ligand [19,20]. An inappropriate activation of NFκB could disturb tissue homeostasis and lead to dysregulated apoptosis. Furthermore, activity of NFκB has been observed in several types of cancers including CRC [21,22], indicating it may play an important role in tumorigenesis [23,24].

NFκB1 (encoding for NFκB) maps to chromosome 4q23-q24 and consists of 24 exons [25,26], and its inhibitory gene NFκBIA (encoding for IkB) is 3.5 kb long, with six exons, and is located on chromosome 1q13 [27,28]. Genetic studies have identified single nucleotide polymorphisms (SNP) in NFκB1 and NFκBIA [29,30]. Recently, a common insertion/deletion (−964 insertion/deletion AT/TTG, rs28362491) polymorphism in the NFκB1 promoter region and a 3′- untranslated region (3′UTR) polymorphism 2758A>G (rs696) in NFκBIA were observed to be significantly correlated with inflammatory bowel disease [31,32] and cancers [33,34,35]. Epidemiological studies have also investigated the association between NFκB1 polymorphisms and risk of CRC in Germans and NFκBIA polymorphism and risk of CRC in the Swedish with conflicting results [36,37].

There has been no previous report on the association between NFκB1 and NFκBIA polymorphisms and CRC risk. As the NFκB/IκB system plays an important regulatory role in the apoptotic pathway and dysregulated expression of the NFκB1 and NFκBIA has been observed in CRC, we hypothesized that combined NFκB1 and NFκBIA polymorphisms may be associated with increased risk of CRC. To test this hypothesis, we genotyped the NFκB1 -944 insertion/deletion AT/TTG and NFκBIA 2758A>G polymorphisms in our ongoing hospital-based case-control study of CRC in a southern Chinese population, and further performed luciferase assays and Western blotting analysis to identify whether genetic variants in NFκB1 alter its gene expressions and functions and thus cancer risk.

Materials and Methods

Ethics statement

The study protocol was approved by the institutional review boards of Sun Yat-Sen University. Written informed consent was obtained from each participant after a full explanation of the study.

Study subjects and sample collection

From July 2002 to April 2010, a total of 1001 patients with histopathologically-confirmed and untreated sporadic CRC were prospectively recruited from the First and Sixth Affiliated Hospital (Gastrointestinal & AnaL Hospital and Cancer Center of Sun Yat-Sen University, Guangzhou, China, the Affiliated Tumor Hospital of Guangzhou Medical College, Guangzhou, China, Guangdong Provincial People’s Hospital, Guangzhou, China, and Panyu People’s Hospital, Guangzhou, China. All these subjects were genetically unrelated ethnic Han Chinese and were from the city of Guangzhou and surrounding regions in southern China. Of the 1001 cases included in this study, there were 169 (16.9%) cases of right colon cancer, 309 (30.9%) cases of left colon cancer, and 523 (52.2%) rectal cancer. According to American Joint Committee on Cancer staging Manual, there were 171 (17.1%) cases of stage I, 320 (31.9%) stage II, 345 (34.5%) stage III, and 165 (16.5%) stage IV. In the interim, a total of 1005 cancer-free controls were randomly selected from a subject pool of more than 10,000 individuals who participated in health check-up programs at the community health stations in Guangzhou, China.

The study participants were interviewed and data on smoking status, alcohol use and other factors including family history of cancer were obtained using a structured questionnaire. Smoking status, alcohol use and family history of cancer were defined as described previously [38]. Subjects whose body mass index (BMI) was <18.5 kg/m2 were categorized as being underweight, subjects whose BMI was from 18.5 to 24.0 kg/m2 were normal body weight, those who have a BMI >24.0 kg/m2 were overweight [39]. Cases belonging to familial adenomatous polyposis and those cases that fulfilled the criteria of Amsterdam for hereditary nonpolyposis CRC were excluded.

Genotyping

Five mL blood was collected from each participant and genomic DNA was extracted using the DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For determination of the NFκB1 promoter (rs28362491) polymorphism, the SNP-containing fragment was amplified using the following primers: 5'-TGGGCAAACTGCTTTATTTAG-3' (forward) and 5'-CTGGAGCCGTTAGGAAG-3' (reverse). PCR was run at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s with a final extension at 72°C for 10 min. The PCR products (281/285 bp in size) were digested with PvuII (Fermentas, Vilnius, Lithuania) at 37°C overnight followed by 2% agarose gel electrophoresis. For determination of the NFκB1 (rs696) polymorphism, the SNP-containing fragment was amplified using the following primers: 5'-GGCTGAAAGACATGAGGT-3' (forward) and 5'-GTACACATTCCAGGGAGG-3' (reverse). The PCR was run at 94°C for 5 min followed by 32 cycles of 94°C for 30 s, 54.3°C for 45 s and 72°C for 60 s with a final extension at 72°C for 10 min. The amplified fragments were digested with HhaIII (Fermentas, Vilnius, Lithuania) overnight at 37°C followed by 2% agarose gel electrophoresis.
Genotype analysis was done by two experimenters independently who were blinded to the status of the subjects as patient or control. To further validate the genotyping results, we randomly selected 10% samples for each of the 2 SNPs to perform repeat assays, and the results were 100% concordant. Additionally, 5% of the PCR products for each target genotype were purified and confirmed by direct sequencing (Figure S1 and Figure S2).

Bioinformatics analysis
To investigate whether genetic variants of NFkB1 could bind to microRNAs (miRNA), we searched for target microRNAs of genetic variants of NFkB1 using the algorithm programs (http://www.targetscan.org and http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl).

Cell culture
Three human colorectal adenocarcinoma cell lines, HCT116, HT29 and SW480 were purchased from Culture Collection of Chinese Academy of Science (Shanghai, China) and routinely cultured in Dulbecco’s Modified Eagle’s Medium or RPMI 1640 medium supplemented with 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 10% fetal bovine serum (FBS). The cells were grown at 37°C with 5% CO2 in a humidified incubator.

RNA interference, transient transfections and luciferase assays

NFkB1 mRNA

miR-449a mimics targeting NFkB1, 5'-UGGGACUGUAAUGUUGUGG-3' (sense) and 5'-CGACGUAACAUAGACUACUUAACUGGCAAU-3' (anti-sense), miR-449a inhibitor, 5'-ACCAGCGU-AAAGAACAGUGCCA-3', miR-34b mimics targeting NFkB1, 5'-CAACUGACUAACUCACCUCCGCAAU-3' (sense) and 5'-GGGAGGGAGUAGUGAGUGU-3' (anti-sense), and miR-34b inhibitor, 5'-AUCUGAGUGGAGUAGUGUUGG-3' were synthesized by GenePharma Co. (Shanghai, China). In addition, the 3'UTR of the NFkB1 2758A allele (2575 to 2955 bp relative to the translation start site) containing the binding site, and family history of cancer. Logistic regression modeling was also used for the trend test. Student's t test was done to examine the difference in levels of luciferase reporter gene expression between different constructs. Kruskal-Wallis one-way ANOVA tests were used for analyzing NFkB1 protein expression in peritumoral tissues of different genotypes. All tests were two-sided by using the SAS software (version 9.1; SAS Institute, Cary, NC). P<0.05 was considered statistically significant.

Results
Characteristics of the study population
The distribution of demographic characteristics of the 1001 sporadic CRC cases and 1005 cancer-free controls are shown in Table 1. Overall, no statistically significant difference was observed in age, sex, and smoking status between the cases and controls (P>0.05 in all). Compared with controls, there were more ever drinkers (cases vs. controls, 45.5% vs. 23.6%) (P<0.01) in the cohort of CRC cases. Moreover, compared with controls, CRC cases were more likely to have a family history of cancer or a higher BMI (P<0.05 in both). Consequently, these variables were further adjusted for in the multivariate logistic regression model to avoid possible confounding on the main effects of the SNPs under study. They were also used in the subsequent stratification and gene-environment interaction analysis.

PCR-RFLP study of the NFkB1 promoter region and the NFkB1 3'UTR region
We carried out analysis of the NFkB1 promoter region by the PCR-RFLP method. Two ATTG repeats are present at the NFkB1 promoter region, and one allele that has an ATTG insertion (ins) was cleaved into a 43-bp and a 240-bp fragment after digestion with PvuII while the other deletion allele (del) that has only one ATTG at its promoter was not cleaved (Figure 1a). Furthermore, after digestion with HaeIII, the 2758GG genotype produced a 316-bp and a 108-bp band whereas the 2758AA genotype produced a single 424-bp band, and heterozygotes displayed all 3 bands (Figure 1b).
Table 1. Frequency distributions of selected variables in CRC patients and cancer-free controls.

| Variables                  | 1001 Patients (n, %) | 1005 Controls (n, %) | P*  |
|----------------------------|----------------------|----------------------|-----|
| Age (years)                |                      |                      |     |
| <50                        | 233 (23.3)           | 231 (23.0)           | 0.4831 |
| 50–60                      | 277 (27.7)           | 302 (30.0)           |     |
| >60                        | 491 (49.0)           | 472 (47.0)           |     |
| Sex                        |                      |                      | 0.9487 |
| Male                       | 604 (60.3)           | 605 (60.2)           |     |
| Female                     | 397 (39.7)           | 400 (39.8)           |     |
| Smoking status             |                      |                      | 0.1964 |
| Ever                       | 482 (48.1)           | 455 (45.3)           |     |
| Never                      | 519 (51.9)           | 550 (54.7)           |     |
| Alcohol drinking           |                      |                      | <0.0001 |
| Ever                       | 456 (45.5)           | 237 (23.6)           |     |
| Never                      | 545 (54.5)           | 768 (76.4)           |     |
| Family history of cancer   |                      |                      | 0.0122 |
| Yes                        | 115 (11.5)           | 82 (8.2)             |     |
| No                         | 886 (88.5)           | 923 (91.8)           |     |
| BMI (kg/m²)                |                      |                      | 0.0290 |
| <18.5                      | 63 (6.3)             | 39 (3.9)             |     |
| 18.5–24.0                  | 662 (66.1)           | 702 (69.8)           |     |
| >24.0                      | 276 (27.6)           | 264 (26.3)           |     |
| Tumor site                 |                      |                      |     |
| Right colon                | 169 (16.9)           |                      |     |
| Left colon                 | 309 (30.9)           |                      |     |
| Rectum                     | 523 (52.2)           |                      |     |
| Tumor Stages               |                      |                      |     |
| I                          | 171 (17.1)           |                      |     |
| II                         | 320 (31.9)           |                      |     |
| III                        | 345 (34.5)           |                      |     |
| IV                         | 165 (16.5)           |                      |     |

*P values for a two-sided χ² test. doi:10.1371/journal.pone.0021726.t001

The NFKB1-94ins/delATTG (rs28362491) and NFKBIA 2758A>G (rs696) polymorphisms are associated with the risk of sporadic CRC.

The genotype and allele distributions of the NFKB1-94ins/delATTG (rs28362491) and NFKBIA 2758A>G (rs696) polymorphisms among the cases and controls are summarized in Table 2. The observed genotype frequencies of these two polymorphisms were all in agreement with the Hardy-Weinberg equilibrium in the control subjects (P > 0.05). As shown in Table 2, for rs28362491, the distribution of the co-dominant genetic model (del/del vs. ins/ins vs. del/ins), and the dominant model (ins/ins vs. del/ins vs. del/del) differed significantly between CRC cases and controls (ins/ins: OR = 1.17; 95% CI = 1.12–2.25; P < 0.01; del/ins: OR = 1.33; 95% CI = 1.03–1.74; P < 0.01; ins/ins vs. del/ins: OR = 1.47; 95% CI = 1.14–1.86; P < 0.01, respectively). For rs696, there was a significant difference in the distribution of the rs696 genotypes between CRC cases and controls (P < 0.01). Significant differences were also noted in the distribution of the recessive model of rs696 (GG versus A+AG) between CRC cases and controls (OR = 1.38; 95% CI = 1.14–1.66; P < 0.05). Consistently, significant association was found between the two SNPs and the risk of sporadic CRC.

Stratification analysis of the association of combined NFKB1 and NFKBIA polymorphisms with risk of CRC

We further analyzed the combined genotypes of the NFKB1 and NFKBIA polymorphisms by examining age, sex, smoking, drinking, BMI, tumor site, and family history of cancer by logistic regression. We combined the NFKB1 and NFKBIA polymorphisms based on the number of variant (risk) alleles (i.e., 2758GG and -94ins/del/del). As shown in Table 3, compared with the NFKB1-94 del/del and NFKBIA 2758A+AG genotype, one variant combined genotype carriers who were <60 years of age had a higher risk of CRC (OR = 1.57; 95% CI = 1.04–2.38; P < 0.05). Further stratification analysis revealed that individuals with two variants had a significantly higher risk of CRC among subgroups of patients aged <60 years (OR = 2.18; 95% CI = 1.37–3.44; P < 0.01), women (OR = 2.36; 95% CI = 1.35–4.10; P < 0.01), never smokers (OR = 2.05; 95% CI = 1.30–3.22; P < 0.01), never drinkers (OR = 1.87; 95% CI = 1.23–2.84; P < 0.01), persons with a normal BMI (OR = 1.80; 95% CI = 1.21–2.69; P < 0.01) and those with a family history of cancer (OR = 4.57; 95% CI = 1.34–15.6; P < 0.05).

Figure 1. Image of the cleavage products of the PfI and HaeIII restriction enzyme on 2% agarose gel. (a) PfI digestion band profile of the NFKB1 gene on 2% agarose gel. Lane 1 Gene Ruler 100 bp Low Range Ladder, Lanes 2 and 7 del/ins heterozygote genotype, Lanes 4 and 6 ins/ins genotype, Lanes3, 5 del/del genotype. (b) HaeIII digestion band profile of NFKBIA on 2% agarose gel. Lane 1 Gene Ruler 100 bp Low Range Ladder, Lanes 3, 5 homoyzogote G/G genotype, Lanes 2 and 6 homoyzogote A/A genotype, Lanes 4, 7 heterozygote A/G genotype.

doi:10.1371/journal.pone.0021726.g001
**Table 2.** Distribution of genotypes in NFkB1 and NFkB1A, and results of logistic regression analysis for associations with risk of colorectal cancer.

| Genotypes                             | Patients n (%) | Controls* n (%) | P   | Crude OR (95% CI) | Adjusted OR (95% CI)* |
|---------------------------------------|---------------|-----------------|-----|------------------|-----------------------|
| Total no. of subjects                 | 1001          | 1005            |     |                  |                       |
| Total no. of alleles                  | 2002          | 2010            |     |                  |                       |
| NFkB1 Rs28362491                      |               |                 |     |                  |                       |
| del/del                              | 138 (13.8)    | 186 (18.5)      | 0.0008 | 1.00 (ref.)   | 1.00 (ref.)           |
| del/ins                              | 500 (49.9)    | 522 (51.9)      |     | 1.29 (1.01–1.66) | 1.33 (1.03–1.74)     |
| ins/ins                              | 363 (36.3)    | 297 (29.6)      |     | 1.65 (1.26–2.15) | 1.70 (1.29–2.25)     |
| ins/ins/del/ins                      | 863 (86.2)    | 819 (81.5)      |     | 1.42 (1.12–1.81) | 1.47 (1.14–1.86)     |
| del/del                              | 138 (13.8)    | 186 (18.5)      |     | 1.00 (ref.)     | 1.00 (ref.)           |
| NFkB1A Rs696 A>G                      |               |                 | 0.0075 |                |                       |
| AA                                   | 233 (23.3)    | 212 (21.1)      |     | 1.00 (ref.)     | 1.00 (ref.)           |
| GA                                   | 460 (45.9)    | 531 (52.8)      |     | 0.79 (0.63–0.99) | 0.78 (0.62–0.98)     |
| GG                                   | 308 (30.8)    | 262 (26.1)      |     | 1.07 (0.83–1.37) | 1.04 (0.81–1.35)     |
| AA+GA                                | 693 (69.2)    | 743 (73.9)      | 0.0196 | 1.00 (ref.)   | 1.00 (ref.)           |
| GG                                   | 308 (30.8)    | 262 (26.1)      |     | 1.38(1.15–1.65) | 1.38(1.14–1.66)      |
| Number of variant genotypes*         |               |                 | 0.0008 |                |                       |
| 0                                    | 100 (10.0)    | 127 (12.6)      |     | 1.00 (ref.)     | 1.00 (ref.)           |
| 1                                    | 631 (63.0)    | 675 (67.2)      |     | 1.19 (0.89–1.58) | 1.22 (0.90–1.63)     |
| 2                                    | 270 (27.0)    | 203 (20.2)      |     | 1.69 (1.23–2.32) | 1.71 (1.23–2.38)     |
| Trend test P value                   |               |                 | 0.0003 | 0.0004        |                       |

*The observed genotype frequencies among the control subjects were in agreement with the Hardy-Weinberg equilibrium (p^2+2pq+q^2 = 1) (χ^2 = 3.539, P = 0.060 for Rs696G->A, P = 0.102 for Rs28362491 -94del/ins ATTG).

**Two-sided χ^2-test for the distribution of genotype frequency.

**Four were adjusted in a logistic regression model that included age, sex, smoking status, alcohol use, family history of cancer and BMI.

**Either the Rs696GG or Rs28362491 ins/ins/del/ins genotypes are risk genotypes as one.

DOI:10.1371/journal.pone.0021726.t002

**Mir-449a mimics suppressed the activities of the NFkB1A 2758 A>G polymorphism**

We further analyzed the 3’UTR of the NFkB1A 2758 A>G polymorphism using a computer algorithm and found that the polymorphism could affect miRNA binding. Mir-449a and miR-34a, which are involved in a wide variety of biological functions, were found to have a binding site within the 3’UTR of the NFkB1A 2758 A>G polymorphism (Figure 2a). To determine the allele-specific effect of the NFkB1A 2758 A>G variant on the activity of the 3’UTR and whether this polymorphism affected miRNA binding, we transfected CRC cell lines SW480, HT29, and HCT116 with reporter plasmids carrying the 2758G or 2758A allele and miR mimics or inhibitors (Figure 2b). We found that, in the absence of miR mimics or inhibitors, compared with the 2758A allele, the 2758G allele exhibited decreased luciferase activities (P<0.05) (Figure 2b). Mir-449a mimics could reduce the relative luciferase activities of the 2758A allele while mir-449a inhibitors significantly up-regulate the relative luciferase activities of the 2758A allele in all the three cell lines (P<0.01 for HT29; P<0.05 for SW480 and HCT116) (Figure 2b). On the other hand, mir-34a mimics failed to exert any noticeable effects on the relative luciferase activities of the 2758G allele in these cell lines (data not shown). Taken together, these results demonstrated that the 2758G allele in the 3’UTR of the NFkB1A reduced normalized luciferase activity compared to the 2758A allele, likely corresponding to reduced mRNA stability or translational efficacy and that miR-449a have the ability to bind and partially repress luciferase expression via the NFkB1A 3’UTR segment when carrying the 2758A allele of NFkB1A 2758 A>G polymorphism.

**Association of the NFkB1A 3’UTR polymorphisms with the NFkB1A protein expression**

We were also interested in investigating whether the NFkB1A 3’UTR polymorphisms (2758A>G) were associated with increased or reduced expression of NFkB1A protein. We collected 32 paired tumor and peritumoral tissues from the untreated CRC patients of different genotypes. Immunoblotting analysis revealed that the levels of NFkB1A (NFkB1A/β-actin protein ratio) in the peritumoral tissues of 12 cases of the 2758A allele was 0.80±0.09 and those of 8 cases of the 2758GG genotype was 0.63±0.11, both of which were significantly higher than those of the other 12 cases of the 2758GG genotype (0.47±0.06) (analysis of variance test, P<0.05) (Figure 3). However, in the tumor tissues, NFkB1A expression levels did not differ significantly among the cases of different genotypes (data not shown). These results indicated that NFkB1A levels were significantly higher in peritumoral tissues from patients of the 2758A allele than those of the 2758GG genotypes.

**Discussion**

In the present study, we investigated the associations of the NFkB1-94del/insATTG and NFkB1A 2758A>G polymorphisms...
with risk of CRC in a southern Han Chinese population. We found that both the \(NFkB1-94\) del/ins \(ATTG\) and \(NFkBIA\) 2758A>G polymorphisms were associated with increased risk of CRC. For the \(NFkB1-94\) del/ins \(ATTG\) polymorphism, with the \(-94\) del/del as the reference, we found that the \(-94\) ins/ins genotype was associated with a statistically significantly increased risk of CRC. For the \(NFkBIA\) 2758A>G polymorphism, with the 2758LA+GA as the reference, we also found that the 2758GG genotype was associated with a statistically significantly increased risk of CRC. Furthermore, when we evaluated \(NFkB1\) and \(NFkBIA\) polymorphisms in combination, we found that the combined 2758GG and -94A+G genotypes were associated with a significantly increased risk of CRC compared with those without the 2758GG and -94A+G genotype, and this increased risk was more pronounced among younger than 60 years, women, never drinkers, never smokers, persons with a normal BMI and those with family history of cancer. In the in vitro assays, we also found that, compared with the 2758A allele, the 2758G variant allele showed significantly decreased mRNA stability and/or translational efficacy. Furthermore, we found that \(NFkBIA\) levels significantly higher in peritumoral tissues from patients of the

---

**Table 3. Stratification analysis of the variant number of genotypes by selected variables in colorectal cancer patients and controls.**

| Patients (\(n = 1001\)) | Controls (\(n = 1005\)) | Adjusted OR (95% CI) \(^a\) |
|--------------------------|--------------------------|---------------------------|
| **Number of variant genotypes** | **Number of variant genotypes** | **Adjusted OR (95% CI) \(^a\)** |
| 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 | \(P_{\text{trend}}\) \(^b\) |
| **Age (years)** | | | | | | | | | |
| \(\leq 60\) | 47 (9.2) | 328 (64.3) | 135 (26.5) | 78 (14.6) | 356 (68.6) | 99 (18.6) | 1.00 (ref) | 1.57 (1.04–2.38) | 2.18 (1.37–3.44) | 0.0015 |
| \(> 60\) | 53 (10.8) | 303 (61.7) | 135 (27.5) | 49 (10.4) | 319 (67.6) | 104 (22.0) | 1.00 (ref) | 0.89 (0.56–1.41) | 1.28 (0.77–2.11) | 0.0923 |
| **Sex** | | | | | | | | | |
| Male | 67 (11.1) | 376 (62.2) | 161 (26.7) | 81 (13.4) | 393 (65.0) | 131 (21.6) | 1.00 (ref) | 1.36 (0.90–2.05) | 1.49 (0.98–2.27) | 0.0530 |
| Female | 33 (8.3) | 255 (64.2) | 109 (27.5) | 46 (11.5) | 282 (70.5) | 72 (18.0) | 1.00 (ref) | 1.30 (0.80–2.11) | 2.36 (1.35–4.10) | 0.0007 |
| **Smoking status** | | | | | | | | | |
| Never | 46 (8.9) | 329 (63.4) | 144 (27.7) | 71 (12.9) | 370 (63.7) | 109 (19.8) | 1.00 (ref) | 1.34 (0.89–2.00) | 2.05 (1.30–3.22) | 0.0066 |
| Ever | 51 (11.2) | 302 (62.7) | 126 (26.1) | 56 (12.3) | 305 (67.0) | 92 (20.7) | 1.00 (ref) | 1.09 (0.68–1.73) | 1.42 (0.87–2.33) | 0.0986 |
| **Drinking status** | | | | | | | | | |
| Never | 53 (9.7) | 338 (62.0) | 154 (28.3) | 94 (12.2) | 950 (69.0) | 144 (18.8) | 1.00 (ref) | 1.09 (0.75–1.59) | 1.87 (1.23–2.84) | 0.0022 |
| Ever | 47 (10.3) | 293 (64.3) | 116 (25.4) | 33 (13.9) | 145 (61.2) | 59 (24.9) | 1.00 (ref) | 1.41 (0.85–2.34) | 1.58 (0.89–2.80) | 0.3055 |
| **Family history of cancer** | | | | | | | | | |
| YES | 10 (8.7) | 72 (62.6) | 33 (28.7) | 15 (18.3) | 55 (67.1) | 12 (14.6) | 1.00 (ref) | 2.50 (0.91–6.87) | 4.57 (1.34–15.6) | 0.0110 |
| NO | 90 (10.2) | 559 (63.1) | 237 (26.7) | 112 (12.1) | 620 (67.2) | 191 (20.7) | 1.00 (ref) | 1.14 (0.59–2.06) | 1.54 (1.09–2.17) | 0.0048 |
| **Tumor site** | | | | | | | | | |
| Right colon | 15 (8.9) | 106 (62.7) | 48 (28.4) | 127 (12.6) | 675 (67.2) | 203 (20.2) | 1.00 (ref) | 1.18 (0.82–1.69) | 1.59 (1.07–2.37) | 0.0107 |
| Left colon | 32 (10.4) | 193 (62.4) | 84 (27.2) | 127 (12.6) | 675 (67.2) | 203 (20.2) | 1.00 (ref) | 1.18 (0.77–1.71) | 1.62 (1.01–2.61) | 0.0238 |
| Rectum | 53 (10.1) | 332 (63.5) | 138 (26.4) | 127 (12.6) | 675 (67.2) | 203 (20.2) | 1.00 (ref) | 1.42 (0.79–2.55) | 1.94 (1.02–3.67) | 0.0293 |
| BMI (kg/m\(^2\)) | | | | | | | | | |
| <18.5 | 11 (17.5) | 38 (60.3) | 14 (22.2) | 37 (5.9) | 287 (81.8) | 8 (20.5) | 1.00 (ref) | 0.28 (0.06–1.36) | 0.22 (0.02–2.40) | 0.5447 |
| 18.5–24.0 | 65 (9.8) | 414 (62.5) | 183 (27.7) | 91 (13.0) | 470 (66.9) | 141 (20.1) | 1.00 (ref) | 1.32 (0.92–1.90) | 1.80 (1.21–2.69) | 0.0022 |
| >24.0 | 24 (8.7) | 179 (64.9) | 73 (26.4) | 33 (12.5) | 171 (67.1) | 54 (20.4) | 1.00 (ref) | 1.10 (0.83–1.47) | 1.84 (0.95–3.54) | 0.0644 |

\(^a\)ORs were adjusted for age, sex, smoking status, and alcohol use, BMI and family history of cancer in a logistic regression models.

\(^b\)Adjusted in a logistic regression model that included age, sex, smoking status, alcohol use, BMI and family history of cancer.

\(^*\)The number represents the numbers of variants within the combined genotypes, ie.0 = no variant (risk) allele and 1–2 = 1–2variant (risk) alleles; the variant (risk) alleles used for the calculation were the -94 ins/ins del/ins and 2758GG alleles.

\(\text{doi:10.1371/journal.pone.0021726.t003}\)
Several association studies have reported that the NFkB1 and NFkB1A polymorphisms is related to the development of inflammatory and other diseases including ulcerative colitis, Graves’ disease, and diabetes mellitus, and susceptibility to tumors including melanoma, bladder cancer and CRC in different ethnic groups [32,36,45,46,47,48]. Gao et al.’s study reported a lack of an association between the NFkBIA2758 A>G polymorphism in northern Chinese population, but showed an association with the Swedish population and CRC risk [36]. Previous studies have provided evidence that the del allele may result in relatively decreased NFkB1 transcript levels and hence decreased p50/p105 NFkB1 protein production [32]. In our study, we further found that the change of the 2758A to G allele in the 3' UTR of NFkBIA decreased luciferase activities as assessed by luciferase assays. Our functional in vitro experiments suggested that NFkBIA 2758 A>G variants may affect mRNA stability. However, that the NFkBIA 2758 A>G variants affects translational efficacy or conduces to differential nuclear RNA processing or export also cannot be completely excluded [49]. Furthermore, it is well known that miRNAs can also cause mRNA cleavage or translational repression by forming imperfect base pairing with the 3' UTR of target genes. In silico analysis of the NFkBIA mRNA sequence predicted the 2758 A>G variant of NFkBIA generates a potential seed site for miR-449a and our ex vivo luciferase data indicated that miR-449a reduced the relative luciferase activities via the NFkBIA 3' UTR target site created by the 2758A allele. The results indicated that the 2758A allele strengthens the binding of miR-449a with 3' UTR of NFkBIA, which in turn inhibits the expression of NFkBIA. Recent evidence indicate that miRNAs can bind to the 3' UTRs of mRNAs and affect their translation, thus regulating cell proliferation, apoptosis and tumorigenesis [50]. Experimental studies concluded that SNPs located in miRNA-binding sites affect miRNA target expression and function, which is potentially associated with cancers [51]. This reconcile with our results that the NFkBIA 2758A-allele may enhance binding of miR-449a and affect NFkBIA gene expression and change of the 2758A to G allele in the 3'UTR of NFkBIA may affect mRNA stability or translational efficacy subsequent diminished protein levels. Consistently, we observed that NFkBIA protein expression was higher in the peritumoral tissues but not in tumor tissues from patients of

Figure 2. Validation of target microRNA predictions. (a) A putative target site of miR-449a and miR-34b highly conserved in the NFkBIA mRNA 3'UTR. (b) psiCHECK-2 Dual Luciferase Assay in three cell lines: SW480, HT29 and HCT116. Cells were transfected with reporter plasmids alone or co-transfected with microRNA. Luciferase expression was measured 48 hrs after transfection. The luciferase activity of each construct was normalized against the internal control of Renilla luciferase. Columns, mean from three independent experiments; bars, SD. *, P<0.05 for all comparisons of each cell line between the activities of the reporter gene constructs.

doi:10.1371/journal.pone.0021726.g002
the 2758AA+GG genotype than those of the 2758GG genotype. These data suggest that wildtype A allele may result in overexpression of NFkBIA in peritumoral normal tissues and hence increased NFkBIA protein production, resulting in inhibition of NFkB activity. However, carrying a mutant 2758G allele result in relatively decreased NFkBIA mRNA stability and hence diminished NFkBIA protein production. As a major inhibitor of NFkB, decreased expression and dysfunction of NFkBIA may be a direct result of the activation of NFkB and thus cancer [16]. This is also consistent with our initial expectations, since CRC has been associated with increased levels of NFkB1 G allele. These findings indicate that the NFkB1-94del/insATTG and NFkBIA 2758 A>G polymorphisms are functional. Our data from this relatively large sample size study further support the notion that the NFkB1 and NFkBIA polymorphisms are potentially implicated in cancer risk.

In the present study, we also observed that the combined effect of the NFkB1 and NFkBIA polymorphisms on risk of CRC was more pronounced among younger than 60 years, women, never drinkers, never smokers, persons with a normal BMI and those with a family history of cancer, suggesting that, in these subpopulations, gene-environment interaction may be very weak and the combined effect was an independent risk factor for these subpopulations. Compared with the published data, our results indicate that the genotype distributions of the NFkB1 and NFkBIA polymorphisms vary with ethnicity. For example, the frequencies of the del/del, del/ins, and ins/ins genotypes of the NFkB1-94del/insATTG among our 1005 southern Han Chinese control subjects were 18.5%, 51.9%, and 29.6%, respectively, compared with 15.6%, 45.9%, and 38.4%, respectively, of 307 Germans in the study by Kathrin et al. [37]. Similarly, the frequencies of the AA, AG and GG genotypes of the NFkBIA 2758A>G in our controls were 18.5%, 51.9%, and 29.6%, respectively, compared with 6%, 45%, and 49%, respectively, of 109 cancer-free Australian controls in the study by Curran et al. [52]. However, the frequencies of the del/del, del/ins, and ins/ins genotypes of the NFkB1-94del/insATTG (18.5%, 51.9%, and 29.6%, respectively) were very similar to the published data by Sun et al. (17%, 50%, and 24%, respectively) [53]. In our southern Han Chinese controls, the frequency of the NFkBIA genotype distributions is also similar to that reported for that of the northern Han Chinese [36]. In this study, we also found that the insertion of NFkB1-94del/insATTG polymorphism increased CRC risk, which was contrary to the results reported by Andersen et al [54]. It is likely that the discrepancy results from the genetic

**Figure 3. Association of the NFkBIA 3′ UTR polymorphism with the NFkBIA protein expression.** NFkBIA protein levels in 32 sporadic CRC peritumoral tissues from individuals with different genotypes of 2758A>G. The NFkBIA protein expression levels were normalized to that of β-actin by calculating the relative expression levels. Individual genotype designation: For 2758A>G, lanes 1–3, 9–11, 17–20, 25–26, AA genotype (n = 12); lanes 4–6,12–14, 21–24, 27–28, GG genotype (n = 12); lanes 7–8, 15–16, 29–32, AG genotype (n = 8).

doi:10.1371/journal.pone.0021726.g003
difference in ethnicity (the frequencies of the del/del, del/ins, and ins/ins genotypes of the NFKB1-94del/insATTG among our 1005 southern Han Chinese control subjects were 18.3%, 51.9%, and 29.6%, respectively, compared with 13.5%, 45.9%, and 40.6%, respectively, of 736 Danes in the study). In addition, environmental effects such as dietary and lifestyle may also contribute to the discrepancy [7]. However, this hypothesis warrants further investigation.

In conclusion, our results suggested that both NfXb1 and NfXb1a polymorphisms have effect on risk of CRC. These findings suggest that the NfXb1 and NfXb1a polymorphisms may jointly contribute to the risk of CRC in a southern Chinese population, which were consistent with the functional assays we performed. Our study indicated that the NfXb1-94fins/insdel/del/ins and NfXb1a G0 polymorphism may be a genetic marker for susceptibility to CRC in Chinese populations. However, additional studies with more detailed data on environmental exposure and survival data are required to verify these findings. Therefore, future population-based studies are needed to verify the findings.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69–90.

2. Cao KJ, Fan QY, Liu YL, Huang R, Yin CZ, et al. (2008) Cancer incidence and mortality in Guangzhou City from 2000 to 2002. Ai Zheng 27: 225–230.

3. Cheah PY (2009) Recent advances in colorectal cancer genetics and diagnostics. Crit Rev Oncol Hematol 69: 45–55.

4. Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, et al. (2000) Epidemiological evidence of the causation of cancer-analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med 343: 78–85.

5. Hayakawa Y, Sekine T, Sasaba T (1993) A case-control study of colorectal cancer and its relation to diet, cigarettes, and alcohol consumption in Saitama Prefecture, Japan. Tokoho J Exp Med 171: 153–165.

6. Tsong WH, Kho WP, Yuan JM, Wang R, Sun CL, et al. (2007) Cigarettes and alcohol in relation to colorectal cancer: the Singapore Chinese Health Study. Br J Cancer 96: 921–927.

7. Huxley RR, Ansary-Moghaddam A, Clifton P, Czeika I, Rinaldi S, et al. (2009) The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. Int J Cancer 125: 171–180.

8. von Roon AC, Reese G, Teare J, Constantinides V, Darzi AW, et al. (2007) The role of ethnicity (the frequencies of the del/del, del/ins, and ins/ins genotype; (b) del/ins genotype; (c) ins/ins genotype. (PPT)

Supporting Information

Figure S1 NfXb1-94fins/delATTG genotyping by direct sequencing: (a) del/del genotype; (b) del/ins genotype; (c) ins/ins genotype. (PPT)

Figure S2 NfXb1a 2758A>G genotyping by direct sequencing: (a) GG genotype; (b) AA genotype; (c) AG genotype. (PPT)

Acknowledgments

The authors would like to thank all the participants for taking part in this research, especially Ms. Qinghua Huang, Peihuang Wu and Dr. Zhengyu Xian; Dechang Diao for their laboratory assistance; and gratefully acknowledge Defeng Chen for revising the manuscript.

Author Contributions

Conceived and designed the experiments: JPW LW JCL. Performed the experiments: SXS DKJ JWL JCL LY. Contributed reagents/materials/analysis tools: SXS DKC YSW XHF. Wrote the paper: SXS LW. Revision of the paper: SXS DKC JWL JCL LY. Contributed reagents/materials/analysis tools: SXS DKC YSW XHF.
36. Gao J, Pfeifer D, He Lj, Qiao F, Zhang Z, et al. (2007) Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations. Scand J Gastroenterol 42: 345–350.

37. Riemann K, Becker L, Struve H, Nuckel H, Duhrsen U, et al. (2006) No association of the NFKB1 insertion/deletion promoter polymorphism with survival in colorectal and renal cell carcinoma as well as disease progression in B-cell chronic lymphocytic leukemia. Pharmacogenet Genomics 16: 783–788.

38. Wei Y, Wang L, Lan P, Zhao H, Fan Z, et al. (2009) The association between −1304T>G polymorphism in the promoter of MKK4 gene and the risk of sporadic colorectal cancer in southern Chinese population. Int J Cancer 125: 1876–1883.

39. Zhou BF (2002) Predictive values of body mass index and waist circumference for risk factors of certain related diseases in Chinese adults–study on optimal cut-off points of body mass index and waist circumference in Chinese adults. Biomed Environ Sci 15: 83–96.

40. Sonenshein GE (1997) Rel/NF-kappa B transcription factors and the control of apoptosis. Semin Cancer Biol 8: 113–119.

41. Richmond A (2002) NF-kappa B, chemokine gene transcription and tumour growth. Nat Rev Immunol 2: 664–674.

42. Biswas DK, Cruz AP, Ganzberger E, Pardee AB (2000) Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. Proc Natl Acad Sci U S A 97: 8542–8547.

43. Doket X, Lloret D, Pallares J, Matias-Guiu X (2005) NF-κB in development and progression of human cancer. Virchows Arch 446: 473–482.

44. Amiri KI, Richmond A (2005) Role of nuclear factor-kappa B in melanoma. Cancer Metastasis Rev 24: 301–313.

45. Sun XF, Zhang H (2007) Importance of polymorphisms in NF-kappaB1 and NF-kappaB1alpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. J Cancer Res Clin Oncol 133: 859–866.

46. Miller MR, Zhang W, Sibbel SP, Langefeld CD, Bowden DW, et al. (2010) Variant in the 3′ region of the IkappaBalpha gene associated with insulin resistance in Hispanic Americans: The IRAS Family Study. Obesity (Silver Spring) 18: 555–562.

47. Bu H, Rosdahl I, Sun XF, Zhang H (2007) Importance of polymorphisms in NFkappaB1 and NF-kappaB1alpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. J Cancer Res Clin Oncol 133: 859–866.

48. Zhou BF (2002) Predictive values of body mass index and waist circumference for risk factors of certain related diseases in Chinese adults–study on optimal cut-off points of body mass index and waist circumference in Chinese adults. Biomed Environ Sci 15: 83–96.

49. Sonenshein GE (1997) Rel/NF-kappa B transcription factors and the control of apoptosis. Semin Cancer Biol 8: 113–119.

50. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.

51. Yu Z, Li Z, Jolicourte N, Zhang L, Fortin Y, et al. (2007) Aberrant allele frequencies of the SNPs located in microRNA target sites are potentially associated with human cancers. Nucleic Acids Res 35: 4535–4541.

52. Curran JE, Weinstein SR, Griffiths LR (2002) Polymorphic variants of NFKB1 and its inhibitory protein NFKBIA, and their involvement in sporadic breast cancer. Cancer Lett 188: 103–107.

53. Sun XF, Zhang H (2007) NFκB and NFκB1 polymorphisms in relation to susceptibility of tumour and other diseases. Histol Histopathol 22: 1387–1398.

54. Andersen V, Christensen J, Overvad K, Tjonneland A, Vogel U (2010) Polymorphisms in NFκB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. BMC Cancer 10: 484.