Expression of NF-kB and COX2 in Colorectal Cancer among Native Indonesians: The Role of Inflammation in Colorectal Carcinogenesis

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ABSTRAK

Tujuan: *untuk menilai ekspresi NF-κB dan COX2 pada orang Indonesia dengan kanker kolorektal (KKR) sporadis.* ***Metode:*** *penelitian kasus kontrol berpasangan dengan menganalisis jaringan tumor KKR dan jaringan usus sehat di sekitar tumor dari pasien yang sama. Pasien KKR yang dioperasi di RS Cipto Mangunkusumo Jakarta atau di RS Hasan Sadikin Bandung pada periode Januari 1998 – April 2008 disertakan sebagai subjek penelitian. Spesimen jaringan diwarnai secara immunohistokimia dengan antibodi terhadap p65 (Re1A) untuk menilai ekspresi NF-κB dan antibodi terhadap protein COX2 manusia untuk menilai ekspresi COX2 pada jaringan.* ***Hasil:*** *masing-masing 67 spesimen jaringan KKR dan jaringan sehat terkumpul dan dianalisis. Ekspresi COX2 positif pada 39 (58%) jaringan KKR tetapi positif hanya pada 19 (28%) jaringan normal (p=0,0002; OR=3,75). Sedangkan ekspresi NF-κB positif pada 47 (70%) jaringan KKR dan 27 (40%) jaringan normal (p<0,0001; OR=5,91).* ***Kesimpulan:*** *jalur inflamasi memegang peranan penting dalam karsinogenesis KKR sporadis pada orang Indonesia. Hasil penelitian ini mendukung kemungkinan penggunaan obat antiinflamasi nonsteorid sebagai agen pencegah KKR.*

Kata kunci: *kanker kolorektal sporadis, karsinogenesis, COX2, NF-κB, inflamasi.*

ABSTRACT

Aim: *to evaluate the expression of NF-kB and COX2 in native Indonesians with sporadic colorectal cancer (CRC).* ***Methods:*** *we conducted a matched-pair case–control study by acquiring both CRC and tumor-adjacent normal tissues from the same subjects. CRC patients who underwent surgery at Cipto Mangunkusumo Hospital, Jakarta, or Hasan Sadikin Hospital, Bandung, were enrolled in the study. The specimens were immunohistologically stained with antibody directed against p65 (RelA) to assess NF-kB expression and against human COX2 protein to assess COX2 expression.* ***Results:*** *sixty-seven specimens consisting of both CRC and tumor-adjacent normal tissues were analyzed. COX2 expression was positive in 39 CRC tissues (58.2%), but in only 19 tumor-adjacent normal tissues (28.4%; p=0.0002). NF-kB expression was positive in 47 CRC tissues (70.1%), but in only 27 tumor-adjacent normal tissues (40.3%; p<0.0001).* ***Conclusion:*** *inflammation plays a role in the carcinogenesis of sporadic CRC in native Indonesians. This support potential use of nonsteroidal anti-inflammatory drugs as chemopreventive agents for CRC.*

Key words: *sporadic colorectal cancer, carcinogenesis, COX2, NF-kB, inflammation.*

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and fourth most common cause of cancer-related mortality in the United States. Based on GLOBOCAN 2002, CRC is the third most common malignant disease in Southeast Asia. The average age of CRC patients in the United States is 67 years.[1] In Western countries, the prevalence of CRC in patients less than 50 years old ranges from 2% to 8%,[2,3] lower than in Asian countries. Kurian et al.[4] concluded that the incidence of CRC in Asian Indian and Pakistani populations under the age of 50 years is substantially higher than that in the white population of the USA. That tendency can also be observed in Indonesia where the prevalence of CRC in patients less than 45 years old is 47.85%.[5]

Inflammatory pathway considered to play a role in colorectal carcinogenesis. Inflammation and cancer have been considered to be closely linked for many years. NF-kB is the most crucial factor in the development of neoplasms. Although the molecular mechanisms are not well understood, recent studies nominate NF-kB as a central molecule responsible for the transition from inflammation to cancer.[6]

COX2 is dynamically expressed in the intestine and required for the maintenance of intestinal homeostasis. Colonic luminal irritation provokes rapid induction of COX2.[7] It increases mucosal defense against injury and initiates mucosal repair.[7] A previous study has shown the important role of COX2 in colorectal tumorigenesis and the strong relationship between COX2/prostaglandin E2 (PGE2) signaling pathway and adenomatous polyposis coli gene (APC) expression in intestinal neoplasia.[8]

The role of COX2 and NF-kB in colorectal carcinogenesis has not been thoroughly studied in the Indonesian population. The aim of this study was to evaluate COX2 and NF-kB expression in CRC and normal colonic tissue of native Indonesians with sporadic CRC.

METHODS

Selection of Patients

This study was a matched-pair case–control study by acquiring both CRC and tumor-adjacent normal tissues from the same subjects. Patients with CRC who underwent surgery at the Cipto Mangunkusumo National Hospital, Jakarta, or Hasan Sadikin General Hospital, Bandung, between January 1998 and April 2008 were enrolled in the study. Patients were eligible to participate in the study if they had sporadic CRC, did not fulfill the Amsterdam/Bethesda revised criteria for hereditary CRC or familial adenomatous polyposis (FAP) syndrome, were aged ≤40 years old or >60 years old, and were native Indonesians (confirmed in second-degree relatives). Patients who refused to give their informed consent or did not meet the requirements of the immunohistochemical examination according to their medical records were excluded. The immunohistochemical examination was conducted between May 2007 and April 2008 and undertaken at the Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia - Cipto Mangunkusumo Hospital and the Anatomic Pathology Laboratory, Faculty of Medicine, Padjajaran University, Bandung. This study was reviewed and approved by the ethics committee of the Faculty of Medicine, Universitas Indonesia.

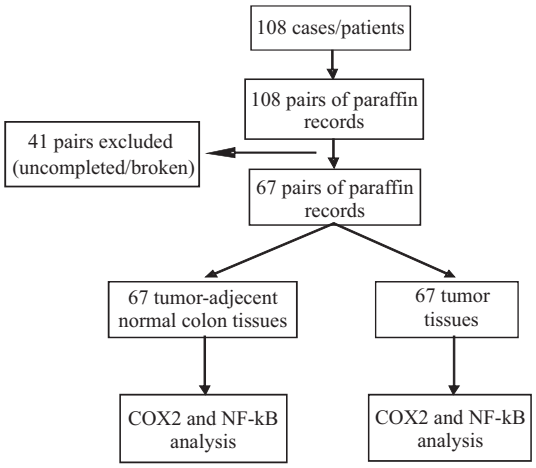

Figure 1. Disposition of patients

Tissue Specimens and Preparation

Tissue specimens taken during surgery for the histopathological confirmation of CRC were fixed in 4% buffered formaldehyde and embedded in paraffin wax. Tissue specimens (4 μm thick) were cut and rinsed with cold water. The sections then were placed on glass slides and put into warm water (50°C). The slides were then placed on a hot plate (70°C) for 15 min and incubated overnight at 35–40°C.

Immunohistochemical Staining for NF-kB and COX2

The expression of NF-kB and COX2 was evaluated using the avidin/biotin complex immunohistochemistry procedure. Rabbit polyclonal antibodies directed against human COX2 protein (cat. no. #ab15191, Abcam, UK) and the human NF-kB p65 subunit (RelA; cat. no. #ab7970, Abcam) were used as the primary antibodies. The tissue sections were sequentially deparaffinized and rehydrated through xylene and graded alcohol solutions. The slides were pretreated with an epitope retrieval system and preheated in a microwave for 5 min. The sections were then immersed in phosphate-buffered saline (PBS) for 5 min. Endogenous peroxidase activity was blocked by immersing the sections in a solution of 3% hydrogen peroxide plus methanol (1:9) for 10 min at 4°C, after which they were rinsed with PBS for 5 min. The slides were marked with and then placed in PBS again. Then, they were removed from PBS and placed in a treatment chamber. Blocking solution was applied to each section and the chamber was closed for 10 min. The primary antibodies were diluted 1:100 for COX2 and 1:150 for NF-kB with distilled water. The sections were incubated with primary antibody in the treatment chamber for 45 min. After the samples were washed with PBS, antibody binding was detected by incubation with a biotinylated secondary antibody for 10 min. The slides were washed again with PBS and incubated with streptavidin–horseradish peroxidase. Staining was visualized with the application of the chromogen 3,3'-diaminobenzidine in distilled water and the sections were counterstained with hematoxylin. The specimens were dehydrated and mounted. Positive and internal negative controls were included for each staining procedure. Internal negative control sections were processed without the addition of primary antibodies. Fibroblast cells, known to express COX2, were used as the positive control.

Sample Assessment

The intensity of staining was divided into four classes: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentages of stained cells were scored as 0 (0%), 1 (<25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%). The final score for each sample was the sum of the intensity and percentage scores, which ranged from 0 to 7. Tumors with a final score of 3 or more were considered positive. All stained slides were reviewed by two pathologists blinded to the clinical data (the kappa values were 0.831 for NF-kB staining and 0.898 for COX2 staining).

Statistical Analysis

The data were analyzed with the STATA version 9.0 software (STATA Corporation, TX, USA). The patients' characteristics and all variables are presented descriptively. The McNemar test was used to examine the relationship between COX2 and CRC and the relationship between NF-kB and CRC. A P value of < 0.05 was considered significant.

RESULTS

Subject Characteristics

One hundred eight patients with CRC and paraffin block records between 1999 and 2007 had been collected. Forty-one specimens could not be analyzed in which 10 specimens included only normal tumor-adjacent tissue samples and 31 specimens included only CRC tissue samples. Therefore, only 67 specimens of CRC and tumor-adjacent normal tissue were analyzed. (**Table 1**)

Forty-three subjects (64%) were ≥60 years old and 24 subjects (36%) were ≤40 years old. Female subjects outnumbered male subjects (1.4:1). Most of the tumors (73%) were located in the distal part of the colon. Late-stage CRC was found in 56 subjects (84%). Adenocarcinoma was the most common histopathological type (91%).

Table 1. Baseline characteristics of patients

Characteristics	N (%)
Age	
- ≤ 40 years	24 (36)
- ≥ 60 years	43 (64)
Sex	
- Male	27 (40)
- Female	40 (60)
Tumor location	
- Distal	49 (73)
- Proximal	18 (27)
CRC stage	
- Early stage	11 (16)
- Late stage	56 (84)
Histopathology	
- Adenocarcinoma	61 (91)
- Other types	6 (9)

Expression of COX2 in CRC and Tumor-Adjacent Normal Tissues

COX2 expression was positive in 39 (58%) and 19 (28%) in CRC and tumor-adjacent normal tissues respectively. This difference is significant (P = 0.0002). (**Table 2**)

Expression of NF-kB in CRC and Tumor-Adjacent Normal Tissues

NF-kB expression was positive in 47 (70%) CRC tissues and 27 (40%) in tumor-adjacent normal tissues, so significantly more CRC tissues expressed NF-kB (P<0.0001). (**Table 3**)

DISCUSSION

The MSI pathway plays a role in hereditary nonpolyposis colorectal cancer (HNPCC) and 15% of sporadic CRCs, which are characterized by mismatch repair deficiency, caused by the inactivation of either the hMLH1 or hMSH2 gene.[9] In contrast to Western countries, most cases of CRC in young patients in Indonesia are sporadic and the expression of hMSH2 or hMLH1 protein does not differ between younger and older native Indonesian patients with nonfamilial CRC. This finding suggests that rather than involving the MSI pathway of colorectal carcinogenesis, sporadic CRC in Indonesia involves another pathway. One pathway that should be considered is the inflammatory pathway. Joo et al.[10] detected COX2 protein in 70% of CRC tissues. COX2 expression was lower in our study, in which only 39 (58%) CRC tissue specimens were positive for COX2 expression.

COX2 expression tends to be low in the gastrointestinal tracts of healthy humans and animals. In our study, 19 (28%) normal tissue specimens were positive for COX2 expression, which might suggest chronic nonspecific colitis in these study subjects. The important clinical implication is that there is a tendency for these tissues to develop into malignancies because colonic epithelial cells that overexpress the COX2 gene have altered adhesion properties and resist apoptosis.[11]

Table 2. Expression of COX2 in CRC and tumor-adjacent normal tissues

		Tumor-adjacent normal tissue		Total N (%)	P value*
		COX2 (+)	COX2 (–)		
CRC tissue	COX2 (+)	15	24	39 (58)	
	COX2 (–)	4	24	28 (42)	0.0002
	Total N (%)	19 (28)	48 (72)	67 (100)	

* McNemar test

Table 3. Expression of NF-kB in CRC and tumor-adjacent normal tissues

		Tumor-adjacent normal tissue		Total N (%)	P value*
		NF-kB (+)	NF-kB (–)		
CRC tissue	NF-kB (+)	24	23	47 (70)	
	NF-kB (–)	3	17	20 (30)	< 0.0001
	Total N (%)	27 (40)	40 (60)	67 (100)	

* McNemar test

Wu et al.[12] have shown that COX2 expression is strong in CRC and weak in the normal mucosa, and that the difference in COX2 expression between CRC tissue and normal mucosa is statistically significant (P<0.001). Our results are consistent with those results insofar as significantly more CRC tissues expressed COX2 than did tumor-adjacent normal tissues (P=0.0002).

NF-kB is an important transcription factor in various biological processes.[11] There is growing evidence of a connection between inflammation and tumor development. NF-kB activation, a proinflammatory transcription factor, has been suggested to promote tumorigenesis.[13] However, the precise role of NF-kB activation in CRC is unclear. Yu et al.[14] reported that the increased expression of NF-kB in colorectal tumorigenesis plays an important role in the pathogenesis of colon cancer in humans by mediating the transition from colorectal adenoma with low-grade dysplasia to adenocarcinoma. Sakamoto et al. reported NF-kB activation in 40% of CRC tissues.[14] The percentage was higher in our study, in which 47 (70%) CRC tissue specimens were positive for NF-kB expression.

Twenty-seven tumor-adjacent normal tissues (40%) were positive for NF-kB expression in our study. This frequency is higher than that in another similar study by Aranha et al.[15] in which only 9.4% of normal tissues were positive for NF-kB expression. NF-kB is not commonly expressed in normal tissue. Like COX2 expression, this high proportion of NF-kB expression might suggest chronic nonspecific colitis in the study subjects. Significantly more CRC tissues than tumor-adjacent normal tissues expressed NF-kB (P<0.0001). We have found no similar studies comparing NF-kB expression in CRC and tumor-adjacent normal tissues.

The results of our study indicate that inflammation plays a role in the carcinogenesis of sporadic CRC in native Indonesians. In Western countries, inflammation-associated CRC is observed in patients with inflammatory bowel disease (IBD).[16] In contrast to IBD patients, who develop CRC from dysplasia or flat adenoma, in most Indonesian patients with CRC, the cancer develops from an adenomatous polyp. Therefore, IBD has not been shown to be the triggering factor that initiates colorectal carcinogenesis in the Indonesian population. We suggest that nonspecific inflammation, rather than IBD, is the key factor responsible for the high expression of COX2 and NF-kB in normal tissues. Nonspecific inflammation can induce epithelial damage in the colon, which favors neoplastic growth.[17]

Observational studies and randomized intervention trials have found that the regular use of aspirin reduces the risk of colorectal neoplasm. Aspirin inhibits COX enzymes, including COX2, which is often overexpressed in CRC. Chan et al.[18] concluded that regular aspirin use significantly reduced the risk of CRCs that overexpress COX2, whereas regular aspirin use had no effect on tumors with weak or no COX2 expression. Latest study by Nakanishi et al.[19] also suggest that COX-2 inhibition may reduce intestinal tumor progression. The antitumor activity of nonsteroidal anti-inflammatory drugs (NSAIDs) is not solely based on COX2 inhibition. Aspirin can also induce the translocation of NF-kB and apoptosis in CRC cells. Din et al.[20] demonstrated that aspirin has a considerable degree of specificity in its apoptotic effect on CRC cells compared with its effects on the other cell lines studied.

Our study shows that COX2 expression in CRC tissues was lower whereas NF-kB expression in CRC tissues was higher than previous study. It still cannot be concluded from these findings that native Indonesians with sporadic CRC will be advantaged by the use of aspirin as a chemopreventive agent. Further studies are required to determine whether routine testing for COX2 and NF-kB expression is appropriate in deciding which CRC patients are suitable candidates for chemoprevention.

Our study has several strengths. It is the first study to report the expression of COX2 and NF-kB in normal and CRC tissues of native Indonesians with sporadic CRC. We also examined the relationship between both COX-2 and NF-kB expression and CRC. Our results support the importance of the continued investigation of the COX2- and NF-kB-related pathways in the development of new treatments and the potential use of NSAIDs as chemopreventive agents for CRC.

The limitation of this study is the probability of selection bias due to significant proportion of inadequate or incomplete specimens. Further study with nationwide samples is needed to confirm the result of this study.

CONCLUSION

Inflammation plays a role in the carcinogenesis of sporadic CRC in native Indonesians. This support potential use of nonsteroidal anti-inflammatory drugs as chemopreventive agents for CRC.

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