Gastroenterology and Hepatology From Bed to Bench

REVIEW ARTICLE

Molecular profile of colorectal cancer in Indonesia: is there another pathway?

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ABSTRACT

Colorectal cancer is an emerging public health problem in Indonesia and currently ranks among the three highest cancers. Lack of a colonoscopy screening and lifestyle changes might contribute to it. In the last few decades, there is an increasing interest towards the contribution of genetic-environment interaction in colorectal carcinogenesis. Some studies have indicated that CRC might develop through several different pathways; the three major routes are chromosomal instability (CIN), microsatellite instability (MSI), and inflammatory pathways. An earlier study on clinical epidemiology of CRC in Indonesia showed that the majority of patients were diagnosed between 45 and 50 years old, with a mean age around 47 years old. Further studies showed that most young Indonesian cases of CRC do not have hereditary characteristics; however, the CRC did not follow the conventional pathways of sporadic CRC (the CIN) pathway. Rather, it is a mixed of MSI and inflammatory pathways. Immunohistochemical studies showed that the proportion of patients with negative mismatch repair proteins was 43.5% for MSH2 and 83.5% for MLH1. Along the sporadic colorectal carcinogenesis pathway, there was a specific role of cyclooxygenase-2 (COX-2) enzyme during the polyp formation. COX-2 expression was reported in about 80% CRC cases worldwide. However, our study found only 49% of COX-2 expression among the CRC patients. Interestingly, an inflammatory marker, the nucleus factor κB (NF-κB), was expressed in about 73.5% cases, in line with a previous study. More recently, *KRAS* has been used as a potential tumor marker to select treatment and its expression was reported to be as high as 30%-40% worldwide. However, we found that *KRAS* gene expression was only 16.3%. Our findings support that CRC patients in Indonesian might follow a distinct pathway, a hypothesis that deserves further exploration.

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Background

Colorectal cancer (CRC) is an emerging public health problem in Indonesia and currently ranks among the three highest cancers. The age-standardized incidence rates of CRC per 100.000 populations in Indonesia were 19.1 for men and 15.6 for women (1). These rates are much lower than the incidence rates in Australia, New Zealand and Western Europe, but the number of cases is high because Indonesia ranks the fourth most populated country in the world with more than 235 million populations.

Previous epidemiological studies have shown that CRC patients in Indonesia are younger than patients in developed countries. More than 30% of cases were 40 years old or younger (2), while patients younger than 50 years in developed countries accounted for only 2-8% (3,4). In

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addition, whereas young or early onset CRC in developed countries are often associated with familial cases (5,6), most Indonesian CRC patients are determined to be sporadic. With the increasing number of cases and the occurrence of CRC during a person's productive years, it is obvious that CRC poses a major public health problem in Indonesia.

Early onset CRC in developed countries showed typical characteristics such as proximal localization (ascending colon), low pathological stage, rarely metastasize, and has better prognosis (7). However, our patients showed otherwise characteristics, i.e. distal localization (rectum), high clinical stage, and poor survival (unpublished data). All these observations have raised questions regarding the carcinogenesis pathways of CRCs in Indonesia.

Genomic instability in colorectal cancer

Classically, CRC is believed to develop from an adenoma, the so-called adenoma-carcinoma sequence (8,9). Epithelial malignant transformation occurs after the cell underwent genomic instability, which promotes the accumulation of genetic and epigenetic alterations without obvious phenotypic changes (10). Genomic instability is the prerequisite of CRC development which allows multiple, gradual genetic mutations (11). There are two distinct pathways that marked the genomic instability, i.e. the microsatellite instability (MSI) and the chromosomal instability (CIN) pathways.

MSI is recognized as genome-wide alterations in repetitive DNA sequence (12). It is found in almost all malignancies from patients with hereditary non-polyposis colorectal cancer (HNPCC) and approximately 15% of the sporadic cancers (13). MSI is caused by defects in the nucleotide mismatch repair (MMR) mechanisms. Mismatch repair enzymes normally recognize errors in nucleotide matching of complementary chromosome strands and initiate segmental excision of the newly synthesized strand to ensure faithful strand replication.

CIN is the most common type of genomic instability and is associated with loss of heterozygosity in various tumor suppressor loci, especially 18q (14). Alterations range from different types of chromosomal rearrangements, to loss and gain of large fragments of the genome and may have abnormal number of chromosomes or DNA content (aneuploidy).

The role of epigenetic alterations

DNA methylation refers to the methyation of cytosine (C) nucleotide preceding guanine (G) nucleotide (CpG islands) throughout the genome, preferentially in gene promoter regions. This alteration may inactivate tumor suppressor genes, thereby promoting cancer. In colorectal cancer, DNA methylation may inactivate the MMR gene *hMLH1* gene leading to microsatellite instability (15). Both hypomethylation of the genome and simultaneous hypermethylation of the promoter regions of strategic genes frequently occur (16).

Pathways to Colorectal Cancers

Colorectal cancer is a heterogeneous disease and may arise through at least three main pathways, namely sporadic, hereditary and inflammation-associated cancers. These pathways harbor distinct genetic alterations although they may share some common mutations.

Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

HNPCC accounts for about 1-8% of all CRC cases based on clinical criteria, i.e. the Amsterdam criteria and the revised Bethesda guidelines (17-19). Pathogenesis of familial or hereditary CRC involved germ-line mutations of mismatch repair (MMR) gene family members and was named

microsatellite instability (MSI) pathway (20,21). Mutation of MMR genes such as *hMLH1* and *hMSH2* range from 0.3% to 3% of total CRC burden (22). However, *hMLH1* and *hMSH2 gene mutations* account for more than 90% of HNPCC cases, with the remaining mutations occur in other MMR genes such as *hMSH6*, *hPMS1*, and *hPMS2* (23).

Sporadic Colorectal Cancer

Most sporadic CRC follow the path of chromosomal instability (CIN) (24) that involved somatic mutation in adenomatous poliposis coli *(APC)* gene and other mutations which have been described as vogelstein adenoma-carcinoma sequel. This pathway is also known as the classical pathway, which involves mutation in *APC, KRAS, and p53 genes,* loss of 18q and deletion of 17p which harbor tumor suppressor gene *p53* locus (8).

Mutations in the *APC* gene on chromosome 5q21 locus are considered the earliest events of colorectal carcinogenesis and constitutes between 60% and 80% of sporadic colorectal carcinoma and adenomas (25). Allelic loss of chromosome 18q21, which contains *DCC, SMAD2,* and SMAD4, is found in up to 60% of CRCs. Mutation of SMAD4 may be more influential in colorectal carcinogenesis and marked the divergence of chromosomal instability (CIN) from the microsatellite instability (MSI) pathway (26). A small proportion of sporadic CRCs, however, contains mutation in MMR genes and showed high MSI (MSI-H).

The reported incidences of *KRAS* and *BRAF* mutations in CRC were 37% and 13% of respectively (27,28). The frequency of *KRAS* mutation was different among HNPCC, MSS, and MSI-H CRC cases. HNPCC and sporadic MSI-H tumors without *hMLH1* hypermethylation shared similar *KRAS* mutation frequency. These findings suggested that *KRAS* mutation frequency depends on the genetic or epigenetic mechanism (29). Mutation in *BRAF* gene, another member of the RAS/RAF/MAPK pathway, is also crucial in colorectal tumorigenesis and co-occurrence with *KRAS* mutation in the same individual patient is a rare case (30,31). The frequency of V600E mutation in *BRAF* has been described in about 35% of sporadic MSI-H CRCs and about 6% in CRC without MMR deficiency or microsatellite stable (MSS) CRCs (32-34). Moreover, *BRAF* mutation is associated with *hMLH1* promoter hypermethylation (34,35). While *KRAS* gene is mutated in about 20% of sporadic MSI-H and of 35% of MSS CRCs negative for the $BRAF^{V600E}$ mutation (32,33,36).

Colitis-Associated Cancer

Inflammation has been associated with cancer since the observation of leukocyte infiltrations in neoplastic tissues by Rudolf Virchow in 1863 (37). Inflammation could alter the dynamics of colonic epithelial cells growth, either their promoting survival or preventing apoptosis (38). Studies have shown a relationship between colonic inflammation and cancer incidence as indicated in context of ulcerative colitis and Crohn disease, the so-called *inflammatory bowel disease* (IBD). Colorectal cancer arising from IBD mostly occurs from a dysplastic precursor forming a flat adenoma, instead of polyp (39,40).

Besides genes classically known to be involved in sporadic colorectal carcinogenesis, patients with IBD also showed overexpression of inflammatory genes, such as the cyclooxygenase 2 *(COX-2)* genes (41) and nuclear factor kappa B (NF-κB) (42). COX-2 enzyme plays important role in producing prostaglandin E2 (PGE2), which stimulates cascades of signaling pathways leading to tumor growth, such as activation of phosphotidilinositol-3-kinase (PI3) and Wnt/β-catenin pathways (43). COX-2 is expressed in 40% of colorectal adenomas and 85% of sporadic CRC (44). Early molecular studies showed that colorectal tumors expressed high level of mRNA

(86%), while in normal colon mucosa, its expression is low or absent (45,46). High expression of COX-2 in sporadic CRC indicated its important role in colorectal carcinogenesis, which probably occurs early during the formation of adenomatous polyp (47).

Activation of NF-κB has been described in Crohn's disease, ulcerative colitis, self-limited colitis, and experimental colitis (48,49). Nuclear factor kappa B is the most important component of inflammatory signaling pathway that may promote tumorigenesis. It is a central transcription factor activated by inflammatory signals in response to infectious agents, cytokines, and necrotic cellular remnants (50). Activation of NF-κB leads to the overexpression of cell cycle genes, apoptosis inhibitor and proteases; all of which may trigger invasive phenotypes. Immunohistochemical staining of RelA protein, the p65 component of NF-κB, found a significant difference of NF-κB expression among normal colorectal mucosa (9.3%), colorectal adenoma (54.0%), and colorectal adenocarcinoma (71.9%) (51).

Molecular characteristics of CRC in Indonesia

MSI and CIN pathways

Most CRC patients in Indonesia are sporadic, even though they were 40 years old or less. Early study showed that the expression of MMR proteins did not differ between young (40 years or less) and old (60 years or more) patients. Moreover, MSH2 and MLH1 were not expressed in 43.5% and 83.5% of cases, respectively hinting toward a defect in DNA mismatch repair system which may promote microsatellite instability (52). However, when tested using BAT26, a surrogate markers of MSI our samples showed very low frequency of MSI that is consistent with sporadic cancer feature. The low expression of normal MMR protein may be caused by epigenetic silencing event of DNA methylation in the promoter regions of MMR genes, which are known to occur in sporadic CRC patients (53). If this is true, then young CRC patients in Indonesia conform to sporadic molecular feature and may not use the MSI pathway, in spite of the large proportion of patients showing low expression of MMR genes. To test this hypothesis, we have evaluated the same specimens for SMAD4 protein expression. None of these tumors stained positive for SMAD4, either young or older patients, confirming that they are not following the MSI pathway.[52]

Oncogenic mutation of KRAS and BRAF genes

Our preliminary study found that only 16.3% of of 43 sporadic CRC cases harbored *KRAS* mutation and no *BRAF* mutation (54). This low *KRAS* mutation may indicate a different pattern of genetic instability in these colorectal tumors. The absence of BRAF gene mutation suggested that the samples may not belongs to the MSI-H group, which also associated with high methylation of *hMLH1* gene promoter. However, we have not analyzed the methylation pattern of our samples, while studies assessing the mutation loci of the *KRAS* gene are still ongoing.

Inflammatory pathways

Using immunohistochemistry technique, we found that NF-κB was expressed in 73.5% of CRC specimens, whereas COX-2 expression was found in only 49.0% cases (55). The COX-2 expression may be considered as low since most studies in sporadic CRC found at least 80% of cases expressed COX-2 in the cytoplasm (44-46, 56). Our further analysis found that there was a significant association between COX-2 and NF-κB expressions (bivariate analysis $p<0.001$ and degree of agreement of 74%). The intensity score was also significantly correlated ($r=0.941$; $p<0.001$) (57). Other study also found a high correlation between COX-2 and RelA (p65) component of NF-κB expression ($r=0.83$; $p<0.05$)

Table 1. Summary of clinicopathology and genetic alterations of various colorectal carcinogenesis pathways

	Sporadic	HNPCC	CAC	Serrated pathway	Sporadic indonesia?
Age	>60 years	<40 years		Young	Young
Location	Distal	Proximal		Proximal	Distal
Pathway	Adenoma-carcinoma	Adenoma-carcinoma	Inflammation-dysplasia-cancer	Hyperplastic polyp- cancer	Adenoma-carcinoma
CIN					No
Defective MMR protein	No	Yes	No	Yes	Yes
MSI	No	Yes	No	Yes	No
Inflammation	No	No	IBD	No	Non-specific?
Adenoma	Polyp	Polyp	No	Serrated adenoma	Polyp
KRAS mutation	Yes	Yes	Yes	Yes	No
BRAF mutation	No	No	No	Yes	No
COX-2		No	High		
NF-κB	Yes	No	Yes	?	Yes

(58). The NF-κB is an important transcription factor to activate *COX-2* gene expression. The 5'-UTR region of *COX-2* gene promoter contains regulatory binding sites of several transcription factors, including two NF-κB binding site motifs (59).

Our study supports the hypothesis that inflammation may play roles in Indonesian CRC patients. However, the role of IBD in our subjects has not been proven yet. COX-2 was also expressed in moderate percentage (23.9%) in normal mucosa of our specimens (57), indicating the presence of chronic inflammation of unknown etiology (non-specific colitis). The role of normal gut flora as the etiology of CRC is difficult to be shown. However, experiment in mice suggested that the normal gut flora may play a role in colitis-associated carcinoma (60).

Is there another pathway?

Studies elucidating the molecular pathway of colorectal carcinogenesis in Indonesia have just begun. Besides the three main pathways mentioned above, there are several minor pathways. Serrated pathway is believed to arise in serrated adenoma and genetically associated with *BRAF* gene mutations exhibiting extensive DNA methylation but lacking *APC* gene mutations (61). The serrated adenoma is a precursor lesion of sporadic CRC with high microsatellite instability (MSI-H), which constitutes approximately 15% of sporadic colon cancers (62). However, the absence

of *BRAF* mutation in our preliminary study does not support the notion that our CRC patients might take this pathway. Studies are still ongoing to tract their genetic alteration patterns. The summary of clinicopathological aspects and genetic mutation associated with various CRC pathways is given in Table 1 along with the proposed unanswered question on Indonesian CRCs. It seems that colorectal carcinogenesis in our population is still far beyond from common understanding and more genetic studies are needed to reveal the heterogeneous mechanisms of its process.

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