



Cigarette Smoke Induces Colorectal Carcinogenesis in Wistar Rats by Decreasing The Expression of APC, MSH2 and MLH1

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DOI: 10.15294/biosaintifika.v9i1.8439

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History Article

Received 11 January 2017

Approved 12 February 2017

Published 1 April 2017

Keywords

cigarette smoke; colitis; colorectal cancer; APC; MSH2; MLH1

Abstract

Colorectal carcinogenesis induced by cigarette smoke requires at least 30-40 years. This long time duration causes an animal research conducted becomes relevant. This research was carried out to observe colorectal carcinogenesis due to cigarette smoke exposure in Wistar Rat. The observations focused on changes in epithelial morphology and expression of APC, MSH2 and MLH1. Twenty male Wistar rats inbreed strain were randomly allocated into control group and experimental group exposure to cigarettes smoke for 14 weeks and 28 weeks sequentially. Colorectal epithelial morphology was assessed on the histopathology examination, whereas the expression of APC, MSH2 and MLH1 was assessed on aspect of immunohistochemistry. The comparative analysis between the two groups was performed using non-parametric Mann-Whitney U test. Histology of colorectal epithelium showed pattern of colitis associated cancer that was significant both in 14 weeks and 28 weeks of treatment. This research indicated negative expression of APC, MSH2 and MLH1 in the colorectal cancer that were significant at 28 weeks of exposure. This research implies that chronic exposure to cigarette smoke can induce colitis associated colorectal cancer through decreased expression of APC, MSH2 and MLH1.

How to Cite

Novrial, D., Riwanto, I., Sumitro, S. B. & Wijaya, I. (2017). Cigarette Smoke Induces Colorectal Carcinogenesis in Wistar Rats by Decreasing The Expression of APC, MSH2 and MLH1. *Biosaintifika: Journal of Biology & Biology Education*, 9(1), 66-72.

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p-ISSN 2085-191X

e-ISSN 2338-7610

INTRODUCTION

Cigarette smoke is a potential risk factor in inducing biological changes in the human body that will trigger various diseases including cancer. Cigarette smoke contains many harmful compounds that are responsible for the occurrence of cancer. The main carcinogens in cigarette smoke are polycyclic aromatic hydrocarbons (PAHs), aromatic amines, nitrosamines, and heterocyclic amines (HCAs) (Kasahara et al., 2008). Nicotine and its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are believed to be the two main causes of smoking-related cancer (Wei, et al., 2011).

In addition to lung cancer, smoking also increases the risk of gastrointestinal cancer. Carcinogens of tobacco can achieve colorectal mucosa through the circulatory system and direct exposure to the gastrointestinal tract (Kasahara et al., 2008). Colorectal carcinogenesis induced by cigarette smoke takes a long exposure (Luchtenborg, et al., 2007). Result in population studies showed that APC mutations caused by cigarette were found in smokers who had smoked more than 40 years (Gay et al., 2012). Strong ties between MSI (+) in patients with colorectal carcinoma with smoking habits were also found in smokers over 35 years (Slattery et al., 2000). This long time duration causes the animal studies are relevant to be carried out since it can shorten the time of observation by converting the time taken from human to animal.

Experimental research on the effect of cigarette smoke on the colorectal mucosa is still minimal. Until today, the mechanism of colorectal carcinogenesis induced by cigarette smoke has not been clear. Thus, this research aimed to observe colorectal carcinogenesis due to exposure to cigarette smoke in Wistar rats. The observations focused on changes in epithelial morphology and expression of APC, MSH2, and MLH1. Further-more, the result of this research can be used as references to conduct other studies.

METHODS

This research was conducted at Animal Facility of The Institute for Integrated Research and Testing Gadjah Mada University Yogyakarta and Anatomical Pathology Laboratory of Medical Faculty, Diponegoro University/ Dr. Kariadi Hospital Semarang, from November 2014 to September 2015. This research was approved by Health Research Ethics Committee, Faculty of Medicine Diponegoro University and Dr. Kariadi Hospital Semarang No.577/EC/FK

- RSDK/2014.

Male Wistar rats aged 1-2 months, weighing between 150 g - 200 g were used in this research. All animals were housed in maintenance cage individually in a well ventilated room, temperature ranging from 25°C-30°C. Lighting was set according to the cycle of day and night. Daily consumption using a standard rat feed from the laboratory and aqua destilata ad libitum. They were quarantined for the first 7 days, then randomized into experimental and control group. Cigarettes used in this research were produced by one of cigarette factories in Kudus, Central Java, Indonesia. Cigarettes have 89 mm in length and 7 mm in diameter. Each cigarette contains 33 mg of tar and 1.9 mg nicotine.

A total of 20 male Wistar rats were divided into 1 experimental group and 1 control group, 10 rats each group. Exposure to cigarette smoke in the experimental group performed daily at a dose of 0.5 cigarettes per day per rat. Exposure to cigarette smoke was done passively in a glass enclosure specifically designed for fogging side stream smoke, while the control group did not get any exposure. Terminations were carried out gradually at the end of the 14th week number 5 rats in each group, and the remaining 5 rats in each group were terminated at the end of the 28th week of exposure. Colorectal tissue were taken for further processing and preparation of routine histopathology.

All tissue samples were fixed with neutral 10% formaldehyde solution. Consecutive 4- μ m thick sections from formalin-fixed-paraffin-embedded (FFPE) tissue blocks were prepared and stained with hematoxylin and eosin (H&E) for histopathological classification. FFPE tissues were cut into 4- μ m thick sections and transferred to positively charged slides for immunohistochemistry staining. Then, sections were subjected to dewaxing, rehydration, blocking with hydrogen peroxide, and antigen retrieval (Dako target retrieval solution, citrate buffer pH 6.0) with microwave. The slides were then incubated overnight at 2-8°C with primary antibodies: anti-APC polyclonal antibody (biorbyt orb 10109; dilution 1:100, UK); MSH2 monoclonal antibody (Biocare Medical CM219; dilution 1:100, USA); and MLH1 monoclonal antibody (Biocare Medical CM220; dilution 1:100, USA). Incubation with secondary antibody and product visualization (Dako) was performed with diaminobenzidine substrate as the chromogen. The slides were finally counterstained with Mayer's hematoxylin and

Table 1. Histopathologic features of colorectal tissue from control and experimental groups

Time	Exposure	Group	Histopathologic Features (%)				p-value
			Normal	Inflammation	Dysplasia	Carcinoma	
14 weeks		Control	100	0	0	0	0.032
		Experimental	20	20	20	40	
28 weeks		Control	80	0	0	20	0.032
		Experimental	0	0	0	100	

washed once each with distilled water and PBS.

Assessment of histopathology and immunohistochemistry were done by two anatomical pathologists. Histopathology of colorectal was assessed by scoring the following conditions: 1. Normal colon, 2. Inflammation without dysplasia, 3. Dysplasia, and 4. Carcinoma. Expression of APC, MSH2, and MLH1 assessed using the Allred score (Allred, et al., 1998).

Continuous variables were expressed as the mean \pm SD and the categorical variables were expressed as numbers (percentages). Continuous variables were checked for normality by using Kolmogorov-Smirnov test. Mann-Whitney U test was used to compare between two groups of non-normally distributed variables and the categorical variables. A p-value $<$ 0.05 was considered significant.

RESULTS AND DISCUSSION

Histopathologic features of the colorectal tissue showed different stages of carcinogenesis at 14 weeks cigarette smoke exposure while 100% carcinoma occurred at 28 weeks exposure, as shown in Table 1. Analysis of Mann-Whitney U test showed a significant difference between colorectal epithelial histology both groups at 14 weeks of exposure ($p = 0.032$) and at 28 weeks of exposure ($p = 0.032$).

At 14 weeks of exposure, it was obtained a mean score of 6.80 ± 1.304 for APC expressions in the control group and 3.40 ± 3.847 in the experimental group. The mean score of the expression of MSH2 in the control group was 3.80 ± 1.095 , while in the experimental group was 2.20 ± 2.280 . MLH1 expression mean score was 3.60 ± 0.894 in the control group and 1.80 ± 2.490 in the experimental group (Figure 1-3). Mann-Whitney U test showed no significant difference in the expression of APC ($p = 0.222$), MSH2 ($p = 0.310$) and MLH1 ($p = 0.421$) between the two groups at 14 weeks of exposure.

At 28 weeks of exposure, it was obtained a mean score of 6.20 ± 3.493 for APC expressions in the control group and 0 (negative) in the

experimental group. The mean score of the expression of MSH2 in the control group was 3 ± 2 whereas 0 (negative) in the experimental group. MLH1 expression means score was 2.80 ± 1.789 in the control group and 0 (negative) to the experimental group (Figure 4-6). The results of the Mann-Whitney U test showed that there were significant differences in the expression of APC ($p = 0.032$), MSH2 ($p = 0.032$) and MLH1 ($p = 0.032$) between the two groups at 28 weeks of exposure.

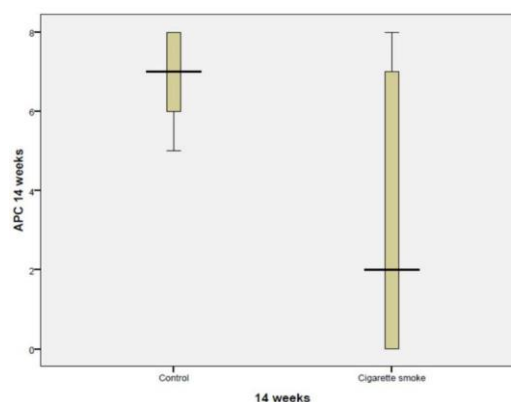


Figure 1. Effect of chronic exposure to cigarette smoke for 14 weeks on APC expression. Data are presented as the ratio of control and experimental group.

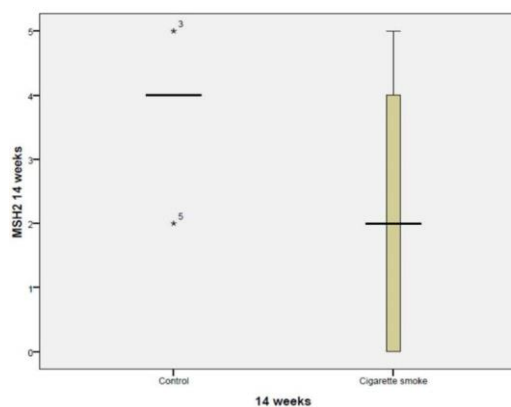


Figure 2. Effect of chronic exposure to cigarette smoke for 14 weeks on MSH2 expression. Data are presented as the ratio of control and experimental group.

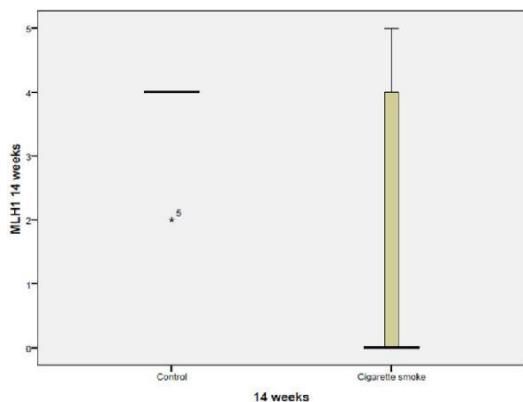


Figure 3. Effect of chronic exposure to cigarette smoke for 14 weeks on MLH1 expression. Data are presented as the ratio of control and experimental group.

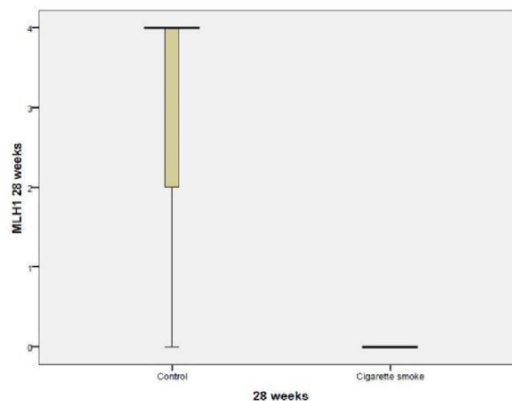


Figure 6. Effect of chronic exposure to cigarette smoke for 28 weeks on MLH1 expression. Data are presented as the ratio of control and experimental group.

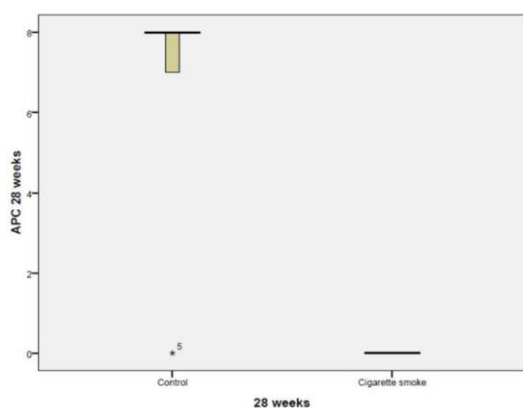


Figure 4. Effect of chronic exposure to cigarette smoke for 28 weeks on APC expression. Data are presented as the ratio of control and experimental group.

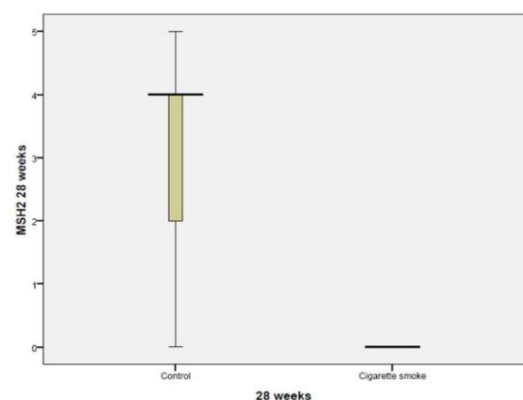


Figure 5. Effect of chronic exposure to cigarette smoke for 28 weeks on MSH2 expression. Data are presented as the ratio of control and experimental group.

This is the first experimental research on the induction of cigarette smoke against colorectal epithelium in rats. The results of this research proved that exposure to clove cigarette smoke 0.5 cigarettes per day on male Wistar rats for 14 weeks has led to the transformation towards malignancy in the colorectal epithelium in form of inflammation, dysplasia and carcinoma. We found 100% incidence of colorectal carcinoma at 28 weeks clove cigarettes exposure in experimental group (Figure 7). The whole carcinoma showed negative immunoexpression on APC, MSH2, and MLH1 (Figure 8).

Previous studies have proved that the intensity and duration of smoking are associated positively with colorectal epithelial damage either the growth of colorectal adenomas, hyperplastic polyps, and carcinoma (Mutch et al., 2009; Wei et al., 2011). Based on population studies, the time required for cigarette smoke to induce colorectal carcinoma ranged from 30 to 40 years (Giovannucci, et al., 1994). This research proved that carcinoma has been arising in 40% of samples exposed to cigarette smoke in the 14th week, or the same as 20 years when converted into humans (Laurence & Bacharach, 1964).

Cigarette smoke contains 7000 chemicals in which 60 of them have been confirmed as a carcinogen in humans and animals. Among these carcinogens, nitrosamines group such as tobacco-specific nitrosamine 4- (methylnitrosamino) -1- (3-pyridyl) -1-butanone (NNK) and N'-nitrosornicotine (NNN), PAHs such as benzo [a] pyrene and aromatic amines such as 4-aminobiphenyl are the main carcinogens that have been verified in animal models and positive found in cigarette smoke (Stepanov et al., 2009). Cigarette smoke also contains free radicals such

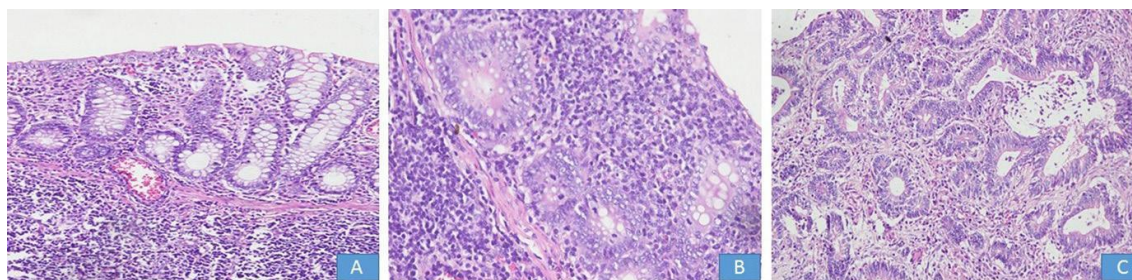


Figure 7. The histopathologic features of colorectal tissue of Wistar rats after chronic exposure to cigarette smoke showed inflammation without dysplasia (A), dysplastic lesion (B) and adenocarcinoma (C). (HE. 200x, 400x, 200x).

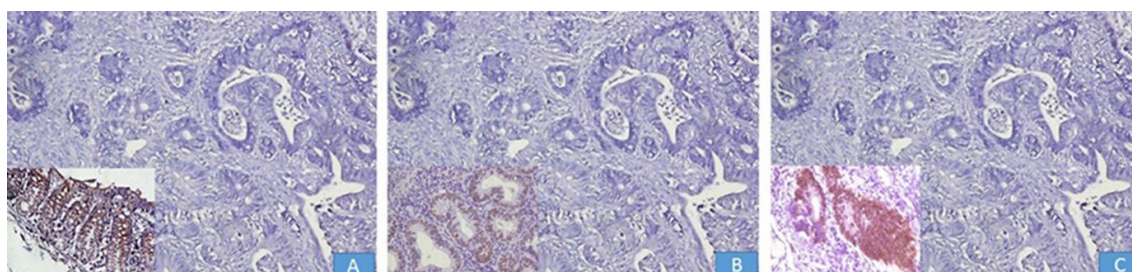


Figure 8. Immunoexpression of colorectal carcinoma of Wistar rats after chronic exposure to cigarette smoke showed negativity on APC (A), MSH2 (B) and MLH1 (C). Insert: positive control. (IHC, 200x)

as nitric oxide and mixed hydroquinone, semi quinone and quinone that can induce redox cycle and contribute to oxidative damage in smokers (Hecht, 2003). Many heavy metals such as Hg, Pb and As also found in cigarette smoke, which can accelerate the pathological process of the disease, cause oxidative stress and chronic inflammatory process that triggers carcinogenesis (Er-cal, et al., 2001).

The results of this research showed the role of APC mutations in colorectal carcinogenesis due to cigarette smoke. In this research, we found significant differences in the expression of APC between control and experimental groups. Significant differences appear in the 28-weeks exposure, although a decrease in the expression of APC has occurred in the exposure of 14 weeks. A number of studies suggest APC mutation have positive correlation to long exposure to smoking. In smokers < 40 years did not obtain a significant effect on APC mutation, but in smokers > 40 years were significantly related to APC mutations (Gay et al., 2012; Naghibalhossaini et al., 2012), as also evidenced by this research where significant differences occurred in the 28 weeks exposure. Long time duration can be due to the mechanism of cigarette smoke induced colorectal carcinogenesis possibly not through sporadic adenoma-adenocarcinoma sequence (CRC), but is more likely to follow colitis-associated colorectal cancer (CAC) pathway through the stages of inflammation,

dysplasia, and carcinoma without the formation of adenoma (Terzic, et al., 2010). In CRC, inactivation mutation of the APC occurs in the early events of carcinogenesis and is found in approximately 90% of cancers that trigger the formation of focal adenoma. While at CAC, inactivation mutation of the APC is found in the later stages of carcinogenesis during the transition between dysplasia towards carcinoma (Barbaro, et al., 2014). This research proved the same thing while the loss of APC expression occurred in dysplasia and carcinoma, in contrast to the normal colorectal and inflammation stage showed positive expression of APC.

Microsatellite instability is one of the major pathways of colorectal carcinogenesis. Microsatellite instability (MSI) occurs due to failure of the mismatch repair system (MMR) correct errors genomic bases and maintain stability, so that the cells with the accumulation of MMR function abnormalities were not able to correct the base error (Wimmer et al., 2014). There are 9 genes with MMR function have identified in human. Five of them play a role in colorectal carcinogenesis, especially in HNPCC / Lynch Syndrome. These five genes are MLH1, MSH2, MSH6, PMS2, and PMS1, where the most frequent mutations occur in the MLH1 and MSH2 genes. Colorectal cancer can be divided into MSI-H (high) when we obtained two or more MMR genes are mutated, and MSI-L (low) when obtained only one MMR gene that is

mutated, known as microsatellite stable (MSS) (Carethers, 2014).

In this research, there were significant differences in the expression of MSH2 and MLH1 between the two groups. Significant differences occur at 28 weeks of exposure. MSH2 and MLH1 expression were significantly reduced in the experimental group than control, so it can be concluded that colorectal carcinomas that occur due to cigarette smoke are MSI-H tumor. Based on the previous studies found that there is a strong relationship between MSI-H with colorectal carcinogenesis (Chen, et al., 2015). There are at least two mechanisms that can lead to defect in MMR. MMR gene mutation can be caused by protein malfunction as seen in HNPCC, or because the protein produced very little as a result of hypermethylation which can be found in sporadic colorectal carcinoma. Hypermethylation of gene often leads to weak or low expression (silencing), so-called epigenetic events (Raskov, et al., 2014). Hypermethylation of MLH1 promoter was found in at least 95% of sporadic colorectal carcinoma (Cunningham, et al., 2001). Cigarette smoke has been shown to stimulate DNA methyltransferase and smoking is also associated with CpG island methylation in lung cancer and head and neck tumor (Marsit, et al., 2009; Wistuba, et al., 2001). In CAC, inflammation can cause the inactivation of genes that encode proteins or DNA proofreading enzymes involved in the mechanism of DNA repair (MMR) (Barbaro et al., 2014).

Hypoxic adaptation has an important role in triggering the development of tumors (Gillies & Gatenby, 2007). Chronic hypoxia conditions that occur in solid tumors including CRC and CAC can improve the signal hypoxia-induced transcription factor (HIF), which if occurs continuously can trigger an inflammatory response through activation of genes that encode pro-inflammatory cytokines (Imtiyaz, et al., 2010). Instead, inflammation can maintain HIF signal at a high level through the mechanism of oxygen dependent or independent (Colgan, et al., 2013). Hypoxia induces epigenetic transcriptional repressor expression silencing through DEC-1 that causes a deficiency of MMR and genomic instability, as happened in mice models of IBD (Edwards, et al., 2009). Thus, the risk of specific subtypes of MSI-H like MSH2 and MLH1 hypermethylation can be explained by epigenetic modifications induced by cigarette smoke. Heavy metal component found in cigarette smoke especially mercury (Hg) can induce hypoxia. Hg binds to hemoglobin inside red blood cells, this bond leads to a reduced capacity of the blood cells to carry oxygen to the tissues that trigger cellular

hypoxia (Myshkin & Khromova, 2000). Further research is needed to clarify how these mechanisms occurred in colorectal cancer.

Based on the result, it can be concluded that chronic exposure to cigarette smoke caused colorectal epithelial morphological changes toward malignancy and inflammation. Changes that occur indicates the line was similar to colorectal carcinogenesis induced by colitis (colitis-associated colon cancer), in the form of 'inflammation-dysplasia-carcinoma'. Our data suggested that the mechanism of cigarette smoke induce colorectal carcinogenesis through decreased expression of APC, MSH2 and MLH1. Loss of protein expression can be caused by gene mutations or DNA hypermethylation. Examination of gene mutations and DNA methylation profiling is required to determine which mechanisms are more dominant in colorectal carcinogenesis due to cigarette smoke between genetic and epigenetic.

CONCLUSIONS

Chronic exposure to cigarette smoke can induce colitis-associated colorectal cancer through decreased expression of APC, MSH2 and MLH1. Changes that occur need long time duration between 14 to 28 weeks in rats or when converted to the human between 20 to 40 years.

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