**AvrA Salmonella Increases TLR4/NF-κB/β-catenin/TGF-β Expressions of Colorectal Cancer Mice Model**

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**Abstract**

**BACKGROUND:** Colorectal cancer (CRC) is reported as the third most frequently diagnosed cancer worldwide. *Salmonella* infection plays a role in developing this cancer, which is chronically related to the avirulence protein A (AvrA) effector protein produced by the bacteria. This study was conducted to point out the effect of AvrA *Salmonella* on the occurrence of CRC through the regulation of TLR4/NF-κB/β-catenin/TGF-β expressions in the azoxymethane (AOM)/dextran sodium sulfate (DSS) CRC model.

**METHODS:** A randomized control group post-test-only study was conducted using male Balb/c mice with 30-gram body weight (10-12 weeks), which were divided into three groups, namely the negative control (normal mice without any treatment), positive control (AOM/DSS-treated mice), treatment 1 (T1) (AOM/DSS-treated mice + AvrA *Salmonella*) groups. Colon tissue was collected and then prepared for immunohistochemistry staining using TLR4, β-catenin, NF-κB, TGF-β, and Ki67 antibodies, whereas apoptotic cells were stained using TUNEL assay.

**RESULTS:** The expressions of TLR4, β-catenin, NF-κB, TGF-β, Ki67, and apoptosis percentage indicated significant differences among the three groups, which statistically showed \( p<0.05 \) in all observed parameters. The mean of all parameters was far more significant in the T1 group than in the negative and positive control groups.

**CONCLUSION:** The findings revealed that AvrA *Salmonella* could increase the expressions of TLR4, β-catenin, NF-κB, TGF-β, and Ki67 and decrease the apoptotic percentage. Thus, AvrA *Salmonella* influences CRC tumorigenesis through TLR4/NF-κB/β-catenin/TGF-β and is suggested as a potential target in future preventive and curative management for CRC.

**KEYWORDS:** AvrA *Salmonella*, carcinogenesis, colorectal cancer

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**Introduction**

Colorectal cancer (CRC), a colon-rectum malignancy, is the third most frequent cancer globally. There were 1.9 million new CRC cases in 2020, with a mortality rate of 935,173 deaths per year, and it is the second most fatal cancer after lung cancer.(1–3) In Southeast Asia, the age-standardized incidence rate (ASR) of CRC was 17.3 per 100,000 men and 11.3 per 100,000 women in 2018.(4) In Indonesia, it is...
reported that the number of new cases of CRC was 34,189 in 2020.(1)

*Salmonella* is one of the most common infectious agents in the world.(5) Approximately 93.8 million people have this infection annually (6), and about 70% of them are caused by serovariant *typhimurium* and *enteritidis*. (7) Most CRCs occur in the ascending and transverse colon. These parts are closest to the terminal ileum, where the bacteria are located. The risk of colon cancer significantly increased in patients with *Salmonella* infection diagnosed under the age of 60. The risk is significantly increased after infection with *Salmonella enteritidis*. (8)

The chronic infection is caused by *Salmonella enterica* bacterial protein secreted as a type 3 secretion system (T3SS) effector protein that influences eukaryotic cell pathways, called avirulence protein A (AvrA). (9) AvrA *Salmonella* is also detected in human CRC (10), which can activate the Wnt/β-catenin signaling pathway (11) and activate the nuclear factor kappa B (NF-κB) signaling pathway via the toll-like receptor 4 (TLR4) receptor. (12) Moreover, NF-κB-β-catenin crosstalk can activate other signaling pathways, such as transforming growth factor-beta (TGF-β), which synergically interfere with cell apoptosis and proliferation (13), increasing carcinogenesis in the colon. This research was conducted to point out the effect of AvrA *Salmonella* on the occurrence of CRC through the regulation of TLR4/NF-κB/β-catenin/TGF-β expressions in the CRC mice model. Thus, it will provide the recent critical role of chronic *Salmonella* infection in CRC tumorigenesis, which can be a potential target for CRC prevention and therapy.

**Methods**

**Animals and Study Design**

A randomized control group post-test-only study was conducted using healthy Balb/c male mice (10-12 weeks) weighing 30 g obtained from the *Pusat Veteriner Farma* (PUSVETMA), Surabaya. The mice were kept at the Laboratory of Tropical Disease Center (TDC), Universitas Airlangga, Surabaya. Twenty-seven mice were divided into three groups, kept in a clean cage, and fed using standard feed AIN-93M (Dyets, Bethlehem, PA, USA). The three groups were the negative control group (normal mice without any treatment), positive control group (azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated mice), treatment group 1 (T1) (AOM/DSS-treated mice + AvrA *Salmonella*). The research was conducted considering the Research Ethics Code and was approved by the Commission on Eligibility for Research Ethics, Faculty of Medicine, Universitas Brawijaya, Malang (No. 224/EC/KEPK-S3/10/2022).

**Culture of *S. enterica* serovar *typhimurium***

*Salmonella typhimurium* was obtained from American Type Culture Collection (ATCC), USA, with catalog number 14028 lot number 58105535. Propagation of *S. typhimurium* on MacConkey’s medium aimed to bring more bacterial cultures, followed by an incubation process at 37°C for 18-24 hours. Then, it was transferred into a biphasic medium, which consisted of brain heart infusion (BHI) liquid medium (Sigma-Aldrich, St. Louis, MO, USA) and thiaproline carbonate glutamate (TCG) slant medium and subsequently incubated at 37°C for 24 hours. The composition of the TCG medium was made as in the previous studies. (14–16)

**Administration of AvrA *S. typhimurium* in a Colorectal Cancer Model**

The water and food of the experimental animals were replaced four hours before oral administration with 7.5 mg streptomycin (Sigma-Aldrich) (100 μL sterile solution) per mouse. After that, the animals were supplied with water and food *ad libitum*. Twenty hours after the streptomycin treatment, water and food were withheld from mice for four hours, after which the mice were either treated with sterile Hanks’ balanced salt solution (HBSS) (Sigma-Aldrich) (as a control) or infected by oral gavage with 10^7 CFU of *S. typhimurium* (100 μL suspension in HBSS). (10) After *Salmonella* gavage, mice were given 10 mg/kg body weight (BW) of AOM (Sigma-Aldrich) by intraperitoneal injection followed by 1% DSS (MP Biomedicals, Santa Ana, CA, USA) administration for five days via oral with a 14-day break. The DSS cycle was repeated three times.(17)

**Colon Tissue Sampling for Immunohistochemistry Preparation**

After the treatment, the mice were anesthetized using 20 mg ketamine (Agrovet, Senasa, Peru) per mouse intraperitoneally. Colon tissue was cut in the tumor area, which indicated colon cancer. Colon tissue was fixed in neutral-buffered formalin for 1-7 days, then submerged in 70% ethanol for at least 24 hours, followed by 80% ethanol for 2 hours, immersed in 90% and 95% ethanol, sequentially, for 30 minutes each, continued three times in absolute ethanol for 30 minutes, and finally submerged in xylol (Merck, Darmstadt, Germany), twice for 30 minutes each. The following process was carried out in an incubator with a 56-58°C temperature. The tissue was soaked in xylol, then
Results

Characteristics of the Research Samples

The distribution of the characteristics of the sample group observed included the mice's body weight, colon length, colon weight (Table 1), and the AvrA expression between three groups. This study showed a significant difference in the mean value of initial \((p=0.000)\) and final body weights \((p=0.000)\) among all groups. The body weight difference before and after the experiment (\(\Delta\) body weight) also showed a substantial finding in the three experimental groups \((p=0.000)\). There were significant differences in the mean body weight comparison between the negative control group \((4.6\pm1.1\) g\) and the positive control group \((-2.8\pm0.6\) g\) \((p=0.000)\), and the T1 group \((-3.4\pm0.7\) g\) \((p=0.000)\). However, there was no significant difference between the positive control and T1 groups \((p=0.082)\). These two groups showed almost the same mean value of the difference in body weight of mice; that is, the negative mean value was practically the same.

There was a significant difference in the mean value of colon length of the mice in all groups \((p=0.000)\). Among the groups, significant differences were observed between the negative \((10.2\pm0.4\) mm\) and the positive control group \((9.2\pm0.4\) mm\) \((p=0.000)\) also with the T1 group \((7.6\pm0.5\) mm\) \((p=0.000)\), then between the positive control group and the T1 group \((p=0.000)\). A significant difference in the mean colon weight of mice \((p=0.000)\) was also observed among the three groups; it was specifically observed between the negative \((29\pm2.9\) mg\) and the positive control group \((40.4\pm1.13\) mg\) \((p=0.000)\) and the T1 group \((57.9\pm1.83\) mg\) \((p=0.000)\), and between the positive control and T1 groups \((p=0.000)\).

AvrA expression on the colon tissue of all groups was confirmed using immunohistochemistry staining. The AvrA expression was more abundant in the T1 group compared to the negative and positive control groups (Figure 1).

TLR4, NF-\(\kappa\)B, \(\beta\)-catenin, TGF-\(\beta\), and Ki67 Expressions

The TLR4, NF-\(\kappa\)B, \(\beta\)-catenin, TGF-\(\beta\), and Ki67 expressions using immunohistochemistry staining were presented in Figure 2. The quantification of TLR4, NF-\(\kappa\)B, \(\beta\)-catenin, TGF-\(\beta\), and Ki67 expressions of all groups was shown in Figure 3. The mean values of TLR4, NF-\(\kappa\)B, \(\beta\)-catenin, TGF-\(\beta\), and Ki67 expressions in the T1 group were higher than those in the control groups, where the T1 group had the highest expression of those markers. In contrast, the negative control group had the lowest value. Statistical results of...
TLR4, NF-κB, β-catenin, TGF-β, and Ki67 expressions in all three groups showed significant differences with \( p=0.000 \) in each parameter.

All tumorigenesis parameters among the three groups in this study showed a similar pattern of the mean quantification value measured. The mean of TLR4 expression between the negative (12.12±2.92%) and the positive control groups (21.23±2.49%) differed significantly (\( p=0.000 \)), as well as the mean of TLR4 expression between the negative control and T1 groups (62.14±3.62%) (\( p=0.000 \)). A significant difference was also observed between the positive control and T1 groups (\( p=0.000 \)).

The mean of β-catenin expression of the positive control group (43.07 ± 3.18%) was significantly higher than that of the negative control group (12.91 ± 2.80%) (\( p=0.000 \)). The mean expression of the T1 group (61.63±4.02%) was also significantly higher than those of the negative control group (\( p=0.000 \)) and the positive control group (\( p=0.000 \)).

It was shown that there were significant differences in the mean expression of NF-κB between the negative (19.22±2.05%) and the positive control groups (32.26±2.47%) (\( p=0.000 \)), as well as between the negative control and the T1 groups (56.53±5.58%) (\( p=0.000 \)). Another significant difference was found between the positive control and the T1 groups (\( p=0.000 \)).

Significant differences in TGF-β expression were specifically demonstrated between the positive control group (52.27±3.38%) and the negative control group (12.82±0.69%) (\( p=0.000 \)), between the T1 group (84.49±8.31%) and the negative control group (\( p=0.000 \)), then the positive control and T1 groups (\( p=0.000 \)).

Lastly, the mean of Ki67 expression between the negative (9.32±0.87%) and the positive (40.21±3.20%) control groups (\( p=0.000 \)), as well as between the negative control and T1 groups (58.83±5.29%) (\( p=0.000 \)) were statistically significant. Moreover, the mean value of Ki67 expression in the T1 group was significantly higher than the one in the positive control group (\( p=0.000 \)).

**Apoptotic Percentage**

The expression of apoptotic cells using the TUNEL assay was presented in Figure 2. The quantification of apoptotic cells (apoptotic percentage data) was shown in Figure 3. All groups significantly differed in the mean apoptotic percentage (\( p=0.000 \)). In contrast to other markers, the negative control group (14.67±2.74%) had the highest
mean apoptotic percentage compared to the positive control (7.89±2.21%) and T1 groups (4.44±1.81%), whereas the T1 group had the lowest apoptotic percentage. Statistical results showed significant differences between negative and positive control groups (p=0.000) and negative control and T1 groups (p=0.000). Moreover, the mean apoptotic percentage between the positive control and the T1 groups showed a significant difference (p=0.004).

Discussion

Using an induced CRC model, AOM/DSS, this study explored the effect of AvrA Salmonella, a bacterial protein secreted as a T3SS effector protein that influences eukaryotic cell pathways, in inducing colon cancer in chronic inflammation.(9,10,18,19) In this study, each group
had differences in body weight changes. The body weight decreased in the treatment and positive control groups compared to the negative control group. Weight loss is an indicator of inflammation in the intestines of mice and is a result of hypercatabolic conditions in malignancy. (20) The treatment group had the highest mean colon weight, followed by the others. This colon weight indicated the tumor size in the mice's colon. So, the weight loss in mice was influenced by the increase in tumor weight in the colon. Mice's colon length is also a colon inflammation parameter; the more severe the inflammation, the shorter the mouse's colon. (20) Colon length differed significantly in all groups; the shortest was in the treatment group. These results indicated that AvrA Salmonella may cause aggravation of AOM/DSS-induced colonic shortening and showed the highest degree of inflammation in all groups.

This study showed that TLR4, NF-κB, β-catenin, TGF-β, and Ki67 expressions were significantly highest in AOM/DSS-treated mice with AvrA Salmonella. It is known that AvrA Salmonella can interact with the TLR4 receptor, a classic pattern recognition receptor responsible for detecting the presence of pathogens, thereby inhibiting the activation of immune signals and surviving it in the host's body, consequently worsening the infections. (12,21,22) TLR4 promotes CRC cell growth by activating other downstream factors, including NF-κB, enhancing the progression of inflammation-associated colorectal neoplasia. TLR4 increases CRC adhesion and metastatic capacity by promoting β1 integrin function involved in tumor-cell-endothelial cell adhesion and invasion through NF-κB-mediated upregulation of β1 integrin. (23) The metastatic capacity is also stimulated via NF-κB-mediated...
transcriptional upregulation of NADPH oxidase-1 (Nox-1) (24) and induced by the release of proinflammatory cytokines like interleukin (IL)-6 and IL-8, which additionally can regulate other metastatic signaling pathways.\(^{(24,25)}\) The modulation of TLR4 overexpression might promote tumorigenesis by facilitating cell proliferation, protecting malignant cells against apoptosis, and accelerating invasion and metastasis to create a tumor-supportive cellular microenvironment.\(^{(22)}\)

Regarding NF-κB expression, it is essential to note that AvrA Salmonella protein can activate the NF-κB pathway, thus increasing cytokine production and inflammatory mediators, elevating gene expression in cell proliferation, and inhibiting apoptosis.\(^{(26)}\) AvrA binds to the inhibitor of kappa B (IκB) protein and prevents IκB degradation by the proteasome complex, inhibiting NF-κB activation.\(^{(27)}\) NF-κB can increase the expression of survival genes like BCL2L1 and BCLXL and tumor-promoting cytokines like IL-6 and tumor necrosis factor (TNF).\(^{(28)}\) This signaling pathway interacts with other protein networks, such as β-catenin, and synergistically upregulates it.\(^{(29)}\) It is consistent with the research showing that S. enterica serovar typhimurium can manipulate the Wnt/β-catenin, influencing cell proliferation and differentiation and enhancing the bacterial ability to survive and cause infection.\(^{(11)}\) AvrA affects the β-catenin signaling pathway by inhibiting the activity of the glycosgen synthase kinase-3β (GSK-3β) kinase.\(^{(27)}\)

Crosstalk between NF-κB and Wnt/β-catenin signaling pathways includes positive and negative regulation of NF-κB by Wnt/β-catenin and vice versa.\(^{(29)}\) Such co-arrangements are critical for increasing or decreasing downstream response. Mutations or increased expression of adenomatous polyposis coli (APC) and β-catenin can control TLR4 activation by lipopolysaccharide (LPS) in colon cancer cell proliferation. Thus, it further reinforces the notion that TLR4/NF-κB/β-catenin may be a potential therapy for inflammation-related cancer.\(^{(29)}\)

Salmonella is known to cause alterations in TGF-β signaling by boosting the number of TGF-receptor type 1 (TGF-RI) receptors and activating the Smad2 and Smad3 signaling pathways in the mouse ileum during both the acute and chronic stages. It is believed that enhanced TGF-β signaling may constitute a compensatory host response to Salmonella infection that promotes mucosal repair or pathogen survival.\(^{(30)}\) TGF-β can suppress the immune system, angiogenesis, apoptosis, cell growth, and epithelial to mesenchymal changes during the development and progression of metastatic cancer through the TGF-β autocrine cycle/loop. However, TGF-β has an antiproliferative effect and functions as a tumor suppressor during the early stages of tumorigenesis.\(^{(31)}\) Mutations that inactivate the Smad receptor and then activate the non-Smad pathway are the primary mechanisms of this pro- and antiproliferative effect.\(^{(32)}\)

Ki67 protein is a core protein widely used as a cell proliferation marker. This study is consistent with most studies that have found an association between tumor rates in human malignancies and Ki67.\(^{(33,34)}\) This protein expression is inhibited by disturbing the Wnt/β-catenin or NF-κB pathway, which the AvrA effector protein regulates and correlates with other proliferation activity.\(^{(35)}\) This protein plays a role during the active phase of the cell cycle (G1, S, G2, and M phases) and vice versa during the resting phase (G0).\(^{(36)}\) Its levels rise in the early mitotic phase and decrease substantially during later mitotic stages.\(^{(34)}\)

Additionally, it was found that there was a decrease in the apoptosis percentage in the group exposed to the AvrA Salmonella effector protein. It is worth noting that AvrA Salmonella can influence the regulation of apoptosis.\(^{(9)}\) In addition, AvrA Salmonella activates the NF-κB pathway, together with crosstalk activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, increases the activity of antiapoptotic proteins such as Bcl-2 and Bcl-X\(_{L}\), and reduces the activity of proapoptotic proteins such as caspase-3 and Bax, and acetylating p53 protein.\(^{(37,38)}\) All previous upregulated interconnected proteins - TLR4, NF-κB, β-catenin, TGF-β, contribute to cell proliferation. Thus, those high expressions correlated with the apoptosis outcome.

This study can open up possibilities for developing new therapies to address the incidence of CRC. Chronic inflammation and oxidative stress conditions in this study were induced by administering AOM/DSS, which is not identical to those in the human colon environment. Further research using human samples is needed to elaborate on the actual CRC pathomechanism in improving CRC prevention and treatment programs.

**Conclusion**

This study reveals the mechanism of AvrA Salmonella in colorectal malignancy through the excessive expression of TLR4, NF-κB, β-catenin, and TGF-β protein, which is confirmed with high expression of Ki67 as a cell proliferation marker and consequently inhibits the apoptotic process demonstrated by decreased apoptotic percentage. Thus, AvrA Salmonella influences CRC tumorigenesis.
through TLR4/NF-κB/β-catenin/TGF-β and is suggested as a potential target for preventive and curative management of CRC.

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Authors Contribution

BPW, HKa, HKh, and HS were involved in the audit’s conception and design. BPW performed data collection and analysis, writing, reviewing, and revising the manuscript. All authors were involved in writing and reviewing the manuscript. All authors have read and approved the final manuscript.

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