RESEARCH ARTICLE

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 10^3 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

paraffin (Merck) three times, and embedded into paraffin. The colon was sliced 5 μm in size using a microtome. The selected part was dried, placed on a hot plate at 38-40°C until dry, and stored in an incubator at 38-40°C for 24 hours.

AvrA, TLR4, NF-κB, β-catenin, TGF-β, and Ki67 Immunohistochemistry Staining

An immunohistochemistry procedure was conducted to observe the colon tissue expressions of AvrA, TLR4, NF-κB, β-catenin, TGF-β, and Ki67—all of the processes undertaken as per manufacturer protocols. The primary antibodies used were anti-AvrA antibody based on amino acid no. 160-259 (Creative Diagnostics, Shirley, NY, USA), TLR4 polyclonal antibody (Catalog no.: BS-20594R, Bioss, Woburn, MA, USA), NF-κB p65 polyclonal antibody (Catalog No. BS-20160R, Bioss), β-catenin antibody (E-5) (Catalog No. sc-7963, Santa Cruz, Dallas, TX, USA), anti-TGF-β monoclonal antibody (Catalog No. MD5A1221, MedikBio, Malang, Indonesia), and anti-Ki67 monoclonal antibody (Catalog No. MD6A1121, MedikBio). All of the primary antibodies were diluted using the optimal dilution at 1:100. The secondary antibody used for TLR4 and NF-κB staining was 1:200 diluted biotin-conjugated goat anti-rabbit IgG (Catalog No. BS-0295G-Biotin, Bioss), while for the remaining primary antibodies (anti-AvrA, anti-β-catenin, anti-TGF-β, and anti-Ki67) used 1:200 diluted biotin-conjugated goat anti-mouse IgG, (Catalog No. BS-0296G-Biotin, Bioss). Then, the application of streptavidin-horseradish peroxidase (SA-HRP) and diaminobenzidine (DAB) (Biocare, Pacheco, CA, USA) was performed, followed by Mayer's hematoxylin counterstaining (ScyTek, Logan, UT, USA) and mounting process using Entellan (Merck). TLR4, NF-κB, β-catenin, TGF-β, and Ki67 expressions were analyzed using light microscopes, and the data was presented as a percentage (%) of the immunoratio results using ImmunoRatio imaging software (ImageJ 1.52p Fiji software, NIH, Bethesda, MD, USA).

Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) Assay for Apoptotic Cell Examination

Tumor TACS In situ Apoptosis Detection Kit (R&D System, Minneapolis, MN, USA) was used for the TUNEL assay. The apoptotic percentage was calculated by the number of apoptotic cells / the total number of cells × 100. The apoptotic cells were analyzed using light microscopes, and their expression was semi-quantitatively measured using ImmunoRatio imaging software (ImageJ 1.52p Fiji software, NIH).

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 (Δ 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±1.1 g) and the positive control group (-2.8±0.6 g) (p=0.000), and the T1 group (-3.4±0.7 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±0.4 mm) and the positive control group (9.2±0.4 mm) (p=0.000) also with the T1 group (7.6±0.5 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±2.9 mg) and the positive control group (40.4±1.13 mg) (p=0.000) and the T1 group (57.9±1.83 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-κB, β-catenin, TGF-β, and Ki67 Expressions

The TLR4, NF-κB, β-catenin, TGF-β, and Ki67 expressions using immunohistochemistry staining were presented in Figure 2. The quantification of TLR4, NF-κB, β-catenin, TGF-β, and Ki67 expressions of all groups was shown in Figure 3. The mean values of TLR4, NF-κB, β-catenin, TGF-β, 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

Table 1. Characteristics of the samples.

Variable	Mean±SD			*p* -value
	Control (-)	Control (+)	T1	
Initial body weight (g)	30.4±0.5[a]	32.7±0.5[b]	32.7±0.5[b]	0.000
Final body weight (g)	35±0.7[a]	29.8±0.4[b]	29.2±0.7[b]	0.000
Δ body weight (g)	4.6±1.1[a]	-2.8±0.6[b]	-3.4±0.7[b]	0.000
Colon length (mm)	10.2±0.4[a]	9.2±0.4[b]	7.6±0.5[c]	0.000
Colon weight (mg)	29±2.9[a]	40.4±1.13[b]	57.9±1.83[c]	0.000

p-values were obtained from the Kruskal-Wallis test for initial, final, and Δ body weight and one-way ANOVA for colon length and weight. Power of letter at the mean±SD data shows the results of the Mann-Whitney test for initial, final, and Δ body weight and the LSD test for colon length and weight. If it contains different letters, it means there is a significant difference (*p*-value<0.05), and if it includes the same note, it means there is no significant difference (*p*-value>0.05). Control (-): Normal mice without any treatment; Control (+) group: AOM/DSS-treated mice; T1 group: AOM/DSS-treated mice + AvrA *Salmonella*.

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

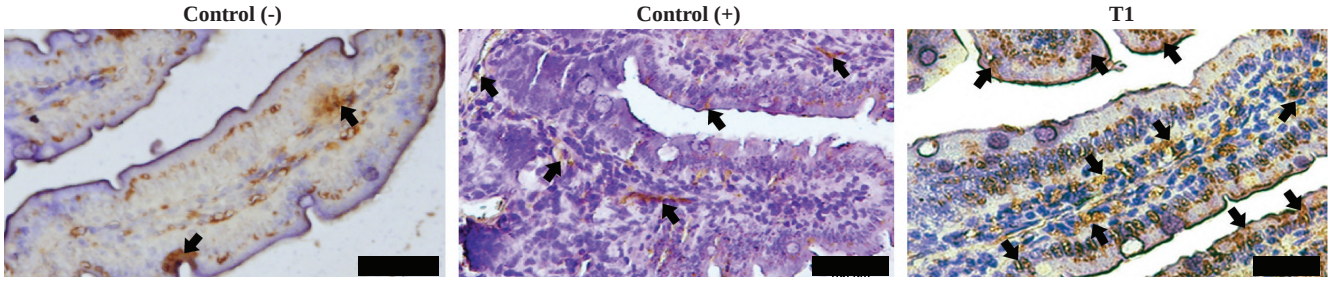

Figure 1. AvrA expression in colon tissue. Black arrows: Cells expressing AvrA. Control (-): Normal mice without any treatment; Control (+): AOM/DSS-treated mice; T1: AOM/DSS-treated mice + AvrA *Salmonella*. Black bar: 100 μm.

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

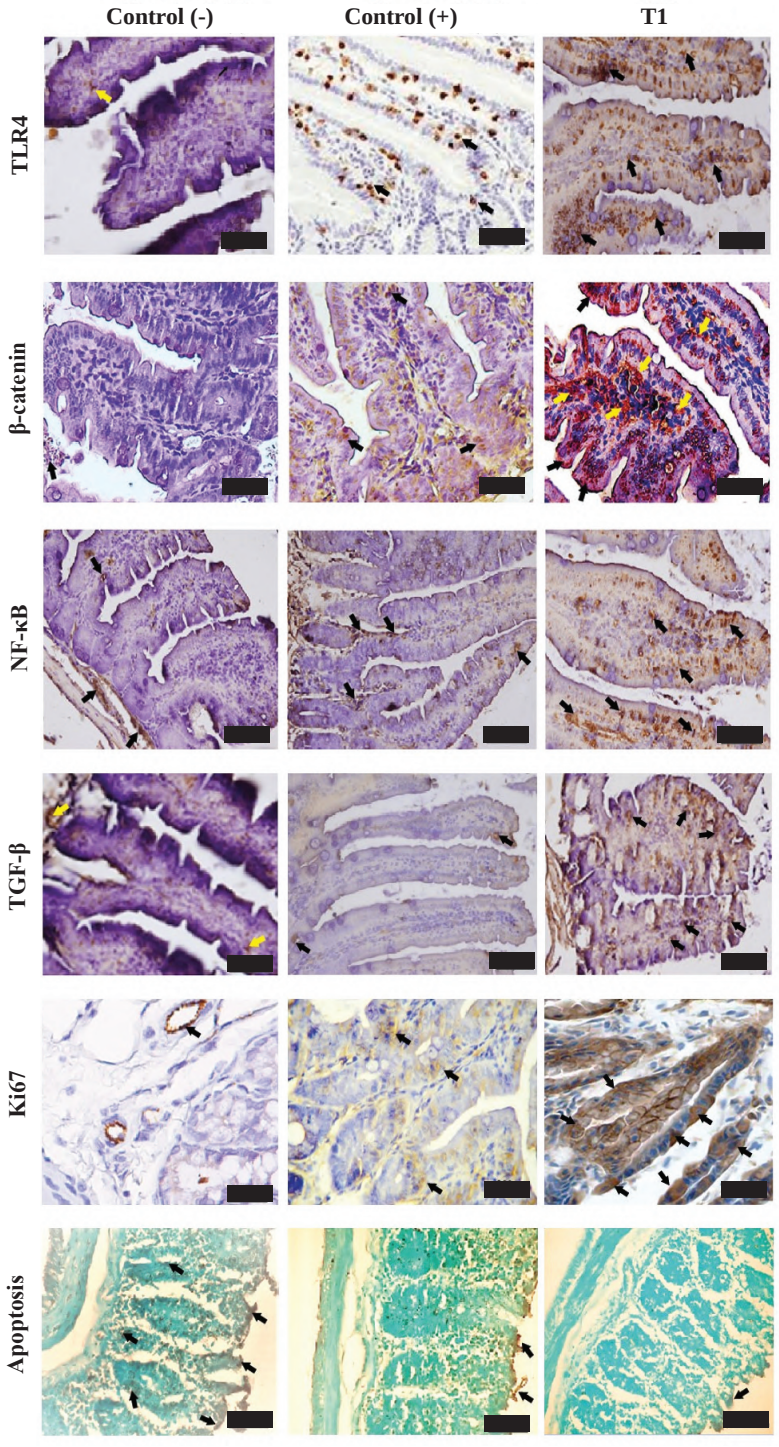

Figure 2. TLR4, β-catenin, NF-κB, TGF-β, Ki67, and apoptotic cells expression. Black and yellow arrows: Cells expressing TLR4, β-catenin, NF-κB, TGF-β, Ki67, and apoptotic cells. Control (-): Normal mice without any treatment; Control (+): AOM/DSS-treated mice; T1: AOM/DSS-treated mice + AvrA *Salmonella*. TLR4: Toll-like receptor 4; NF-κB: Nuclear factor kappa B; TGF-β: Transforming growth factor-beta. Black bar: 100 μm.

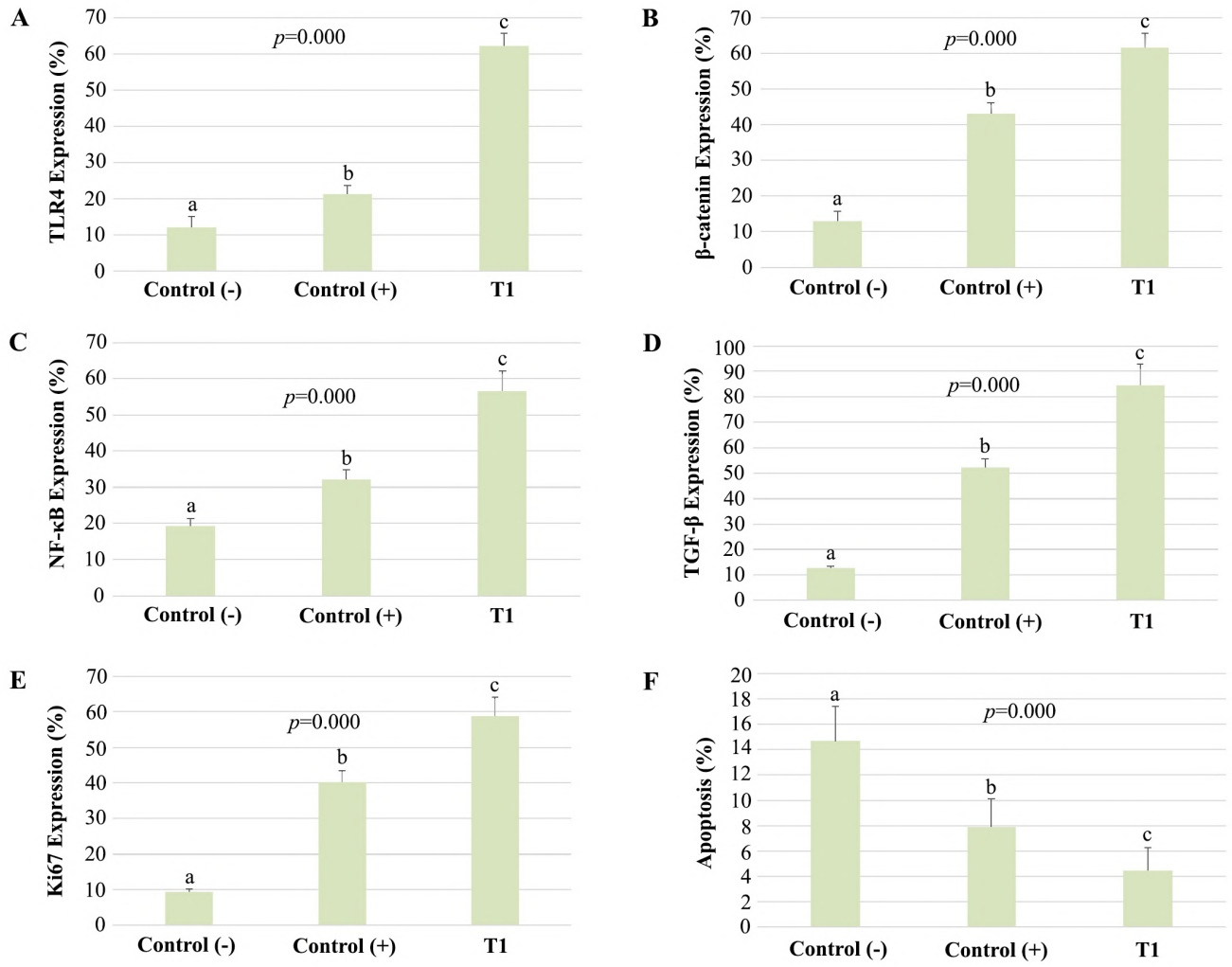

Figure 3. The quantification of TLR4, β-catenin, NF-κB, TGF-β, Ki67 expressions, and apoptosis percentage. The *p*-values described in each graph were analyzed using the one-way ANOVA test. The a, b, and c labels on each bar indicate a significant difference between groups with the different labels as analyzed using multiple comparisons LSD test. Control (-): Normal mice without any treatment; Control (+): AOM/DSS-treated mice; T1: AOM/DSS-treated mice + AvrA *Salmonella*. TLR4: Toll-like receptor 4; NF-κB: Nuclear factor kappa B; TGF-β: Transforming growth factor-beta.

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 glycogen 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.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al*. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021; 71(3): 209-49.
2. Wulandari F, Hanifa M. Integrative bioinformatics reveals the lactate dehydrogenase B (LDHB) significance in colon adenocarcinoma. Mol Cell Biomed Sci. 2023; 7(2): 98-108.
3. Harjanti DA, Murtono C, Suryawinata K, Halim A, Wiryokusuma MF, Djitro TB. John Cunningham virus T-antigen expression on mild and severe dysplasia adenomatous polyp, low and high grade adenocarcinoma of the colon. Mol Cell Biomed Sci. 2019; 3(1): 7-12.
4. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68(6): 394-424.
5. Nurtamin T, Nurman RY, Hafizah I. Antibacterial activity of eel (Anguilla spp.) mucus against Salmonella typhi. Indones Biomed J. 2016; 8(3): 179-82.
6. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, *et al*. The global burden of nontyphoidal Salmonella gastroenteritis. Clin Infect Dis. 2010; 50(6): 882-9.
7. Havelaar AH, Ivarsson S, Löfdahl M, Nauta MJ. Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009. Epidemiol Infect. 2013; 141(2): 293-302.
8. Mughini-Gras L, Schaapveld M, Kramers J, Mooij S, Neefjes-Borst EA, Pelt WV, *et al*. Increased colon cancer risk after severe Salmonella infection. PLoS One. 2018; 13(1): e0189721. doi: 10.1371/journal.pone.0189721.
9. Liu X, Lu R, Xia Y, Wu S, Sun J. Eukaryotic signaling pathways targeted by Salmonella effector protein AvrA in intestinal infection in vivo. BMC Microbiol. 2010; 10: 326. doi: 10.1186/1471-2180-10-326.
10. Lu R, Bosland M, Xia Y, Zhang YG, Kato I, Sun J. Presence of Salmonella AvrA in colorectal tumor and its precursor lesions in mouse intestine and human specimens. Oncotarget. 2017; 8(33): 55104-15.
11. Lu R, Wu S, Zhang YG, Xia Y, Liu X, Zheng Y, *et al*. Enteric bacterial protein AvrA promotes colonic tumorigenesis and activates colonic beta-catenin signaling pathway. Oncogenesis. 2014; 3(6): e105. doi: 10.1038/oncsis.2014.20.
12. Shibolet O, Podolsky DK. TLRs in the gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: Addition by subtraction. Am J Physiol Gastrointest Liver Physiol. 2007; 292(6): G1469-73.
13. Oshima H, Nakayama M, Han TS, Naoi K, Ju X, Maeda Y, *et al*. Suppressing TGFβ signaling in regenerating epithelia in an inflammatory microenvironment is sufficient to cause invasive intestinal cancer. Cancer Res. 2015; 75(4): 766-76.
14. Utsunomiya A, Naito T, Ehara M, Ichinose Y, Hamamoto A. Studies on novel pili from Shigella flexneri. I. Detection of pili and hemagglutination activity. Microbiol Immunol. 1992; 36(8): 803-13.
15. Satuman, Sari DS, Eva R, Tanggo EH, Notobroto HB, Sudiana K, *et al*. The effect of acute and chronic infection-induced by AvrA protein of Salmonella typhimurium on radical oxygen species, phosphatase and tensin homolog, and cellular homolog expression during the development of colon cancer. Open Access Maced J Med Sci. 2021; 9(A): 343–51.
16. Mufida DC, Handono K, Prawiro SR, Santoso S. The effect of intranasal immunization with Streptococcus pilus protein on nasopharyngeal pIgR and IgA expression in rats. Turkish J Immunol. 2018; 6(3): 87–94.
17. Snider AJ, Bialkowska AB, Ghaleb AM, Yang VW, Obeid LM, Hannun YA. Murine model for colitis-associated cancer of the colon. Methods Mol Biol. 2016; 1438: 245-54.
18. Lee SK, Hwang JH, Choi KY. Interaction of the Wnt/β-catenin and RAS-ERK pathways involving co-stabilization of both β-catenin and RAS plays important roles in the colorectal tumorigenesis. Adv Biol Regul. 2018; 68: 46-54.
19. Park CH, Eun CS, Han DS. Intestinal microbiota, chronic inflammation, and colorectal cancer. Intest Res. 2018; 16(3): 338-45.
20. Lu R, Wu S, Liu X, Xia Y, Zhang YG, Sun J. Chronic effects of a Salmonella type III secretion effector protein AvrA in vivo. PLoS One. 2010; 5(5): e10505. doi: 10.1371/journal.pone.0010505.
21. Crame EE, Nourmohammadi S, Wardill HR, Coller JK, Bowen JM. Contribution of TLR4 to colorectal tumor microenvironment, etiology and prognosis. J Cancer Res Clin Oncol. 2023; 149(7): 3009-21.
22. Guo J, Liao M, Wang J. TLR4 signaling in the development of colitis-associated cancer and its possible interplay with microRNA-155. Cell Commun Signal. 2021; 19(1): 90. doi: 10.1186/s12964-021-00771-6.
23. Wang EL, Qian ZR, Nakasono M, Tanahashi T, Yoshimoto K, Bando Y, *et al*. High expression of Toll-like receptor 4/myeloid differentiation factor 88 signals correlates with poor prognosis in colorectal cancer. Br J Cancer. 2010; 102(5): 908-15.
24. O'Leary DP, Bhatt L, Woolley JF, Gough DR, Wang JH, Cotter TG,

et al. TLR-4 signalling accelerates colon cancer cell adhesion via NF-κB mediated transcriptional up-regulation of Nox-1. PLoS One. 2012; 7(10): e44176. doi: 10.1371/journal.pone.0044176.

25. Hu X, Fatima S, Chen M, Xu K, Huang C, Gong RH, *et al*. Toll-like receptor 4 is a master regulator for colorectal cancer growth under high-fat diet by programming cancer metabolism. Cell Death Dis. 2021; 12(8): 791. doi: 10.1038/s41419-021-04076-x.
26. Kurtz JR, Goggins JA, McLachlan JB. Salmonella infection: Interplay between the bacteria and host immune system. Immunol Lett. 2017; 190: 42-50.
27. Oguma K, Oshima H, Aoki M, Uchio R, Naka K, Nakamura S, *et al*. Activated macrophages promote Wnt signalling through tumour necrosis factor-alpha in gastric tumour cells. EMBO J. 2008; 27(12): 1671-81.
28. Schwitalla S, Fingerle AA, Cammareri P, Nebelsiek T, Göktuna SI, Ziegler PK, *et al*. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. Cell. 2013; 152(1-2): 25-38.
29. Ma B, Hottiger MO. Crosstalk between Wnt/β-catenin and NF-κB signaling pathway during inflammation. Front Immunol. 2016; 7: 378. doi: 10.3389/fimmu.2016.00378.
30. Zhang YG, Singhal M, Lin Z, Manzella C, Kumar A, Alrefai WA, *et al*. Infection with enteric pathogens Salmonella typhimurium and Citrobacter rodentium modulate TGF-beta/Smad signaling pathways in the intestine. Gut Microbes. 2018; 9(4): 326-37.
31. Bellam N, Pasche B. Tgf-beta signaling alterations and colon cancer. Cancer Treat Res. 2010; 155: 85-103.
32. Zhang BH, Wang C, Dong W, Chen X, Leng C, Luo X, *et al*. A novel approach for monitoring TGF-β signaling in vivo in colon cancer. Carcinogenesis. 2021; 42(4): 631-9.
33. Mulyawan IM. Role of Ki67 protein in colorectal cancer. Int J Res Med Sci. 2019; 7(2): 644-8.
34. Li LT, Jiang G, Chen Q, Zheng JN. Ki67 is a promising molecular target in the diagnosis of cancer (review). Mol Med Rep. 2015; 11(3): 1566-72.
35. Sun X, Kaufman PD. Ki-67: More than a proliferation marker. Chromosoma. 2018; 127(2): 175-86.
36. de Azambuja E, Cardoso F, de Castro G Jr, Colozza M, Mano MS, Durbecq V, *et al*. Ki-67 as prognostic marker in early breast cancer: A meta-analysis of published studies involving 12,155 patients. Br J Cancer. 2007; 96(10): 1504-13.
37. Hernández-Luna MA, Muñóz-López P, Aguilar-González CA, Luria-Pérez R. Infection by Salmonella enterica promotes or demotes tumor development. In: Mascellino MT, editor. Salmonella: A Re-emerging Pathogen. London: IntechOpen; 2018. p.55–71.
38. Hernández-Luna MA, López-Briones S, Luria-Pérez R. The four horsemen in colon cancer. J Oncol. 2019; 2019: 5636272. doi: 10.1155/2019/5636272.