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THE ROLE OF *P53* AND *K-RAS* IN REGULATING SPLEEN INNATE MEDIATORS IN MICE WITH COLON CANCER

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ABSTRACT

The spleen is a secondary lymphoid organ that embraces several immune cells. It possesses an anti-tumor impact where splenectomies correlated directly to a significant increase of malignant tumor induction. The unmutant *p53* and *K-ras* genes are expressed at low levels and play an essential role in the apoptosis of corrupted cells. We studied the impacts of *p53* and *K-ras* transcriptions in some innated mediators' expressions on the spleen of mice bearing colon cancer. A number of Swiss mice were categorized into the untreated negative control group, and mice were injected with Azoxymethane carcinogen for colon cancer induction (AOM group). Both groups were subjected to *TLR1*, *TLR2*, *IL1β*, *TNFα*, *IFNγ*, *p53*, and *K-ras* expression levels estimations after 4, 8, and 10 weeks from colon cancer induction. A significant increase in the *p53* transcription level was observed at week 10, whilst K-ras transcription level upregulated significantly only at week 8 in the AOM group compared to the untreated group. The AOM spleen cells showed a late (weeks 8 and 10) significant upregulation and transcriptions on *TLR1*, *TLR2*, *TNFα*, and *IFNγ*, levels, however, the *IL1β* transcription showed an early significant downregulation in comparison to those of the untreated group. Moreover, *p53* transcription showed a significant correlation with *TLR1* and *TLR2* expressions, while *K-ras* transcription showed a significant correlation to the *IL1β*, *TNFα*, and *IFNγ*, transcriptions levels. The study gave an insight into the confirmed correlation of *p53* and *K-ras* transcriptions of mice spleen to some innate mediators during the induction of colon cancer.

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Introduction

The spleen is the biggest lymphoid member in the body and besides its role in hematopoiesis and red blood cell clearance it has a wide range of immunologic functions [1]. Spleen is also considered the largest natural reservoir of immune cells including natural killer cells, neutrophils, monocytes, and lymphocytes [2]. The main immunological function of the spleen is to mount an immune response and remove micro-organisms from circulation; these functions are accomplished through two functional compartments of spleen; the first one is the red pulp region which removes pathogens and cellular debris and the second one is the white pulp regions of the spleen that contain the lymphocytes which initiate adaptive immune response [3, 4]. The spleen also has a very important role in the anti-tumor immune system and this role has become clear since many modern studies linked between splenectomy and cancer development in mice and rats, species; splenectomies rats and mice showed a significant increase of malignant tumor induction. Epidemiological studies also have found that the risk of cancer development is increased when the spleen is removed [5, 6].

Cancer is a global health problem that is associated with high mortality rates around the world [7, 8] and colon cancer, in particular, accounts for approximately 11% of all annually diagnosed cancer and cancer-related deaths around the world [9]. The development of colon cancer depends on many factors where both hereditary and environmental play important roles.

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The risk factors include decreased physical activity, obesity, excessive consumption of processed food and red meat, alcohol, and smoking [10-12]. Epidemiological studies show that male gender and age increasing have a strong association with colon cancer incidence where statistics show that approximately 10-20% of all patients with colon cancer have a positive family history [11].

A variety of factors have contributed to the process of transformation of a normal cell towards a cancerous cell. This process has several stages of epigenetic and genetic changes that confer selective advantages upon the altered cells [13]. Generally, cancer-related genes are divided into 2 classes, tumor suppressor genes and proto-oncogenes [14]. *P53* gene is one of the most important tumor suppressor genes that play a key role in the regulation process of various signaling pathways, as a tumor suppressor, *p53* plays various significant roles including the ability to arrest cell cycle, apoptosis senescence, and DNA repair [13, 15]. The *p53* gene is inactive and commonly mutated in the majority of cancers it is considered the second most frequent mutation observed in colorectal cancer and many *p53* mutants can promote tumor growth through several ways [13, 16]. Proto-oncogenes are involved in pathways that promote cell growth; these genes lead normal cells to be cancerous when they are activated by alterations or mutations, mutations in proto-oncogenes are usually dominant, and their mutated versions are known as oncogenes [14]. *K-ras* gene is an oncogene that encodes *K-ras*, a small GTPase transductor protein involved in regulating cell division due to its ability to transform external signals into the cell nucleus [17]. *K-ras* mutations are predominant in several human cancers and have been found in 52% of colorectal cancer patients [18]. Since the mice spleen considered as the occupants of many immune cells in particular monocytes and macrophages, this study investigated the correlation between spleen *p53* and *K-ras* transcriptions and some innate mediators during the early stages of colon cancer induction.

Materials and Methods

Induction of Colon Cancer in Experimental Animal

Forty Swiss male mice (20-25 g, 6-8 weeks old) were maintained in the Animal House Unit at the King Fahd Medical Research Center, King Abdul-Aziz University, KSA. Azoxymethane (AOM; Sigma-Aldrich St. Louis, MO, Cat NO A5486) is a chemical carcinogen used to induce colon cancer in animals [19]. The mice were randomly sorted into two groups: negative untreated control (C group), and mice group received four IP injections of 10mg/kg AOM over eight weeks, from week 0 to week 7, to induce colon cancer tumorigenesis; then, they were left untreated till the end of week 10 (AOM group). Next, five mice from C and AOM groups were sacrificed at weeks 4, 8, and 10, and all efforts were made to minimize the suffering. Finally, their spleen samples were collected and stored at -80°C until use.

Estimation of Gene Expression Levels in Mice

The mRNA extractions were performed using the RNeasy Mini kit (QIAGEN, Cat No. 75162) from the mice spleen tissues preserved in RNA later (QIAGEN, Cat NO. 76106) according to the manufacturer's protocol. The QuantiTect SYBR Green RT-qPCR Kit Master Mix reagents (QIAGEN Cat No. 204243) and sets of IL-1β, TLR1, TLR 2, IFN-γ, TNFα, *p53*, and *K-ras* specific primers **(Table 1)** were used to evaluate the transcriptions levels as described by Elbanna *et al.* [20] and Hadad *et al.* [21]. Transcriptions relative ratio levels of the target genes were estimated according to the 2-ΔΔCt method in comparison with the GAPDH gene transcription as a housekeeping gene [22].

Table 1. Primers Used for Genes Expression Quantitation Using SYBER Green qRT-PCR

Gene	Polarity	Primer sequence (5'---'3)	Primer length	Nucleotide positions	GenBank References
GAPDH	F	GTTGTCTCCTGCGACTTCA	19	1356-1374	XM_017321385
	R	GGTGGTCCAGGGTTTCTTA	19	1649-1631	
TLR1	F	TCAAGCATTTGGACCTCTCCT	21	824-844	NM_001276445
	R	TTGTACCCGAGAACCGCTCA	20	933-914	
TLR2	F	AACCTCAGACAAAGCGTCAAATC	23	323-345	NM_011905.3
	R	ACCAAGATCCAGAAGAGCCAAA	22	387-366	
IFNγ	F	GGCCATCAGCAACAACATAAGCGT	24	364-387	NM_008337.4
	R	TGGGTTGTTGACCTCAAACTTGGC	24	481-458	
TNFα	F	ATGAGCACAGAAAGCATGA	19	157-175	AB185894.1
	R	AGTAGACAGAAGAGCGTGGT	20	308-289	
IL1β	F	TCATGGGATGATGATGATAACCTGCT	26	960-985	XM_006498795
	R	CCCATACTTTAGGAAGACACGGATT	25	1462-1458	
P53	F	ACACCTGATCGTTACTCGGCTTGT	24	674-697	X_60470.1
	R	AAATTACAGACCTGGTGGCTCA	22	887-865	

Kras	F	AGGCCTGCTGAAAATGACTG	20	242-261	XM_032905399.1
	R	TCTATCGTAGGGTCGTACTCATC	23	146-168	

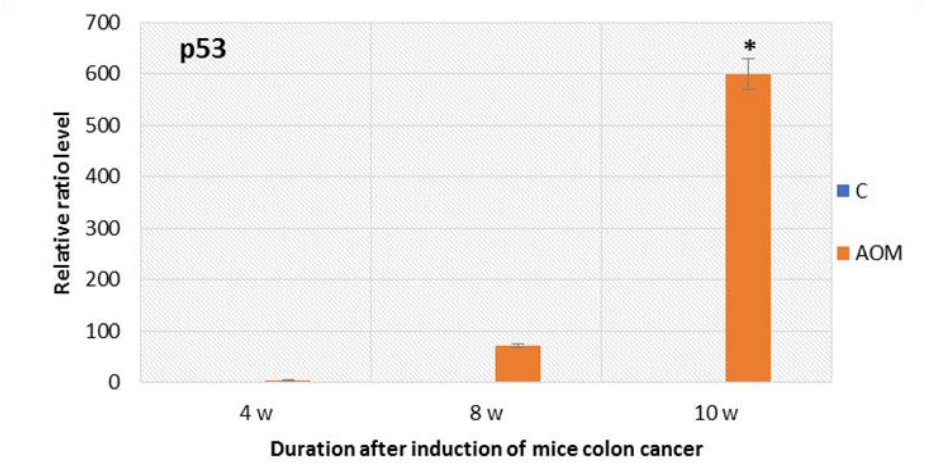
Statistical Methods

The statistical evaluations between the groups were performed using Megastat software version 10.1. One-way ANOVA parametric tests were performed for obtaining the relative ratios of the gene expressions. A P value <0.05 was deemed significant. In addition, the correlation matrix analysis test was used for obtaining the correlation analysis between the *p53, K-ras* and the current immunological markers.

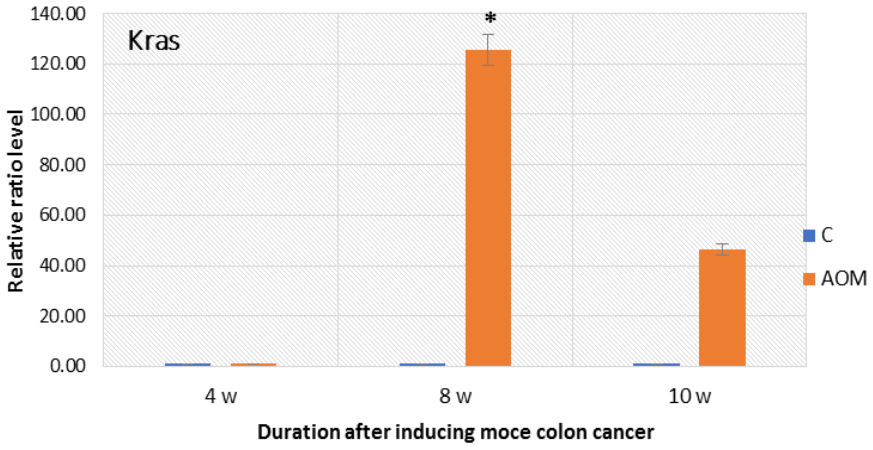
Results and Discussion

Influence of AOM p53 and K-ras Transcriptions

By weeks 4, the *p53* expression level diminished non significantly in the spleen cells of the AOM group in comparison with its level in the untreated group, whereas its expression was increased non significantly at week 8. Finally, by week 10, the *p53* transcription level increased extremely and significantly in the AOM group compared to its corresponding levels in the control group (*P=0.0000*) **(Figure 1)**. Moreover, the transcription level of *K-ras* in the spleen cells of the AOM group fluctuated non-significantly when compared with its levels in the untreated group at weeks 4 and 10. By week 8, the transcription of the current oncogene was upregulated significantly in the AOM group when compared with its level in the untreated group (*P=0.005*) **(Figure 1)**.



a)



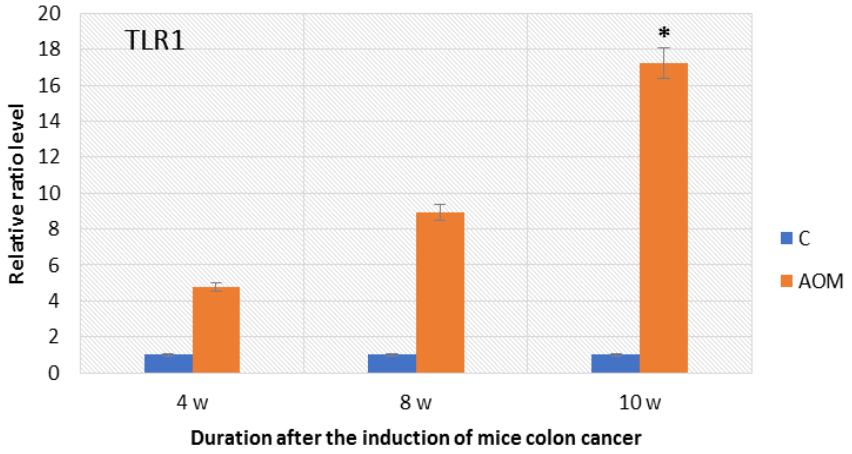
b)

Figure 1. The Relative Ratio of *p53/mRNA* and *K-ras/mRNA* Transcriptions Levels in Different Mice Spleen

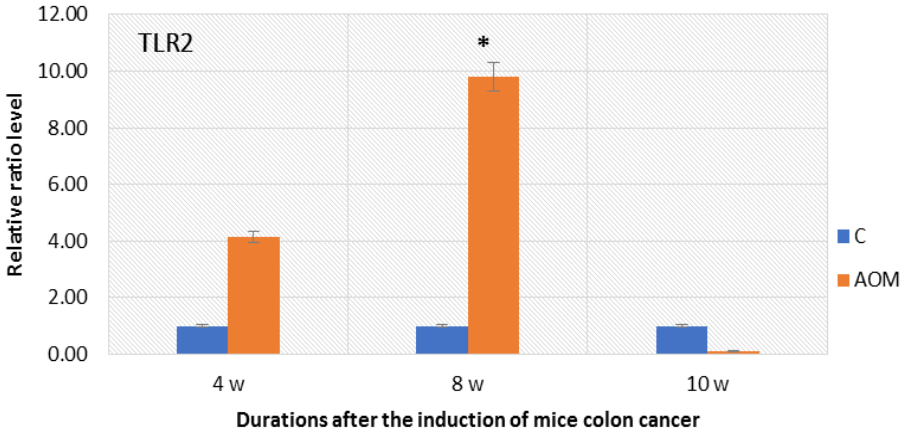
Where group C represented mice untreated negative control, and the AOM group represented mice injected with AOM for inducing colon cancer. (*) Significant at *P<0.05* as determined by ANOVA and One-factor ANOVA test. Each point represented the mean value of 3 tests and the vertical bars denote the mean±5%.

Influence of AOM on Toll-like Receptors 1 and 2 Transcriptions

By weeks 4 and 8, the transcription levels of *TLR1* increased in the spleen cells of the AOM mice group in comparison to the levels in the untreated group; however, this change was non-significant. On the other hand, and by week 10, the *TLR1* transcription level was significantly enhanced in the AOM group when compared with the levels in untreated mice (*P=0.0024*) **(Figure 2)**. Furthermore, and by weeks 4 and 10, the *TLR2* transcription level fluctuated non-significantly in the spleen cells of the AOM group when compared with its level in the control group; meanwhile, the expression levels significantly increased in the AOM group in comparison to the levels in the untreated group after eight weeks from inducing mice colon cancer (*P=0.019*) **(Figure 2)**.



a)



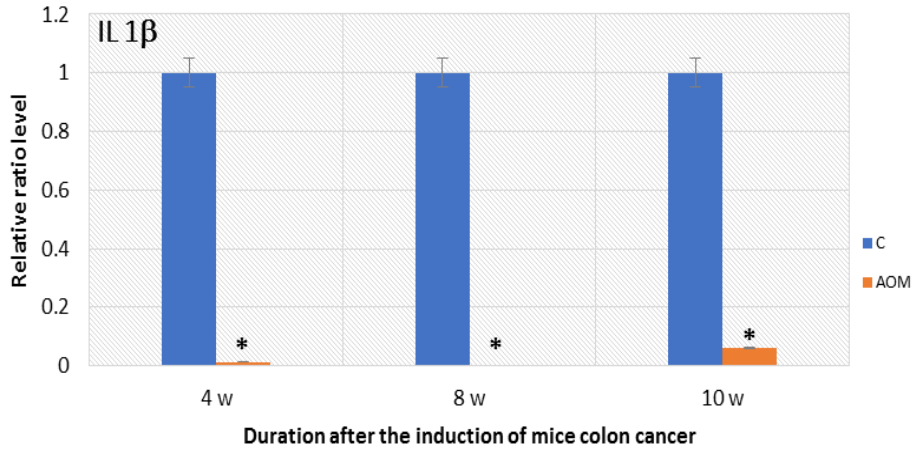
b)

Figure 2. The Relative Ratio of *TLR1/mRNA and TLR2/mRNA* Transcriptions Levels in Different Mice Spleen

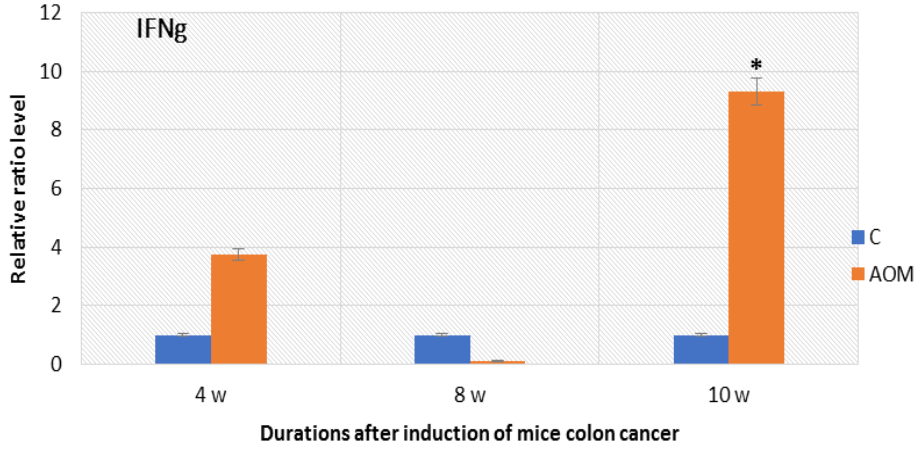
Where group C represented mice untreated negative control, and the AOM group represented mice injected with AOM for inducing colon cancer. (*) Significant at *P<0.05* as determined by ANOVA and One-factor ANOVA test. Each point represented the mean value of 3 tests and the vertical bars denote the mean±5%.

Influence of AOM on Pro-inflammatory Cytokines Transcriptions

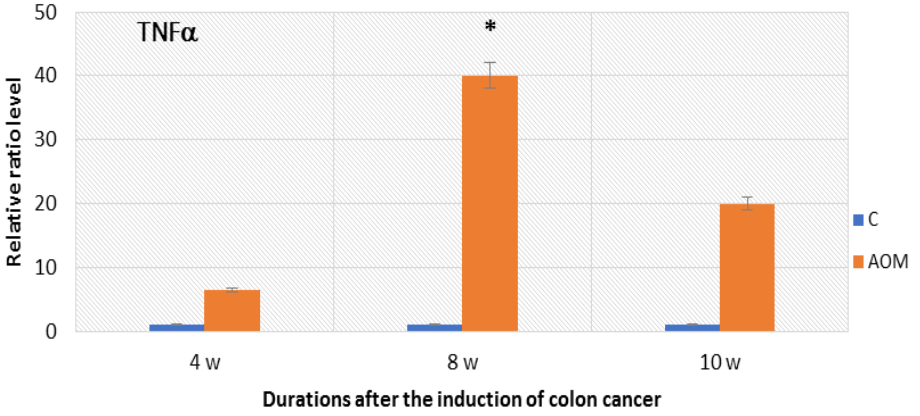
During all the current experiment durations, the transcription level of the cytokine, *IL Iβ*, downregulated extremely significantly in the AOM group when compared with its level in the untreated group (*P=0.0000* per each duration) **(Figure 3)**. By weeks 4 and 10, the transcription of *TNFα* was upregulated non-significantly in the spleen cells of the AOM group in comparison to the control group; Moreover, it was upregulated significantly after eight weeks compared to the *TNFα* level in the control group (*P=0.0237*) **(Figure 3)**. Furthermore, by weeks 4 and 8, no noticeable differences were remarked between the expression levels of *IFNγ* in the spleen cells of either AOM, or the untreated groups, whereas, at week ten, it was significantly upregulated in the spleen cells of the AOM mice group in comparison with its transcription levels in the control group (*P=0.0019*) **(Figure 3)**.



a)



b)



c)

Figure 3. The Relative Ratio of *IL1β/mRNA, TNFα/mRNA and IFNγ/mRNA* Transcriptions Levels in Different Mice Spleen

Where group C represented mice untreated negative control, and the AOM group represented mice injected with AOM for inducing colon cancer. (*) Significant at $P<0.05$ as determined by ANOVA and One-factor ANOVA test. Each point represented the mean value of 3 tests and the vertical bars denote the mean±5%.

Transcription Relative Ratio Correlation Analysis between the p53 and the Current Innate Mediators

At week 4, the correlation transcriptions analysis of *p53* in spleen cells of mice bearing colon cancer and the present immunological markers demonstrated a positive significant correlation to the *TLR2* ($P<0.01$) transcription; meanwhile, it was remarked negative significant negative correlation to *TLR1* ($P<0.01$) transcription. A nonsignificant correlation was observed between the current tumor suppressor gene transcription to both *IL1β* and *TNFα* gene expression (Positive manner), and *IFNγ* gene expression (negative manner) **(Table 2)**. By week 8, the transcription of the AOM-*p53* gene in mice

spleen cells presented an extremely significant positive correlation to *TLR2* transcription (*P>0.01*). In addition, it was still demonstrated a non-significant correlation to the *TNFα* gene (Positive manner), and to *TLR1, IFNγ,* and *IL1β* expressions (negative manner) **(Table 2)**. At week 10, *p53* transcription on the spleen cells of the AOM group verified a positive correlation to *TLR2* and TNFα transcriptions and correlated negatively with *IFNγ, IL1β* transcription; however, this correlation was in a nonsignificant manner. The current tumor suppressor gene illustrated a significant negative correlation to *TLR1* transcription (*P>0.05*) **(Table 2)**.

Table 2. Correlations Analysis of the Relative Ratio Transcription Levels of the Present *p53* Expressions and the Immunological Markers in the Spleen of Mice Bearing Colon Cancer

Durations	*TLR1*	*TLR2*	*IFNγ*	*IL1β*	*TNFα*
4 w	-0.911	0.989	-0.055	0.072	0.062
8 w	-0.655	0.899	-0.072	-0.298	0.485
10 w	-0.795	0.675	-0.102	-0.159	0.135

Number of samples =8, Red numbers represented negative correlations; Black numbers represented positive correlations; 0.707 critical value <0 .05 (two-tail); 0.834 critical value< 0 .01 (two-tail)

Transcription Relative Ratio Correlation Analysis between the K-ras and the Current Innate Mediators

At week 4, *K-ras* transcription verified a positive correlation with either *TLR1*, or *IFNγ* expressions, and a negative correlation with either *TLR2, ILIβ*, or *TNFα* transcriptions; however, these correlations were nonsignificant **(Table 3)**. These correlations were reversed by week 8; but this oncogene transcription demonstrated a nonsignificant correlation to *TLR2, IL1β,* and *TNFα* in a positive manner, and a negative correlation to *TLR1*, and *IFNγ* transcriptions **(Table 3)**. At week 10; *K-ras* gene expression was reported to have a strong significant positive relationship with the expression of the *IL1β* gene (*P<0.01*) and a non-significant positive relation with *IFNγ* expression. Meanwhile, *K-ras* has a significant negative correlation with *TNFα* and a non-significant negative correlation with *TLR1* and *TLR2* expressions (*P<0.05*) **(Table 3)**.

Table 3. Correlations Analysis of the Relative Ratio Transcription Levels of the Present K-ras Expressions and the Immunological Markers in the Spleen of Mice Bearing Colon Cancer

Durations	*TLR1*	*TLR2*	*IFNγ*	*IL1β*	*TNFα*
4 w	0.085	-0.020	0.349	-0.499	-0.443
8 w	-0.387	0.322	-0.690	0.320	0.538
10 w	-0.341	-0.683	0.420	0.857	-0.787

Number of samples =8, Red numbers represented negative correlations; Black numbers represented positive correlations; 0.707 critical value <0 .05 (two-tail); 0.834 critical value< 0 .01 (two-tail)

When the growth rate of the cancer is offensive, the host immunological response is defeated or destroyed easily due to the uncontrolled rise in the number of cancer cells [23]. The spleen is not only an immune organ that provides an antitumor effect because of its enrichment with numerous types of lymphocytes and macrophages [24] but also, it seems to be a very antagonistic area for the establishment of cancerous cells from different organs [25]. *p53* is a nuclear transcription factor that transactivates several target genes involved in the apoptosis and/or cell cycle arrest [26-28]. K-RAS is one of the RAS family isoforms - an example of an oncogene- [29], which have many cellular functions, such as proliferation, growth, migration, differentiation, apoptosis, and division of the cells. The natural, unchanged form of the gene is called wild-type K-RAS [29]. Mutations in the RAS gene were first reported in cancer over 30 years ago, while mutations in K-RAS account for about 85% of all RAS mutations in human tumors [30]. In the current study, the most significant upregulation of the *p53* transcription in the spleen cells of the AOM group was observed at week ten from the induction of colon cancer. Meanwhile, the present *K-ras* transcription upregulation fluctuated during the current experiment, whereas the most significant upregulation was observed in the AOM spleen cells group after eight weeks from inducing colon cancer compared to the normal control group. In normal circumstances, *p53* is a transcript at a remarkably low level [31] and as a functionally inert form. When DNA is damaged, p53 accumulates in the nucleus throughout post-translational modifications. These modifications activate *p53* [32]. Under specific conditions related to the induction of colon cancer using AOM, the present *p53* transcription of the AOM spleen cells group succeeded in activating the functional form of *p53*. The active form of *p53* transactivates target genes and induces cell cycle arrest and apoptosis, which is dependent on the type and extent of DNA damage [26, 28, 33]. *p53*-mediated cell cycle suppression permits cells to repair damaged DNA that has been confirmed. When DNA is repaired, the cell re-enters its normal cycle. However, when cells have severe DNA damage, *p53* exerts its pro-apoptotic function and eliminates the cell and thereby inhibits the transfer of damaged DNA to daughter cells. Therefore, *p53* can maintain genomic integrity [34]. Otherwise, little is known about the correlation between the unchanged/wiled type *K-ras* transcription activity and the early stages of colon cancer induction. We analyzed the associations between *p53, K-ras*, transcription, and the TLRs -specifically *TLR1* (CD281) and *TLR2* (CD281)-, as well as their correlations with the pro-inflammatory cytokines as an immune guardian against tumor development. Both *TLR1* and *TLR2* activation are essential for

innate immune monitoring, which activates and regulates either the macrophages or the dendritic cells to respond in the systemic areas [35]. Commonly, *TLR1* and *TLR2* play crucial roles in this immunomodulatory effect. From our results analysis, the current *K-ras* transcription showed a non-significant correlation with either *TLR1* and *TLR2* transcriptions in the spleen cells of the AOM group, whilst *p53* transcription – a tumor suppressor gene- was correlated significantly with *TLR1* (in a positive manner) and *TLR2* transcriptions (in a negative manner). Our result confirmed the suggestion of several studies about the vital role of the *p53* as a DNA damage regulator and as a TLRs gene transcription controller [36] in the spleen cells during the induction of colon cancer. However, the current *K-ras* transcription results did not announce any role in the *TLR1* or *TLR2* expression regulation under the same circumstances. The upregulation of the *pro-inflammatory* cytokine cascade is commonly correlated directly to the upregulation of TLRs expression levels. The early activation of the *pro-inflammatory* cytokines expressions is a revolution against tumorigenesis and protecting its development by increasing the apoptosis pathway of cancerous cells [37]. Although TNFα and IL1β are generated by monocytes, in particular, the macrophages (the most effective antigen-presenting cells) [38, 39]. The spleen is the main residential area of monocytes and macrophages, so it is considered an essential source of different *pro-inflammatory* cytokines that are released in response to several pathogenic diseases and tumor induction [40]. The Th1 lymphocytes are the main producers of the IFN-γ cytokine, where it is providing an immunological shield against tumor survival [41]. In this investigation, a nonsignificant relationship was reported between the *p53* transcription and the present pro-inflammatory cytokines -*IL1β*, *TNFα*, and *IFNγ*- expressions during the periods of the experiment. Besides, the further current analysis confirmed a significant correlation between *K-ras* expression and the *IL-1β* (in a positive manner) and *TNFα* (in a negative manner) expressions on the spleen cells of the AOM group. These current results supported multiple studies that discussed the effects of macro-autophagy inhibition in the spread of K-ras-driven malignancies, which may be resulted from the increase of disabled mitochondria, and raised levels of reactive oxygen species, which also restricts tumor survival and expansion [42]. Macro-autophagy or autophagy -a process of degrades the large macromolecular complexes and eliminates corrupted organelles- is a tumor-suppressive mechanism, however, it also can improve oncogenesis in established K-ras dependent cancers [43]. Autophagic proteins have significant actions in the regulation of inflammatory mediators, so it will influence cytokines produced from macrophages [44]. It has been established that autophagy inducement is affected by numerous cytokines, including TGFβ, IL-1, IL-2, IL-6, TNFα, IFNγ, and [45, 46]. Stimulation of macrophages with IFNγ may lead to the elevated pathogens intracellular killing. However, TNF blockers suppress the maturation of the IFNγ-induced phagosome, implying that maturation and autophagy of the IFNγ-induced phagosome might be TNFα dependent. Notably, TNFα is demonstrated to pretend a role in activating autophagy in several types of cells by using different actions and mechanisms [47]. Moreover, IL 1 has been demonstrated to stimulate autophagy [12]. However, insulin-like growth factor 1 [48] and fibroblast growth factor 2 both can inhibit autophagy [8].

Conclusion

In conclusion, the current study gave an insight into the immunomodulatory effect of *p53* and *K-ras* transcriptions of mice spleen - a part of the systemic immune system- pro-inflammatory cytokines and TLRs during the induction of colon cancer using Azoxymethane. The correlation results analysis verified a significant correlation between the *p53* and the *TLR1* and *TLR2*, whilst it correlated non-significantly with the *pro-inflammatory* cytokines' transcription. Moreover, *K-ras* transcription correlated significantly to the present *pro-inflammatory* -*IL1β* and *TNFα*- cytokines transcriptions whilst it correlated non-significantly *TLR1*, *TLR2*, and *IFNγ* transcriptions on the spleen cells during the induction of colon cancer.

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Conflict of interest: None

Financial support: None

Ethics statement: The experimental protocol was established, according to the ethical guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) of King Abdulaziz University and Faculty of science (IACUC NO 2019/432067/4). The animals were obtained from the King Fahad for the Medical research. The detail of mice euthanasia and scarification methods is following the IACCU guideline. This research is not an application for clinical research, an institutional review board (IRB) is not applicable.

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