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Comparison of NOD-like and Toll-like receptors gene expression levels in blood monocytes of colon cancer patients

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ABSTRACT

Colon cancer is one of the most common cancers affecting many people worldwide. This disease can be treated if diagnosed in the early stages. Therefore, with the hypothesis that the level of expression of inflammatory genes in peripheral blood monocytes of patients with colon cancer is different from that of healthy people, this research was done to find out the role of inflammation in the development of colon cancer by relying on its immunopathological profile to help diagnose it in the early stages. In this case-control study, the expression levels of TLR4, TLR2, NLRP3, and NOS2 genes in 15 patients with confirmed stage II colon cancer were determined by the TNM method. Also, 15 healthy people referred for this cancer screening were selected as the control group. First, RNA was extracted from the blood monocytes of two groups, and after making cDNA, the comparison was created using the qPCR method. In this study, the β-actin gene was used as a reference gene. The expression levels of TLR2 and TLR4 at the mRNA level were significantly lower in colon cancer patients compared to the healthy control group ($P<0.05$). The expression level of NLRP3 in the group of colon cancer patients showed a relative increase. Still, it was not significant, while the expression level of the NOS2 gene in the group of colon cancer patients increased significantly compared to the healthy control group ($P<0.05$). Considering the significant changes in TLR4, TLR2, and NOS2 gene expression in monocytes of patients with grade II colon cancer and the role of inflammatory reactions in the development of this cancer, these findings can be used to diagnose and determine the prognosis. However, this requires further studies.

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Introduction

Colon cancer is one of the most common cancers affecting many people worldwide. Although colon cancer is the most related to chronic inflammation among all types of cancer, the relationship between inflammation and the pathogenesis of this cancer has not yet been correctly determined (1). This disease is generally associated with immune dysregulation in the presence of cells and molecules that create an inflammatory environment (2). Colonoscopy is the most critical method in screening and early diagnosis of colon cancer. However, this diagnostic screening method is not very welcome due to the invasive nature of this method and causing problems such as the possibility of intestinal rupture and the resulting pain and discomfort (3). Therefore, the current research is necessary to find an easy and non-invasive method for screening this cancer in its early stages. In general, the use of different cellular and molecular components of blood as a non-invasive tool in the early screening of colon cancer and its timely diagnosis is discussed (4). Since chronic inflammation plays an essential role in cancer development, especially colon cancer. Various inflammatory markers have been reported as useful markers in patients with cancers, including colon cancer (5). Meanwhile, counting peripheral blood monocytes and examining the expression profile of erysipelas genes in them are relevant markers in the poor prognosis of colon cancer (3).

The activity of inflammation in the matrix of invasive cancers depends on the type of immune cells involved, the number of cells per square millimeter, and the location of immune cells are factors that are effective in the prognosis of this cancer (6). The gene expression profile of peripheral blood cells, predominantly neutrophils, monocytes, and macrophages, of people with colon cancer differs from that of healthy people (7). In this regard, a group of immune system cells plays an important role in the initial and rapid response of the host to infections and cancers. Monocytes are 2 to 10% of human white blood cells. Monocytes are made from myeloid precursors in the bone marrow, known as monoblasts (8). Then they enter the bloodstream, and in the presence of inflamed cytokines in the tissue space, they turn into macrophages or dendritic cells, which play an important role in the body's defense against cancer (9). Based on their phenotypic receptors, these cells include at least three subsets:

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1. High expression of CD14 surface receptor (10)
2. Low expression of CD14 with CD16 expression (11)
3. High expression of CD14 and low expression of CD16 (12, 13).

Through phagocytosis, secretion of inflamed cytokines, production of oxygen free radicals, and NO (nitric oxide), monocytes initiate inflammatory responses in the form of the innate immune system (14). Controlling the homeostasis of inflammatory responses, such as decreasing or increasing the expression of specific genes in cases of inflammatory responses by monocytes, is particularly important in regulating immune responses. In addition to their role in the homeostasis of immune responses, these cells are also important in tissue repair and fighting cancers such as colon cancer (15). On the surface of monocytes, there are various pattern recognition receptors, especially TLRs, which, by recognizing the presence of microbes and their products in their signaling pathway, initiate primary immune responses against infection and tissue damage (16). NLRs, as another group of pattern recognition receptors in monocytes, by starting several inflammatory messenger pathways, lead to the initiation of the inflammasome complex and the secretion of mature forms of IL-18 and IL-1β, which create chronic inflammatory conditions in the microenvironment of cancer cells (17).

On the other hand, pro-inflammatory factors such as NO play an essential role in regulating inflammatory responses. The production and secretion of large amounts of NO by activated macrophages infiltrating and residing around cancer cells may cause survival and prolong the time of tissue damage (18). Following the stimulation of monocytes and macrophages by lipopolysaccharide or inflammatory cytokines such as TNF-a, the inducible nitric oxide synthase enzyme is activated, which causes the release of nitric oxide. Activating this enzyme leads to the production of increased and potentially cytotoxic amounts of NO in the long term (19). Also, it leads to increased angiogenesis around cancer cells and facilitating their metastasis. Therefore, it is necessary to investigate the expression profile of inflammatory genes expressed by monocytes of people with colon cancer to examine the role of chronic inflammation in developing this cancer and to contribute to the standard screening methods based on the existing immune-pathological profile (15). Based on this, the present study evaluated the expression levels of TLR4, TLR2, NLRP3, and NOS2 genes in two groups of colon cancer patients and controls at the mRNA level by qPCR method.

Materials and Methods

The type of this study was a case-control study that was done in a descriptive-analytical manner according to the purpose of this study.

Selection of study groups and entry and exit criteria

In this study, 15 patients were selected and included from patients with confirmed stage II colon cancer according to TNM 1 classification, who were diagnosed by colonoscopy, CT scan, and histology methods before chemotherapy and radiotherapy. Also, 15 of the clients who were male and aged between 60 and 70 years old were considered the control group.

The criteria for entering this research were the same gender of cancer patients and healthy people, the same stage of cancer patients was confirmed by TNM method, and no chemotherapy and radiotherapy were performed on the patients. Those cancer patients who had received chemotherapy or radiotherapy, whose gender was different from the control group, or who had died during the study were excluded.

Taking peripheral blood samples from cancer patients and healthy people

After filling out the relevant forms and obtaining the consent of the people of both groups following medical ethics, venous blood samples were taken in 10 ml each and sent to the laboratory as soon as possible to isolate monocytes and RNA extraction.

Isolation of peripheral blood monocytes

This work used the Faicol gradient (p=1.077) and concentration gradient centrifugation (20). Then isolation of monocytes was done by sticking to plastic. Briefly, peripheral blood mononuclear cells (PBMC) were washed twice with cold RPMI-1640 medium after harvesting. Their number and viability were determined by the trypan blue method. Then, the number of 5x106 cells per ml was poured into 24-well plates, and after 2 hours of incubation at 37°C, 5% CO2, and 95% humidity, the non-adherent cells were separated, and the monocytes attached to the bottom of the plate were separated in RPMI-medium. 1640 enriched with 10% FBS 100 unit/ml penicillin, 100 g/ml gentamicin, and 0.30 mg/ml L-glutamine were cultured and isolated.

RNA extraction and cDNA preparation

To extract RNA and make DNA of desired genes from peripheral blood monocyte samples, RNA was extracted using the RNX kit of Synaclon company using Trizol reagent, and using the Revert Aid FIRST Strand DNA synthesis kit using Oligo-dT primers, and the random hexamer was converted into cDNA according to the relevant instructions.

The quantitative polymerase chain (qPCR) reaction

In the PCR technique, first, to find the optimal binding temperature of primers, two cDNA samples prepared from monocytes of cancer patients were tested using a master mix kit of Qiagen Company (Germany), two binding temperatures of 58°C and 61°C. According to the melting curves, the binding temperature of 61°C was optimal for the above genes. Also, to ensure the designed primers' optimal performance and the qPCR performance accuracy, the PCR product's amplified genes were electrophoresed on a 1.5% agarose gel. PCR test with a protocol of 10 minutes at 95°C, cycles of 10 seconds at 95°C, 20 seconds at 61°C and 20 seconds at 72°C, 40 cycles with the specified program was done for TLR2, TLR4, NLRP3, and NOS2 using Rotor-Gene Q series software. The reference gene was β-actin and $2^{-\Delta\Delta ct}$ of genes was evaluated by Mann-Whitney statistical method. Table 1 shows Primer sequences and product length for studied genes.

Results

In this study, after taking blood from the group of colon

Table1. Primer sequences and product length for TLR2, TLR4, NLRP3, NOS2 and β-ACTIN.

Gene	Primer Sequence (5'-3')	Product length
TLR2	Forward: ATCCGATGATTCTTACCAGCG	103 bp
	Reverse: GCTGACTGTCTCTTGAGGAT	
TLR4	Forward: CGGATGATTCAGTGCGATCGA	211 bp
	Reverse: CGATGTCGGTGTTATCATGGCGA	
NLRP3	Forward: AGGCTCAGTCATGGATC	88 bp
	Reverse: GCGCTACATGACTGCGTATA	
NOS2	Forward: TCGTCAGTGTCAGGTACTGCATAT	156 bp
	Reverse: AGCAGTGTACACGGCTAA	
β-actin	Forward: TGCACGTCAGTCATACGTA	134 bp
	Reverse: GGATGCACGTTAGCAGCTAC	

cancer patients and healthy control subjects, the peripheral blood monocytes of the two groups were separated using the plastic adhesion method. After dissolving and confirming the survival rate of 95% of them, smears were prepared and stained with Giemsa dye. The other molecular work was done. To ensure the performance of the primers used in this study, after performing PCR on the relevant genes, electrophoresis was also performed on 1.5% agarose gel. The gel electrophoresis results confirmed the melting curves. Each single band represented the specific function of the corresponding primers, and a single band was obtained for all genes. After extracting RNA and making DNA from monocytes of both groups of cancer patients and healthy control and performing PCR tests, the test results were analyzed by SPSS statistical software system and the Mann-Whitney method. These results can be seen in the graphs of Figure 1A and 1B.

Based on graphs A and B, the expression level of TLR2, TLR4, and NOS2 genes in monocytes of the cancer patients group significantly decreased, decreased, and increased, respectively (P<0.05). The NLRP3 gene showed a relative increase in cancer patients, which was insignificant compared to the healthy control group. In cancer patients, TLR2 had the most significant decrease in expression compared to the healthy control group. In other words, in this series, NOS2 gene expression changes in colon cancer patients were associated with up-regulation. However, the expression of TLR2 and TLR4 genes in cancer patients was down-regulated compared to controls. NLRP3 gene expression was up-regulated in cancer patients but was insignificant compared to the control group.

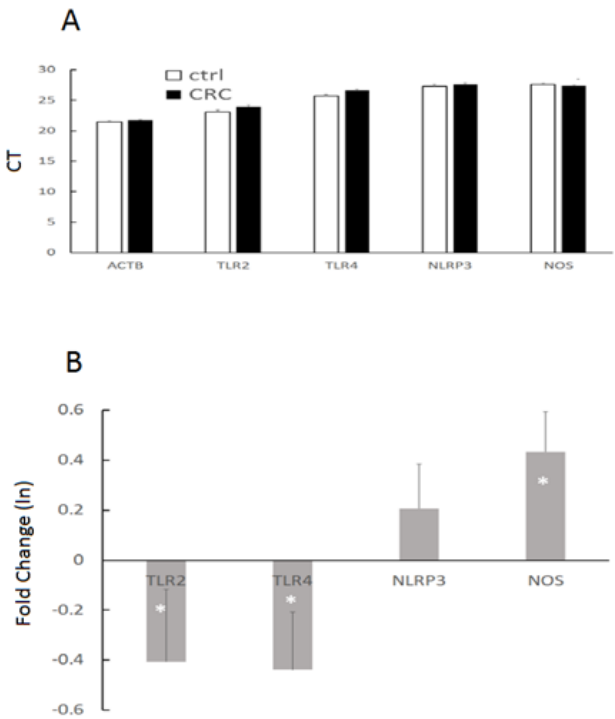

Figure 1. A) The graph of the threshold cycle (CT) of the studied genes in the groups of colon cancer patients and healthy controls in the qPCR test, the white rectangles are the mean threshold cycle of the control group, and the black rectangles are the mean threshold cycle of the cancer patients group. B) Fold change diagram (ΔΔct) expression level of NLRP3, TLR4, TLR2, and NOS2 genes resulting from PCR test on different cDNAs obtained from peripheral blood monocytes of cancer patient groups (n=12) compared to the healthy control group (n=12). The fold change data compared to the control group is displayed based on the base two logarithms (ln), and the sign * indicates the significance (p<0.05) of the gene expression level of the cancer patients group compared to the healthy control group.

Discussion

This research aimed at the timely diagnosis and treatment of colon cancer based on the immunopathological profile of this cancer in its early stages. The results of this research described the relative immunopathological status of 15 monocyte samples obtained from stage II colon cancer patients. The evaluation of the expression of essential inflammatory genes in these cells showed significant expression changes in TLR2, NOS2, and TLR4 genes compared to the monocytes of healthy controls.

The genes studied in this study were each somehow involved in immune responses. Of course, the results of this research were partially consistent with the results of Chang *et al*. (21). The most precise result of this research was the increase in the expression of the NOS2 gene and the decrease in the expression of the TLR2 and TLR4 genes in the monocytes of patients with colon cancer. The amount of fold change of the mentioned three genes in the monocytes of the cancer group had a significant difference (p<0.05) compared to the control, and this may be indicative of the production of variable or unstable mRNA levels of the mentioned genes in the monocytes of the patient group.

According to the study of Honda *et al*. (22), the gene expression profile of monocytes of patients with colon cancer is different from that of ordinary people. Based

on the findings of this research, the expression of three genes, TLR4, TLR2, and NOS2, in the monocytes of cancer patients was significantly decreased, decreased, and increased, respectively, compared to regular patients. However, the level of NLRP3 gene expression in monocytes of cancer patients did not increase significantly compared to ordinary people. This issue may be due to intermittent splicing in the mRNA of this gene and as a result of ineffective mRNA production in the monocytes of cancer patients or affected by the increased expression of the NOS2 gene, which of course, needs further investigation. According to the proposal of Fridman *et al*. (23), the change in the amount of gene expression indicates the immune system's reaction in fighting cancer. On the other hand, the type of tumor cells infiltrated into the cancer microenvironment depends on the type of cancer and its stage, and this information can be used in the prognosis of timely diagnosis and even immunotherapy of the patient. On the other hand, since the count of peripheral blood monocytes reflects the number of macrophages associated with cancer per square millimeter of the cancer microenvironment, it is an important prognostic factor in the search for the initial stages of the development of this cancer (24, 25).

According to the increase in NOS2 gene expression in monocytes of cancer patients, it seems that after monocytes are attracted to the colon cancer microenvironment due to the loss of the epithelium barrier and, of course, the penetration of microbes located in the intestinal lumen into the lamina propria area at first The purpose of identifying these microbial agents may be to increase the expression of TLR2 mRNA and TLR4 as well as NLRP3 to initiate the immune response. Then, by signaling downstream of these receptors, the production of inflammatory cytokines and NO, H2O2, superoxide radicals, etc., occurs in monocytes. Of course, the cooperation of these factors with TNF-a in increasing angiogenesis in the cancer microenvironment can be justified. On the other hand, the high expression of NO has an inhibitory effect on cancer. But due to the high level of immunosuppressive cytokines such as 1-TGF-B, IL, and PGE2 in the cancer microenvironment, which are mainly secreted by M2 macrophages and also by the calling of MDSC 1 cells from the bone marrow in the cancer microenvironment and enter the blood of lymphatic tissues and become cancerous and the inflammatory inhibition applied by TGF-L10 NO and PGE decreased the expression of TLR2 and TLR4 genes on the level of monocytes as the main executive cells of innate immunity. According to Green *et al.* (26), in some cancers such as colon cancer and breast cancer, circulating cancer cells (CTCS) can have a reducing effect on the TLR2 and TLR4 gene expression of blood cells, including monocytes, by secreting inhibitory cytokines.

According to Kesselring *et al*.'s study (27), it was found that the IRAK-M molecule, which is expressed by cancer cells, is one of the negative regulators of TLRs expression, reducing its downstream signaling and reducing NF-KB levels. This, in turn, may be another justification for reducing the expression of TLR2 and TLR4 genes in the monocytes of cancer patients. Another finding of this research was the insignificant increase in NLRP3 gene expression in the monocytes of cancer patients compared to the control group. It seems to be due to the activation of the inflammasome complex and the production of two cytokines, IL-1 and IL-18, to create inflammation in dealing with the ligands resulting from the destruction of unstable cancer cells such as migratory cancer cells in the blood circulation and creating anti-cancer immunity. But apparently, the expression of this molecule was decreasing, which is probably one of the reasons for the release of inhibitory factors from these cancer cells, as Green *et al.* (26), have expressed in their study. On the other hand, according to Qin He *et al*.'s study (28), the exact role of inflammasomes in cancers is strongly dependent on the heterogeneity of cancer cells and their accompanying cells, such as fibroblasts and endothelial cells, as well as the lack of oxygen in the cancer microenvironment, which affects the function of inflammasomes. This may be another reason why the increase in NLRP3 expression is insignificant in cancer patients. From the point of view of the instability of the mRNA of this gene, it is worth pondering. Of course, proving this issue requires more studies.

In this research, according to the increase in NOS2 gene expression in monocytes of stage II cancer patients, it seems that the increase in the expression of genes related to signaling downstream of TLRs in peripheral blood monocytes is probably associated with the infiltration of monocytes from the bloodstream to the cancer tissue along with the transformation of these cells into macrophages or the initial upregulation of TLRs expression on the surface of these cells is due to their stimulation towards cancer cell growth. This causes the stimulation of more NO secretion. As mentioned in the study of Ye Xu *et al*. (29). In addition, according to Sara Benkhelifa *et al*.'s study (30), NOS2 mRNA expression has a significant relationship with the colon cancer stage. In the present study, the exact relationship is significant in the case of stage II colon cancer. Of course, more research is still needed in this case. In all the sources that have been used in the implementation of this research, only some limited criteria have been emphasized in colon cancer immunopathology. In the present study, the expression of four critical genes of inflammation in patients with stage II colon cancer has been evaluated. Considering the relationship between the development of colon cancer and cancer, studying the gene expression changes of immune cells, mainly monocytes, as the leading players of immunity, is an intrinsic diagram of the immunopathological status of colon cancer because the expression of genes is different according to the epigenetic conditions that govern them. In one way, these conditions have an inhibitory effect on the expression of genes in the microenvironment of cancer cells by releasing cytokines that inhibit the immune system. In such a way, these inhibitory cytokines, by secretion in blood and lymph, also have an inhibitory effect on the expression of peripheral monocyte edema genes. Therefore, these changes can be used according to the pathological grade of colon cancer as a prognosis of the disease and a diagnostic field in the future, along with other diagnostic methods for targeted treatment of this cancer in the early stages.

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