

LPS Induces microRNAs -9, -192, and -205 Overexpression in Colorectal Cell Lines SW480, HCT116

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Abstract

Background: Today, cancer is one of the serious health issues worldwide. In USA, colorectal cancer is the third cause of death. Reportedly, some risk factors, including family history of colorectal cancer, age, and infectious diseases can deteriorate the progression of colorectal cancer. One of the most common specifications in all gram-negative bacteria is bacterial cell wall, known lipopolysaccharide. Moreover, LPS probably can affect some microRNAs and cancer progression. We studied the effects of LPS on microRNA-9, -192, and -205 expressions in colorectal cell lines [SW480, HCT116], which are considered oncogene.

Methods: SW480 and HCT116 cell lines were treated with LPS to analyze microRNA-9, -192, and -205 expressions by quantitative real-time PCR in 48 hours at 10 ug/L of LPS.

Results: Quantitative real-time PCR illustrated that microRNA-9, -192, and -205 were upregulated after treating LPS. There was an increase in microRNA-9 level, six and eight times in SW480 and HCT116 cell lines, respectively. Furthermore, upregulation in the expression of microRNA-192, six times in HCT116 and four times in SW480 was observed. Moreover, there was an upregulation expression [almost four times in both cell lines] in microRNA-205. Our results show that treating LPS increases microRNA-9, -192 and -205, which may be related to cancer in colorectal cell lines [SW480 and HCT116].

Conclusion: Therefore, disrupting the balance of bacterial flora can be influential in colorectal cancer progression and increase the chances of getting colorectal cancer that further investigation is required.

Keywords: MicroRNA-9, MicroRNA -192, MicroRNA -205, LPS, Colon cancer

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Introduction

Cancer is one of the most terminal health problems considered as the first cause of death in the developed countries and the second one in the

developing world.¹ About 12.7 million people in 2008 and 7.98 million people in 2010 have died due to cancer.² Lung, prostate, colorectal, breast, and stomach cancer are of the

most common types of cancer.^{3,4}

Colorectal cancer is one of the most common malignant diseases, which is statistically the third type of cancer and the fourth cause of malignant death in both men and women.⁵ The progression of this disease may depend on the nutrition conditions, particularly the consumption of red meat, alcohol, and aspirin.⁶⁻⁸ Other risk factors including age and family history of colorectal cancer can deteriorate the progression of this disease.⁹

There are a lot of bacterial floras in human gastrointestinal (GI) tract which if disturbed, may cause colorectal cancer to progress.¹⁰ Estimations show that 10 bacteria over 500 microbial species can colonize in colorectal track, most of which are gram-negative.¹¹⁻¹³

The most common specification in all gram-negative bacteria is their cell wall called lipopolysaccharide LPS. This molecule comprises three hydrophilic parts - outer various O antigen, core, and inner lipid site, which are responsible for fever, diarrhea, and probably the fatal endotoxin shock (also called septic shock).¹⁴ TLR-4 is one of the well-known ligand of this molecule which if stimulated by LPS, innate immune signaling pathways (TRIF-dependent and MyD88-dependent) will be initiated; therefore, TNF- α , IFN- β , and IL-1 β will be secreted and inflammation will occur.^{15,16}

Moreover, most of microRNAs are themselves oncogenes for unknown reasons.¹⁷ MicroRNA is a non-coding RNA of viruses, animals, and plants regulating protein and gene expression after transcription.¹⁸ This type of RNA contains almost 22 nucleotides transcribed by RNA polymerase II (Pol II).¹⁹ 40% of microRNAs are located in intron or exon of genes.²⁰ It has recently been estimated that human genes encode almost 600 microRNAs.²¹

Some microRNAs are also known as tumor suppressor including miR-15a and miR-16-1 in different types of cancers and colorectal cancer is not an exception.²²⁻²⁴ However, some studies claimed microRNA-9, -192, and -205 are upregulated in colon cancer tissues; therefore, they can affect the colon cancer progression and

these microRNAs may be used as biomarkers.^{25,26} Based on the background, we studied the expression of microRNA-9, -192, and -205 in colorectal cell lines (SW480, HCT116) when LPS induces inflammatory signaling pathways.

Methods

Ethical statement

We conducted the study with the approval of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1396.879).

Cell culture

In this experimental study, we obtained colorectal cancer cell lines (SW480 and HCT116) from National Cell Bank of Iran (Pasteur institute of Iran) and suspended them in culture medium RPMI-1640 (Gibco) with 2 mM/L-glutamine (Sigma-Aldrich, MO, USA) supplemented with penicillin/streptomycin (Invitrogen, Life Technologies, Camarillo, CA, USA) and 10% fetal bovine serum (Life Technologies, Camarillo, CA, USA). All cells were incubated in a humidified atmosphere, containing 5% CO₂ at 37°C on plastic petri dishes.

Lipopolysaccharides preparation

Escherichia coli O111:B4 LPS has been prepared from (Sigma-Aldrich, MO, USA). To make three different concentrations [1, 2, and 5 ug/L], we used phosphate buffered saline (PBS) to dissolve LPS.

Proliferation assay

Escherichia coli O111:B4 LPS concentration was evaluated by 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT assay). SW480 and HCT116 cell lines were incubated in 96 well-plates overnight with RPMI, containing 10% serum. After 24 hours, cells were treated with LPS at concentration of 1, 5, and 10 ug/L and incubated with previous condition. Then, MTT was added at 24, 48, and 72 hours and after adding DMSO, the absorbance was determined at 570 nm and 625 nm. HCT116 and SW480 cell lines without treatment were used as control cell lines.

RNA extraction

SW480 and HCT116 cell lines were seeded in 12 well-plates. After reaching the 80 percent of confluence, cells were treated with LPS in RPMI containing 10 ug/L LPS for 48 hours. After 48 hours, RNA has been extracted. Briefly, to discharge protein, RNX-plus solution [Cinnagen, Tehran, Iran] and chloroform were used. The propanol precipitated RNA, and RNA was washed by 70% alcohol. Nanodrop spectrophotometry [Eppendorf, Humburg, Germany] examined the quality and quantity of RNA at the wavelength of 260 nm. 1% agarose gel electrophoresis has been used to evaluate the purity of RNA.

CDNA synthesis

In this study, we designed primers, and synthesized microRNA cDNA by combining 10 µl of RNA, 9 µl of reverse transcriptase BioFACT [Daejeon, South Korea], and 0.5 µl primer 10pmol of RTmicroRNA-9[5'GTCGTATCCAGT-GCAGGGTCCGAGGTATTCGCACTGGATAC GACTCATAAC-3'], 0.5 µl primer 10pmol of RTmicroRNA-192[5' GTCGTATCCAGT-

GCAGGGTCCGAGGTATTCGCACTGGATAC-GACGGCTGT-3'], and 0.5 ul primer 10pmol of RTmicroRNA-205[5'-GTCGTATCCAGT-GCAGGGTCCGAGGTATTCGCACTGGATAC GACCAGACT-3'] to make microRNA -9,192, 205 cDNA, respectively. All microRNAs have the same common reverse primer to synthesize cDNA: u6[5'-ATATGGAACGCTTCACGAAT TTGC- 3'].

According to BioFACT kit, total cDNAs solutions were incubated in 95°C for 5 minutes; then, cDNAs were prepared in 50°C for 40 minutes in Bio Intellectica PCR. All cDNAs were diluted (twice in sterile water) and employed as a template for quantitative real-time PCR analysis.

Quantitative real-time PCR

Quantitative real-time PCR has been done by Rotor-gene 6000 (Corbett life sciences, Sydney, Australia) in 36-well Gene Discs. MicroRNAs expression (micro RNA -9,-192, and -205) were evaluated by using the BIOFACT™ 2X real-time PCR master mix [SYBR Green I; BIOFACT, South Korea].

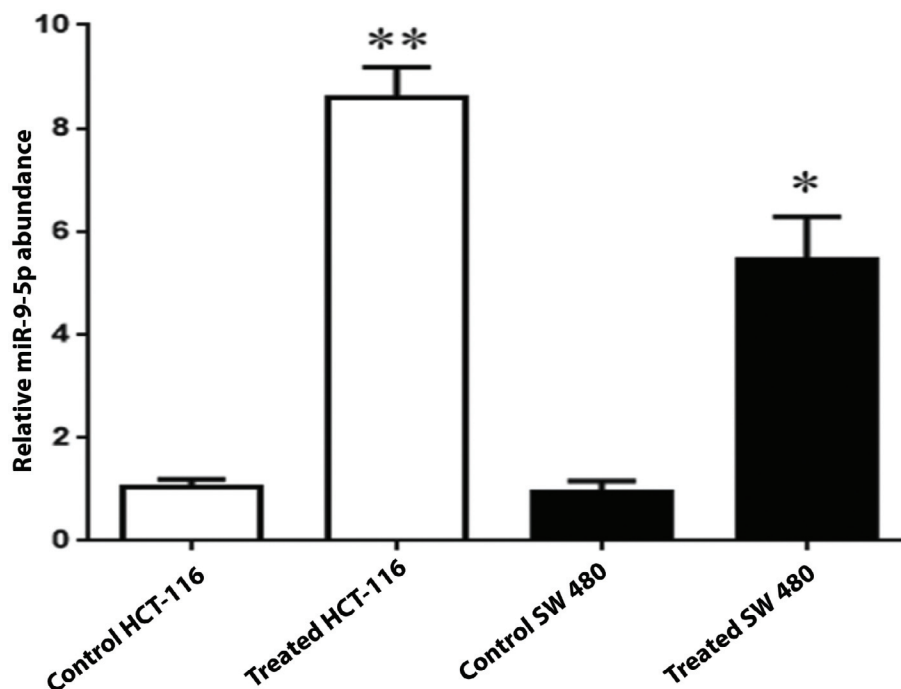


Figure 1. MicroRNA-9 has been up-regulated in both SW480 and HCT116 cell lines almost six and eight times, respectively. (* indicate P -value<0.05 and 0.01).

Table 1. The real-time PCR forward and reverse primers of miRNAs -9, -192, and -205

Forward miRNA -9-5P	5'-CTTTGGTTATCTAGCTGTATGAGTCGT-3'
Forward miRNA -192-5P	5'-CTGACCTATGAATTGACAGCCGT-3'
Forward miRNA -205-5P	5'-TCCTTCATTCCACCGGAGTC-3'
Universal Reverse	5'-ATCCAGTGCAGGGTCCGA-3'

In this study, 10 μ l of SYBR master mix, 1 μ l of forward primer, 1 μ l of reverse primer, 2 μ l of 1/2 diluted cDNA, and 6 μ l of sterile water were combined. All experiments were performed triplicate to verify our results. The designed primers were shown in table 1.

The real-time PCR temperature program was 95°C for 10 min; 40 cycles of 95°C for 20 sec, 55°C for 20 sec, and 72°C for 20 sec. Melting curve program was between 60 and 95°C.

The threshold cycle (ct) of three microRNAs in treated cell lines were compared by the ct of control cell lines. Then all microRNAs expression were normalized with housekeeping gene u6 by the $2^{-\Delta\Delta ct}$ method, respectively.

Statistical analysis

The differences between microRNAs were evaluated via Graph-Pad Prism software and ANOVA test. P -value<0.05 was considered as statistically significant for the differences.

Results

Optimization of the LPS concentration

MTT assay showing the concentration at 10 μ g/L of LPS in 48 hours had a high cell viability (more than 50%) and was the best to evaluate the expression of microRNAs.

LPS up-regulates the expression of microRNA-9

There was an increase in microRNA-9 in both SW480 and HCT116 cell lines, showing that this microRNA has a remarkable increase, when LPS

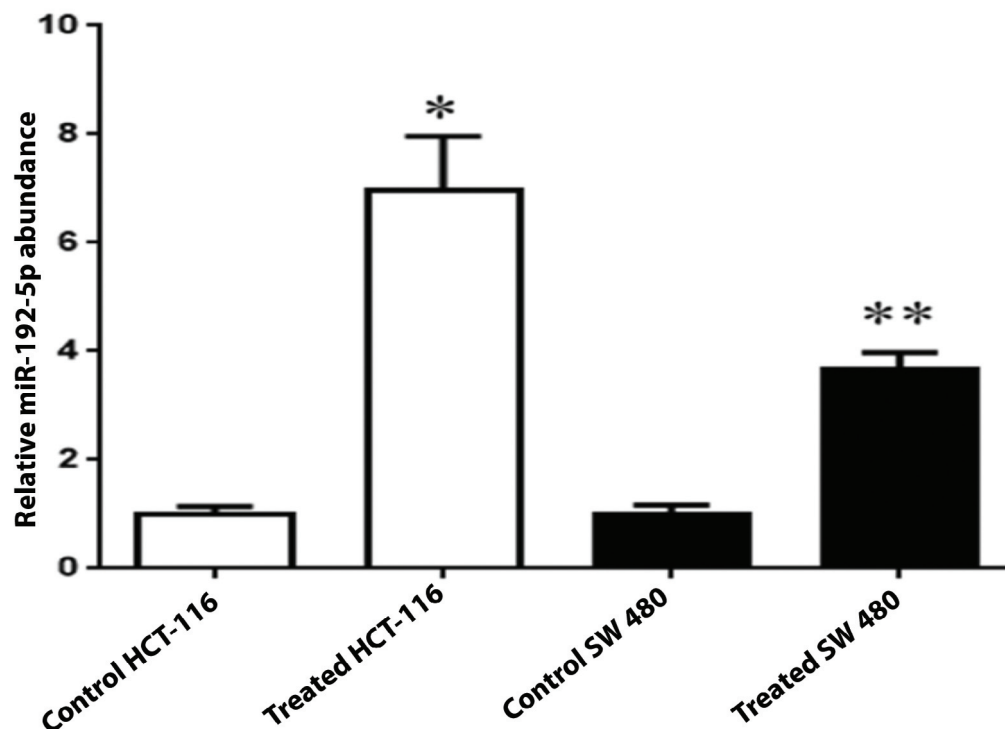


Figure 2. microRNA-192 has been raised six times in HCT116 and four times in SW480, when LPS induces inflammation signaling pathways. (*indicate P -value <0.05 and **indicate P -value<0.01)

signaling pathways initiate in characterize colorectal cell lines (SW480 and HCT116). This increase was six and eight times in SW480 and HCT116, respectively. (P -value <0.05 and 0.01). The diagram is showed in figure 1.

MicroRNA-192 was augmented after treating LPS

When LPS was treated in 10% serum RPMI medium at 10 ug/L of LPS in 48 hours, there was a noticeable increase in microRNA-192 expression in HCT116 six times and SW480 four times ($P < 0.05$ and 0.01) (Figure 2).

LPS induces expression of microRNA-205

MicroRNA-205 expression has a same increasing level after treating LPS in SW480 and HCT116. LPS leads about four times increase in microRNA-205 expression in both two cell lines ($P < 0.05$) (Figure 3).

Discussion

Most of microRNAs progress cancer signaling pathways and accelerate cell division.²⁶⁻²⁸ The results of some studies confirm that microRNAs

in different cancers are up-or down-regulated.²⁹ One of the most serious types of diseases is colorectal cancer and microRNAs have remarkable roles in its progression. However, experiments show that the expression of microRNAs in normal and colorectal cancer tissues is different.³⁰⁻³²

MicroRNAs are strongly affected by LPS which is a major component of bacteria and a key element in the survival of microRNAs.³³ LPS contains a number of receptors, especially TLR-4 that initiate some inflammatory signaling pathways; thereby, inducing side effects.^{34,35} Nowadays, there is a strong relationship between auto-immune disease,³⁶ obesity,³⁷ cancer, and LPS.

In this study, we evaluated the effects of LPS in colorectal cell lines (SW480, HCT116) on microRN-9, -192, and -205. These microRNAs have a lot of functions in different metastases; for example, colorectal lymph node,³⁸ breast,³⁹ and stem cell cancer.⁴⁰

Some microRNAs act similar to oncogenes. We evaluated the expression of microRNA-9 after treating LPS. Our observations have a remarkable

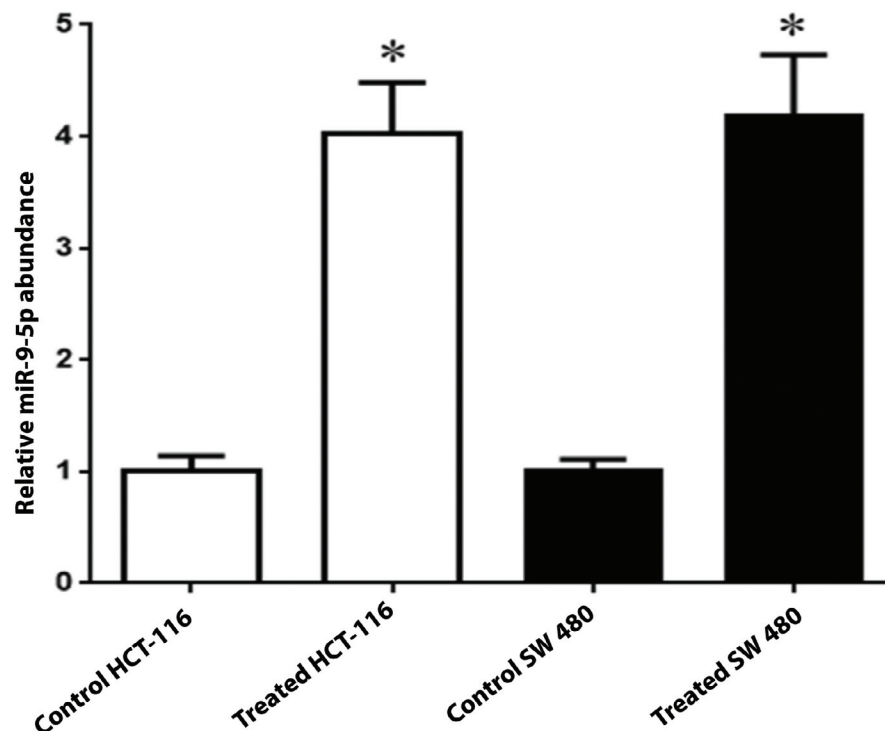


Figure 3. microRNA-205 has been raised almost four times in both cell lines, after inducing LPS. (* indicate P -value < 0.05)

increase in SW480 and HCT116 cell lines in response to LPS. Our result is similar to others; for example, with two mechanisms there is an increase in the level of microRNA-9 after treating LPS. On one hand, activation of TLR-4 by LPS, regarding MyD88/NF- κ B-dependent signaling pathways, may increase in microRNA-9 expression. On the other hand, TLR7/8 and TLR-2 induce microRNA-9 followed by inflammatory cytokines TNF- α and IL-1 β secretion.⁴¹ Some mechanisms can relate cancers pathways with upregulation of microRNA-9. Gang Li has found microRNA-9 acts as an oncogene and inhibits TGFBR2 overexpression; therefore, down-regulation of TGFBR2 prevents control cell division and induces metastasis and cell proliferation.⁴² Reportedly, microRNA-9 is able to target CDH1, encoding E-cadherin. Metastasis can occur with the downregulating E-cadherin and downexpression of intercellular protein.^{43,44} Hence, we supposed that LPS with different signaling pathways may raise microRNA-9, leading to colorectal cancer.

In this study, LPS upregulates microRNA-192, six and four times in HCT116 and SW480 cell lines. LPS in macrophages with PIK3-Akt, MAP kinase, ErbB pathways are able to induce the expression of microRNAs.⁴⁵ LPS also increases microRNAs by initiating NF-kappaB p65 caused inflammatory signaling pathways in human biliary epithelial cells.⁴⁶ It seems that microRNA-192 acts as an oncogene in colorectal cell lines and there are a lot of mechanisms causing cancer progression. For instance, MicroRNA-192 may prevent the activation of leukocyte cell adhesion molecule expression; thereby, separating the cancer cells can separate from other cells and spreading all over the body.⁴⁷ This microRNA downregulate one of the apoptosis protein called Bim by directly blocking 3'-UTR of Bim gene.⁴⁸ Therefore, our results showed that LPS is likely to increase the progress of cancer by increasing microRNA-192 level.

We also investigated microRNA-205 expression in colorectal cell lines. The results of our experiment showed that this microRNA has been upregulated four times in both cell lines.

MicroRNA-205 is supposed to be able to suppress the 3'-UTR region of SMAD4. Thus, SMAD4 is repressed and cancer happens.⁴⁹ In addition, this microRNA can interact with the lncRNA small nucleolar RNA host gene 16 and zeb-1; thereby, proliferating cell division and accelerating the progression of cancer.⁵⁰ We supposed that the effect of LPS may promote the cancer by increasing microRNA-205.

Conclusion

Since we supposed that LPS is able to change the expression of microRNAs regarding the colorectal cancer; hence, gram-negative bacterial infectious diseases in colorectal may have the same mechanisms and they raise the chance of getting colorectal cancer. Therefore, appropriate antibiotics to inhibit colorectal cancer progression are recommended.

Acknowledgement

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Conflict of Interest

None declared.

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