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Evaluation of changes in expression of muc19 and muc21 genes in colorectal cancer tissues

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Abstract

Background: Colorectal cancer is the third most common cause of cancer deaths in the world. Mucins are large glycoproteins that are expressed by epithelial cells in tubular organs. They recreate important functions in the regeneration and differentiation of epithelium, cell adhesion, immune response, and cellular signaling. In this study, mucin-19 and 21 gene expression were investigated in cancerous tumor samples, and tumor margins in patients with colorectal cancer. The purpose of working on these genes is their potential biomarker for the diagnosis of colorectal cancer. After extraction of RNA from 30 pairs of tumor and peripheral tumors of the colorectal tumor, cDNA was synthesized using a thermosetting kit, and RT-PCR was used to find the optimum temperature and specificity of primers. Then, real-time qPCR was performed to examine the difference between the gene expression of mucin on the tumor and tumor margins. This study showed an increase in the expression of mucin 19 in colorectal cancer tissue samples with tumor margins.
Conclusion: The upregulation levels of mucin 19 gene can correlate with colorectal cancer progression. Therefore, Mucin 19 may be used as a biomarker for detecting colorectal cancer development.

Keywords: Colorectal cancer, Biomarker, Mucins, Muc19 gene

1- Introduction

Colorectal cancer is the third most common type of cancer, and accounts for 10% of all cancer cases. Abnormal cell growth in the colon wall generates polyps, which can turn into cancer. Approximately 75% of colorectal cancer cases are sporadic, and less than 25% are genetic [1, 2]. Accordingly, extensive studies have been conducted to identify biomarkers for diagnosis or treatment of the disease, including mucins. Increased mucins expression has been reported in some types of cancers. Increased mucins expression can be in favor of cancer cells due to several reasons. Due to a particular glycosylation pattern, mucins act as a platform for different types of growth factors and cytokines. Therefore, they enhance cellular proliferation and metastasis of cancer cells by cellular signal cascades [3]. Mucins are large glycoproteins involved in mucous adhesion, hydrophobicity, and mucus viscosity. Thus, they protect the epithelium from chemical, enzymatic, and mechanical damage [4, 5]. Extensive clinical studies have introduced mucins as prognostic or therapeutic indices, and diagnostic markers for cancers. Mucins are large glycoproteins containing 75% carbohydrates and 25% amino acids. More than 22 genes of this family have been identified so far. Based on their physiological nature, mucins are fall into two groups, membrane and secretion. Different studies have introduced mucins as valuable biomarkers to distinguish normal and illness conditions [6]. Human mucins 19 and 21 are protein-encoding genes, which are located in chromosomes 12 and 6, respectively [7]. Mucin 19 is considered among the gel-forming secretion mucins secreted at the intestine, stomach, salivary glands, gastric tubes. In addition, to the extensive glycosylation of the second PTS, it is also found in the N-terminal and C-terminals, which are rich in cysteine amino acids and vital for its function [8, 9].

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Mucin 21 is also among the membrane mucins. It is part of type 1 proteins anchored to the membrane. With one domain passed through the plasma membrane once, the N-terminal part of the protein is located outside the cell and is rich in carbohydrates, and the C- terminal domain is inside the cytosol. The extracellular domain of mucin contains a tandem-repeat (TR) domain, Sea urchin sperm protein, enterokinase, agrin (SEA) domain, and epidermal growth factor (EGF)-like domain [10]. Several studies have reported changes in the expression of genes and the structure of mucin proteins in colorectal cancer [11]. Among adenocarcinoma, there is a different pattern of sterilization and sulfidation in mucins. In adenocarcinoma, mucins show reduced acetylation and sulfidation and a significant increase in sialic acid in their structure. Compared to adenocarcinoma, mucin phenotypes are different in carcinoma. In colorectal carcinoma cancer, increased mucus secretion is associated with changes in the content of acetylation and sialic acid [12]. Mucin 19 and 21 play an important role in tissue hemostasis, cellular joints, and many other functions. Owing to different roles in physiological and pathological conditions, change in their expression is also likely in colorectal cancer. The objective of this study was to evaluate MUC19 and MUC21 expressions in patients with colorectal cancer. It is hoped that, these genes to be introduced as suitable diagnostic biomarkers for patients with colorectal cancer.

2- Methodology

2.1- Sampling

In this investigation, all patients collected from the hospital. All paitients were examined by a physician. After following the clinical symptoms and tests, they were selected as cases or control. Samples were purchased from the tumor bank of Imam Khomeini Hospital. In this study, 30 colorectal cancer patients and 30 healthy control subjects (tumor margin) were participated. Tumor margin samples were prepared from a site far from the tumor, without tumor involvement.

2.2- RNA extraction

The Qiagen extraction kit was used to extract RNA according to the kit manufacturer's protocol. The Nanodrop was used at 260 nm to examine the quantity of extracted RNA. In addition, the quality of RNA was also studied by examining the absorption ratio of 260:280. If the absorption ratio of 260:280 is equal to 2, the sample quality is correct, and the RNA is free of contamination with the protein and DNA. In addition, for a closer examination of RNA quality, about 2 µL of RNA was fused to the Agarose gel, and the presence of 3 bands 5S, 18S, and 28S indicated the correct separation of RNA.

2.3- cDNA synthesis

After extraction of RNA, and as RNA disappears very quickly, cDNA synthesis was performed immediately. According to the manufacturer company protocol. The oligo dT primer, RNAs were converted to cDNA using the reverse transcriptase enzyme in the thermocycler device during the reverse transcription reaction. The used time program was 15 minutes at 45 ° C and 5 seconds at 85 ° C. Finally, by performing the RT-PCR reaction, and single-band observation, the construction, and quality of the cDNA and primer were approved.

2.4- Primer design

The primers were designed for each gene using Primer software 3. Table 1 shows the primers sequence. The Primer-BLAST software (NCBI site) was used to confirm the particularity of the primers.

Table 1. Primers Used for Quantitative Real-Time PCR Assessments

Genes	Primer sequence	length
MUC 19	F: 5'GACTGTAAACCTGAGGAGTGCC-3'	191bp
	R: 5'CAGCAGTAGGAGACACAGGGG3'	
MUC 21	F:5'-CCCGAGATTCTCATAAATCCCCG-3'	147bp
	R: 5'GAGCCCTCCATTACCTTGTGG-3'	
β-actin	F: 5'-GATCAAGATCATTGCTCCTCCTG-3'	151
	R: 5'-CTAGAAGCATTTGCGGTGGAC-3'	

2.5- Real-Time PCR:

To test the Real-Time PCR, a kit, cap, and strip of Gene Amitidis Company (Roche) were used. In this method, beta-actin gene primers, synthesized as a housekeeping gene (beta-actin internal control gene) from normal tissues and cDNA for all RT test were used, and PCR products were electrophoresed in 1% gel. The materials required to conduct the Real Time reaction including 1µ cDNA, 1µ primer, Real-Time PCR 10µ cyber-green master mix Time PCR 10 µ, and water without RNase 8µ were poured into the strips. All steps of Real-Time PCR were performed under conditions without RNase and below the hood. All samples were performed with two replications.

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To prepare the control group, β-actin gene with Tm equal to Tm of considered genes was used. Additionally, a group was also prepared as a negative test of control (NTC) in which all the materials except the cDNA were added. After preparation, all groups were placed inside the Real-Time PCR (ABI. Step One). The reaction of the considered samples in 37 cycles and each cycle in 3 steps was performed in this way: the denaturation step for 15 seconds at 95 ° C, the primer binding step with 30 seconds at 60 ° C and the end of the synthesis step for 10 seconds at 72 ° C. The CtΔΔ method is a mathematical model to determine the changes in the relative expression of the target gene. In this method, the target gene changes are measured in comparison with internal control gene changes in two experimental samples and a control sample. To implement this method, having a Ct target gene and internal control in experimental and control samples is necessary. Internal control is used to normalize the data. The genes with consistent expression levels in all tissues and cellular conditions are used as an internal control. Therefore, observing any changes in the rate of internal control expression can be considered as an error term during the procedure (such as taking a different rate of RNA in each reaction). Genes such as β-actin, GAPDH, rRNAs, and snRNA are good examples of internal control.

To determine ΔΔCt, the following equation is used:

$$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct_{sample} - \Delta Ct_{control})} = \frac{2^{-(\Delta Ct)\ sample}}{2^{-(\Delta Ct)\ control}}$$

In this equation, the ΔCt sample is the difference between target genes' threshold cycles and internal control in the experimental sample, and ΔCt control is also the difference between target genes' threshold cycles and internal control in the control sample. It should be noted that, this model considers the efficiency of primers in the PCR reaction completely, which means that the rate of products in each cycle doubles. Accordingly, to determine the level of difference between the experimental sample and the control sample, the following equation is used: 2 ΔΔCt

Analysis of data obtained from the Real-Time PCR reaction

Genex v6.04 software was used to calculate the difference among the genes and SPSS v21 software was used for statistical analysis, and Graph Pad 7 software was used to plot the diagrams. P <0.05 was considered as a significant difference.

3- Results

In the current study, 30 subjects with colorectal cancer and 30 healthy control samples were examined. The clinical and pathological factors were extracted and classified according to the information inserted in the patient's medical records. Among pathologic factors, factors of tumor grade, tumor size, and tumor stage in terms of a TNM system were examined. Information on the samples is given in Table 2. Results of the quantitative examination of RNA extracted from tissues indicated that the concentration of RNA for all samples is appropriate and different from each other, and the A260 / A280 absorption ratio for all samples is between 1.8 and 2, indicating the absence of contamination of protein, chloroform, as well as the degradation of RNA molecules. All RNAs extracted from the tissues have good quality. Genex v6.04 software was used to calculate the difference in gene expression. Also, SPSS v21 software was used for statistical analysis, and Graph Pad 7 software was used to plot the diagrams. To analyze RT-PCR data with other clinical factors, the association between RT-PCR data for MUC19 and MUC21 genes at different stages, different degrees, and sizes of tumor were examined, and the significance of their association was tested with T-test, Anova, Pearson, and Chi-square tests (P <0.05). Results of the RT-PCR test with specific primers for MUC19 and MUC21 genes examined by the T-test showed that MUC19 gene expression in tumor tissue was higher than in healthy tissue however, MUC21 gene expression in tumor and healthy tissues did not show a significant change (Diagram 1). Due to find out relationships between RT-PCR results and pathologic data, statistical analysis was used. In comparing MUC19 and MUC21 genes in two healthy and patient in grades I, II and III, IV groups, none of the MUC19 and MUC21genes showed significant change in expression in Grade I and II, while in Grade III and IV, MUC19 gene showed a significant change and MUC21 did not show any expression change (Diagram 2). Comparison of MUC19 gene expression in healthy tissue and tumor tissue at stages 0 and III / IV of the tumor showed significant changes, but in the I / II stage, changes were not significant. MUC21 gene expression was not significant in stages 0, I / II. Also, the differential expression was significant in the III / IV stage. Diagram 3 shows the significant differences. In examining the gene expression based on the size of the tumor, MUC19 expression gene in size one and less than 1 showed a significant difference compared to healthy tissue. While in tumor size larger than 1, a significant difference in expression was not observed between healthy tissue and tumor tissue, and no significant difference was not seen between expression of MUC21 gene in healthy tissue and tumor tissue in different tumor sizes (Diagram 4).

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Table 2. Characteristics of the studied population

Variable		Patients number (%)
Grade	I	30.00
	II	33.30
	III	6.70
	IV	30.00
Tumor size	≤5	79.31
	>5	20.69
Stage	0	26.70
	I	23.30
	II	30.00
	III	10.00
	IV	6.70

Table 3. Expression changes of MUC19 and MUC21 genes in the grouping based on clinical and pathological parameters of the tumor

Variable		Expression changes MUC19	Expression changes MUC21
Grade	I/ II	No Significant	No Significant
	III/ IV	Significant	No Significant
Tumor size	<1	Significant	No Significant
	1-4	No Significant	No Significant
	>4	No Significant	No Significant
Stage	0	Significant	No Significant
	I/ II	No Significant	No Significant
	III/ IV	Significant	Significant

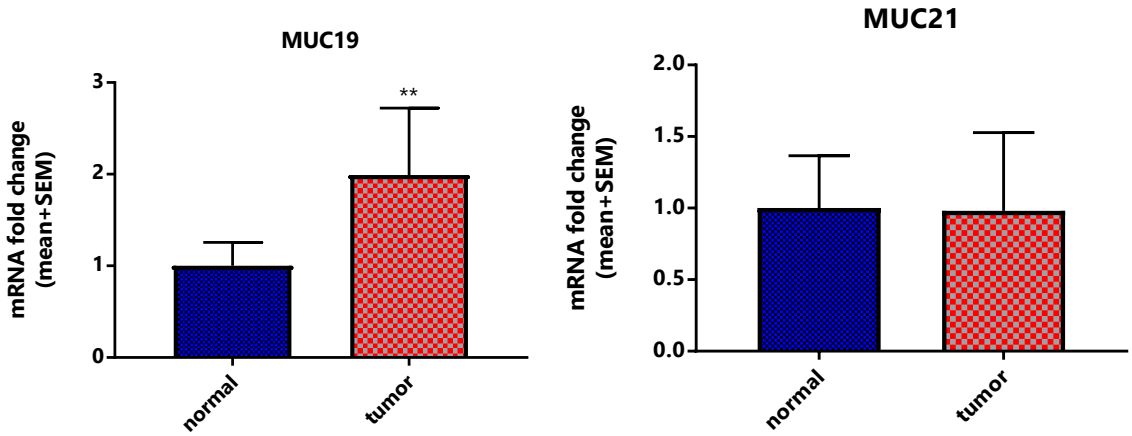

Diagram 1- shows the difference between normal and tumor tissue samples in terms of muc19 and muc21 genes expressions

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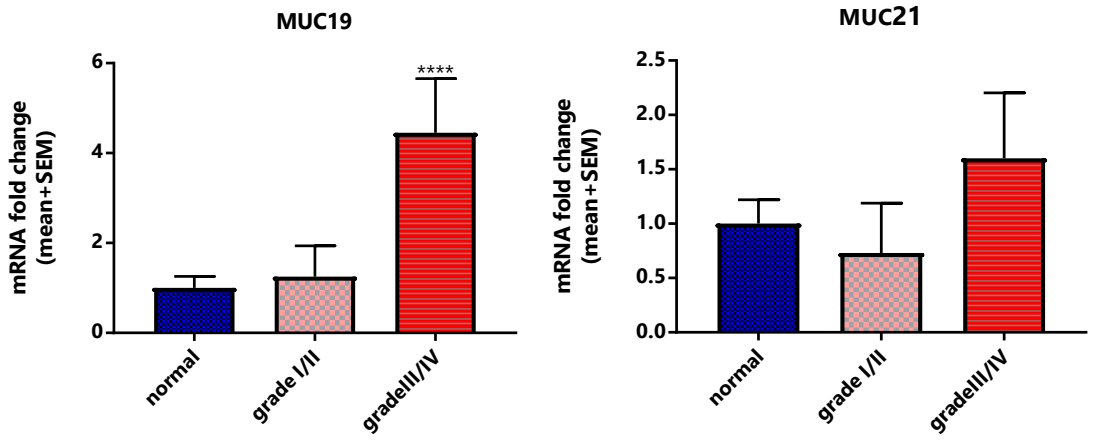

Diagram 2- *shows the difference between normal tissue samples and different tumor grades in terms of muc19 and muc21 genes expression*

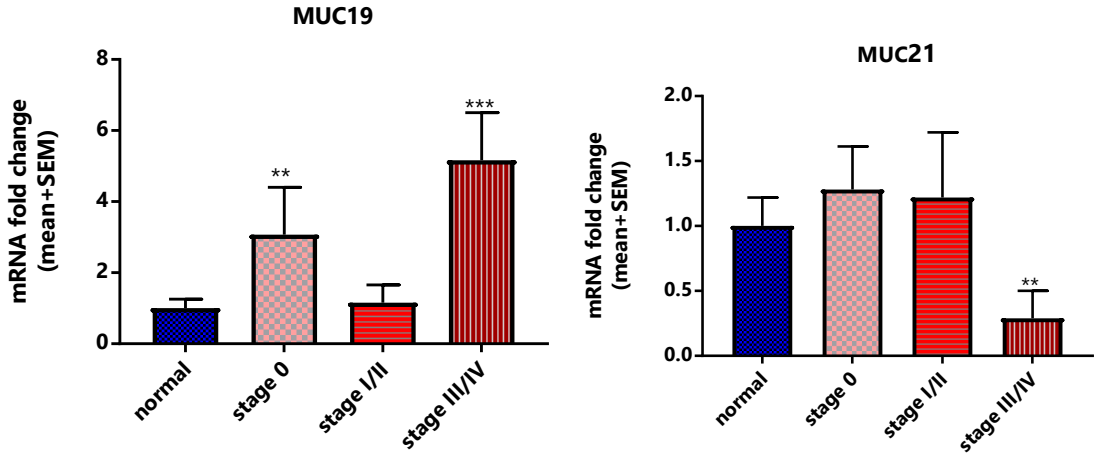

Diagram 3- *shows the difference between normal tissue samples and different stages of tumor in terms of muc19 and muc21genes expression*

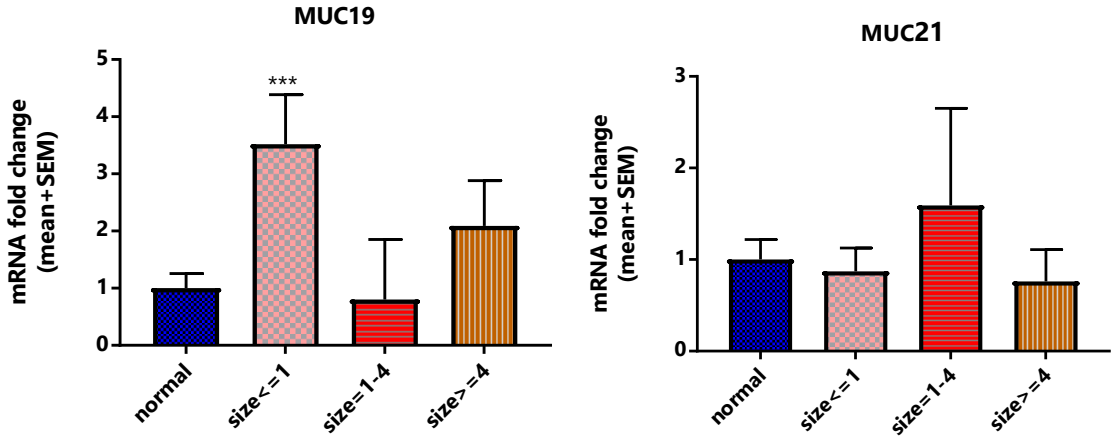

Diagram 4- *shows the difference between normal tissue samples and different sizes of tumor in terms of muc19 and muc21genes expression*

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4- Discussion

Mucins are glycoproteins with high molecular weight expressed mainly in the tissue epithelial surface, leading to the protection of the colon surface in normal physiological conditions. There are evidences on the biological role of mucins in colorectal cancer, including observation of a mucinous in colorectal cancer in vivo and in vitro experiments, and a change in the structure of mucin in polyps and cancer. Although colorectal cancer mucinous is at a high level in the diagnosis stage, it shows a bad prognosis. Mucin plays an essential role in protecting epithelial cells and preserving homeostasis by coating the surface of the human colon through a gel molecular layer. Low cell adhesion might be involved in the incorrect organizing structure of the cells, proliferation, survival, and ultimately variable gene expression. Correct cell adhesion is essential for numerous physiological processes, such as inflammation and cancer. The objective of this study was to evaluate the expression of MUC19 and MUC21 genes in colorectal cancer patients whose ultimate goal is to use it as a biomarker to evaluate the malignancy and early diagnosis of colon cancer. There is currently adequate information on the involvement of mucins in malignancies. It has been recognized that the expression of mucins significantly changes in various pathological and tumorization conditions. It has been reported that, the expression of mucins increases in some types of cancers. For this reason, they are used as biomarkers to diagnose diseases and for therapeutic goals. Increased secretion of mucins can be in favor of cancer cells.

Due to a special glycosylation pattern, mucins act as a platform for a variety of growth factors and cytokines, so they enhance the cellular proliferation and metastasis of cancer cells through cellular signal cascades [3]. Only a small subset of mucins has been studied. Due to their significant role in the cancer biology and survival of patients, it is necessary to examine the role of multiple mucins in cancer. For a better understanding of the genomic importance of mucins, 37 tissue subgroups were examined in 12 cancers for mRNA mutation, number of copies, methylation status, and new expression. Given the recent findings, membrane mucins play a role in cellular signal events. It is thought that, the function of these molecules in response to changes in the layer of mucins or near molecular environments is creating a signal to the epithelial cells. This signal may be in response to changes in the conformation or the state of ligands of extracellular domains. Signals may be transmitted to indicate the normal state of the cell surface (for example, a cell has undergone class differentiation morphology). Thus, mucins might act as an external signaling device, which makes changes in the state of epithelial cell proliferation, differentiation, or adhesion [13].

Thus, the rate of changes in mucin 19 and 21 genes expression in colorectal cancer were evaluated. Based on the results of this study, MUC19 gene expression in colorectal tumors showed a significant change compared to healthy tissue. The change in expression of this gene was also evaluated in different degrees, stages, and sizes of the tumor. It was found that gene expression change is non-significant in grade I / II, significant in grade III / IV, significant in stage 0, and non-significant in III / IV. In addition, in sizes one and less than one, an increase in expression was significant, but the significant increase in expression was not seen in larger sizes. Based on the present study on MUC19 expression changes in the degree, size, and different stages of the tumor, it can be stated that, the MUC19 gene in different tumor stages showed a significant increase and reduction in expression, which might be involved in the malignancy of the colon. In a study conducted by Abulí et al in 2013, they examined genetic mutations in different genes and mucins. They examined 515 normal and colorectal cancer samples in stage 1 and 901 control and tumor samples in stage 2. Genotyping results on mucin 19 showed that this gene did not play a role in the development of colorectal cancer [14]. The changes in MUC19 gene expression were observed in diseases such as Crohn's disease, middle ear inflammation [16], and lung carcinoma [17]. Based on this study, the reduction and increase in the expression of the MUC19 gene in degree, stages, and size of the colorectal tumor, as well as expression changes in various diseases, and as a biomarker should have specificity; this gene cannot be considered as a diagnostic biomarker for colon cancer. The rate of MUC21 gene expression in the tumor tissue did not change significantly in comparison to healthy tissue, but in a study conducted by Ryan J et al. a significant correlation was found between the expression of MUC21 gene with colorectal cancer, which is inconsistent with our results. [18]. In another study conducted by Veronika et al on CC genotype of rs886403 in MUC21, it was found that the MUC21 gene polymorphism showed a greater mortality risk than the TT genotype [19]. The changes in the expression have been examined in various cancers, including lung cancer, thyroid, and esophagitis [22], and a significant relationship has been observed.

Conflicts of interest

The authors declare no conflict of interest.

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