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Original Article

Investigating Association between Membrane-Bound Mucin (MUC12) Expression Level and Clinicopathological Characterization of Colorectal Cancer

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Abstract

Introduction: Colorectal cancer (CRC) is one of the most prevalent canceres throughout the world and has a high mortality rate. It is accepted that dysfunction in the expression of mucins is associated with the occurrence and development of CRC. Given that, the purpose of the current study was to analyze the expression of the MUC12 gene in CRC and its relationship with clinicopathological variables.

Materials and Methods: This research was a prospective case-control study. Tumors from CRC patients were collected from the Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. RNA extraction and cDNA synthesis were performed using the corresponding kits. The gene primer was designed and RT-PCR was used to evaluate gene expression. ANOVA analysis and the t-test were employed to examine the differences between the different groups. Prism8 software was also used for data analysis.

Results: The results of the present study showed that the expression of MUC12 (P=0.0012) gene in patients with CRC was significantly different from tumor margin samples. Associations between the expression of the studied gene and clinicopathological variables such as grade and stage of CRC tumor as well as the age of the patients were also observed. The area under the curves (AUC) for the MUC12 0.953 was calculated by ROC analysis.

Conclusion: It can be stated that malignant transformation of colorectal cells is accompanied by changes in the expression of MUC12 in CRC, which has a biomarker value for the diagnosis of CRC.

Keywords: MUC gene, CRC, Gene expression

1. Introduction

CRC is the third most common disease and the fourth deadliest cancer in the world [1]. It is the second most common cancer in women after breast cancer, and the most common cancer in men after lung and prostate cancer [2]. Most cases of CRC are due to environmental factors and in about 5% of cases, hereditary factors play an important role [3]. One of the major clinical challenges of this malignancy is the late diagnosis or progression of the disease to metastasis. Therefore, diagnostic or predictive biomarkers are of clinical importance. Finding new biomarkers can enhance accuratediagnosis

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anddifferentiation, or confirm early stage of the disease [4].

Mucins, whose roleis to protect and lubricate the epithelial tissue,are a large group of glycoproteins secreted by the epithelium on the cell surface that are identified by the central polymorphic tandem repeat structure.As cell surface receptors, Mucinsare involved in guiding cellular signals generated in response to external stimuli that cause proliferation, differentiation, and apoptosis in cells.Mucins expression levels increase in a variety of cancersincluding adenocarcinoma [5], and their prognostic value in CRC has been studied [6]. Membranes-bound mucins(MUC12) are expressed in organs such as colons, small intestine and lymph nodes [7]. They both transduce the external stimuli message to the cells and act as a protective barrier [8]. Epithelial to mesenchymal transition (EMT) has been shown to affect cancer progression and metastasis. The role of MUC genes such as MUC1, Muc5ac, Muc4 and Muc12 in the EMT process has been reported in various cancers [9].

It has also been shown that the level of mucin secretion increases in various cancers, especially in adenocarcinoma [10]. These molecules have high molecular weights and contain tandem repeat sequences of amino acids.

Taken together, the aim of this study was to investigate the expression of MUC12 gene in normal tissues and colorectal tumors and their relationship with invasion, metastasisand clinicopathological variables.

2. Materials and Methods

2.1. Experimental Procedure

The presentstudy adopteda case-control approach. The participants of this study were28 patients who referred to Taleghani Hospital, Tehran, Iran for treatment or diagnosis. After a colonoscopy and confirmation of the results by a pathologist, these patients were further referred by a gastroenterologist, for genetic testing. The participantswere asked to answer demographic questions on a questionnaire. Thereafter, specimens were obtained individually from their seemingly healthy margins during a colonoscopy by a gastroenterologist specializing in CRC, and then placed in RNAlater solution [Sigma, Germany].The samples were immediately transferred to liquid nitrogen and stored until the time of RNA extraction. The current study was approved by the hospital. Furthermore, written consent was obtained from the patients participating in the study. Exclusion criteria included the patients suffering from T1 cancer and treated by endoscopic polypectomy [2], the ones undergoingneoadjuvant chemotherapy [4], and the patients diagnosed with invasive cancers [synchronous or metachronous]originatingfrom the colorectum or other areas [11]. The current study has received approval from the Ethics Committee of the Research Center for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran: code IR.SBMU.RIGLD.REC.1396.180

2.2. RNA Extraction and cDNA Synthesis

RNA was extracted using a Total RNA Extraction Kit (Yekta Tajhiz Azma Co., Iran). After extraction, the quantity and quality were evaluated using Nanodrop (Thermo, NANO 300) and gel electrophoresis. The RevertAid RT Kit (Thermo scientific) was used to synthesize the cDNA following manufacturer instructions for cDNA synthesis.

2.3. Primer Design

In this study, the β-actin gene was selected as the internal control. To design primers for MUC12 gene, two pairs of primers were designed using Gene Runner software. The primers were synthesized by the Sinagen Co.,Tehran, Iran. The primer sequences used for RT-PCR in terms of genes are listed below :

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Table 1. Sequences of primers used in the current study

Genes	Sequences	TM	PCR Product Size
Muc12-F	5'- CTGCGCGTCCGTTACTACAG -3'	61.1	
Muc12-R	5'- GGGTCGCTTGAACTGGGTG -3'	60.9	96
β-actin-F	5'- CACCATTGGCAATGAGCGGTTC-3'	59.5	
β-actin-R	5'- AGGTCTTTGCGGATGTCCACGT-3'	59.8	151

2.4. Real Time PCR

Relative Quantitative Real Time PCR was used to study the expression of MUC12 gene. For this purpose, the Takara SYBR® Premix Ex Taq II (TliRNaseH Plus) kit was used. Kit manufacturer instructions were followed to perform RT-PCR. For initial denaturation, annealing, and extension stages, RT-PCR was run at 95°C for 10 min, 95°C for 40 cycles of 10s, and 60°C for 30 min, in turn.

2.5. Statistical Analysis

In this study, the rate of change in the expression of the studied gene compared to that of the control group was investigated by $2^{-\Delta\Delta Ct}$method. Quantitative and qualitative values were shown as mean ± SD and percentage (%), respectively. The t-test was used to investigate significant differences in gene expression in tumor and healthy tissues. The statistically significant level was considered to be <0.05. GraphPad software Prism version 8 was used to analyze the data.

3. Results

Patient Clinicopathological Attributes

The clinopathological features of CRC patients (28) are given where the median age of CRC patients was 59.5(32-82) years. Overall, 64.29% (18) were male patients and 35.71% (10) female. Thirty percent (9) of the tumor was located in the proximal colon, 36.7(11) percent in the distal colon, and 26.6(8) percent in rectum. As for tumor grade, 33.3% (10) of the cases were moderately differentiated and 23.3% (7) and 28.57% (8) showed poorly- and well-differentiated, respectively. Most patients were in stage II 33.3% (10) and stage III 26.7% (8), and stage I 16.7 % (5) and only 6.7% (2)of patients showed stage IV .

Comparison of gene expression in normal and cancer cells

Significant differences were observed in the expression of MUC12 gene between normal and tumor cells so that the expression of MUC12 gene in normal cells was higher than in tumor cells (P <0.001).

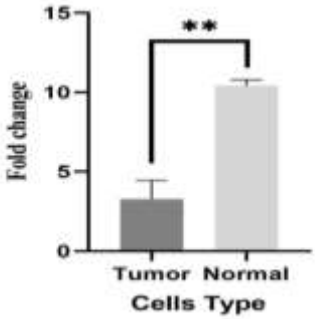

Figure 1. Comparison of MUC12 genes expression in normal and cancer cells
**: Significant at the probability level of 0.01.

In the present study, the expression of the MUC12 gene depended on patient's age. There was a significant difference in the expression of this gene in CRC patients with respect to age (P = 0.0028). The expression of the MUC12 gene was higher in patients over 50 and lower in thoseunder 50.

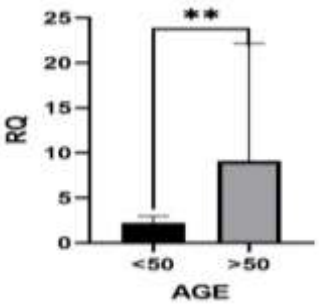

Figure 2. MUC12 gene expression in different patients with colorectal cancer by age
**: Significant at the probability level of 0.01.

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The Kruskal-Wallis test showed that there was no significant difference in the expression of the MUC12 gene in different grades of CRC(P = 0.190). However, expression of the MUC12 gene in poorly and moderately differentiated tumors was higher than well-differentiated CRCtumors.

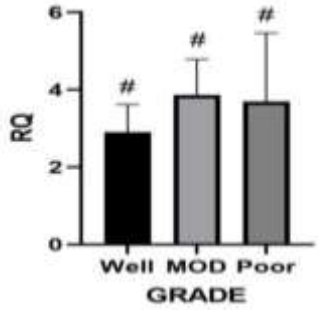

Figure 3. MUC12 gene expression in different patients with colorectal cancer by grade .
: no significant difference at the probability level of 0.01.

CRC tumors were observed, at different stages, to express the MUC12 gene differently. (P = 0.021). The highest expression of the MUC12 gene was observed in Stage IV. There was no significant difference in the expressions of the MUC12 gene between Stages II and III tumors, and the lowest expression of this gene was seen at Stage I.

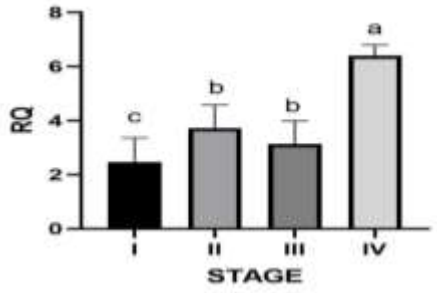

Figure 4. MUC12 gene expression in different patients with colorectal cancer by stage .
Different letters indicate significant differences.

There was no significant difference considering expression of the MUC12 gene between men and women with CRC (P = 0.725).

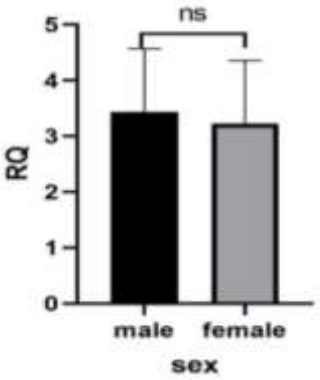

Figure 5. MUC12 gene expression in different patients with colorectal cancer by gender .
ns : no significant difference at the probability level of 0.01.

The receiver operating characteristic (ROC) curve was used to assess the potential use of MUC12 gene as a predictor of CRC. The area under the curve (AUC) value for MUC12 was 0.840 at a 95% confidence interval (CI = 0.6963 to 0.9837, P = 0.001). At the cut-off point, the sensitivity and the specificity for the MUC12 gene was 90% and 84%, respectively.

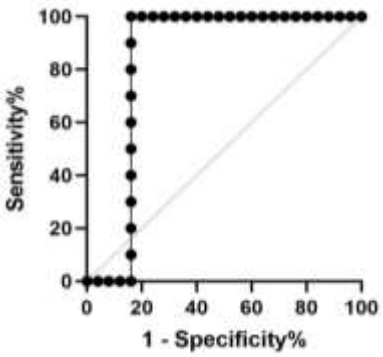

Figure 6. ROC curve for the potential use of MUC12 gene to identify patients with colorectal cancer

4. Discussion

CRC as a common, fatal, yet preventable disease has always attracted the attention of health centers around the world. It is more common in men than women and increases with ageso that the age of diagnosis in developed countries is about 70 years. Screening has been shown to significantly reduce the incidence and mortality rate of this cancer, but there are currently no organized screening programs in most

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countries [22]. Over the past three decades, molecular genetics methods have been developed based on the analysis of fecal proteins, DNA, and RNA [23]. There is little information on the expression of MUC genes in CRC tumors and its association with clinical variables. Therefore, the present study was undertaken to investigate the relation between the expression of secretory MUC genes and clinical variables.
The downregulated of MUC12 gene was observed in CRC tumor cells. Young patients also showed downregulation of this gene. In poorly differentiated tumors, overexpression of MUC12 was detected and MUC12 was upregulatedin stage IVof CRC. In our study, it is revealed that MUC12experienced downregulation in metastatic tumors. MUC12, as a novel membrane- associated mucin gene, is located on chromosome 7q22. It is postulated that membrane- associated mucins contribute to cancerous invasion through destroying the connections between opposing cells (anti-adhesion effect) as well as dysregulating tumor differentiation and proliferation [12]. Although the clinical importance or function of MUC12 has not been yet investigated, itis indicated that the gene is downregulated in CRC [13]. In the presentstudy, for the first time, the clinicopathological and prognostic significance of the MUC12 gene has been includedin CRC through the analysis oftissue samples. Based on our findings, the expression of MUC12 is a novel independent prognostic variable in the patients diagnosed with Stages III andIVof CRC. This study is the first report indicating a relation between the expression of MUC12 mRNA and the survival rate.
While the expression of MUC12 is normally at a high level in the colon, it is weakly expressed in the uterus, prostate, and pancreas. It is reported that MUC12 is downregulated in CRC [12, 13] and the mechanisms related to aggressive behavior of CRC have not been determined. Nonetheless, it is believed that low MUC12 expression contributes to degrading adherens junctions, leading to the migration, invasion, and metastasis of tumor cells, as well as the aggressive behavior of CRC [14].
The current study had its own limitations. Gene expression analysis was performed solely using molecular analysis and not by immunohistochemical staining. However, one of the strengths of this study is that it is a prospective study. In addition, this study showed that the expression of MUC12 could be a prognostic marker in colorectal cancer. Our study proposes that the low expression level of MUC12 is a novel postoperative relapse marker. Patients with a low expression level of MUC12 are recommended to apply adjuvant chemotherapy and more intensive chemotherapy in stage III and stage IV, respectively.

5. Conclusion

Based on the obtained results, it can be stated that malignant transformation of colorectal cells is accompanied by changes in the expression of the membrane-bound MUC gene (MUC12) which can be used for diagnostic purposes.It was also found that these genes have biomarker value in colorectal cancer diagnosis. More research, however, is required in this regard to reach the optimal approach.

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Conflict of interest

The authors declare no conflict of interest.

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