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Kolorektal Kanserde *RYBP* ve *MDM2* Genlerinin Ekspresyonu

Expression Levels of *RYBP* and *MDM2* Genes in Colorectal Cancer

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Öz

Giriş ve Amaç: Gastrointestinal kanserler arasında sıklıkla görülen kolorektal kanser, dünya genelinde mortalite ve morbiditenin en önemli nedenlerinden biridir. Bu çalışmada kolorektal kanserde *RYBP* ve *MDM2* genlerinin ekspresyon düzeylerini araştırmayı amaçladık. Ayrıca kolorektal kanserde *RYBP* ve *MDM2* ekspresyonları arasındaki olası korelasyonu ve bu genlerin ekspresyonu ile kolorektal kanserli hastaların klinikopatolojik özellikleri arasındaki ilişkiyi inceledik.

Gereç ve Yöntemler: Bu çalışmada, cerrahi operasyon sonucunda 43 kolorektal kanserli hastanın tümörlü kolon/rektum dokuları ve komşu sağlıklı dokuları toplandı. *RYBP* ve *MDM2* mRNA ekspresyonları Real-Time PCR kullanılarak incelendi.

Bulgular: Çalışmanın sonucunda tümörlü dokularda normal dokulara kıyasla hem *RYBP* hem de *MDM2* ekspresyonlarında azalış görüldü fakat bu azalış istatistiksel açıdan anlamlı değildi (sırasıyla p=0.673 ve p=0.721). Ayrıca kolorektal kanserde *RYBP* ve *MDM2* mRNA ekspresyonları arasında korelasyon bulunmazken, bu genlerin ekspresyonları ile klinikopatolojik veriler arasında istatistiksel olarak anlamlı bir ilişki saptanmadı (p>0.05).

Sonuç: Sonuç olarak, *RYBP* ve *MDM2* ekspresyonlarının kolorektal kanser ile ilişkili olmadığı söylenebilir, ancak bu çalışmadan elde edilen sonuçları doğrulamak için daha büyük örneklem grupları ile yapılacak çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Gen ekspresyonu, Kolorektal kanser, *MDM2*, *RYBP*, Real-Time PCR.

Abstract

Objective: Colorectal cancer, frequently seen among gastrointestinal cancers, is one of the major causes in mortality and morbidity worldwide. In this study, we aimed to investigate the expression levels of *RYBP* and *MDM2* genes in colorectal cancer. We further examined the possible correlation between *RYBP* and *MDM2* expressions in colorectal cancer and the relationship between the expression of these genes and the clinicopathological features of patients with colorectal cancer.

Materials and Methods: In this study, fresh tumor colon/rectum tissues and the adjacent healthy tissues collected from 43 patients with colorectal cancer during a surgical operation. *RYBP* and *MDM2* mRNA expressions were examined using Real-Time PCR.

Results: In this study results showed that the expression levels of both *RYBP* and *MDM2* were decreased in tumor tissues compared to normal tissues with colorectal cancer patients, but these decreases were not statistically significant (p=0.673 and p=0.721, respectively). Moreover, while there was no correlation between *RYBP* and *MDM2* mRNA expressions in colorectal cancer, no statistically significant relationship was found between the expressions of these genes and clinicopathological data (p>0.05).

Conclusion: Consequently, it can be said that *RYBP* and *MDM2* expressions are not related to colorectal cancer, however, future studies with larger sample groups are needed to validate the results obtained from this study.

Keywords: Colorectal cancer, Gene expression, *MDM2*, *RYBP*, Real-Time PCR

1. Introduction

Cancer has been among the most vital health concerns in the world. According to GLOBOCAN data in 2020, there are an estimated 19.3 million cancer cases worldwide, and 10% of these cases are colorectal cancer. Colorectal cancer is the second leading cause of cancer-related deaths (9.4% of a total of 9.9 million cancer-related deaths) [1]. It is also the third most common cancer in both men and women in our country [2]. Moreover, its prevalence is 3-4 times more common in developed countries compared to developing countries. Ageing, unhealthy diet, smoking, alcohol use, and a lifestyle without physical exercise significantly increase the risk of colorectal cancer [1-3].

Evolutionarily conserved Polycomb group (PcG) proteins have similar sequences preserved from plants to mammals, and are the enzyme groups that transcriptionally suppress the modification of chromatin. They have very critical roles in maintaining the balance between differentiation and growth during the normal development process of living things. These proteins in fact play also an important role in many biological events including regeneration, differentiation and cancer via inactivating their target genes transcriptionally during the cell division throughout development [4-6]. Ring 1 and YY1-binding protein (RYBP) is a member of polycomb proteins which has a transcriptional suppressive function. RYBP prevents uncontrolled proliferation of malignant cells by suppressing the activity of some specific transcription factors such as YY1, GABPB1 and E2F6 [5]. Several studies have reported that RYBP expression increases or decreases in different cancer types including cervical, prostate, hepatocellular and esophageal squamous cell cancers [7-10].

The Mouse double minute 2 (*MDM2*) gene contains 11 exons on chromosome 12q14.3-15 and was first discovered in mouse fibroblast cells, and it was identified as oncogene [11,12]. The *MDM2* gene includes various protected functional domains providing it an oncogenic feature. It plays a vital role in the control point of the cell cycle which regulates the functions of p53 stopping the cell division when DNA is damaged. In normal cells, p53 and MDM2 are synthesized in a balanced way, and MDM2 acts as the negative regulator of p53. The increase in the expression of MDM2 decreases the protein level of p53 and thereby reducing the function of p53. The decrease in p53 protein level increases cancer risk through increasing tumor formation and progression [11,13,14]. MDM2 further affects other mechanisms in the tumor formation process independently from regulating the level of p53. Such mechanisms include proteosomal degradation and ubiquitination of proteins involved in tumor suppression, genomic instability, and the stability of mRNA molecules that are effective in tumor formation by genomic instability and DNA damage, and encode metastatic properties [15]. Several studies showed the high expression of MDM2 in various human tumors including osteosarcoma, acute lymphoblastic leukemia, melanoma, breast, lung, and ovarian cancers, and the low MDM2 expression in primary tumors of childhood neuroblastoma, bladder cancer, and head and neck squamous cell carcinoma [16-23].

Chen et al. [13] previously reported that RYBP was bound to MDM2 in different mammalian cell lines which causes an alteration in the conformation of MDM2. They further showed that the interaction between MDM2 and p53 changed and the ubiquitination and degradation of p53 were prevented as a result of such alteration. Motivated from this, we aimed to assess both *MDM2* and *RYBP* gene expression levels in colorectal cancer and whether there is an association between the expressions of these genes. We also aimed to determine the possible relationship between *MDM2* and *RYBP* expressions and clinical and pathological data of patients with colorectal cancer.

2. Materials and methods

2.1. Tissue samples

This study was approved by the Local Ethics Committee of Gaziantep University, Turkey in accordance with the Helsinki Declaration (No: 2017/77). After obtaining a signed consent form, we collected fresh tumor and adjacent healthy tissues (as a control group) from a total of 43 colorectal cancer patients that did not receive radiotherapy and chemotherapy from General Surgery Department of Gaziantep University, Turkey. Tissue samples taken from patients were stored in RNA Later solution (Thermo Fisher Scientific) at -80^{0}C until RNA isolation. The average age of patients, of which 14 of them were women and 29 were men, was 53.65 ± 0.26. The demographic and clinical findings of the patients are shown in Table 1.

2.2. Total RNA isolation from the fresh tissues

Total RNA isolation was performed by using PureLink RNA Mini Kit (Thermo Fisher Scientific, catalog no: 12183018A) following the manufacturer's instructions. Concentrations and purities of the isolated RNA samples were measured with Nanodrop Spectrophotometer.

2.3. cDNA synthesis

cDNA synthesis was performed by using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, catalog no. 4368814) by the Reverse Transcriptase PCR method. Reverse transcription of RNA was performed in a final volume of 20 µL mixed solution containing 2 µL 10X RT buffer, 0.8 µL 25X dNTP Mix (100 mM), 2 µL 10X RT Random primary, 1 µL Reverse transcriptase (50U / µL), 1 µL RNase inhibitor, 10 µL RNA (30 ng / µL) and 3.2 µL Nuclease-free water with following conditions: 10 min at 25ºC, 120 min at 37ºC, and 5 min at 85ºC.

2.4. Quantitative-Real Time Polymerase Chain Reaction (qRT-PCR)

Real-Time PCR is method of choice for quantitative analysis of nucleic acids. The general steps performed during a Real-Time PCR experiment are RNA isolation, cDNA synthesis, and Real-Time PCR [24]. We performed Real-Time PCR by using the Taq Man Primer

Table 1. The demographic and clinical findings of colorectal cancer patients

Characteristics	Number of Patients (%)
Tissue type	
Colon	17 (39.5)
Rectum	26 (60.5)
Age groups (years)	
50≥ age	26 (60.5)
<50 age	17 (39.5)
Gender	
Female	14 (32.6)
Male	29 (67.4)
Smoking status	
Yes	16 (37.2)
No	27 (62.8)
Alcohol status	
Yes	0 (0)
No	43 (100)
Metastasis	
Yes	10 (23.3)
No	33 (76.7)
TNM Stage	
I- II	23 (53.5)
III - IV	20 (46.5)
Lymph node involvement	
Yes	19 (44.2)
No	24 (55.8)

Probe (Thermo Fisher Scientific) to determine the expression levels of *RYBP*, *MDM2* and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (as Housekeeping gene) genes.

The reaction mixture of 20 µL contained 1 µL of 20X Taq Man Gene Expression Assay, 10 µL 2X Taq Man Gene Expression Master Mix (Thermo Fisher Scientific, catalog no. 4369016), 2 µL cDNA, and 7 µL RNase-free water. The following reaction protocol was performed: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60 ° C for 1 min. The experiment was performed in duplicate, and $2^{-\Delta\Delta Ct}$ was used to determine the expression levels of *RYBP* and *MDM2* genes [25]. Finally, the expression levels in tumor tissues were compared to expression levels in normal tissues. The expression level with a $2^{-\Delta\Delta Ct}$ value greater than 1 were grouped as high expression level while those less than 1 grouped as low expression level [26].

2.5. Statistical analysis

All the statistical analyzes were performed by using SPSS for Windows 22.0 version. Following the calculation of the ΔCt values, which determine *RYBP* and *MDM2* expression levels, paired t test was used to compare between ΔCt values in tumor and normal tissues. Results were reported as mean ± SD, and the relationships between expression levels and clinical and pathological data were determined through chi-square analysis. The correlation between *RYBP* and *MDM2* expression levels was examined with Spearman correlation test. The p value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Results

In this study, in a total of 43 patients with colorectal cancer, expression levels of *RYBP* and *MDM2* were determined using Real-Time PCR in tumor tissues compared to adjacent healthy tissues counterparts and the values of ΔCt (means ± SD) were 3.84 ± 2.70 and 3.36 ± 2.16 in tumor tissues, and 3.60 ± 2.56 and 3.20 ±1.79 in normal tissues, respectively. It was observed that the expression levels of both *RYBP* and *MDM2* decreased in tumor tissues compared to normal tissues, but the difference was not statistically significant (p = 0.673 and p = 0.721, respectively) (Figure 1). When all the $2^{-\Delta\Delta Ct}$ values of 43 patients were examined together, we showed that 53.4% decrease in the expression of *RYBP* gene and 51.2% decrease in the expression of *MDM2* gene in tumor tissues compared to normal tissues.

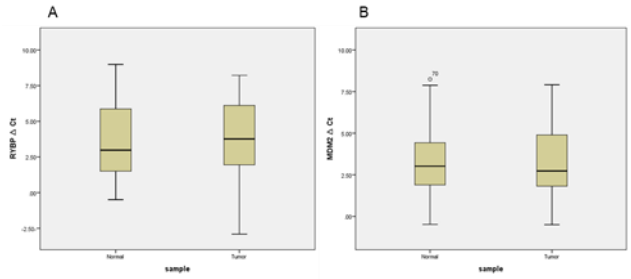

Figure 1. Expressions of *RYBP* (A) and *MDM2* (B) in normal and tumor tissue samples of colorectal cancer patients

We further found that there were no statistical relationships between both *RYBP* and *MDM2* expression levels and clinical and pathological data of the patients (Table 2). Moreover, no correlation was found between the expression levels of *RYBP* and *MDM2* genes (r = 0.159, p = 0.308) (Figure 2).

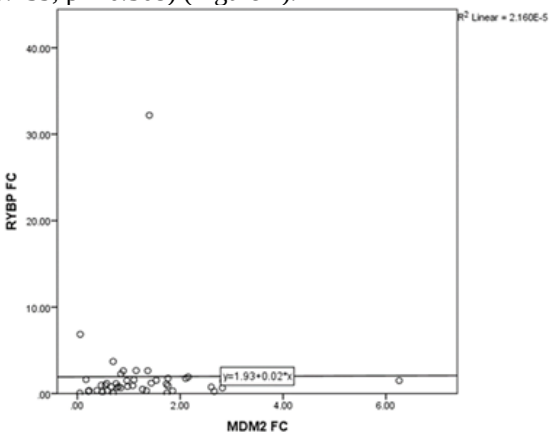

Figure 2. *Correlation between RYBP and MDM2 expressions in colorectal cancer. FC, fold change*

Table 2. Association between the expression levels of *RYBP* and *MDM2* and clinicopathological features of patients with colorectal cancer

Clinicopathological paramaters		*MDM2 expression*			*RYBP expression*		
		Low	*High*	*p value*	*Low*	*High*	*p value*
Age (years)	<50	8	9	0.663	8	9	0.494
	≥50	14	12		15	11	
Sex	Female	6	8	0.449	8	6	0.739
	Male	16	13		15	14	
Tissue type	Colon	14	12	0.663	14	12	0.233
	Rectum	8	9		9	8	
Smoking status	+	9	7	0.607	6	10	0.106
	-	13	14		17	10	
Metastatis status	+	18	15	0.420	4	6	0.473
	-	4	6		19	14	
TNM Stage	I-II	13	10	0.541	14	9	0.298
	III-IV	9	11		9	11	
Lymphatic invasion	+	12	12	0.864	12	7	0.258
	-	10	9		11	13	

3.2. Discussion

It has been widely known that various factors such as both genetic and environmental lead to the high incidence of colorectal cancer worldwide. However, there have been still many unidentified factors causing in colorectal cancer, in which numerous genetic parameters affect its occurrence. Motivated from this, the expression levels of *RYBP* and *MDM2* genes in colorectal cancer and whether there was any correlation between these two genes were investigated in this study. RYBP, the member of the PcG family, has been known to play a transcriptional suppressive role in embryonic development, apoptosis, and cancer formation. Therefore, *RYBP* expression levels have been studied in various cancer types and the findings of previous studies suggest RYBP functions differently in different type of cancers. In our study, the expression of *RYBP* decreased in 53.4% of tumor tissues compared to normal tissues, although this decrease was not statistically significant. As a result of the deletion on chromosome 3p, where the *RYBP* gene is located, decreased RYBP expression was reported in cervical and prostate cancers [7-8]. Similarly, in the previous study on the patients with hepatocellular carcinoma, it was found that high expression of RYBP inhibited the growth, migration and apoptosis of tumor cells [5]. In addition, in another research, it was shown that RYBP was downregulated in esophageal squamous cell carcinoma [10]. Zhu et al. [9] showed that RYBP expression level was low in hepatocellular carcinoma (HCC) and more importantly there was a relationship between negative expression of RYBP and poor prognosis of HCC patients. The findings of all aforementioned studies was suggested that RYBP has a tumor suppressor role. Contrary to these data, it was revealed that RYBP expression increased in acute leukemia, oligodendroglia tumors and pituitary adenoma, and *RYBP* functioned as an oncogene [6, 27]. On the other hand, it was reported that KLF4 and SP1, which are among the transcription factors, played an important role in the regulation of RYBP levels in hepatocellular cancer. The same study showed that the different levels of expression of RYBP in various pathological conditions might be due to the fact that the KLF4 and SP1 binding sites act as repressors and activators, respectively [28].

In addition, some studies have shown that microRNAs have an effect on the expression level of *RYBP*. However, miR-29, miR-1 and miR-206 were found to be effective in decreased RYBP expression in melanoma [29]. Therefore, in future studies, determination of the functions of transcription factors that bind to the *RYBP* promoter and microRNAs that play a role in *RYBP* expression in colorectal cancer may be critical data to reveal the relationship between colorectal cancer and *RYBP* expression. Additionally, the difference between the results of our study and previous studies may be due to the formation of undetermined *RYBP* isoforms and the differential synthesis of *RYBP* by the alternative splicing

mechanism. When considering the role of RYBP in the cell, RYBP may perhaps act as a tumor suppressor in colorectal cancer, as our findings suggest.

MDM2, defined as an oncogene, can function as dependent on p53 or independently [14]. Our study revealed that *MDM2* mRNA expression was decreased in 51.2% of tumor tissues compared to normal tissues. Similar to the finding herein, a previous study, investigated p53, MDM2 and p14ARF pathways in colorectal cancer, reported that expression of *MDM2* was decreased in 55% of tumor samples [30]. Furthermore, some studies published that MDM2 expression was decreased in head and neck squamous cell carcinoma, primary tumors of childhood neuroblastoma and bladder cancer [21-23]. Contrary to these findings, various previous research also showed that there was a relationship between high expression of MDM2 and melanoma, lung, breast, and ovarian cancers [17-20].

In fact, *MDM2* has over 70 different splice variants which have different roles in oncogenesis. Some of these variants have been known to be associated with cancer [31-33]. In tumor formation, some of these variants have an initiator role, while the other is suppressive [33]. Based on this information, it is also important to determine which *MDM2* isoform is present when reaching a clear decision regarding the expression of the *MDM2* gene in colorectal cancer. In a previous research, it has been also reported some mutations and polymorphisms in the *MDM2* gene. However, the effects of these genetic differences on *MDM2* expression have not been clearly demonstrated yet [31]. In addition, the presence of *MDM2* transcripts without a p53 binding site was demonstrated in some tumor types. As commonly known, MDM2 proteins of different sizes can be formed as a result of proteolytic cut, alternative splicing or post-transcriptional modifications. More importantly, among these, the shorter MDM2 proteins were previously reported to increase tumor suppressor activity of p53 [34]. Therefore, future studies aiming to determine alternative splice models of this gene and also to investigate protein expression levels are still required in determining the exact role of *MDM2* in colorectal cancer. In this study, we did not observe significant relationship between clinicopathological findings of patients and *RYBP* and *MDM2* expressions. However, previous studies have reported positive or negative correlations between MDM2, RYBP expressions and the several clinical findings such as the stage, size, invasion and metastasis of the tumor in different types of cancer [9, 22, 23, 30, 35].

While we did not find correlation between *RYBP* and *MDM2* mRNA expressions in colorectal cancer, it was shown that these two proteins were involved in the p53 pathway by interacting with each other in the U2OS, A549, HCT116 and PC3 cell lines. [13].

4. Conclusion

Taking all into consideration, we showed a decrease in both gene expression levels in tumor tissues, especially in *RYBP*, compared to adjacent healthy tissues, but these decreases did not reach a statistically significant level. Therefore, more detailed future studies are still needed to detect the protein levels of genes with larger sample numbers in order to more clearly determine the roles of these two genes, their importance on clinopathological findings, and their relationships with each other in colorectal cancer.

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