Differential Expressions of Cancer-Associated Genes and Their Regulatory miRNAs in Colorectal Carcinoma

Murat Kara[1], Onder Yumrutas[2], Onder Ozcan[3], Ozgur Ilhan Celik[4], Esra Bozgeyik[5], Ibrahim Bozgeyik[2]*, Sener Tasdemir[6]

[1]Department of Medical Genetics, Faculty of Medicine, Mugla Sıtkı Kocman University, Mugla, Turkey

[2]Department of Medical Biology, Faculty of Medicine, Adiyaman University, Adiyaman, Turkey

[3]Department of General Surgery, Faculty of Medicine, Mugla Sıtkı Kocman University, Mugla, Turkey

[4]Department of Medical Pathology, Faculty of Medicine, Mugla Sıtkı Kocman University, Mugla, Turkey

[5]Department of Medical Biology, Faculty of Medicine, University of Gaziantep, Gaziantep, Turkey

[6]Department of Medical Genetics, Faculty of Medicine, Ataturk University, Erzurum, Turkey

*Corresponding Author

Ibrahim BOZGEYIK

i.bozgeyik@gmail.com

Phone: +904162231690

Adiyaman University, Faculty of Medicine, Department of Medical Biology

Adiyaman, Turkey

ABSTRACT

Colorectal cancer is one of the frequently seen malignancy in the world. To date, several oncogenes and tumor suppressor gene have been identified and linked to colorectal cancer pathogenesis. Although recent advances in the diagnosis and therapy of colorectal cancer are promising, identifying novel genetic contributors is still high priority. In the present study, expression profile of some cancer-related genes and their regulatory miRNA molecules were evaluated by using a high-throughput real-time PCR method. For the study, a total of 54 patients diagnosed with CRC and normal colon tissue samples of 42 healthy controls were included. For the expression analysis, total RNA was extracted from FFPE tissue samples and converted to cDNA. All expression analysis were assessed by using Fludigm Microfluidic Dynamic Array chips for 96 samples and the reactions were held in Fludigm BioMark™ HD System Real-Time PCR. As a result of the study, expression of the ADAMTS1, FHIT, RUNX1, RUNX3 and WWOX genes were shown to be significantly altered in CRC tissues in contrast to normal tissue samples. Moreover, miR-378a-3p, miR-155-5p, miR-193b-3p, miR-96-5p, miR-17-5p, miR-27a-3p, miR-133b, miR-203a, miR-205-5p, miR-34c-5p, miR-130a-3p, miR-301a-3p, miR-132-3p, miR-222-3p, miR-34a-5p, miR-21-5p, miR-29a-3p and miR-29b-3p were found to be significantly deregulated in CRC. Consequently, results of the current study strongly suggest the involvement of novel cancer-related genes and their regulatory miRNA in CRC physiopathology.

Keywords: Colorectal cancer, miRNA, ADAMTS1, FHIT, RUNXs, SIRT1, WWOX

Introduction

Colorectal cancer (CRC) is a complex heterogeneous disease and responsible for the 10 % of the cancer-related deaths [1]. Molecular pathogenesis of CRC involves gradual accumulation of mutations in the DNA repair genes, proto-oncogenes, and tumor suppressor genes [2]. Despite recent promising diagnostic and therapeutic methods, CRC-related mortality is very high all over the world. Hence, identifying novel biomarkers with diagnostic and therapeutic potentials for the diagnosis of CRC and developing alternative treatment methods is very important.

Moreover, it is believed that cancer arose from the accumulating mutations of oncogenes and tumor suppressor genes. These facts about the cancer pathogenesis have been changed by the discovery of novel regulatory molecules, called miRNAs [3-5]. miRNAs are a family of non-coding regulatory RNAs and regulate gene expression by targeting mRNAs [5]. These tiny RNA molecules play key roles in a variety of biological processes including cell proliferation, differentiation, angiogenesis, and apoptosis and they also participate in the invasion and metastasis of cancer cells [3-8].

Also, a growing number of evidence suggests that deregulated miRNA expressions are one of the important hallmarks of cancers including the CRC [4, 6, 7, 9]. miRNAs with oncogenic potentials have been well characterized in CRC [10]. For instance, miR-21-5p, miR-29-3p, and miR-148-3p were reported to be associate with CRC [11]. Additionally, expression of miR-29a-3p was shown to be elevated in CRC [12]. In addition, it is reported that expression levels of miR-148a-3p were significantly decreased in advanced stages of CRC [13].

Accordingly, it is well known that increased or decreased expression of a miRNA leads to deregulation of its target genes and results in complete deregulation of the regarding pathway. Thus, determining the interrelation between cancer-associated genes and miRNAs is very important and will provide much information for the CRC pathology. Thus, in the present study, we aimed to determine the expression levels of some cancer related genes and their regulatory miRNAs in FFPE tissue samples of CRC patients and normal FFPE tissues.

Material and Methods

Study Population and Sample Collection

In this study, cancerous tissue samples of 54 patients who were diagnosed with CRC and normal colon tissue samples of 42 healthy controls were included. The demographic characteristics and clinical findings of patients and controls were presented in Table 1. The study was ethically approved by the local ethics committee of Mugla Sitki Kocman University (accession number: 17.02.2015/02) in accordance with the ethical standards of Helsinki Declaration. All study participants gave a written informed consent prior to inclusion in the study. 5–20 μm thick, formalin-fixed, paraffin-embedded tissue sections of patients and controls were obtained from the pathology unit and stored at -20 until RNA isolations.

Determination of validated miRNA regulators

Determination of validated miRNAs that target A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1), fragile histidine triad protein (FHIT), repair of DNA double strand breaks RAD51, Runt-related transcription factor 1-3 (RUNX1-3) Sirtuin 1-2 (SIRT1-2), WW domain-containing oxidoreductase (WWOX) genes were carried out by literature scanning of earlier studies. Validated miRNAs of selected genes were presented in Table 2.

Isolation and Quantification of total RNA from FFPE Tissues

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were obtained from the archive of pathology unit. Total RNA isolation from FFPE samples were carried out by using miRNeasy FFPE Kit (QIAGEN Sample & Assay Technologies, Germany) according to instructions of the manufacturer. The concentration and purity of RNA samples were determined by using a spectrophotometer (NanoDrop ND-100, Thermo Fisher Scientific Inc. Wilmington, USA). Concentrations of RNA samples were adjusted according to spectrophotometric measurements and equal aliquots of samples were stored at -80 ºC.

cDNA Synthesis of RNA samples

The reverse transcriptions reactions were achieved by using Qiagen miScript II RT Kit (QIAGEN Sample & Assay Technologies, Germany). Single-stranded cDNA synthesis were carried out in accordance with the manufacturer's recommended protocols. Equal aliquots of cDNA samples were stored at -80 ºC until gene expression analysis.

Real-Time PCR (qPCR) by Fluidigm BioMark™ HD System

The expression levels of selected genes and miRNAs were held in high-throughput Fludigm BioMark™ HD System Real-Time PCR. Nano-technology based Fludigm Microfluidic Dynamic Array chips (Fluidigm, South San Francisco, Calif., USA) for 96 samples were used for the expression analysis. Briefly, diluted cDNA samples and reaction mixtures were mixed and loaded on chips. Also, primers pairs for the amplification of selected mRNAs and miRNAs were loaded on chips. The cDNA samples and primers were allowed to mix on Dynamic Array chips by the help of IFC (integrated fluidic circuits) machine (Fluidigm, South San Francisco, Calif., USA). Finally, chips were placed into Fludigm BioMark™ HD System Real-Time PCR for the analysis.

Collection of Expression data and Statistical analysis

Pre-analysis and collection of expression results were attained by using Fluidigm Real-Time PCR Analysis software (Fluidigm, South San Francisco, Calif., USA). Expression levels of mRNAs were normalized with *Beta-2-microglobulin* (B2M) gene. For the normalization of miRNA expressions SNORD61, SNORD68, SNORD72, and SNORD95 small RNAs were used. In the analysis of qRT-PCR results, $2^{-\Delta Ct}$ (ΔCt = Target gene – Reference gene) formula was used. Resulting data were statistically tested by using GraphPad Prism (v6.02) program and Wilcoxon signed rank test. Additionally, fold-change analysis were carried out for both mRNA and miRNA expressions. Fold-changes were analyzed by using online program of RT² Profiler PCR Array Data Analysis Version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php?target=upload). P values for all statistical tests were two-tailed and p <0.05 accepted as statistically significant.

Results

Differential expression of cancer-associated genes

To determine the expression levels of ADAMTS1, RAD51, RUNX1, RUNX2, RUNX3, SIRT1, SIRT2, FHIT and WWOX gene a high-throughput real-time PCR method was used. As a result of the expression analysis of selected genes, ADAMTS1, RAD51, RUNX1, RUNX2, RUNX3, SIRT1, and WWOX were shown to be significantly upregulated in CRC tissues as compared to normal tissues samples (Figure 1, Table 3, Figure 2). In particular, a 51-fold increase was detected in ADAMTS1 gene by fold-change analysis (p=0,000014). In addition, fold-change elevations of RAD51, RUNX1, RUNX2, SIRT1, and WWOX were found to be 9, 19, 17, 6, and 3, respectively (Figure 1, Table 3, Figure 2). In addition to,

expression increase in RUNX3 gene was found to be 68-fold. On the other hand, FHIT and SIRT2 genes were found to be downregulated in CRC tissues as compared to normal tissues (Figure 1, Figure 2). Fold-change analysis of FHIT and SIRT2 genes revealed 7.5 and 2 increase, respectively (Table 3).

Differential expression of miRNAs that target cancer-associated genes

miRNAs that target selected cancer associated genes were analyzed for expression changes. Expression changes of miRNAs were presented in Figure 3 and Table 4. Expression of hsa-miR-378a-3p which targets ADAMTS1 was found to be downregulated in CRC tissues (Table 4). Additionally, expression levels of hsa-miR-143-3p which target FHIT and hsa-miR-148a-3p which target RUNX3 were found to be downregulated in CRC as compared to normal tissue samples (Table 4).

Moreover, validated miRNAs of RAD51 gene, hsa-miR-155-5p, -193b-3p, and -96-5p were found to be upregulated in CRC tissues. Also, expressions of hsa-miR-17-5p, -27a-3p, and -30c-5p which targets RUNX1 were upregulated in CRC tissues. In addition, expression levels of hsa-miR-133b, -203a, -205-5p, and -34c-5p which targets RUNX2 were determined to be upregulated. In addition to, miRNA regulators of RUNX3, hsa-miR-130a-3p and hsa-miR -301a-3p were found to be upregulated in CRC tissues. Expressions of hsa-miR-132-3p, -222-3p, -34a-5p which targets SIRT1 were found to be upregulated. Also, miRNAs of hsa-miR-134-5p, -29a-3p, -29b-3p that targets WWOX were shown to be upregulated. Lastly, hsa-miR-21-5p which targets SIRT2 were shown to be upregulated in CRC tissues (Figure 3, Table 4).

Discussion

Previous studies indicated that increased ADAMTS1 activity stimulates tumor promoter events such as increased proliferative signaling, inhibition of apoptosis and increased metastatic potential [14]. They also reported tumor suppressor properties of ADAMTS1. However, in present study, expression levels of ADAMTS1 were found to be elevated in CRC tissues. Consistent with the findings of pro-tumorigenic functions of ADAMTS1, our study results strongly suggest ADAMTS1 as a tumor promoter gene in CRC. Also, in a study, miR-378-3p was found to be negative regulator of ADAMTS1 in granulosa cells [15]. Our study results revealed that miR-378-3p was downregulated in CRC tissues as compared to normal tissues. Suggesting that miR-378-3p may have a key role in the CRC pathogenesis through

regulating ADAMTS1. Downregulation of miR-378-3p results in the upregulation of one of its targets, ADAMTS1. Yet, these findings must be supported by the future investigations of miRNA mimic and inhibitor studies.

FHIT is one of the tumor suppressor gene which is commonly downregulated and/or lost in various types of malignancies [16]. Consistent with the results of the previous reports, in our study, the expression of FHIT gene was shown to be significantly downregulated in CRC tissues. Indicating that FHIT may have important roles in the pathogenesis of CRC. Also, *Lin et. al.* reported that FHIT is one of the direct target of miR-143-3p [17]. Also, expression level of miR-143-3p was also found to be insignificantly downregulated in CRC tissues.

Deregulation of DNA repair system plays a central role in the carcinogenesis [18]. DNA double-strand break repair and homologous recombination protein Rad51 was reported to be frequently overexpressed in cancers [19]. Consistent with the previous reports, RAD51 was upregulated in CRC tissues compared to normal tissues. However, expression changes in RAD51 gene was found to be statistically insignificant. In addition, several miRNAs that target Rad51 were found to be upregulated in CRC tissues. *Wang et. al.* reposted miR-96-5p as a direct target of RAD51. Overexpression studies of miR-96-5p resulted significant downregulation of RAD51 gene [20]. However, in our study, expression of miR-96-5p was found to be significantly overexpressed in CRC tissues. Indicating potential role of this miRNA in the CRC pathogenesis, but not dependent to RAD51 expression. In another study, RAD51 gene was found to be one of the target of miR-155 [21]. Also, miR-155 is well known of fine-tuner of inflammatory responses and frequently deregulated in various human cancers [22]. In breast cancer, overexpression of miR-155 was correlated with downregulation RAD51 gene expression [21]. Consistent with previous studies, miR-155 is found to be significantly upregulated in CRC tissues. Indicating this miRNA plays key role in the CRC progression and may be associated with the cancer related inflammation (CRI). However, RAD51 overexpression may be independent from miR-155 overexpression. As a result, these findings have to be confirmed by the independent future studies to clearly understand RAD51 and miR-155 interaction. Furthermore, inhibition of miR-193b expression was found to be associated with the RAD51 overexpression in ovarian cancer [23]. Also, it was reported that miR-193b overexpression decreased the RAD51 expression [23]. In our study, miR-193b was found to be significantly overexpressed in CRC tissues. Suggesting possible clinical significance of this miRNA in CRC physiopathology. However, RAD51 overexpression seems not to be related with miR-193b overexpression.

Runt-related transcription factors (RUNXs) have been implicated in tumor development [24]. There are three RUNX genes in mammalian cells designed as RUNX1-3 and they have tissue specific expression patterns [25]. They have been shown to play key roles in the process of carcinogenesis [25]. Also, several studies reported the relationship between RUNX genes and cancer-associated miRNAs [26-29]. It was reported that miR-17-5p targets 3′UTR of RUNX1 gene and negatively regulates its expression. In particular, anti-miR-27 transduction experiments revealed significant upregulation of RUNX1 in granulocytes [27] . In the present study, expressions of both miR-17-5p and its target RUNX1 were found to be upregulated. However, statistical analysis of RUNX1 expression changes was insignificant. On the other hand, elevated expression of miR-17-5p was shown to be statistically significant. Suggesting an important role of miR-17-5p in the colorectal carcinogenesis, yet this expression increase is independent from RUNX1. Similarly, expression of miR-30c-5p which targets RUNX1 [26] was found to be increased in CRC tissues. However, miR-30c-5p expression change was statistically insignificant.

Interestingly, expression levels of miR-203a, miR-133b, miR-205-5p and miR-34c-5p was found to be elevated in CRC tissues. All of these miRNAs are known to target RUNX2 gene. Studies investigated that downregulation of miR-203a is correlated with increased cell proliferation, metastasis and invasion. [30]. Also, previous studies reported miR-203a as potential biomarker in metastatic prostate cancer [30]. In addition, miR-133b was reported to inhibit RUNX2 translation [31]. In addition to, Zhang et. al. reported that miR-34c-5p strongly inhibits RUNX2 and miR-205-5p moderately inhibits RUNX2 translation [32]. In our study, expression levels of RUNX2 were found to be insignificantly upregulated in CRC tissues. Suggesting that miR-203a, miR-133b, miR-205-5p and miR-34c-5p miRNAs have important roles in CRC progression. However, their mechanism of action is not related to RUNX2 gene.

Furthermore, expression of RUNX3 was found to be upregulated in our study. miR-130a, miR-148a and miR-301a-3p were reported to be target RUNX3 gene. In a study, ectopic expression of miR-148a was reported to be interfere with proliferation of gastric cancer cells [33]. As it is correlated with overexpression of RUNX3, miR-148a expression is downregulated in CRC tissues in our study. However, this expression change was statistically insignificant. On the other hand, expression of miR-130a and miR-301a-3p were shown to be significantly upregulated in our study. Indicating potential role of these miRNAs in CRC physiopathology.

Silent information regulator 1 (SIRT1) is a well known member of sirtuin family of genes [34]. SIRT1 is NAD-dependent histone deacetylase which is activated in response to oxidative and genotoxic stresses [35]. Also, it is known to participate in a variety of physiological processes including cell proliferation, inflammatory responses, cell cycle and cell migration [35]. SIRT1 have been reported to have dual functions in tumor development [35-37]. In some studies, increased SIRT1 activity was reported to be associate with tumor promoting events [37, 38]. As consistent with these findings, SIRT1 expression was shown to be elevated in our study. However, this expression change was statistically insignificant. On the other hand, SIRT1 targeting miRNAs; miR-132, miR-222 and miR-34a-5p were found to be significantly upregulated in CRC tissues. In previous studies, increased miR-222 expression was reported in prostate cancer cell lines (PC3 and LNcaP) [39]. Downregulation of miR-222 was shown to inhibit cell proliferation, migration and increase apoptotic cell death [39]. Also, miR-34a activity was reported to have tumor suppressor effects in breast cancer [40]. All in all, these miRNAs appears to be significant regulators of CRC pathology.

Another well characterized member of the sirtuin family of genes is SIRT2 and it was reported to be downregulated in glioma tissues and various cancer cell lines [41]. Also, miR-21 have been shown to target SIRT2 and regulate its expression [41]. In our study, an inverse relationship was found SIRT2 and miR-21-5p expression. In particular, expression of SIRT2 was reduced and miR-21-5p was increased in CRC tissues. Indicating deregulation of SIRT2-miR-21-5p axis may contribute to CRC physiopathology.

Lastly, WW domain-containing oxidoreductase (WWOX) is known to be tumor suppressor gene [42]. In our study, WWOX expression was found to be insignificantly upregulated. Several WWOX targeting miRNAs have been shown to be associate with cancers. In a study, miR-134 was shown to be upregulated in head and neck squamous cell carcinoma (HNSCC) and negatively regulate WWOX expression. Also, miR-29s (-29a,-29b) were shown to be upregulated in lung cancer cell lines and their expression inversely associated with WWOX expression. In our study, mir-29a-3p and miR-29b-3p were found to be significantly upregulated and miR-134-5p insignificantly upregulated in CRC tissues. Suggesting that mir-29a-3p and miR-29b-3p are the important contributors of CRC physiopathology.

Conclusions

In conclusion, significant interactions between miRNAs and their target genes were determined. The findings of the present study will shed light into future upcoming studies to understand more about CRC physiopathology. On the other hand, there are several limitations of our study. One of them is the lack of protein expression data and the validation of the protein expression of selected genes will be great of interest for the future investigators. Also, the results of the present study have to be supported by the expression of selected genes and miRNAs in CRC cell lines. Moreover, in the future studies the results of this study have to be confirmed in an independent cohort form different ethnic groups. In addition to, miRNA mimicry and inhibitory studies in cell lines will be more informative for to understand these miRNA-mRNA interactions.

Acknowledgements

Declaration of interest

The authors report no conflicts of interest.

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Table 1: Demographic and clinical characteristics of patients and controls

Colorectal Cancer Patients		n=54 (%)
	> 60	34 (62,96)
Age	40-60	20 (37,04)
	Female	20 (37,04)
Gender	Male	34 (62,96)
	Right Colon	10 (18,52)
	Sigmoid Colon	14 (25,93)
Localization	Rectum	30 (55,56)
	Positive	7 (12,96)
	Negative	16 (29,63)
Lenf nodule	Non specified	31 (57,41)
	II	20 (37,04)
	III	25 (46,3)
Stage	IV	9 (16,67)
Controls		**n=42 (%)**
	> 60	12 (28,57)
Age	40-60	30 (71,43)
	Female	17 (40,48)
Gender	Male	25 (59,52)

Table 2. Selected cancer-associated genes and their validated miRNAs

No	Target Gene	Functions	miRNA	References
1	**ADAMTS1**	Tumor promoter	hsa-miR-378a-3p	[15]
2	**FHIT**	Tumor suppressor	hsa-miR-143	[17]
3	**RAD51**	DNA during double strand break repair	hsa-miR-155-5p, -193b-3p, -96-5p	[20, 21, 23]
4	**RUNX1**	Transcription factor	hsa-miR-17-5p,-27a-3p, -30c-5p	[26, 27, 29]
5	**RUNX2**	Transcription factor	hsa-miR-133b, -203a, -205-5p, -34c-5p	[30-32]
6	**RUNX3**	Transcription factor	hsa-miR-130a-3p, -148a-3p, -301a-3p	[33, 43-46]
7	**SIRT1**	Anti-apoptotic protein	hsa-miR-132-3p, -222-3p, -34a-5p	[39, 47, 48]
8	**SIRT2**	Survival, cell cycle regulation	hsa-miR-21-5p	[41]
9	**WWOX**	Tumor suppressor	hsa-miR-134-5p, -29a-3p, -29b-3p	[49, 50]

Table 3. Fold change analysis of selected genes

Genes	Fold change	p value	$2^{-\Delta Ct}$ p value	Up/Down regule
ADAMTS1	51,1776	**0,000014**	**0,0005**	Upregule
FHIT	-7,5813	**0,001089**	**0,0137**	Downregule
RAD51	8,9475	0,80972	0,1506	Upregule
RUNX1	19,0399	0,520305	**0,0032**	Upregule
RUNX2	16,8635	0,838392	0,1242	Upregule
RUNX3	68,5737	**0,000616**	**0,0030**	Upregule
SIRT1	6,5697	0,979087	**0,0335**	Upregule
SIRT2	-2,0031	0,278904	0,2942	Downregule
WWOX	3,5157	0,416325	**0,0055**	Upregule

Table 4. Fold change analysis of miRNAs and representation of their target mRNAs.

Target gene	miRNA	Fold change	p value	Up/Down regule
ADAMTS1	hsa-miR-378a-3p	-2,0464	**0,013107**	Down-regule
FHIT	hsa-miR-143-3p	-2,0226	0,098385	Downregule
RAD51	hsa-miR-155-5p	9,5344	**0,003666**	Upregule
RAD51	hsa-miR-193b-3p	2,0226	**0,03595**	Upregule
RAD51	hsa-miR-96-5p	29,9234	**0,0010975**	Upregule
RUNX1	hsa-miR-17-5p	8,4896	**0,001783**	Upregule
RUNX1	hsa-miR-30c-5p	1,4967	0,098385	Upregule
RUNX1	hsa-miR-27a-3p	2,1788	**0,007623**	Upregule
RUNX2	hsa-miR-133b	1,9819	**0,019298**	Upregule
RUNX2	hsa-miR-203a	8,5246	**0,000345**	Upregule
RUNX2	hsa-miR-205-5p	4,1074	**0,005377**	Upregule
RUNX2	hsa-miR-34c-5p	2,1593	**0,008549**	Upregule
RUNX3	hsa-miR-130a-3p	3,4678	**0,003971**	Upregule
RUNX3	hsa-miR-148a-3p	-2,0226	0,482742	Downregule
RUNX3	hsa-miR-301a-3p	6,3519	**0,019431**	Upregule
SIRT1	hsa-miR-132-3p	2,9894	**0,034038**	Upregule
SIRT1	hsa-miR-222-3p	3,6624	**0,025468**	Upregule
SIRT1	hsa-miR-34a-5p	2,1307	**0,004859**	Upregule
SIRT2	hsa-miR-21-5p	1,3418	**0,017928**	Upregule
WWOX	hsa-miR-134-5p	4,5984	0,05832	Upregule
WWOX	hsa-miR-29a-3p	3,1147	**0,00046**	Upregule
WWOX	hsa-miR-29b-3p	1,951	**0,013832**	Upregule

Figure legends

Figure 1. Representation of the expression of selected genes. ADAMTS1, RAD51, RUNX1-3 SIRT1 and WWOX genes were shown to be upregulated and FHIT and SIRT2 were shown to be downregulated. P: Colorectal cancer patients, C: Normal colon tissue. *$p<0.05$ accepted as significant.

Figure 2. Representation of the expression levels of selected genes in CRC tissues group according to the fold-change analysis. Results were compared to control group. $p<0.05$ accepted as significant.

Figure 3. Representation of the differential expression of miRNAs in CRC tissues as compared to control group. These miRNAs are the validated targets of selected genes. $p<0.05$ accepted as significant.
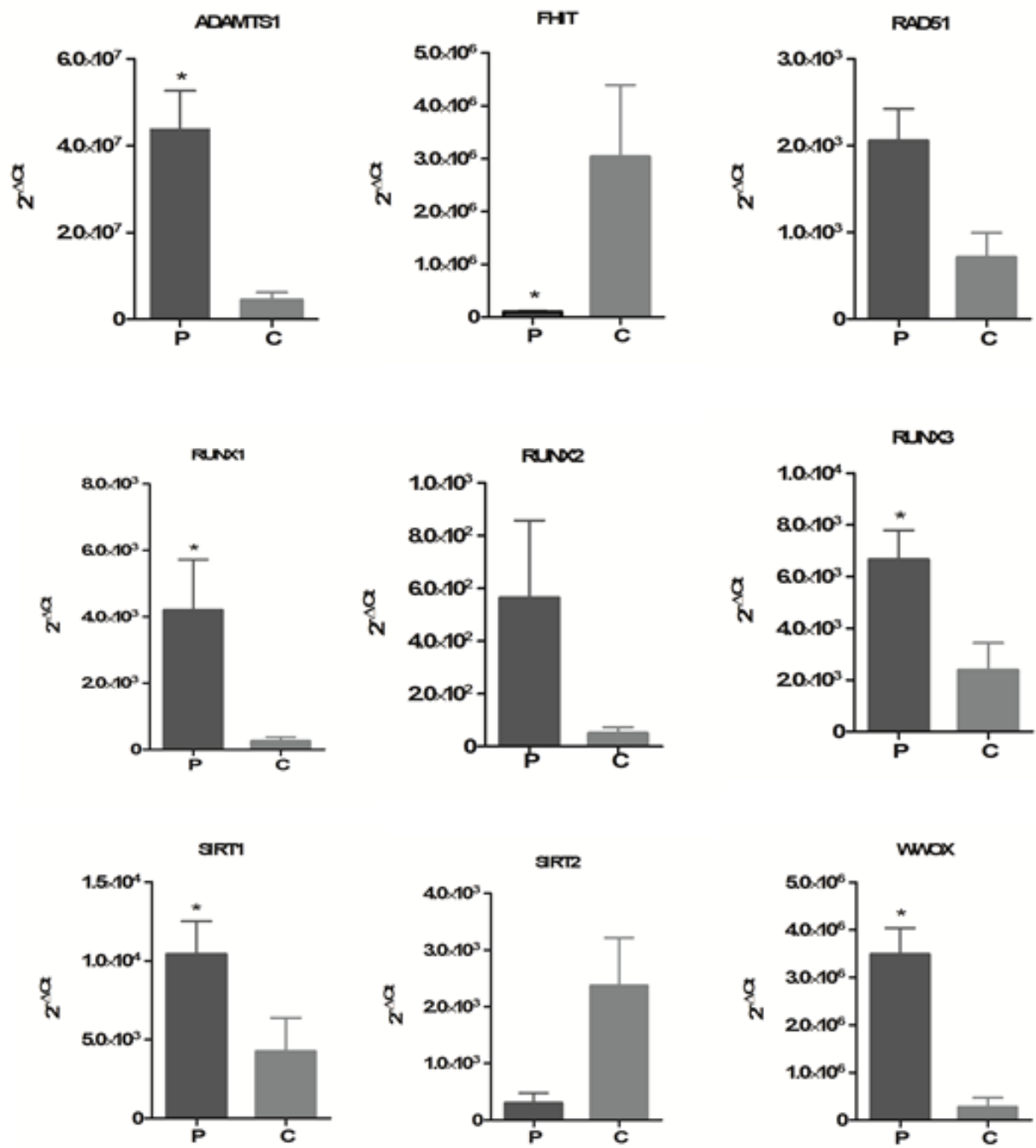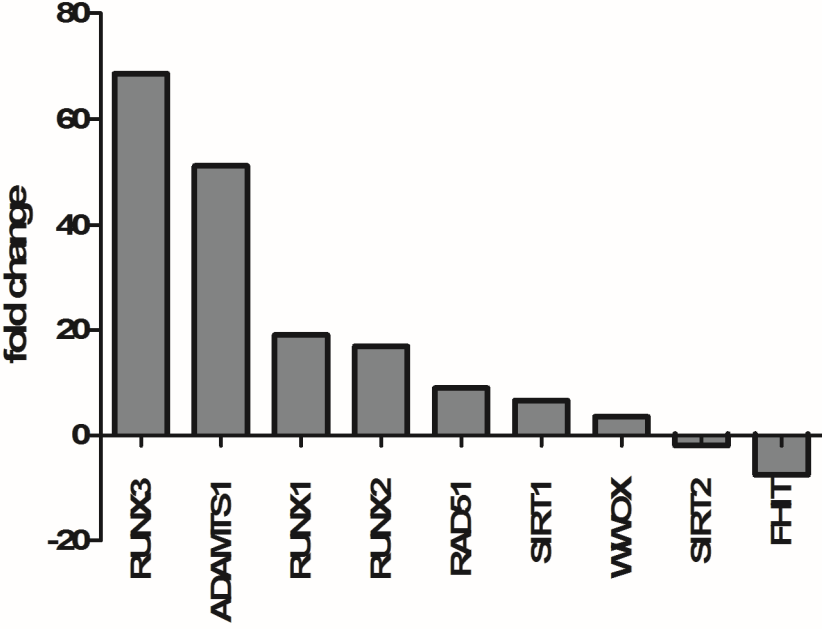
Figure 1.

Figure 2.

Figure 3.