



miRNA Profile and Bioinformatic Analysis for Diagnosis in Patients with Stage IIIA Colon Cancer

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Received: 31 August 2020 / Accepted: 9 June 2021 / Published online: 19 June 2021

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Abstract

Early diagnosis is a critical factor in deciding the outcome of colon cancer, as is the case with other types of cancers. Recent scientific developments have enabled the use of biomarkers for diagnosis and for designing treatment strategies for various cancer types. Further, identification of potential targets of these biomarkers will facilitate a better understanding of molecular processes. The aim of this study is to analyze microRNA expression profile, and through bioinformatic analyses determine the cellular processes of potential target genes and understand their molecular mechanism in stage IIIA colon cancer patients. The microRNA expression profiles of both normal and tumor tissues of seven patients were analyzed using the Affymetrix microarray system. The target genes were identified by performing a KEGG pathway analysis on eight miRNAs (hsa-miR-362-3p, hsa-miR-34c-5p, hsa-miR-34c-3p, hsa-miR-34a-3p, hsa-miR-19b-1-3p, hsa-miR-371a-5p, hsa-miR-941 and hsa-miR-7-5p), which were selected through an array scan by using DIANA-miRPath v.3 bioinformatic analysis tool. Biological pathway and cellular component analyses were performed on 30 genes targeted by miRNAs using FunRich Gene Enrichment tool. These analyses indicated that the genes targeted by these eight miRNAs played a role in either cell communication (53%), signal transduction (60%) or apoptosis (20%) in stage IIIA colon cancer. Taken together, these data suggest that these miRNAs can be used as biomarkers in Stage IIIA colon cancer.

Keywords Colorectal cancer · Biomarker · Stage IIIA · Bioinformatic analysis · miRNA

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Introduction

Understanding the molecular mechanism of colorectal cancer enables the use of new biomarkers for diagnosis and treatment (Xiao et al. 2019). As the tumor is disseminated but not metastasized in stage IIIA colon cancer, biomarkers can improve the treatment outcome (Jepsen et al. 2016; Ahmed et al. 2019).

Recent studies have reported that microRNAs play a significant role in the development of cancer and in anti-cancer therapy outcomes (Zhang et al. 2017). In the last 10 years, studies have increased on the relationship of miRNAs to cancer formation mechanisms and their ability to be used as biomarkers at an early stage (Falzone et al. 2019). MicroRNAs (miRNAs) are single-stranded RNAs containing about 20–23 nucleotides, that regulate post-transcriptional gene expression by mainly binding to 3' UTR region of target mRNAs (Pang et al. 2010). By binding miRNAs to target mRNAs, it can lead to degradation of mRNAs, thereby affecting translation suppression and regulation of expression. miRNAs are known to play an important role in the regulation of key biological processes including cell growth, cell differentiation, and apoptosis (Yang et al. 2016; Bajan and Hutvanger 2014). In the process of CRC formation, miRNAs regulate many molecular pathways by taking many oncogenic or tumor suppressor roles [9, 10]. miRNAs regulate the proteins (such as Wnt/beta-catenin, phosphatidylinositol-3 kinase pathway, KRAS, p53), which are one of the important signal pathways in colorectal cancer formation (Faber et al. 2009; To et al. 2018). Studies in this field have gained speed, especially since it can help determine the course of chemotherapy with the use of prognostics in the diagnosis before metastasis (Masuda et al. 2017).

It has been reported that differential expression levels of miRNAs can be associated with the stage, molecular sub-type, and clinical characteristics of the tumor. These reports indicate the potential application of miRNA expression levels as biomarkers in colorectal cancers (Baraniskin et al. 2012; Schetter et al. 2012). For this, it is necessary to determine the expression differences of miRNAs in carcinomas. The miRNA profiles obtained will allow the understanding of the molecular mechanism of the genes affected by these miRNAs by bioinformatic calculations.

In this study, we have attempted to profile miRNA using microarray analysis on normal and tumor tissues of seven patients with stage IIIA colon cancer. Further, we attempt to perform a bioinformatic assessment for the selection of miRNAs that might be critical in the pathogenesis of colon cancer. In addition, we intended to establish the correlation between histopathological features of colon cancer and the expression levels of miRNAs.

Material and Methods

Microarray Analysis

This study was performed using tissues obtained from Turkey Gaziantep University Faculty of Medicine, Research and Application Hospital, Department of General Surgery. Ethics committee approval was obtained for the study by Harran University Ethics committee with the number 2019/02/12. Seven patients with colorectal carcinoma who met the relevant criteria under Stage IIIA were included in the study. Both control and tumor tissues of around 0.5 cm³ in size, collected from patients for the study were preserved at – 80 °C in 200 ml RNAlater solution. GeneChip miRNA 4.0 Array (Affymetrix, USA) was used for this study, according to the manufacturer's protocol. miRNAs were isolated from tissue samples that were homogenized in QIAzol (Qiagen, Germany) at 4 °C, using Qiagen miRNeasy (Qiagen, Germany) mini kit. The quality of isolated RNAs was measured using a nano-spectrophotometer (Implen, Germany). The miRNAs were profiled using the commercial GeneChip miRNA expression 4.0 Microarray kit on Affymetrix Microarray (Affymetrix, Santa Clara, CA, USA) system. As per the recommendation of manufacturer, the below mentioned steps were followed for RNA Labeling, Poly(A) Tailing and Ligation, and Hybridization protocol: Briefly, 1 µg of RNA was labeled using a FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, USA). The labeled RNA samples were loaded onto microarray chips and hybridization was performed with continuous agitation using a GeneChip Hybridization Control Kit (Affymetrix, USA) at 48 °C at 60 rpm for 16 h (Gungormez et al. 2019). Then, microarray chips were washed and stained on a Fluidics Station 450 with AGCC Fluidics Control Software. Fluorescence from the array was scanned using an Affymetrix® GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA). Finally, the hybridization data (CEL files) was normalized by the Affymetrix GeneChip Command Console software and imported into probe-level summarization files (*CHP files) for data extraction. Analyses of these CHP files were performed using Affymetrix® GeneChip Command Console® Software (AGCC). CEL and CHP extension files and patient clinical information were uploaded to GEO (<https://www.ncbi.nlm.nih.gov/geo/>). The data was recorded by GEO with the number GSE136020.

Initially, image analyzes were performed with AGCC software where the quality of the images exited from the scanner was determined in the microarray analysis and the artifacts on the array were excluded. Then, data obtained from the analysis were statistically normalized. After log₂ conversion of the signal intensities of the normalized arrays, the data were compared statistically. According to the results of one-way analysis of variance (ANOVA), miRNAs were detected in the array results showing statistically significant ($p < 0.05$) increase and decrease in expression level between the control and tumor groups.

Pathway and Target Gene Analysis

Meta-analysis was performed using multiple software in connection with bioinformatics analysis.

DIANA-mirPath is a tool for predicting miRNA targets (in CDS or 3' UTR regions) that uses the DIANA-microT-CDS algorithm or the experimentally validated miRNA interactions derived from DIANA-TarBase for its functioning (Web site Diana tool). These interactions (predicted and/or validated) are subsequently combined with sophisticated merging and meta-analysis algorithms to determine target genes and pathways. A KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was performed on the genes targeted by differentially expressed miRNAs by using DIANA-miRPath v3.0 tool.

FunRich Gene Enrichment Analysis

FunRich (Functional enrichment) is a stand-alone software tool used mainly for functional enrichment and interaction network analysis of genes and proteins (Gungormez et al. 2019). FunRich Gene enrichment analysis tool was used for biological pathway and biological process analyzes which are functional analysis with genes targeted by 8 miRNAs registered in DIANA database (Diana web site accessed 2019).

Analysis and Construction of Protein–Protein Interactions (PPI) Network

STRING is a database of direct (physical) and indirect (functional) relationships of known and predicted protein interactions. Interactions of target genes were shown by using String Functional protein–protein association Networks (<https://string-db.org/>).

cBioPortal Database Analysis

CBio Cancer Genomics Portal (cBioPortal) (<http://cbioportal.org>) is a type of software that integrates genomic data such as mRNA and microRNA (miRNA) expressions, DNA methylation-mutation, protein enrichment.

In Bowel at Colorectal Adenocarcinoma (DFCI, Cell Reports 2016), Colorectal Adenocarcinoma (Genentech, Nature 2012), Colon Adenocarcinoma (CaseCCC, PNAS 2015), Colon Cancer (CPTAC-2 Prospective, Cell 2019) 832 samples analyses in 30 genes were performed in the OncoPrint and histogram using cBioPortal V3.6.8.

Results

Array Result

This study was conducted to profile differentially expressed miRNAs in Stage IIIA colon cancer samples and to determine their target genes and their biological processes. Tumor and normal (control-clean surgical limits) colon tissues of seven

Table 1 Clinical information of colon cancer stage IIIA patients included in the study

| Characteristics (<i>n</i> = 7, control and tumor tissue) | Number | |
|--|------------|------------|
| Gender | Male | 6 |
| | Female | 1 |
| Age (age range) | | 53 (51–57) |
| Locations of Tumors | Colon | 6 |
| | Rectum | 1 |
| Pathologic T classification | T1 | 2 |
| | T2 | 5 |
| | T3 | 0 |
| Pathologic N classification | N0 | 0 |
| | N1 | 7 |
| | N2 | 0 |
| Metastatic classification | M0 | 7 |
| | M1 | 0 |
| AJCC classification | Stage IIIA | 7 |

patients who were diagnosed with colorectal cancer by laboratory and pathological findings as well as clinical evaluation were chosen for this study. Information about these patients is provided in Table 1.

Array scanning was performed with GeneChip[®] miRNA 4.0 Array (Affymetrix, USA) containing a total of 30,424 mature miRNA probes, 2,578 human mature miRNAs and 2,025 human pre-miRNAs, that are present on normal and tumor tissues of seven patients, and miRNA profiles were compared. Values of the fold-change, chromosomal location, and genomic position of miRNAs according to the array analysis, are shown in Table 2.

As a result of the comparison of the control and tumor groups, miRNAs were listed which gave the best results with $p < 0.05$ and log fold change value of at least 2. The expression variation was calculated by ANOVA based on logarithmic values in the AGCC software, and statistically, the fold change was at least 2 and $p < 0.05$. Cluster Analysis provides the collection of miRNAs with similar expression patterns into a single group. Hierarchical clustering analysis was performed to monitor the miRNA expression pattern between the groups. The red low expressed area shows the green high expressed miRNAs.

Of the 13 miRNAs that were found to be differentially expressed according to the analysis results, 10 miRNAs (hsa-miR-362-3p, hsa-miR-34c-5p, hsa-miR-34c-3p, hsa-miR-34a-3p, hsa-miR-19b-1-3p, hsa-miR-2276, hsa-miR-371a-5p, hsa-miR-941, hsa-miR-7-5p, and hsa-miR-1280) increased while expression of 3 miRNAs (hsa-miR-10b-5p, hsa-miR-451, and hsa-miR-486) decreased (Fig. 1). Taken together, our study provides evidence for the first time that, of 13 differentially expressed miRNAs, the expression of hsa-miR-2276, hsa-miR-451 and hsa-miR-486 have been altered in stage IIIA colon cancer. These three miRNAs have not yet been recorded for GEO software using the Mirbase database. The first registration of these miRNAs was set using GEO with the number GSE136020.

Table 2 Information of miRNAs whose expression of the array changes

| Transcript ID (array design) | Accession number | Fold change (linear) (tumor vs. control) | ANOVA p-value (tumor vs control)* | Chromosomal location | Genomic position |
|------------------------------|------------------|--|-----------------------------------|----------------------|-------------------------|
| hsa-miR-10b-5p | MIMAT0000254 | - 3.34 | 0.005 | Chr 2 | 177,015,031–177,015,140 |
| hsa-miR-362-3p | MIMAT0000705 | 2.96 | 0.0106 | Chr X | 49,773,572–49,773,636 |
| hsa-miR-34c-5p | MIMAT0000686 | 5.19 | 0.0125 | Chr 11 | 111,384,164–111,384,240 |
| hsa-miR-34c-3p | MIMAT0004677 | 2.08 | 0.0253 | Chr 11 | 111,384,164–111,384,240 |
| hsa-mir-34a-3p | MIMAT0000255 | 2.29 | 0.0301 | Chr 1 | 9,211,727–9,211,836 |
| hsa-miR-19b-1-3p | MIMAT0004491 | 2.41 | 0.0304 | Chr 13 | 91,351,192–91,351,278 |
| hsa-miR-371a-5p | MIMAT0004687 | 2.02 | 0.0478 | Chr 19 | 54,290,929–54,290,995 |
| hsa-miR-941 | MIMAT0004984 | 11.69 | 0.0480 | Chr 20 | 63,919,449–63,919,520 |
| hsa-miR-7-5p | MIMAT0000252 | 9.22 | 0.0489 | Chr 9 | 83,969,748–83,969,857 |
| hsa-miR-1280 | MIMAT0019074 | 2.80 | 0.0492 | Chr 3 | 128,081,008–128,081,101 |
| hsa-miR-2276 | MIMAT0011775 | 2.71 | 0.0369 | Chr 13 | 24,736,555–24,736,643 |
| hsa-miR-451a | MIMAT0001631 | - 7.92 | 0.0435 | Chr 17 | 27,188,387–27,188,458 |
| hsa-miR-486-5p | MIMAT0002177 | - 5.05 | 0.0475 | Chr 8 | 41,517,959–41,518,026 |

* $p < 0.05$

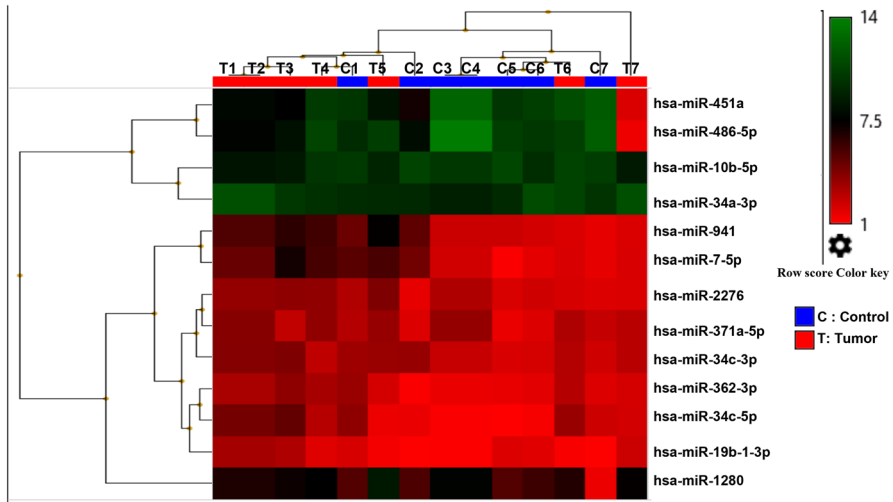


Fig. 1 Hierarchical clustering of miRNAs that were statistically significant ($p \leq 0.05$ fold change ± 2) as a result of array screening in 7 patients. Tumor: T (red labeled) and control: C (blue labeled) (Color figure online)

Bioinformatic Analyses

KEGG analysis of downregulated 3 miRNAs (hsa-miR-10b-5p, hsa-miR-451, and hsa-miR-486) was performed with DIANA miRpath software. As a result of this analysis, it was determined that these miRNAs were linked with colorectal cancer on 7 genes (BRAF, BCL2, TP53, SMAD4, MSH6, MYC and CASP9) ($p < 0.05$).

Of 13 differentially expressed miRNAs, eight miRNAs (hsa-miR-362-3p, hsa-miR-34c-5p, hsa-miR-34c-3p, hsa-miR-34a, hsa-miR-19b-1-3p, hsa-miR-371a-5p, hsa-miR-941 and hsa-miR-7-5p) recognized in DIANA-miRPath database were selected and their targets in colorectal cancer were determined via DIANA-miRPath analysis. The analysis indicated that 30 genes were targeted by these miRNAs. Since the genes targeted by these three miRNAs with decreased expression are included in these 30 genes, the analyzes were continued with 30 genes. The biological pathways and biological process properties of 30 genes targeted by all eight miRNAs selected by expression different from FunRich Gene Enrichment analysis are shown in Fig. 2. As a result of this analysis, it was seen that these genes influenced cell communication (53%), signal transduction (60%), and apoptosis (20%) among the biological processes (Fig. 2A). In terms of biological pathways, these 30 targeted genes affected cell signaling pathways such as ALK1 and SMAD2/3 TGF-beta (Fig. 2B). When the PPI of the 30 targeted genes were examined, it was seen that the genes interacted with CCND1, BCS, and TP53 which play a crucial role in cellular signal transduction (Slattery et al. 2019), as seen in Fig. 3. In addition, the functional relationship of the target 30 gene to each other is shown in relation to colors. The TP53 gene seems to function as the hub gene of expression, as many genes interact in many functions such

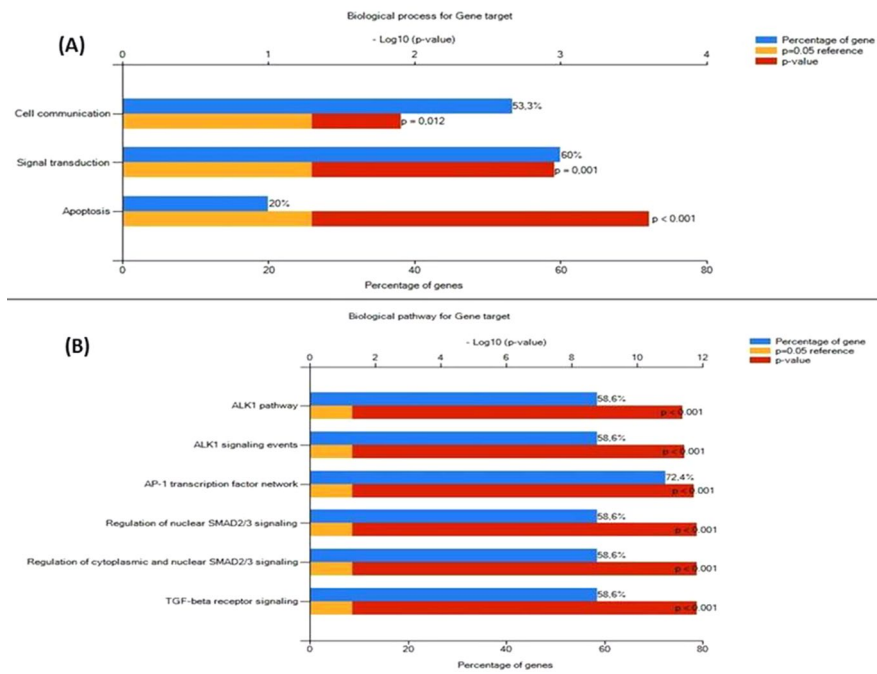


Fig. 2 Identified biological processes **A** and biological pathway **B** analysis in the target gene using Fun- Rich Gene Enrichment

as activation, inhibition, and binding (Fig. 3). In the analysis of 30 genes with 832 samples with cBioPortal, it was shown that expression differences were affected by different mutation rates (Fig. 4).

Discussion

As CRC is a terminal cancer with a high incidence, the studies have been directed towards obtaining new markers for the diagnosis and treatment of CRC (Dong et al. 2019). The studies have shown that miRNAs are key molecules in multiple biological events such as cell proliferation and death mechanisms (Meng et al. 2015). With the understanding that miRNA might be involved in carcinogenesis, techniques that are used for detecting expression levels of miRNAs in specific cell types in different types of cancer have been started to be used also for identifying the potential functions of miRNAs in cancer (Meng et al. 2015; Zhang et al. 2019). In particular, a new perspective on cancer therapy has been gained through the identification and bioinformatic analyses of differentially expressed miRNAs, and identification of target genes and determining their roles in biological processes (Zhang et al. 2019; Chen et al. 2019a, b; Li et al. 2018; Han et al. 2018).

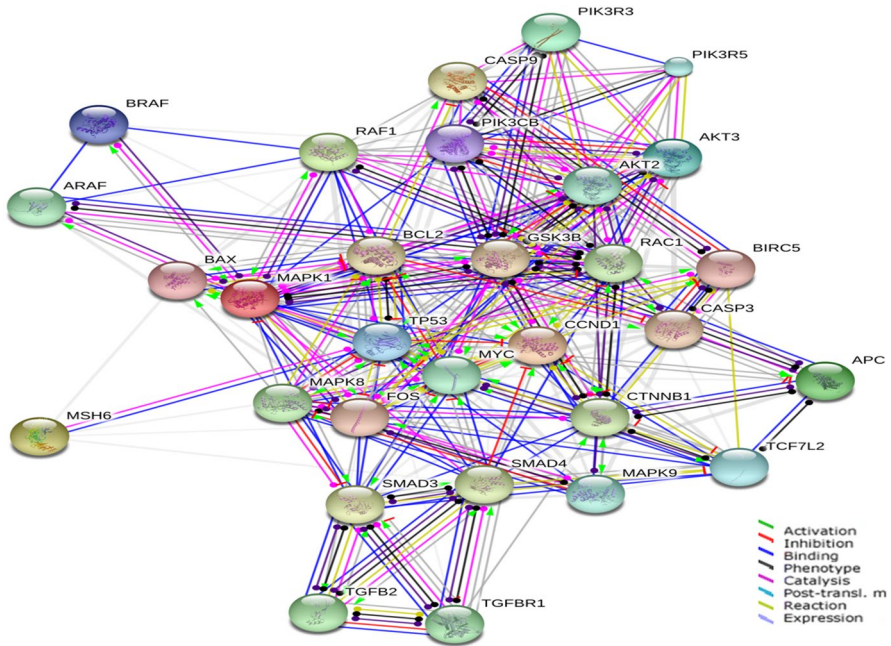


Fig. 3 PPI networks of the potential target genes of eight different expression miRNA. Interaction between 30 genes was demonstrated using STRING online software

First, Micheal et al. pioneered an increase in studies in this field by demonstrating the difference of expression of a miRNA in CRC cancer with downregulation of miR-143 and miR-145 (Micheal et al. 2003). Researches have revealed new approaches to the molecular mechanism by analyzing the microarray and sequencing data of miRNAs and bioinformatics calculation (Zhang et al. 2014; Low et al. 2017). For example, miRNA data from oral cancer can be analyzed and bioinformatic calculations show that miR-196a-5p and miR-196b-5p increased expression, the effect of miR-99a-5p decreased expression on molecular pathways. They suggested that they can be used as biomarkers (Falzone et al. 2019). Its most important role in the use of miRNAs as biomarkers comes from its being an oncogenic and tumor suppressor. However, this may not always be valid. Masuda et al. reported that miR-27a was both a tumor suppressor in the CRC and downregulated and targeted Stat3 and Smad2, as well as functioning as oncogenic miRNA (Masuda et al. 2017). In addition, miRNAs (miR-140, miR-215, miR-224 ve miR-20) have been shown to affect drug-agent communication in chemotherapy as well as the use of biomarkers only in early diagnosis, Masudda et al. and Chen et al. shown in the review.(Masuda et al. 2017; Chen et al. 2019a, b).

In this study, it was found that the expression levels of 13 miRNAs were altered when normal and tumor tissues of seven patients were compared to identify microRNAs that could be effective in the pathogenesis of colon cancer. The expression of 10 miRNAs increased while the expression of three miRNAs decreased. Bioinformatic assessment and meta-analysis were performed on

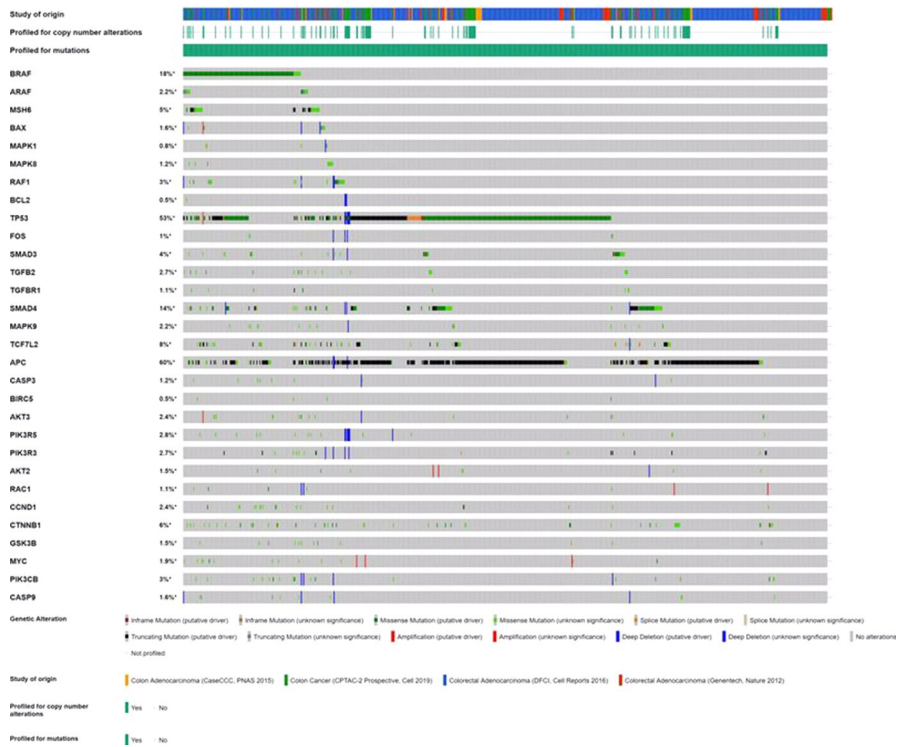


Fig. 4 cBioPortal Oncoprint analysis

shortlisted eight miRNAs. As a result of the bioinformatic analysis, it was found that the genes targeted by miRNA differentially expressed in stage IIIA colon cancer were regulating cellular signal transduction both in the biological pathway and biological process analyses.

The fact that *P53*, *BCL2*, and *TGFB2* genes, which were also targeted by eight miRNAs in our study, have antiproliferative and pro-apoptotic functions suggests that the miR-34 family might have great importance in terms of early diagnosis in stage IIIA colon cancer patients. Conrey et al. defined the miR-34 family [27] as a p53 effector and demonstrated that it had antiproliferative and pro-apoptotic functions (Conrey et al. 2007; Falzone et al. 2019; Wu et al. 2012). Grady et al. demonstrate in their studies that TGF-Beta receptors, one of the important pathways in carcinogenesis, target SMADs (Grady and Markowitz 2002). This supports our study of targeting SMADs as a result of the bioinformatic analysis of 8 miRNAs (*hsa-miR-362-3p*, *hsa-miR-34c-5p*, *hsa-miR-34c-3p*, *hsa-miR-34a*, *hsa-miR-19b-1-3p*, *hsa-miR-371a-5p*, *hsa-miR-941* and *hsa-miR-7-5p*). In addition, we stated in our study that miR-378 may be a determinative marker before metastasis in the array study we conducted in CRC Stage II patients (Gungormez et al. 2019). The expression of *hsa-miR-34a* one of the miRNAs selected for bioinformatic analysis increased in tumor tissue compared to normal tissue in colon cancer cells HCT116 and RKO and it directly affected the *P53* gene with an effect

that influenced on cell invasion and migration (Zhang et al. 2017; Ahmed et al. 2019). In their study on the various stages of colon cancer, Aherne et al. showed that the miR-34a and miR-7 expressions increased in correlation with the stages of colon cancer (Aherne et al. 2015).

It was shown that target genes obtained by the bioinformatic analysis were impinging particularly on cell communication, apoptosis and signal transduction in biological process. Another study on miR-34c in prostate cancer patients showed that increased expression of this miRNA induces apoptosis in tumor cells and also affects cell proliferation and invasion, a finding that supports our study (Krajewska et al. 2018; Rokhlin et al. 2008; Wu et al. 2013).

The highlights feature of our study was the serial scanning using technology from fresh frozen tissues taken from each patient's own tissue and normal tissue defined by surgical borders. Microarray technology is a technique based on the nucleotide base pairing (hybridization) principle. One of the most important features of this technique is that it makes it possible to determine the expression of all known human miRNAs in a single reaction. Taken together, in our study, a meta-analysis was performed by profiling miRNA isolated from normal and tumor tissues of stage IIIA colon cancer patients, and the target genes of the selected miRNAs and the impact of these miRNAs on biological processes was determined. Thus, identification of miRNAs during the early diagnosis of colorectal cancer, dissecting their effects on the biological processes and increasing the amount of research that focuses on using the target genes in cancer therapies will highly contribute to the understanding of the pathogenesis of cancer. The best of our knowledge, although the mechanism between miRNA and carcinoma, continues to be understood, there is no study that is used as a biomarker in the diagnosis of patients. To use miRNA as a marker in colorectal cancer, if necessary, in all types of cancer, it is possible by repeating the analysis and meta-analysis with various software. With this aspect, we will continue our studies and continue to offer our contributions.

Declarations

Conflict of interest None of the authors declare any competing interests in the matters related to this paper. All authors contributed to the study conception and design.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study. Ethics committee approval was obtained for the study by Harran University Ethics committee with the number 2019/02/12.

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