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Differential Expression of miRNAs in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the deadliest diseases in the world and current screening methods are still limited. In past decade, it has been discovered that miRNAs play an important role in every cell process including CRC initiation and progression. The aim of this study was to identify differentially expressed miRNAs in normal colon (CRN) and colorectal cancer (CRC) by using microarray system. This includes discovery of new miRNAs related to CRC. Total RNA was extracted from total 12 tissue samples of 7 patients with colorectal cancer undergoing surgical resection of the colon for studying tumor specific changes in miRNA expression compared to 5 matched as normal tissue samples using the Qiagen miRN easy Kit. MiRNA was polyadenylated by using PolyA Tailing master mix. After Flash Tag Biotin HSR Ligation samples were hybridized, stained and washed. The arrays were finally scanned using AGCC Scan control programmer according to the manufacturer's protocol of Affymetrix Gene Chip software. It was found that some of miRNAs were found up or down regulated in studied CRC tissues compared to non-tumor tissues. MiR-1201, miR-181a, miR-7, miR-188, miR-552, miR-183, miR-941, U71d, ACA3-2 and miR-34c were over expressed and miR-486, HBII-85-6, miR-550-1, miR-635, miR-10b, miR-550-2, ENSG00000238430 and miR-548a were down regulated in patients with CRC. We have also found that some dysregulated miRNAs, which to our knowledge have not previously been associated with colorectal carcinogenesis. Consequently, the results of this study will increase our understanding of development, progression and earlier detection and personnel treatment of colon cancer. This work was supported by grants from the Turkish Ministry of Development (2009K120780 and 2012K120690).

Keywords: Colon cancer; miRNAs; Microarray; Transcriptomics

Introduction

Colorectal cancer (CRC) is one of the deadliest diseases in the world and it is the second most common cause of cancer-related deaths in women, and the third in men. Although CRC mortality rates have declined in Western population, incidence rate of CRC has been increasing in Asia. Identified risk factors for CRC include: aging, gender, family history, lifestyle, pre-existing conditions such as, lynch syndrome, inflammatory bowel disease. CRC is most of the time asymptomatic until the late stages and can be only diagnosed when the cancer is already metastasized [1].

CRC was used to be considered as a disease caused by genetic mutations. However, it is currently viewed as a complex malignancy including epigenetic abnormalities in addition to genetic mutations. Three different pathogenic pathways have been identified which play role in CRC development: chromosomal instability, microsatellite instability and CpG island methylator phenotype. Chromosomal instability (CI) means deregulation of oncogenes such as, KRAS (functions as a transmitter of key extracellular signals) and TP53 (p53 functions as a key transcriptional regulator in cell cycle regulation) and also tumor suppressor genes. Microsatellite instability (MI) is caused by the inactivation of mismatch repair genes such as, hMLH1, hMSH2, hMSH6, and hPMS2. MI can result in Lynch syndrome and sporadic tumors. The CpG island methylator phenotype is caused by hypermethylation of CpG islands at tumor suppressors gene promotors [2,3].

The most common screening methods for CRC are colonoscopy, flexible sigmoidoscopy, guaiac-based fecal occult blood tests, and fecal immunochemical tests. Having the fact that these methods are still limited by unsatisfactory sensitivity and specificity; researchers have investigated the potential use of miRNAs as diagnostic and prognostic biomarkers for CRC screening [1].

During the past decade, it has been discovered that miRNAs play important roles in cancer initiation, progression and metastasis. Functionally, miRNAs can mediate cell proliferation, cell cycle progression, differentiation, metabolism, apoptosis, invasion to trigger tumor formation and angiogenesis [4].

MiRNA is a ≈ 20 nucleotides long non-protein-coding single-stranded RNA molecule. They are involved in almost all biological pathways, including gene expression, cell cycle regulation, cellular development, proliferation, differentiation and apoptosis [5-9].

MiRNAs are transcribed by RNA polymerase II in nucleus into long primary transcripts of miRNA as hairpins (pri-miRNAs, 1-3 kilobases). Nuclear RNase III enzyme (also called Drosha) cleaves pri-miRNA into a precursor (pre-miRNA, around ≈ 70 nucleotides in length) hairpin-shaped dsRNA. Pre-miRNAs are then exported from nucleus into cytoplasm by a nuclear export factor, Exportin-5 (XPO5). Then, protein complex of RNase III, Dicer, trims pre-miRNA in the

cytoplasm to produce mature double-stranded (miRNA/miRNA complex) miRNA duplexes (≈ 20 nucleotides in length). Next, these two strands of the duplexes are unwounded by helicase, and then combined with RNA-induced silencing complex (RISC) containing an argonaut protein. One strand of the duplex is removed and degraded while the other one (single-stranded miRNA, ssmiRNA) is actively interacts with target mRNA to regulate the expression [6,8-13]. The seed region (6-8 nucleotides) at 5' ends of miRNAs mainly bind to the 3'-untranslated region (3'-UTR) of their target mRNAs with imperfect sequence complementarity. However, recent studies have reported that miRNAs can also bind to 5'UTR, or open reading frame (ORF) of the target mRNA [8]. By binding to their target mRNA, miRNAs can inhibit expression of proteins from mRNA (suppression of translation) or causing mRNA degradation [12,14-18].

Since miRNAs can function as oncogenic (oncomir) as well as tumor suppressor depending on the function of their target genes, miRNAs are known to play an important role in tumorigenesis. A particular miRNA may be found up regulated in some cancer types as oncogenic function, but down regulated in other cancers, indicative of tumor suppressor function [19]. It is possible that the function of a single miRNA is cell or tissue specific. Recent studies indicate that miRNAs may have more oncogenic than tumor suppressive function in CRC [11,20,21]. 1.900 precursor and 2.600 mature miRNA have been identified up to now and these miRNAs are able to regulate the expression of almost 60% of all protein-coding genes in human genome [6,8,9,14,22-24]. Due to its small size, a single miRNA may act on expression of thousands of mRNAs. However, a single gene can be regulated only by multiple different miRNAs [11,13,23,25]. Therefore, miRNA expression profiles have been shown as potential signatures which are highly tissue specific for the classification, diagnosis and progression of cancer. Early detection of CRC by using miRNAs as diagnostic biomarkers provides the best chance for predictive diagnosis and successful treatment of diseases [1,21,26].

In 2003, it was first reported by Michael that differentially expressed upregulated and downregulated miRNAs were associated with CRC development and progression [27]. Since then more than 100 miRNAs have been shown to be associated with CRC since then. It was also shown that downregulation of let-7a-1 in CRC samples. MiR-34a has been shown to block cell proliferation and it is downregulated in colon cancer cells, whereas miR-31, miR-96, miR-31, miR-135b, and miR-183 were up regulated in CRC cells in the same study [28].

MiRNA associations with specific tumor markers also have been identified. Loss of miR-34 expression has been shown to deteriorate TP53-mediated cell death while overexpression of miR-34 results in apoptosis. MiRNAs also have been exhibited to be associated with regulation of oncogenes and with tumor suppressor genes. MiR-143 and let-7 expression levels have been associated with KRAS2 mutations [21]. Furthermore, miRNA-21 and miRNA-183 were found to be up-regulated in CRC and miRNA-143, miRNA-145 and miRNA-497 are down-regulated when compared to healthy subjects as stated by different studies [3,6,7,14,17,21,29-32]. Compared with those in normal tissue, miRNAs expression levels are down-regulated in malignant tissue, only several miRNAs are up regulated; most of them play oncogenic roles [8,15,33]. On the other hand, tumor suppressor's miRNA-497 and miRNA-378c are mainly downregulated in colorectal cancer [29,30].

The first miRNA mimic entered the clinic for cancer therapy is synthetic miRNA-34 (tumor suppressor) loaded in liposomal nanoparticles [34]. In general, there are two strategies to developing miRNA-based therapeutics either by direct inhibition of the interaction between oncogenic miRNA and mRNA or replacement of target tumor suppressor miRNA genes to restore a loss of function [15]. Oncogenic miRNAs could be therapeutically targeted by repression. A simple method to block oncogenic miRNAs is the use of anti-miRNA oligonucleotides complementary to the sequence of the targeted mature oncogenic miRNAs (antagomirs). These oligonucleotides disrupt miRISC complex and prevent degradation of an mRNA which can then be translated. Another approach to more specifically inhibit the miRNA function is to use of miRNA masks which are complementary to binding sites in 3'UTR of target mRNA. MiRNA replacement therapy aims to substitution of tumor suppressor miRNAs absent or expressed at lower levels by using oligonucleotide mimics containing the same sequence as the mature miRNA. Restoration of silenced tumor suppressor miRNAs might produce beneficial effects on cell migration, invasion and increasing sensitivity to therapeutic agents [6,15,21,35].

Materials and Methods

Ethics statement

This study was approved by the Medical Ethical Committee of the Harran University, Turkey and has been performed in accordance with the ethical principles of the 2008 revised Declaration of Helsinki. The samples were used with written informed consents from patients and the approval of the Turkish Academy of Medical Sciences. Tissue samples (~0.5 cm^3) from CRC patients were collected at surgery after the pathologist had confirmed the histopathology at the Hospital of Gaziantep Medical University. Normal tissue adjacent to the cancer tissue was used as control for this study. Subjects were signed by participants in a written informed consent form for this study. None of this CRC patients received chemotherapy or radiotherapy before the surgical resection. Each sample was placed in a cryovial and covered with the RNALater (Applied Biosystems) that prevents fragmentation of the fragile mRNA. Due to small sizes of miRNA (20-22 nt.), miRNA levels are remarkably stable in tissue samples when compared to mRNA. Tissue samples were homogenized in liquid nitrogen by using precellys homogenizer (Bertin) and stored at -86°C for subsequent analysis.

Total RNA isolation and reverse transcription of mature miRNAs

Total RNA containing miRNA was purified from homogenate by using the microRNAsy mini isolation kit (Qiagen) according to manufacturer's instructions. The purity and concentration of total RNA was assessed by using NanoDrop spectrophotometer p360 (Implen). Total RNA samples were aliquoted and stored at -86°C until used.

Microarray profiling of miRNAs

MiRNA expression was profiled by using GeneChip miRNA 3.0 Array (Affymetrix) for five controls and seven CRC with stage I-IV samples. About 1,000 ng RNA from each sample were labeled by biotin using the FlashTag Biotin HSR RNA labeling kit (Affymetrix). Then, the labeled samples were hybridized with the human probes on the microarray chips for 16 hours at 48°C with a rotation speed of 60 rpm. After hybridization, the probe arrays were washed and stained by using the Fluidics Station 450 according to AGCC Fluidics Control Software.

Then the fluorescence on the array was scanned using the Affymetrix® GeneChip® Scanner 3000 with a high resolution 6 g patch. The probe cell intensity files (*CEL files) generated by Affymetrix GeneChip® Command Console® software were transferred into probe level summarization files (*CHP files) using a robust multi-array (RMA) detection algorithm workflow. The *CHP files were further analyzed using Transcriptome Analysis Console (TAC) software, version 3.0, to identify and visualize the differentially expressed genes.

We identified the level of expressed miRNA with statistical significance from a volcano plot filtering between the colorectal cancerous and normal miRNAs from the experiment. Expression data of all probe sets detected by microarray analysis have been deposited in ArrayExpress, E-MTAB-4573.

Number	Systematic name	Tumor Bi-weight Avg Signal (log2)	Control Bi-weight Avg Signal (log2)	Fold Change (linear) (Tumor vs. Control)	ANOVA p-value (Tumor vs. Control)
1.	hsa-mir-1201	6.65	1.77	29.64	0.000641
2.	hsa-miR-181a-star	6.03	1.56	22.21	0.000461
3.	hsa-miR-7	6.17	1.77	21.16	0.020387
4.	hsa-mir-188	7.05	2.75	19.63	0.001616
5.	hsa-mir-552	7.67	3.49	18.11	0.014192
6.	hsa-mir-183	8.17	4.02	17.67	0.021005
7.	hsa-miR-941	6.36	2.31	16.64	0.000003
8.	U71d	6.29	2.33	15.57	0.021340
9.	ACA3-2	7.68	3.81	14.67	0.000048
10.	hsa-mir-34c	4.74	1.15	12.06	0.000309
11.	hsa-miR-7-1-star	5.91	2.34	11.9	0.000627
12.	hsa-mir-148a	5.38	1.81	11.9	0.001089
13.	hsa-mir-409	6.08	2.54	11.61	0.000224
14.	hsa-mir-769	5.19	1.67	11.49	0.000261
15.	hsa-mir-331	6.2	2.68	11.43	0.007081
16.	hsa-mir-10a	5.75	2.26	11.27	0.017370
17.	ACA9	5.55	2.09	11.03	0.000002
18.	hsa-mir-181d	8.24	4.81	10.75	0.004900
19.	U71d	6.43	3.02	10.58	0.015358
20.	hsa-mir-30e	5.55	2.15	10.55	0.047163
21.	hsa-mir-455	5.84	2.47	10.36	0.000016
22.	hsa-mir-495	5.29	2.01	9.74	0.000285
23.	hsa-mir-941-3	5.37	2.11	9.52	0.007112
24.	hsa-mir-146b	5.13	1.89	9.44	0.003417
25.	hsa-mir-181c	5.51	2.3	9.3	0.003289
26.	hsa-mir-299	5.04	1.87	9.03	0.036420
27.	ACA9	6.14	2.96	9.02	0.000125
28.	hsa-mir-18a	7.04	3.87	9	0.004278
29.	hsa-mir-493	5.64	2.52	8.73	0.001531
30.	hsa-miR-92a-1-star	5.82	2.73	8.49	0.005380

31.	hsa-mir-301a	5.25	2.19	8.34	0.000267
32.	ACA6	6.26	3.2	8.32	0.001425
33.	HBII-99	8.66	5.61	8.28	0.020411
34.	hsa-mir-431	5.44	2.43	8.05	0.000265
35.	U70	7.25	4.25	7.99	0.013214
36.	hsa-mir-487a	5.34	2.35	7.91	0.000055
37.	hsa-mir-505	6.69	3.72	7.85	0.003072
38.	ACA34	7.03	4.1	7.65	0.004997
39.	hsa-mir-34a	6.1	3.18	7.54	0.000132
40.	U65	6.29	3.38	7.5	0.013446
41.	hsa-mir-224	7.95	5.06	7.44	0.006594
42.	hsa-mir-154	6.18	3.29	7.41	0.014806
43.	hsa-mir-181c	7.5	4.63	7.29	0.001522
44.	hsa-mir-629	5.61	2.75	7.28	0.033213
45.	hsa-mir-542	5.09	2.26	7.1	0.005752
46.	hsa-mir-183	6.08	3.29	6.95	0.000749
47.	hsa-mir-454	5.22	2.42	6.93	0.000161
48.	U71c	4.83	2.07	6.79	0.001399
49.	ACA55	5.51	2.74	6.79	0.005687
50.	ENSG00000206903	10.38	7.62	6.76	0.005521
51.	hsa-miR-181a-2-star	7.28	4.53	6.73	0.019146
52.	hsa-mir-654	5.61	2.86	6.72	0.003405
53.	hsa-mir-337	6.98	4.25	6.66	0.007153
54.	hsa-miR-29b	8.03	5.29	6.66	0.012884
55.	hsa-mir-622	4.84	2.11	6.65	0.046174
56.	hsa-mir-135b	4.53	1.8	6.64	0.027261
57.	U71a	6.63	3.91	6.56	0.011939
58.	ACA24	5.95	3.3	6.29	0.000034
59.	hsa-mir-429	7.3	4.65	6.26	0.001175
60.	hsa-mir-411	5.79	3.15	6.21	0.000330
61.	hsa-mir-192	9.41	6.83	5.97	0.013712
62.	ACA52	8.2	5.64	5.87	0.010732
63.	ACA5	5.05	2.51	5.84	0.000047
64.	ENSG00000201199	5.57	3.04	5.8	0.001431
65.	U53	7.44	4.9	5.78	0.008812
66.	U67	5.21	2.74	5.55	0.000761

67.	U71c	4.61	2.14	5.55	0.018617
68.	hsa-mir-374b	5.65	3.2	5.46	0.049707
69.	ENSG00000201592	5.64	3.21	5.4	0.000223
70.	U46	8.64	6.21	5.39	0.020504
71.	hsa-let-7i	5.38	2.95	5.38	0.000545
72.	hsa-mir-376c	6.83	4.41	5.37	0.002284
73.	hsa-mir-485	4.04	1.66	5.21	0.011868
74.	ENSG00000207130	10.59	8.22	5.15	0.008658
75.	hsa-mir-936	3.92	1.56	5.12	0.000137
76.	hsa-mir-377	4.04	1.68	5.12	0.012927
77.	hsa-mir-489	5.71	3.35	5.11	0.021337
78.	hsa-mir-502	4.25	1.92	5.01	0.003544
79.	hsa-mir-941-4	5.76	3.45	4.94	0.034572
80.	hsa-mir-34c	4.73	2.44	4.9	0.000645
81.	hsa-miR-550-star	6.61	4.34	4.82	0.032494
82.	hsa-let-7g	3.66	1.39	4.8	0.000264
83.	hsa-mir-361	5.31	3.05	4.77	0.002347
84.	hsa-mir-362	3.85	1.6	4.75	0.000054
85.	SNORA11B	3.94	1.71	4.69	0.009034
86.	ACA66	3.49	1.29	4.58	0.002080
87.	hsa-mir-217	3.54	1.35	4.56	0.046813
88.	U71b	4.59	2.4	4.55	0.007406
89.	hsa-mir-127	4.88	2.7	4.53	0.022632
90.	HBII-316	7.19	5.03	4.48	0.023347
91.	hsa-mir-370	6.01	3.85	4.47	0.011705
92.	U107	5.22	3.07	4.46	0.019918
93.	hsa-mir-433	5.7	3.56	4.43	0.004833
94.	U103	4.83	2.69	4.4	0.014816
95.	ACA10	7.17	5.03	4.39	0.002936
96.	hsa-mir-543	4.86	2.73	4.39	0.006302
97.	hsa-mir-140	6.87	4.74	4.37	0.024446
98.	U46	6.65	4.54	4.34	0.002078
99.	hsa-mir-501	7.43	5.31	4.34	0.009283
100.	hsa-mir-326	4.34	2.23	4.32	0.000050
101.	HBI-100	4.78	2.68	4.28	0.000311
102.	hsa-mir-1254	4.86	2.76	4.28	0.001672

103.	ACA62	5.54	3.45	4.26	0.001151
104.	hsa-mir-196b	6.9	4.86	4.13	0.020518
105.	ACA33	6.89	4.84	4.12	0.001894
106.	hsa-mir-941-2	5.68	3.64	4.12	0.007328
107.	hsa-mir-17	8.91	6.87	4.09	0.000788
108.	hsa-mir-147b	3.4	1.4	4.02	0.049155
109.	U67	4.25	2.25	3.99	0.017309
110.	ACA3-2	9.62	7.63	3.96	0.000053
111.	U31	9.14	7.16	3.96	0.002425
112.	ACA3	7.61	5.62	3.95	0.005823
113.	ACA24	10.34	8.36	3.95	0.011863
114.	ACA5	5.73	3.75	3.93	0.000789
115.	hsa-mir-299	4.47	2.52	3.87	0.001478
116.	HBII-99B	5.39	3.44	3.87	0.008777
117.	hsa-mir-96	3.45	1.5	3.87	0.012393
118.	hsa-mir-1291	3.91	1.97	3.85	0.002163
119.	hsa-mir-425	8.46	6.52	3.82	0.008893
120.	ACA26	4.8	2.87	3.8	0.002390
121.	hsa-mir-3200	5.02	3.1	3.79	0.011422
122.	hsa-miR-3130-5p	3.48	1.58	3.73	0.004953
123.	hsa-mir-200a	10.98	9.09	3.7	0.004609
124.	ACA31	4.53	2.64	3.7	0.009703
125.	ACA17	3.57	1.68	3.69	0.000557
126.	ACA16	7.48	5.62	3.64	0.001931
127.	hsa-mir-199b	5.25	3.39	3.62	0.004455
128.	hsa-mir-221	5.35	3.49	3.62	0.033553
129.	hsa-mir-3138	3.82	1.97	3.61	0.035768
130.	U58C	6.04	4.2	3.57	0.003545
131.	ACA43	5.15	3.32	3.56	0.000473
140.	U68	8.18	6.35	3.56	0.007408
141.	hsa-mir-21	8.47	6.63	3.56	0.036590
142.	U19	6.04	4.21	3.54	0.015532
143.	hsa-mir-1201	4.95	3.13	3.53	0.004735
144.	hsa-let-7d	3.5	1.71	3.45	0.049116
145.	ENSG00000206913	9.34	7.58	3.4	0.000693
146.	ACA23	3.48	1.73	3.36	0.000177

147.	hsa-miR-103-2-star	3.69	1.94	3.35	0.000714
148.	ACA46	3.92	2.19	3.3	0.004512
149.	U15B	7.04	5.32	3.3	0.005781
150.	ENSG00000206603	3.64	1.93	3.27	0.031852
151.	ACA66	3.39	1.69	3.26	0.000431
152.	ACA16	4.97	3.27	3.25	0.003536
153.	hsa-miR-1285	5.76	4.06	3.25	0.008728
154.	hsa-mir-660	8.76	7.07	3.22	0.000667
155.	U18A	4.04	2.36	3.22	0.011729
156.	hsa-mir-1292	4.7	3.02	3.22	0.020186
157.	hsa-miR-24-1-star	3.14	1.46	3.2	0.002881
158.	snR38B	5.43	3.77	3.15	0.005555
159.	hsa-mir-1183	4.44	2.78	3.15	0.013904
160.	HBII-180C	8.22	6.56	3.14	0.010323
161.	HBI-6	6.33	4.7	3.1	0.000781
162.	hsa-miR-103-as	2.93	1.29	3.1	0.003721
163.	hsa-mir-424	3.39	1.76	3.1	0.013516
164.	ACA41	9.86	8.24	3.09	0.043374
165.	hsa-mir-4317	4.24	2.66	3	0.007898
166.	U14B	5.12	3.55	2.98	0.000241
167.	hsa-mir-18b	8.01	6.44	2.97	0.048861
168.	hsa-mir-1259	4	2.43	2.96	0.005410
169.	E2	5.41	3.84	2.95	0.011803
170.	U23	3.44	1.91	2.9	0.042505
171.	U71b	3.72	2.19	2.89	0.000953
172.	14ql-4	3.61	2.09	2.88	0.003175
173.	hsa-mir-2276	3.69	2.17	2.87	0.000172
174.	U99	6.76	5.24	2.87	0.019247
175.	hsa-mir-4257	2.88	1.37	2.86	0.008870
176.	ACA34	7.2	5.7	2.83	0.038452
177.	v49_ENSG00000206633	3.43	1.93	2.83	0.039065
178.	hsa-miR-181b	11.76	10.27	2.8	0.019370
179.	ACA15	4.6	3.11	2.8	0.030689
180.	ENSG00000238956	7.95	6.47	2.79	0.006921
181.	U68	10.87	9.39	2.79	0.009064
182.	ENSG00000222489	5.95	4.48	2.77	0.005702

183.	hsa-mir-500	9.15	7.69	2.75	0.013823
184.	ENSG00000252213	4.59	3.13	2.75	0.013963
185.	U48	9.58	8.12	2.75	0.026235
186.	U64	4.88	3.43	2.74	0.000422
187.	U96b	4.67	3.22	2.74	0.005474
188.	ENSG00000200879	8.8	7.35	2.73	0.004711
189.	U54	9.23	7.78	2.73	0.006385
190.	U27	8.39	6.95	2.71	0.009984
191.	U44	11.36	9.92	2.7	0.005082
192.	U23	8.9	7.47	2.7	0.039786
193.	hsa-mir-532	9.26	7.83	2.69	0.001247
194.	ACA67B	3.23	1.8	2.69	0.015256
195.	ACA7B	9.29	7.88	2.66	0.011805
196.	U60	5.58	4.18	2.65	0.000133
197.	U18C	3.27	1.86	2.65	0.001589
198.	hsa-mir-148a	9.72	8.31	2.65	0.002850
199.	U42B	3.7	2.3	2.64	0.000125
200.	ENSG00000207187	7.21	5.81	2.64	0.002058
201.	ACA32	5.95	4.55	2.64	0.007992
202.	U103B	5.17	3.79	2.61	0.001744
203.	U17a	5.01	3.63	2.6	0.001582
204.	U106	4.59	3.22	2.6	0.012716
205.	snR39B	8.08	6.7	2.6	0.015181
206.	hsa-mir-93	3.41	2.04	2.58	0.001656
207.	SNORA11B	3.63	2.27	2.56	0.006011
208.	14qII-26	4.28	2.93	2.56	0.023833
209.	U35A	9.92	8.56	2.56	0.033458
210.	ACA2b	3.83	2.48	2.54	0.005727
211.	mgU6-53	3.85	2.51	2.53	0.000996
212.	snR38A	4.86	3.52	2.53	0.001270
213.	ACA18	10.27	8.94	2.52	0.017484
214.	SNORA84	4.26	2.93	2.52	0.021507
215.	hsa-mir-22	5.96	4.62	2.52	0.034913
216.	HBII-180A	9.58	8.25	2.5	0.013371
217.	hsa-mir-185	4.1	2.78	2.5	0.023328
218.	U46	7.63	6.32	2.49	0.011107

219.	hsa-mir-141	9.85	8.55	2.47	0.033946
220.	ACA41	4.48	3.18	2.47	0.043068
221.	hsa-mir-132	2.56	1.26	2.46	0.013196
222.	hsa-mir-493	2.47	1.17	2.46	0.030161
223.	hsa-mir-654	3.16	1.87	2.45	0.002769
224.	U92	4.26	2.97	2.45	0.003824
225.	ACA47	3.02	1.73	2.45	0.013111
226.	ACA32	5.71	4.42	2.44	0.016438
227.	mgh28S-2411	11.22	9.95	2.42	0.002499
228.	hsa-mir-744	2.99	1.72	2.41	0.017194
229.	HBI-61	4.28	3.01	2.41	0.025907
230.	U13	6.73	5.48	2.38	0.035739
231.	ACA50	4.89	3.65	2.37	0.000397
232.	hsa-miR-376a	3.39	2.15	2.37	0.029751
233.	hsa-mir-612	2.9	1.66	2.36	0.000370
234.	hsa-mir-671	3.19	1.95	2.36	0.004397
235.	U72	2.43	1.21	2.34	0.007780
236.	U18A	4.46	3.27	2.29	0.016004
237.	ACA48	8.5	7.31	2.27	0.040439
238.	U42B	5.91	4.74	2.24	0.006167
239.	U30	11.43	10.27	2.22	0.009547
240.	U75	11.24	10.1	2.2	0.003475
241.	hsa-mir-421	8.25	7.11	2.2	0.034311
242.	U56	10.08	8.95	2.18	0.011403
243.	hsa-mir-492	2.79	1.68	2.16	0.019203
244.	hsa-mir-182	2.6	1.5	2.15	0.034690
245.	HBII-95	2.71	1.62	2.12	0.005868
246.	U109	4.11	3.03	2.12	0.009534
247.	ACA11	5.71	4.62	2.12	0.023544
248.	U36A	6.28	5.2	2.11	0.001139
249.	hsa-mir-106b	12.64	11.58	2.1	0.002558
250.	hsa-mir-711	2.97	1.9	2.09	0.009272
251.	U106	5.36	4.3	2.09	0.041728
252.	U47	3.68	2.62	2.08	0.028603
253.	ACA53	3.31	2.26	2.07	0.002782
254.	hsa-mir-1291	3.42	2.37	2.07	0.008497

255.	U58C	8.26	7.23	2.05	0.025618
256.	hsa-mir-222	2.56	1.53	2.05	0.030812
257.	hsa-mir-212	7.03	6	2.05	0.041135
258.	hsa-mir-222	13.12	12.09	2.04	0.014307
259.	ENSG00000238807	1.23	2.23	-2.01	0.005883
260.	ENSG00000238804	1.08	2.1	-2.02	0.018261
261.	ENSG00000238430	1.35	2.38	-2.04	0.013001
262.	14ql-1	1.46	2.5	-2.06	0.010180
263.	v49_ENSG00000201733	1.36	2.51	-2.22	0.022257
264.	ENSG00000239140	1.22	2.38	-2.24	0.023096
265.	ENSG00000201619	4.34	5.52	-2.26	0.021703
266.	hsa-mir-744	8.21	9.39	-2.27	0.015659
267.	hsa-mir-497	9.66	10.86	-2.3	0.021457
268.	hsa-mir-4314	1.74	2.95	-2.31	0.012926
269.	hsa-mir-3141	8.28	9.5	-2.33	0.029632
270.	hsa-mir-631	1.24	2.49	-2.38	0.006627
271.	ENSG00000252921	2.81	4.06	-2.38	0.010667
272.	ENSG00000252921	1.69	2.98	-2.45	0.033509
273.	ENSG00000221345	1.41	2.71	-2.46	0.036777
274.	hsa-mir-548x	1	2.3	-2.47	0.002773
275.	hsa-mir-1281	7.03	8.42	-2.6	0.025311
276.	ENSG00000239080	1.72	3.1	-2.6	0.047346
277.	hsa-mir-606	1.29	2.73	-2.72	0.041951
278.	ENSG00000238549	1.7	3.15	-2.74	0.003969
279.	hsa-miR-548a-3p	2.29	3.8	-2.84	0.037466
280.	ENSG00000238430	1.26	2.78	-2.85	0.024357
281.	hsa-mir-550-2	2.09	4.04	-3.87	0.000059
282.	hsa-mir-10b	9.04	11.05	-4.04	0.002456
283.	hsa-mir-635	1.28	3.48	-4.58	0.019899
284.	hsa-mir-550-1	4.85	7.12	-4.82	0.001534
285.	HBII-85-6	6.54	9.08	-5.83	0.045268
286.	hsa-mir-486	8.5	11.05	-5.89	0.024529
287.	hsa-mir-451	8.32	11.04	-6.62	0.036823

Table 1: Microarray Analysis: Differentially expressed miRNAs in CRC samples compared with samples of controls in the training and validation sets. Statistical analysis was performed using TAC software one way analysis of variance (ANOVA) was used. The statistical significance level was set at p-value<0.05. Greater than 2-fold changes were analyzed for up/down regulated miRNAs. A total of miRNAs were found to be differentially expressed in the above table.

Number	Systematic name	Tumor Bi-weight Avg Signal (log2)	Control Bi-weight Avg Signal (log2)	Fold Change (linear)
1.	hsa-mir-1201	6.65	1.77	29.64
2.	hsa-miR-181a-star	6.03	1.56	22.21
3.	hsa-miR-7	6.17	1.77	21.16
4.	hsa-mir-188	7.05	2.75	19.63
5.	hsa-mir-552	7.67	3.49	18.11
6.	hsa-mir-183	8.17	4.02	17.67
7.	hsa-miR-941	6.36	2.31	16.64
8.	U71d	6.29	2.33	15.57
9.	ACA3-2	7.68	3.81	14.67
10.	hsa-mir-34c	4.74	1.15	12.06

Table 2a: The most important significant increase and decrease rates of expression in miRNA transcripts, some upregulated miRNA transcripts.

Number	Systematic name	Tumor Bi-weight Avg Signal (log2)	Control Bi-weight Avg Signal (log2)	Fold Change (linear)
1.	hsa-mir-606	1.29	2.73	-2.72
2.	ENSG00000238549	1.7	3.15	-2.74
3.	hsa-miR-548a-3p	2.29	3.8	-2.84
4.	ENSG00000238430	1.26	2.78	-2.85
5.	hsa-mir-550-2	2.09	4.04	-3.87
6.	hsa-mir-10b	9.04	11.05	-4.04
7.	hsa-mir-635	1.28	3.48	-4.58
8.	hsa-mir-550-1	4.85	7.12	-4.82
9.	HBII-85-6	6.54	9.08	-5.83
10.	hsa-mir-486	8.5	11.05	-5.89

Table 2b: The most important significant increase and decrease rates of expression in miRNA transcripts, some down-regulated miRNA transcripts.

Results and Discussion

In microarray analysis, we identified 287 aberrantly expressed miRNA genes that either have increased (250 miRNAs), or reduced (29 miRNAs) expression levels as shown in Table 1. We found that MiR-1201, miR-181-a, miR-7, miR-188, miR-552 and miR-183 were highly expressed in colon carcinoma tissues compared with the normal tissues (Table 2a). Among the overexpressed miRNAs, miR-1201 is the highest expressed one in CRC tissues. MiR-181, miR-183, 34a, miR-92, miR-21, miR-431 and miR-487a were identified as on co mirRNA in various tumor tissues [36-39]. MiR-21 has been shown to be overexpressed in many cancer types [18,24,37,39,40]. On the other hand, five miRNAs (miR-451, miR-486, miR-550, miR-635 and miR-10b) had decreased expression significantly in CRC tissue samples compared with the normal samples (Table 2b). These down regulated miRNAs were found to be tumor suppressors in CRC tissues in previous studies [29,30,39,41,42]. MiR-486 was found to be the lowest expressed one CRC tissues compared with the normal tissues. We also found some new aberrant expression miRNAs in colorectal cancer tissues (Tables 1-2b). Finally, we could expect that miRNAs have important role as tumor suppressors or oncogenic factors in the network of carcinogenesis. The genes with similar expression patterns are grouped together and connected by a series of branches (clustering tree or dendrogram). The threshold we used to screen up-regulated or down-regulated miRNAs is fold change ≥2.0 and p value<0.05. The results of hierarchical clustering show aberrantly expressed miRNAs among CRC tissues and normal CR tissues (Figure 1). Principal component analysis was also used to find out the similarities and differences between CRC samples and normal tissues (Figure 2). Total RNA was obtained from tissues of control and CRC and hybridized to Affymetrix GeneChip® miRNA 3.0 Arrays. After normalization,

differential miRNA expression data was analyzed by unsupervised hierarchical clustering.

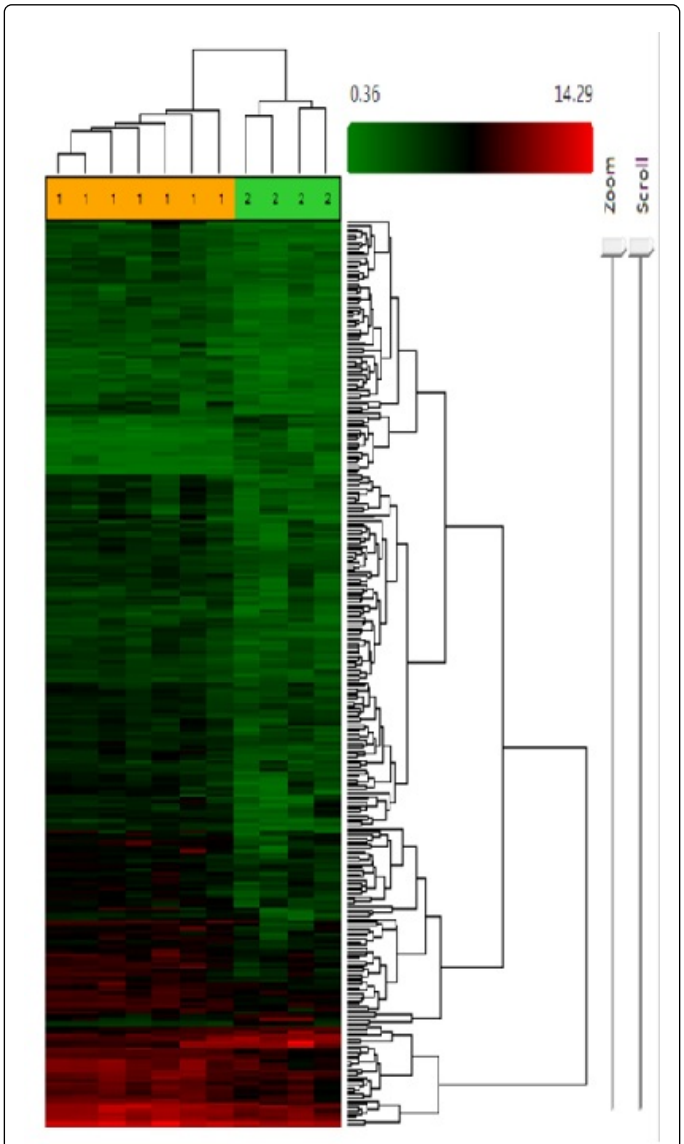

Figure 1: Hierarchical Clustering of Gene Expression.

Each row represents individual miRNA. In hierarchical clustering, genes with similar expression patterns are grouped together and connected by a series of branches (clustering tree or dendrogram). The red colors on the dendrogram indicate expression levels higher than the median. While green colors show expression levels lower than the median.

Conclusion

Our results show that most of miRNAs differentially expressed are up regulated miRNAs in CRC tissues compared to control tissues. Evidences have been accumulating that some miRNAs are overexpressed in CRC cells and might function as inhibitors of different tumor suppressor genes. There are three strategies for miRNA-based therapies are to inhibit oncogenic miRNAs or restoring tumor suppressor miRNAs. The first strategy is to direct inhibition of oncogenic microRNAs and this can be achieved by using single-stranded antisense oligonucleotides (approximately 20-22 nucleotides in long) that act through complementar base-pairing with target miRNAs. The second strategy is tumor suppressor miRNA replacement. This involves reintroducing synthetic miRNA or expression vectors that will produce the miRNA of interest to restore a loss of function. The third strategy is to develop drugs that decrease the levels of oncogenic miRNAs or increase the levels of tumor-suppressor miRNA for cancer prevention [21,43].

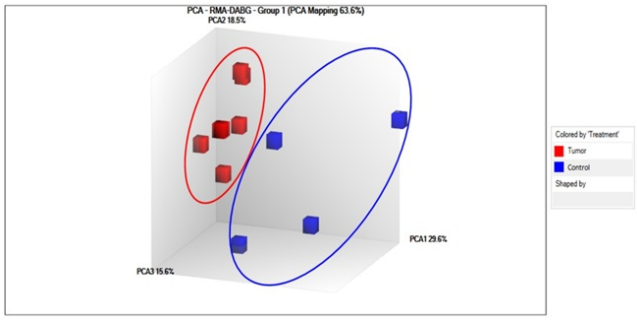

Figure 2: Principle Component Analyses (PCA). Each red dot represents a tumor sample and each blue dot represents control sample. According to the graph tumor and control samples were clustered at different localities.

The most important challenge is the identification of definitive miRNA signatures for CRC by large and comprehensive profiling studies. This will allow the identification of certain diagnostic and prognostic biomarkers that can help physicians with patient evaluation and also prospective markers for the early therapy [8,12,15,44]. In summary, miRNAs are extremely important regulators of oncogenes and tumor suppressor genes that are responsible for pathologic processes associated with malignant progression. Although the roles of many different miRNA have been identified in various tumors, more study is still needed to fully understand the role of each miRNA and assessing their roles in personalized miRNA targeted cancer therapy [24]. In future cancer therapy, miRNAs may be important in deciding which drugs are selected for a patient and in determination of whether the patient has responded to the drug [23]. In conclusion, further efforts to get more sensitive, fast and effective methods are needed to address the role of miRNAs biomarkers in clinical diagnostic, prognostic and therapy. Finally, miRNAs will emerge as a powerful resource to advance the diagnosis and management of cancer including colorectal cancer.

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