RESEARCH ARTICLE

microRNA Expression Profile in Patients with Stage II Colorectal Cancer: A Turkish Referral Center Study

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Abstract

<u>Background</u>: There are increasing data about microRNAs (miRNA) in the literature, providing abundant evidence that they play important roles in pathogenesis and development of colorectal cancer. In this study, we aimed to investigate the miRNA expression profiles in surgically resected specimens of patients with recurrent and non-recurrent colorectal cancer. <u>Materials and Methods</u>: The study population included 40 patients with stage II colorectal cancer (20 patients with recurrent tumors, and 20 sex and age matched patients without recurrence), who underwent curative colectomy between 2004 and 2011 without adjuvant therapy. Expression of 16 miRNAs (miRNA-9, 21, 30d, 31, 106a, 127, 133a, 133b, 135b, 143, 145, 155, 182, 200a, 200c, 362) was verified by quantitative real-time polymerase chain reaction (qRT-PCR) in all resected colon cancer tissue samples and in corresponding normal colonic tissues. Data analyses were carried out using SPSS 15 software. Values were statistically significantly changed in 40 cancer tissues when compared to the corresponding 40 normal colonic tissues (p<0.001). MiR-30d, miR-133a, miR-143, miR-145 and miR-362 expression was statistically significantly downregulated in 40 resected colorectal cancer tissue samples (p<0.001). When we compared subgroups, miRNA expression profiles of 20 recurrent cancer tissues were similar to all 40 cancer tissues. However in 20 non-recurrent cancer tissues, miR-133a expression was not significantly downregulated, moreover miR-133b expression was significantly upregulated (p<0.05). <u>Conclusions</u>: Our study revealed dysregulation of expression of ten miRNAs in Turkish colon cancer patients. These miRNAs may be used as potential biomarkers for early detection, screening and surveillance of colorectal cancer, with functional effects on tumor cell behavior.

Keywords: Colorectal cancer - microRNA expression profiling - cancer recurrence

Introduction

Colorectal cancer is the third most common cancer and the fourth leading cause of deaths due to cancer (Soerjomataram et al., 2012) Novel treatment interventions of CRC involving surgery, chemotheraphy and/or radiotherapy have increased the overall survival rates in early stages. Unfortunately, tumor recurrence especially in lymph-node metastatic cancers is still frequent (Bozkurt et al., 2014). As a literature data, nearly one-third of CRC patients develop synchronous or metachronous metastases in the liver (Fritzmann et al., 2009). In localized CRC surgical resection is the first step. Even after a successful resection, the risk of recurrence is high in stage II and III CRC and most of these patients need addi¬tional treatments in the future (Omranipour et al., 2014). Therefore, prevention and successful management of recurrence is the main topic in CRC follow-up.

MicroRNAs (miRNA) are short non-coding RNAs which comprise a broad class of small (17-22 nucleotides) endogenous RNAs. They regulate gene expression, and by means of that they play crucial roles in various physi¬ological and pathological processes in human beings (Bentwich et al., 2005; Farh et al., 2005; Carthew et al., 2009; Farooqi et al., 2014). A single miRNA may regulate multiple targets in cellular environment and thus act as an orchestra chief of gene expression. The miRNAs expression is a dynamic process and related to many situations in the physiological consequences. In the light of this fact, it may be expressed that tumor tissue derived miRNAs may be used as diagnostic or prognostic biomarkers in many cancers (Calin et al., 2006; Ahmed 2007; Rosenfeld et al., 2008; Aghanoori et al., 2014). Multiple types of miRNAs have been found to be overexpressed in several malignancies, including colorectal cancers (Berber et al., 2014; Ma et al., 2014; Mo et al., 2014). The association between miRNA expressions and CRC outcome has been explored by a number of studies. In these studies prognostic role of miRNAs in CRC remains controversial (Xi et al., 2006; Schepeler et

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Table 1. Clinicopathological Features of All Patients

		All Patients (n)	Patients with recurrence	Patients without recurrence	p value
Age (mean)		65.8±11.9	65.7±10.4	65.9±13.6	p>0.05
Gender	Female	20 (50%)	10 (25%)	10 (25%)	p>0.05
	Male	20 (50%)	10 (25%)	10 (25%)	
Location	Right Colon	9 (22%)	2 (5%)	7 (17%)	
	Left Colon	20 (50%)	10 (25%)	10 (25%)	0.014
	Rectum	11 (28%)	8 (20%)	3 (8%)	
Examined	<12	21 (55%)	11 (30%)	10 (25%)	p>0.05
Lymph Node	≥12	19 (45%)	9 (20%)	10 (25%)	
Follow up (median)		44.2(4.2-127.5)	33.8 (4.7-92.5)	68(4.2-127.5)	p<0.05

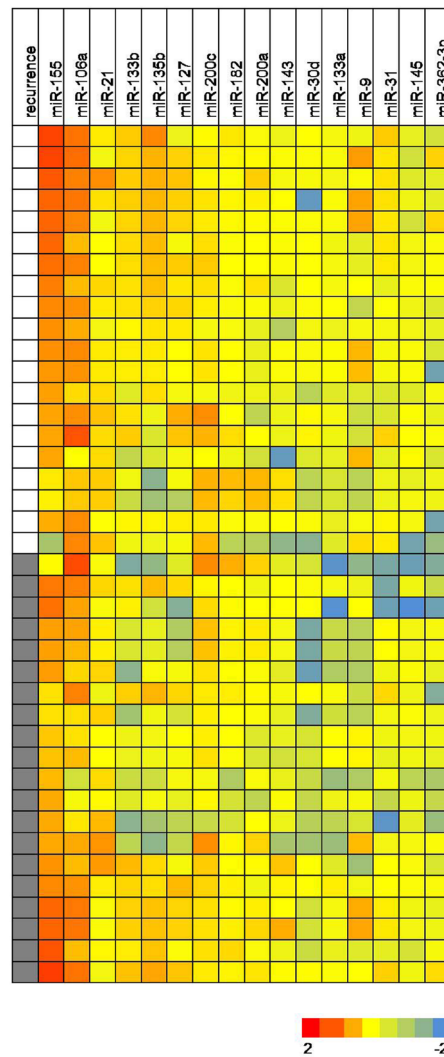**Table 2. The list of miRNAs and Mature miRNA Sequences Used in the Study**

miRNA	Mature miRNA sequence
Hsa-miR-9-3p	AUAAAGCUAGAUAACCGAAAGU
Hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
Hsa-miR-30d-5p	UGUAAACAUCCCCGACUGGAAG
Hsa-miR-31-5p	AGGCAAGAUGCUGGCAUAGCU
Hsa-miR-106a-5p	AGGCAGUGUAGUUAGCUGAUUGC
Hsa-miR-127-5p	CUGAAGCUCAGAGGGCUCUGAU
Hsa-miR-133a-3p	UUUGGUCCCUUCAACCAGCUG
Hsa-miR-133b-5p	UUUGGUCCCCUUCAACCAGCUA
Hsa-miR-135b-5p	UAUGGCUUUUCAUUCCUAUGUGA
Hsa-miR-143-3p	UGAGAUGAAGCACUGUAGCUC
Hsa-miR-145-5p	GUCCAGUUUUCCCAGGAAUCCCU
Hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGU
Hsa-miR-182-5p	UUUGGCAAUGGUAGAACUCACACU
Hsa-miR-200a-5p	UGGCAGUGUCUUAGCUGGUUGUU
Hsa-miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA
Hsa-miR-362-3p	AACACACCUAUUCAAGGAUUCA

Figure 1. Heatmap Colors Represents Standardized miRNA Expression Levels by log2 Transformation in Tumor Samples Normalized to Benign Colonic Tissues. Each line represents a case. Gray boxes in the first column indicate recurrent disease

al., 2008; Karaayvaz et al., 2011)

In this study, we aimed to investigate the miRNA expression profiles in the surgically resected specimens of patients with recurrent and non-recurrent colorectal cancer.

Materials and Methods

Patients: Pairs of colon tumor and adjacent non-tumorous tissues of 40 Stage II (according to the AJCC staging system) patients with colorectal cancer (20 patients with recurrent tumor, sex and age matched 20 patients without recurrence) from the GATA Haydarpasa Training Hospital, between 2004 and 2012, were included in the study. Cases with familial adenomatous polyposis or hereditary non-polyposis colorectal cancer were excluded from the study. All patients were underwent curative resections with clear margins. Detailed backgrounds for each case, including age, sex, tumor localization, survival times from diagnosis, and receipt of adjuvant chemotherapy have been collected. Patients were followed-up with uniform methods for detection of recurrence. The cases who received any postoperative adjuvant therapy were also excluded from the study. A minimum follow-up of two years for all patients was required. Following the study's approval by the institutional ethics committee, the study was performed in GATA Haydarpasa Training Hospital.

Follow-up: According to our hospital's follow-up protocol, patients were followed every three months in the first two years. Our cases were then followed-up every six months for three years and yearly afterwards. In follow-up visits whole and routine blood examinations performed, serum carcinoembryonic antigen (CEA) levels were tested besides complete physical examination. Patients had also abdominal ultrasound and chest X-rays or chest, abdominal and pelvic computerized tomog¬raphy

(CT) scan once yearly. Colonoscopy was also performed one year later from the surgical resection and every 3-5 years afterwards. Patients were evaluated as 'cases with recurrence' and 'cases without recurrence'.

Pathology: Formallin-fixed, paraffin-embedded tissue blocks of the patients were retrieved from the archives of our Pathology Department. The blocks containing at least 50% tumor and >0.5 cm in diameter were chosen for evaluation. From each block, 5 slices of 5 μm each were collected in one 1.5 ml tube for microRNA analysis. Slides were reviewed by the same expert pathologist.

miRNA, qRT-PCR: For miRNA extraction, high-tumor-containing areas were selected from formallin-fixed paraffin-embedded archival tissues. After 5 μm-thick tissue sections were cut, tumor areas were macrodissected from the slides to maximize the tumor cell content. In each case, corresponding benign colonic tissues were also selected for miRNA extraction. Total RNA was isolated by using miRNeasy FFPE Kit (QIAGEN, Germany), and the quantity of the isolated RNA was assessed by using NanoDrop 1000 spectrophotometer (Thermo Scientific,

DE). For reverse transcription and cDNA synthesis from isolated total RNA, the miScript (QIAGEN, Germany) technology kits were used according to the manufacturer's instructions. Selected mature miRNAs and their sequences are listed in Table 2. As reference, we used RNU6B small nuclear RNA with serial dilutions of template cDNA. PCR amplifications were carried out on RotorGene Q-5 Plex (QIAGEN, Germany) in a final volume of 20 μl containing 2 μl of synthesized cDNA. All reactions were performed in duplicate by including no DNA template and reverse transcriptase minus controls. We performed melting curve analysis to evaluate PCR amplicons by melting data acquired from 50°C to 85°C, at a ramping rate of 1°C/ sec. MiRNA expression levels were calculated using the ΔΔCt method as previously described (Livak et al., 2001). Expressions of 16 miRNAs (miRNA-9, 21, 30d, 31, 106a, 127, 133a, 133b, 135b, 143, 145, 155, 182, 200a, 200c, 362-3p) were examined in all colon cancer tissue samples, and in corresponding normal colonic tissues (Table 2). Clinicians involved in the miRNA measurement were blinded to the patients' clinical data.

Table 3. miRNA Profiles Comparison of Tumor Tissues and Corresponding Normal Tissues

miRNA	miRNA expression					
	All tumor tissues	All corresponding normal tissues	p value	Non-recurrent tumor tissues	Corresponding normal tissues	p value
Hsa-miR-9	0.147 (0.01-.0.80)	0.198 (0.05-1.14)	0.126	0.178 (0.02-0.8)	0.180 (0.08-1.02)	0.888
Hsa-miR-21	1.865 (0.01-10.34)	1.466 (0.01-3.36)	<0.001	2.483 (0.01-10.34)	1.615 (0.01-3.36)	0.003
Hsa-miR-30d	0.500 (0-1.79)	1.090 (0.01-2.81)	<0.001	0.572 (0.01-1.74)	0.909 (0.01-2.03)	<0.001
Hsa-miR-31	0.274 (0-3.49)	0.512 (0.01-3.93)	0.502	0.407 (0-3.49)	0.593 (0.01-1.88)	0.575
Hsa-miR-106a	0.280 (0-5.21)	0.094 (0-0.75)	<0.001	0.318 (0-2.69)	0.084 (0-0.55)	<0.001
Hsa-miR-127	0.159 (0-2.95)	0.276 (0-1.60)	0.53	0.244 (0-2.78)	0.163 (0-1.36)	0.107
Hsa-miR-133a	0.439 (0-2.64)	1.085 (0.01-3.24)	<0.001	0.597 (0-2.64)	1.138 (0.01-3.24)	<0.001
Hsa-miR-133b	0.324 (0-2.06)	0.442 (0.02-2.04)	0.326	0.376 (0-1.68)	0.371 (0.02-1.04)	0.021
Hsa-miR-135b	0.263 (0-1.45)	0.306 (0.01-1.02)	0.179	0.524 (0.02-1.45)	0.377 (0.06-0.91)	0.054
Hsa-miR-143	0.234 (0-5.45)	0.462 (0-3.13)	<0.001	0.477 (0-1.92)	0.644 (0-2.16)	0.003
Hsa-miR-145	0.672 (0-3.73)	1.197 (0.02-9.73)	<0.001	0.752 (0.01-3.73)	1.445 (0.02-9.73)	<0.001
Hsa-miR-155	0.369 (0.01-1.93)	0.102 (0-0.53)	<0.001	0.388 (0.01-1.93)	0.063 (0.01-0.53)	<0.001
Hsa-miR-182	0.832 (0-2.99)	0.356 (0-3.04)	0.361	0.316 (0-2.76)	0.317 (0-2.25)	0.679
Hsa-miR-200a	0.261 (0-3.36)	0.396 (0-2.49)	0.914	0.990 (0.01-3.36)	0.347 (0-2.49)	0.794
Hsa-miR-200c	0.480 (0-7.91)	0.303 (0-4.70)	<0.001	0.466 (0-7.91)	0.311 (0-4.7)	0.003
Hsa-miR-362-3p	0.086 (0-0.98)	0.468 (0.01-1.47)	<0.001	0.122 (0-0.77)	0.569 (0.03-1.47)	0.002

miRNA	miRNA expression					
	Recurrent tumor tissues	Corresponding normal tissues	p value	Non-recurrent tumor tissues	Recurrent tumor tissues	p value
Hsa-miR-9	0.087(0.01-0.76)	0.211 (0.05-1.14)	0.057	0.178 (0.02-0.80)	0.087(0.01-0.76)	0.063
Hsa-miR-21	1.587 (0.01-6.44)	1.027 (0.01-3.11)	0.04	2.483 (0.01-10.34)	1.587 (0.01-6.44)	0.253
Hsa-miR-30d	0.330 (0-1.79)	1.322 (0.12-2.81)	<0.001	0.572 (0.01-1.74)	0.330 (0-1.79)	0.327
Hsa-miR-31	0.157 (0-0.99)	0.489 (0.01-3.93)	0.135	0.407 (0-3.49)	0.157 (0-0.99)	0.035
Hsa-miR-106a	0.184 (0.01-5.21)	0.104 (0-0.75)	<0.001	0.318 (0-2.69)	0.184 (0.01-5.21)	0.495
Hsa-miR-127	0.149 (0-2.95)	0.498 (0-1.60)	0.501	0.244 (0-2.78)	0.149 (0-2.95)	0.799
Hsa-miR-133a	0.088 (0-1.83)	0.988 (0.02-3.24)	0.001	0.597 (0-2.64)	0.088 (0-1.83)	0.108
Hsa-miR-133b	0.140 (0.01-2.06)	0.492 (0.05-2.04)	0.526	0.376 (0-1.68)	0.140 (0.01-2.06)	0.221
Hsa-miR-135b	0.189 (0-1.27)	0.296 (0.01-1.02)	0.911	0.524 (0.02-1.45)	0.189 (0-1.27)	0.06
Hsa-miR-143	0.095 (0-5.45)	0.154 (0.01-3.13)	0.038	0.477 (0-1.92)	0.095 (0-5.45)	0.947
Hsa-miR-145	0.672 (0-1.86)	1.030 (0.02-2.21)	<0.001	0.752 (0.01-3.73)	0.672 (0-1.86)	0.429
Hsa-miR-155	0.344 (0.01-1.86)	0.120 (0-0.24)	<0.001	0.388 (0.01-1.93)	0.344 (0.01-1.86)	0.947
Hsa-miR-182	0.441 (0-2.99)	0.363 (0-3.04)	0.108	0.316 (0-2.76)	0.441 (0-2.99)	0.947
Hsa-miR-200a	0.211 (0-3.23)	0.445 (0-1.76)	0.794	0.990 (0.01-3.36)	0.211 (0-3.23)	0.989
Hsa-miR-200c	0.448 (0-4.34)	0.214 (0-2.00)	0.002	0.466 (0-7.91)	0.448 (0-4.34)	0.738
Hsa-miR-362-3p	0.071 (0-0.98)	0.432 (0.01-1.28)	0.001	0.122 (0-0.77)	0.071 (0-0.98)	0.718

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<u>Data analysis and statistic</u>: We used Mann Whitney U and Wilcoxon Signed Rank tests to compare the differences in expression levels of miRNAs between recurrent, non-recurrent cancer tissue samples and corresponding normal colonic tissues. Data are expressed as the mean±standard deviation for clinicopathological features and expressed as median (minumum-maximum) for miRNA measurements. Statistical calculations were performed using SPSS software (version 15.0, SPSS, Chicago, IL, USA). A p value of <0.05 was considered to be statistically significant.

Results

We compared microRNA profiles of 40 pairs of colon tumor and adjacent non-tumorous tissues in cases with recurrent CRC (n=20) and non-recurrent CRC (n=20). The clinicopathological characteristics of the study population are summarized in Table I. The mean age of the study group was 65.8±11.9 years and 50% were (20 patients) males. Mean age of patients with recurrence was not statistically different from patients without recurrence (p>0.05). All patients had stage II disease. The median follow-up time was 44.2(4.2-127.5) months for all patients. The median follow-up time was 33.8(4.7-92.5) months for patients with recurrence and 68(4.2-127.5) months for patients without recurrence (p<0.05). Expression levels of 16 miRNAs in recurrent, non-recurrent and overall cancer groups are presented in Table 3. The individualized miRNA expressions for each case are shown by heatmap colors in Figure 1. The list of miRNAs and mature miRNA sequences used in the study are displayed in Table 2. The comparison of miRNA expression levels in non-recurrence group and recurrence disease group are exhibited in Table 3.

MiR-21, miR-106a, miR-155 and miR-200c levels were statistically significantly upregulated in 40 cancer tissues when compared to their corresponding 40 normal colonic tissues (p<0.001). Moreover, miR-30d, miR-133a, miR-143, miR-145 and miR-362-3p expressions were statistically significantly downregulated in 40 resected colorectal cancer tissue samples (p<0.001). When we compared subgroups, miRNA expression profiles of 20 recurrent cancer tissues were similar to all 40 cancer tissues (Table 3). However in 20 non-recurrent cancer tissues, miR-133a expression was not significantly downregulated, moreover miR-133b expression was significantly upregulated (Table 3) (p<0.05). Only miR-31 expressions were statistically different between recurrent and non-recurrent colonic tumor tissue samples (Table 3) (p=0.035). Finally, microRNA expression profiles were not different among all 40 corresponding normal colonic tissues (p>0.05).

Discussion

In agreement with previous studies, our findings have shown that some miRNAs are upregulated and some miRNAs are down-regulated in resected stage II colon cancer samples relative to normal colonic tissue. Also our study suggests a potential role of down-regulated and up-regulated miRNA levels as 'recurrence markers' among CRC patients. Thus, our findings about dysregulated miRNAs are overlapping with previous studies which suggested concordant expression.

Recently, there is emerging data about dysregulated expression of multiple miRNAs in CRC. In this regard, expressions of various miRNAs have been found to be up or down-regulated in colon cancer tissues. As reported in previous papers, through their interactions with intracellular signaling networks, miRNAs can change cell proliferation, apoptosis and metastasis (Wu et al., 2011). Despite the growing knowledge about the likely role of miRNA in diagnosis (Huang et al., 2010; Link et al., 2010), prognosis (Diaz et al., 2008) and response to treatment of (Nakajima et al., 2006; Zhang et al., 2011) CRC, the data is still controversial and the association between miRNAs and CRC warrants further investigation.

In this current study, we investigated the possible role of 16 miRNAs in 40 resected stage II CRC tissue samples and furthermore concentrated on association between miRNAs and CRC recurrence. At the end of our investigation, miR-21, miR-106a, miR-155 and miR-200c levels were significantly upregulated (p<0.001). Until the present time, miRNA-21 has been the most frequently studied miRNA in this context. Previously, upregulation of miR-21 was shown to correlate with bad patient outcomes including advanced stage, lymph node or distant metastases and poor survival (Slaby et al., 2007; Kulda et al., 2010). On the other hand, miR-106a has been exhibited to be upregulated in cancer tissues and stool samples of CRC patients (Diaz et al., 2008); however, the exact role of miR-106 in CRCs is still unknown. On the other side, in Zhang et al. study it has been shown that miR-155 levels are upregulated in CRC tissues. They also suggested that upregulation of microRNA-155 promotes the migration and invasion of colorectal cancer cells (Zhang et al., 2013). From the other point of view, it has been demonstrated that miR-200c levels are significantly upregulated in CRC tissues (Della Vittoria Scarpati et al., 2014).

In our current miRNA research, miR-30d, miR-133a, miR-143, miR-145 and miR-362-3p expressions were significantly downregulated (p<0.001). Previously, Su et al. reported that miR-30d was significantly downregulated in colon cancer tissue samples (Su et al., 2013). On the other hand, Wang et al. demonstrated that expression of miR-133a was significantly downregulated in CRC tissues compared with corresponding non-cancerous tissues (Wang et al., 2014). On the other side, Bauer et al. suggested that miR-143 and miR-145 levels are greatly reduced in colorectal cancer tissues and deregulation of miR-143/-145 are associated with cancer-related events such as proliferation, invasion, and migration (Bauer et al., 2012). From the other point of view, Christensen et al. exhibited the deregulation of miR-362-3p in CRC tissue samples (Christensen et al., 2013).

As seen above, our miRNA results are compatible with previous researches. However, there are some limitations of the present study. Firstly, our study group was relatively small but, as a valuable feature of our research, we evaluated 16 miRNA expressions in all 40 colon cancer tissue samples and in their 40 corresponding

normal colonic tissues. Secondly, the study population was determined strictly: all participants were stage II colon carcinoma, operated in our General Surgery Department, none of them received adjuvant therapy, followed-up by the same physicians. Although these limitations, we found statistically significant deregulation of several miRNAs in CRC tissue samples containing patients with recurrence and without recurrence and this finding may give new insights to researchers.

In conclusion, our study revealed dysregulation of ten miRNAs expression levels in Turkish stage II colorectal cancer patients. In the light of our findings, these miRNAs may be used as potential biomarkers for early detection, screening and surveillance of colorectal cancer and they may have a functional effect on tumor cell behavior.

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