ORIGINAL

Differential expression of *miR-143*, *miR-145* and *miR-150* in colorectal cancer

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Abstract

Background: Recent reports indicated the involvement of microRNAs (miRNAs) deregulation in colorectal adenomatous mucosa and different stages of colorectal cancer (CRC). **Aims:** The purpose of this study was to analyze a panel of miRNAs previously selected by bioinformatics analysis from SAGE human colorectal cancer library compared to normal colorectal library in order to identify microRNA species with altered expression in colorectal cancer. **Methods:** We analyzed the expression of let-7a, *miR-21*, *miR-15a*, *miR-141*, *miR-143*, *miR-145* and *miR-150* in 15 tumor tissues from patients who undergone surgery for CRC and 4 non-tumor adjacent tissues. The expression of miRNAs was measured by real-time PCR. Relative quantification of miRNA expression was calculated using the 2(-Delta Delta C(T)) method. **Results:** Our findings showed under expression of *miR-143*, *miR-150* and *miR-145* in about 6-fold ($p = 0.05$), 5-fold and 4-fold ($p = 0.01$), respectively, in CRC compared to normal colorectal tissue. In addition, there was no association between each miRNA expression and tumor stage or tumor localization. **Conclusion:** Our results support that under expression of *miR-143*, *miR-150* and *miR-145* might be involved in colorectal carcinogenesis. However, the lack of knowledge about miRNA target genes postpones full understanding of the biological functions of downregulated miRNAs in CRC.

Keywords: colorectal neoplasms, gene expression regulation, micrornas, neoplastic.

INTRODUCTION

Colorectal cancer (CRC) is among the most prevalent and preventable forms of cancer worldwide. It is the second leading cause of cancer related death in the United States. The cumulative lifetime risk of developing colorectal cancer is approximately 5-6%[1]. In Brazil, the estimated cases per 100,000 of new cancer cases by sex are 12,490 and 14,500 for male and female, respectively, according to the Ministry of Health/National Cancer Institute, in 2008. CRC tumorigenesis appears to be the result of a progressive transformation of colorectal epithelial cells. A molecular genetic model based on adenoma to adenocarcinoma progression suggests that successive accumulation of mutations in oncogenes and tumor suppressor genes drives the transition from normal colonic epithelia through increasing dysplastic adenoma to malignant cancer[2]. Inactivation of tumor-suppressor gene adenomatous polyposis coli (APC) and activation of oncogene *K-ras* are considered important determinants of early carcinogenesis[3], while inactivation of tumor-suppressor gene *TP53*[4] and deleted colorectal cancer gene[5] is thought to occur during late carcinogenesis[6].

MicroRNAs (miRNAs) represent a recently identified class of endogenous non-coding RNAs that act as negative regulators of the protein-coding gene expression[7,8]. A rapidly growing number of studies has provided evidence for miRNA deregulation in carcinogenesis of different human cancers and specifically in colorectal cancer[9,10]. Although the biological processes are not yet fully understood, precise regulation of miRNA expression seems to be a crucial factor on intracellular pathways regulation, including cell development, differentiation, proliferation and apoptosis[8,11-13]. Therefore, these findings support the notion that alterations in the expression of miRNAs and their regulatory genes are involved in carcinogenesis.

The expression of *miR-143* and *miR-145* were consistently reduced in the adenomatous and different cancer stages of CRC[14]; similar findings were also demons-

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trated by differential display using microRNA-array[15]. Recent studies in CRC cell lines and in tumor of patients with CRC confirmed the differential expression of several miRNAs[16]. Studies on miRNA expression and function are exponentially growing in order to identify patients at high risk of more invasive tumors. Therefore, in the current study, we examine the profile of expression of let-7a, *miR-15a*, *miR-21*, *miR-141*, *miR-143*, *miR-145* and *miR-150*, previously selected by bioinformatics analysis from SAGE human colorectal cancer library compared to normal colorectal library in order to identify microRNA differently regulated in colorectal cancer.

MATERIALS AND METHODS

Patients

The study was approved by the Institutional Review Board for Human Research of the University Hospital of the School of Medicine of Ribeirão Preto - University of São Paulo and all participants gave their informed consent.

Fifteen colorectal tumor tissues were collected from patients who had undergone surgery for treatment of CRC. We classified the tumors using the Duke's system, which are Duke's stage A, B, C or D depending upon the extent of tumor development and spread. Duke´s stage A: tumor is limited to the bowel wall, lymph nodes negative; Duke´s stage B: tumor spread beyond muscularis propria, lymph nodes negative; Duke´s stage C: any tumor involving the lymph nodes; and Duke´s stage D: presence of distant metastasis. In addition, we also classified tumors according the colorectal cancer TNM classification. Stage I: Tumor invades muscularis propria, but has not spread to nearby lymph nodes; Stage II: Tumor spread into the subserosa and/or perirectal tissues with up to 3 regional lymph nodes, or directly invades adjacent tissues without lymph node involvement; Stage III Any depth of tumor invasion with four or more positive lymph nodes, without distant metastases; and Stage IV: any depth of tumor involvement; any number of involved lymp nodes, with distant metastases.

Samples were snapped-frozen; processed for routine histopathological examination and an aliquot was stored at -70°C until used. As control, four non-tumor adjacent tissues were also obtained during surgery from patients with CRC. Tumor and non-tumor adjacent tissues were microdissected by an experienced pathologist in order to avoid any tissue contamination.

SAGE Analysis

We had previously performed two cDNA SAGE libraries obtained from normal colorectal tissue or colorectal cancer, using I-SAGE kit (Invitrogen Life Technologies, Inc.). Sequences were further analyzed using SAGE2000 software. The annotation of tag sequences was based on the SAGEmap database (http://www.ncbi.nlm.nih.gov/SAGE). Statistical analysis was carried out with the H2G software (http://gdm.fmrp.usp.br) to evaluate hyper- and hypo-expressed genes. Then, let-7a, *miR-15a*, *miR-21*, *miR-141*, *miR-143*, *miR-145* and *miR-150* were identified based on a list of miRNAs differentially expressed in CRC library in comparison with a normal colorectal library.

RNA extraction and Real-time PCR quantification

After microdissection, all 15 samples were disrupted using a *Polytron*™ homogenizer and total RNA was isolated by TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA). The RNA quality was evaluated by spectrophotometry and by agarose gel electrophoresis.

Approximately 2.5 ng of total RNA were used in a reverse transcriptase reaction with final volume of 10 mL, using 20x miRNA specific stem-loop primer (TaqManMicroRNA Assay, Applied Biosystems Foster City, CA), 5.5 mM $MgCl_2$, 2.0 mM dNTP, 20U RNAse Inhibitor, 50U MultiScribe enzyme and 10x Buffer (TaqMan® RT reagents, Applied Biosystems, Branchburg, New Jersey, USA). The reverse transcription PCR cycle sequence was 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes.

Quantitative PCR was performed using 7500 Real-Time PCR System (Applied Biosystems). The final volume 10 µL of PCR included 4.0 µL reverse transcriptase product, 2x TaqMan® Universal PCR master mix (Applied Biosystems, Branchburg, New Jersey, USA) and 0.5 µl of primers and probe mix of the TaqMan MicroRNA Assay protocol (hsa-*miR-141*, Part No. 4373137; hsa-*miR-143*, Part No. 4373134; hsa-*miR-145*, Part No. 4373133; hsa-let 7a, Part No. 4373169; hsa-*miR-15a*, Part No. 4373123; hsa-*miR-21*, Part No. 4373090; and RNU-44, Part No. 4373384) all purchased from Applied Biosystems Foster City, CA. The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 10 min. The cycle threshold (Ct) data were obtained using default threshold settings. The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Normalization and data analysis

Data were presented as $\log_{10}$ of relative quantity (RQ) of target miRNA, normalized by endogenous control RNU44 Ct-median expression and calibrated using a DeltaCt-median value obtained from all normal colorectal tissue. Relative quantification of miRNA expression was calculated using the 2(-Delta Delta C(t)) method[17]. Fold change of the expression of each miRNA observed in CRC samples in relation to normal colorectal tissues was determined by the median of 2(-Delta Delta C(t)) values of CRC tissues related to median of 2(-Delta Delta C(t)) values of normal colorectal tissues.

Statistical analysis

The expression of each miRNA in the CRC samples are presented as the mean, standard error deviation (x ± SE), median, and range. Statistics were carried out using Mann--Whitney test for continuous variables or Fisher`s exact test for categorical data. To analyze the association of each differential miRNA expressed in CRC tissues and tumor stage or tumor localization, we divided tumors samples in two subgroups (expression lesser or equal than the median and greater than the median) based on the 2(-Delta Delta C(t)) values. Data were analyzed by *GraphPad Prism 4* software and differences were considered significant at $p < 0.05$.

RESULTS

Table 1 shows the clinical characteristics, including age, gender, tumor location, Duke´s classification, and TNM stage of our series of patients with CRC. The mean age of the colorectal cancer patients at diagnosis was 62.4 ± 12.4 years. TNM stage varied from I to IV and Duke´s classification from A to D. CRC localization was more frequently observed in rectum.

Table 2 shows the mean-DeltaCt and standard error deviation, median and range of each miRNA obtained from CRC normalized by DeltaCt-median of normal colorectal tissue and also the fold-change of the expression of each miRNA observed in CRC samples in relation to normal colorectal tissues. It also shows the miRNAs putative target genes associated with carcinogenesis. We observed no changes in let-7a, *miR-21*, *miR-15a* and *miR-141* expression in CRC. However, *miR-143*, *miR-150* and *miR-145* expression were down-regulated in about 6- ($p = 0.05$), 5- and 4-fold ($p = 0.01$), respectively, in CRC compared to normal colorectal tissue. There was no association between the expression of *miR-143*, *miR-145* and *miR-150* and tumor stage as well as the expression of these miRNAs and tumor localization.

DISCUSSION

In the present study, we examined the expression of let-7a, *miR-15a*, *miR-21*, *miR-141*, *miR-143*, *miR-145* and *miR-150* in CRC samples compared to normal colorectal cancer. We did not observe differences in the let-7a, *miR--15a*, *miR-21* and *miR-141* expression between normal and tumor tissues. On the other hand, our analysis by Real-time PCR, showed an underexpression of *miR-143*, *miR-145* and *miR-150* in CRC samples.

Deregulation in miRNA expression has been associated with different human tumors, such as chronic lymphocytic leukemia[18], lung cancer[19], breast cancer, glioblastoma[20], hepatocellular carcinoma[21], and thyroid cancer[22,23]. The different biologic effects of any particular miRNA in different cells could be dependent on the cell-specific repertoire in target genes.

Let-7a might act as an anticancer miRNA by repressing *RAS* and/or *c-MYC* expression at the translational level. Therefore, reduced expression of let-7a could result in cell

Table 1. Clinical findings of patients with colorectal adenocarcinoma.

Patient	Age (years)	Gender	Tumor Localization	Duke´s Classification	TNM Classification/(Stage)
1	70	F	Caecum	D	T4N1M1/(IV)
2	54	M	Rectum	A	T2N0M0/(I)
3	51	M	Rectum	B	T4N0M0/(II)
4	68	M	Ascending colon	A	T2N0M0/(I)
5	74	F	Rectum	C	T2N1M0/(III-a)
6	50	M	Descending colon	/	T3NxMx
7	66	M	Rectum	A	T2N0M0/(I)
8	71	M	Rectum	D	T3N1M1/(IV)
9	39	F	Caecum	C	T4N1M0/(III-a)
10	80	F	Rectum	B	T3N0M0/(II)
11	74	M	Descending colon	B	T4N0M0/(II)
12	54	F	Ascending colon	/	T3N1Mx
13	76	M	Caecum	B	T3N0M0/(II)
14	60	M	Rectum	D	T4N0M1/(IV)
15	49	F	Rectum	/	T3N3Mx

F: female, M: male; Duke´s A: tumor is limited to the bowel wall, lymph nodes negative; B: tumor spread beyond muscularis propria, lymph nodes negative; C: any tumor involving the lymph nodes; and D: presence of distant metastasis. Tumor, nodules and metastasis (TNM) classification; Stage I: tumor invades muscularis propria, but has not spread to nearby lymph nodes; Stage II: Tumor spreads into the subserosa and/or perirectal tissues with up to 3 regional lymph nodes, or directly invades adjacent tissues without lymph node involvement; Stage III: any depth of tumor invasion with four or more positive lymph nodes, without distant metastases; and Stage IV: any depth of tumor involvement; any number of involved lymp nodes, with distant metastases.

Table 2. MiRNA expression in colorectal cancer compared to normal colorectal tissue.

miRNA	Mean DeltaCt normal tissue	Mean DeltaCt CRC tissue	x ± SE, median, range of normal tissue, 2(-Delta Delta C(t))	x ± SE, median, range of CRC tissues, 2(-Delta Delta C(t))	Fold change CRC/normal	Putative targets associated with carcinogenesis
let 7a	-2.4 ± 0.5	-2.1 ± 0.3	0.8 ± 0.3; 0.6 (0.3 to 1.7)	-1.7 ± 0.8; -1.6 (-7.5 to 2.1)	-1.1 (p = 0.8)	RAS, c-MYC
miR-15a	2.1 ± 1.0	3.0 ± 0.5	2.2 ± 1.2; 1.4 (0.2 to 5.8)	-2.2 ± -1.7; -1.3 (-20.0 to 5.8)	-1.7 (p = 0.5)	BCL2
miR-21	-4.8 ± 0.9	-4.4 ± 0.5	2.3 ± 1.3; 1.6 (0.32 to 5.9)	-0.2 ± 1.3; 1.5 (-11.5 to 7.9)	-1.1 (p = 0.8)	Tropomyosin 1
miR-141	-0.2 ± 0.9	0.7 ± 0.5	1.6 ± 1,0; 0.8 (0.2 to 4.6)	-5.7 ± 3.4; -1.7 (-44.3 to 5.6)	-1.5 (p = 0.5)	APC, MSH2
miR-143	0.3 ± 0.6	3.5 ± 0.6	1.1 ± 0.5; 0.6 (0.5 to 2.7)	-37.8 ± 19.3; -9.6 (-288.4 to -1.3)	-5.9 (p = 0.01)	ERK5, MAPK
miR-145	-2.3 ± 0.7	0.1 ± 0.5	1.7 ± 0.9; 0.9 (0.5 to 4.6)	-11.8 ± 6.1; -4.5 (-93.5 to 1.5)	-4.2 (p = 0.05)	TGFRII, APC
miR-150	-2.7 ± 0.4	-0.3 ± 0.5	0.9 ± 0.2; 0.9 (0.3 to 1.5)	-13.1 ± 4.2; -5.8 (-52.9 to -1.1)	-5.1 (p = 0.01)	Fms-like tyrosin kinase-3, IL-7R, E2A, EBF, Myb, Foxp1, Pax5

Normal: normal colorectal tissue; CRC: colorectal cancer tissue; Ct: cycle threshold; Fold change of the expression of each miRNA observed in CRC in relation to normal tissue was determined by the median of 2(-Delta Delta C(T)) values of CRC related to median of 2(-Delta Delta C(T)) values of normal tissue.

proliferation leading to carcinogenesis[24]. In the present study, we found no difference in the let-7a expression in CRC tumors compared to normal colorectal tissue, similarly to the data from Michael et al.[14]. However, a reduced expression of let-7a was previously observed in lung cancer[19], as well as in a small series of CRC[13,25,26]. Further studies on let-7a expression in a greater series of CRC will help to clarify the involvement of this miRNA in colorectal carcinogenesis.

We also observed no changes on *miR-15a* expression in CRC tumors compared to normal colorectal tissue. *miR-15a* gene is located at chromosome 13q14, a region which is deleted in more than 50% of B cell chronic lymphocytic leukemia[18] and also in many pituitary adenomas[27-29]. *miR-141*, another studied miRNA, showed no difference in expression in CRC tissue compared to normal colorectal tissue. Previous reports demonstrated up-regulation of *miR-141* in human cholangiocarcinoma cell lines, whereas inhibition of *miR-141* in response to chemotherapy decreased cell growth[30].

In glioblastoma cell lines, an antiapoptotic role of *miR-21* has been described[16]. We did not find alterations in *miR-21* expression in CRC tumors compared to normal colorectal tissue in our small subset of CRC. Recently, microRNA microarray expression profiling of tumors and paired nontumorous tissues was performed on a US test cohort of 84 patients with incident colon adenocarcinoma, recruited between 1993 and 2002. In that study, *miR-20a, miR-106a, miR-181b*, and *miR-203* were also selected for validation. The authors demonstrated that high *miR-21* expression was associated with poor survival, independent of clinical covariates, including TNM staging, and was associated with a poor therapeutic outcome. These data were compared and validated in a second, independent Chinese cohort of 113 patients[20]. Apart from interference with the PI-3-K pathway, *miR-21* has been shown to act on PDCD4, a tumor suppressor gene that is an independent prognostic factor in colorectal cancer. In addition, silencing of *miR-21* by anti-*miR-21* resulted in increased levels of PDCD4 in colorectal cell lines and increased invasion in a chicken-embryo-metastasis assay. These results argue for an important function of *miR-21* in the pathogenesis of CRC and also as a prognostic marker[30]. All studies with identical as well as with conflicting findings need to be further confirmed.

In the present study, using a subset of patients with CRC, we observed an underexpression of *miR-143*, *miR-145*, and *miR-150*. In addition, we observed, in this small series of CRC patients, no association between the expression of *miR-143*, *miR-145* and *miR-150* and tumor stage and/or tumor localization. Reduced *miR-143* and *miR-145* expression has been previously reported in adenomatous and different CRC stages compared to normal colorectal tissue[14,15,31]. These findings are important since several gene transcripts encoding proteins involved in signal transduction have been described as putative targets for *miR-143* and *miR-145* repression[32-36]. Based on seed site analyses (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi), we found miRNA binding sites in the 3'-untranslated regions (UTRs) of different genes involved in cell death, cellular growth and proliferation, cell cycle, gene expression and cancer. Indeed, decreased levels of *miR-143* in cancer may be directly involved in carcinogenesis through activation of the mitogen-activated protein kinase (MAPK). ERK5 is a recently characterized MAPK, which is mostly similar to the well-studied ERK1/2 subfamily, but is substantially higher in molecular weight and uses distinct mechanisms to elicit responses[37].

Our data also demonstrate underexpression of *miR-145* in our series of CRC, in accordance to previous studies[14-16,31]. More recently, down-regulation of *miR-145* has also been reported in lung cancer[38] and breast cancer[39]. *miR-145* target genes encode proteins with potential oncogenic functions, such as MYC, KRAS, FOS, YES, and FLI, as well as cyclins D2, and MAPK transduction proteins,

such as MAPK3K3 and MAPK4K4[39]. Using microarrays to study the expression of several human miRNAs in normal mucosa and stage II colon cancer, it was demonstrated that *miR-145* showed the lowest expression in cancer. Furthermore, a biomarker based on miRNA expression profiles could predict recurrence of disease with an overall performance accuracy of 81%, indicating a potential role of miRNAs in determining tumor aggressiveness[40].

We also found an underexpression of *miR-150* in CRC compared to normal colorectal tissue. To our knowledge, this is the first report showing such differential expression in CRC. Overexpression of *miR-150* has been observed in hematopoietic progenitor/stem cells. Among many genes, *miR-150* targets cytokine genes and transcription factors, such as Fms-like tyrosin kinase-3 (flt3) and IL-7 receptor (IL-7R)-mediated signaling, E2A, early B cell factor (EBF), Myb, Foxp1, and Pax5, which have critical effects on the early stages of B lymphopoiesis[41,42]. Further studies are necessary to understand the role of *miR-150* in human general carcinogenesis and specifically in CRC. It is important to point out that miRNAs are expressed in a tissue specific manner[7,8].

A recent study demonstrated that both *miR-17-3p* and *miR-92* were significantly elevated in the patients with CRC in plasma and tissue samples. The plasma levels of these markers were significantly reduced after surgery in 10 patients with CRC. Further validation with an independent set of plasma samples indicated that *miR-92* differentiates CRC from gastric cancer and normal subjects. This marker yielded a receiver operating characteristic curve area of 88.5%. The authors concluded that *miR-92* can be a potential non-invasive molecular marker for CRC screening[43].

Altogether, these data suggest that reduced expression of *miR-145* and *-143* may have an oncogenic role in CRC. Indeed, functional studies showed that overexpression of *miR-145* potently suppressed growth of three different colon carcinoma cell lines[27]. In addition, similar to our present data, recent observation confirmed that *miR-143* and *miR-145* were down-regulated in CRC[31]. In addition, *in vitro* functional studies indicated that *miR-143* and *miR-145* appear to function in opposing manner to each other, inhibiting or augmenting cell proliferation in a metastatic CRC model[31]. Small RNAs affect gene expression posttranscriptional, however, more recently, it has been suggested that they also mediate gene regulation and induction of epigenetic silencing in the promoter region in plants and mammals by DNA methylation, which would depend on the ratio of the miRNA and its target RNA[44].

In conclusion, our results support, in additional to several other reports, that under expression of *miR-143*, *miR-150* and *miR-145* might be involved in colorectal carcinogenesis. However, the lack of knowledge about miRNA target genes and the precise mechanisms of how RNA mediated transcriptional gene silencing or activation function postpones full understanding of the biological functions of miRNAs in CRC.

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