Tennessee Medicine E-Journal

Volume 2
Issue 3 *Issue 3*

Article 11

February 2017

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Cover Page Footnote
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INTRODUCTION

Colorectal cancer (CRC) is ranked number four in the world in cancers causing death. An approximate of 610,000 patients die of CRC per year (1). According to the 2008 statistics; 1.24 million people have been diagnosed with CRC, which make up 10% of the entire cancer diagnosis of the same year (2). The survival rate of the patients with early diagnosis is high. Approximately 90% of the patients with CRC have a survival rate of 5 years. However, in the later stages of cancer and the metastatic cases, the prognosis can be poor (3). There are many biomarkers discovered in different cancer pathways that might be useful for the diagnosis and treatment of colorectal cancer. As these findings are limited and need further research.

miRNA is a small non-coding protein and is made up of 20-25 nucleotides. The miRNA connects with the target mRNA containing the nucleotide sequence complementary to its own and perform translational repression or regulation of gene expression post-transcriptional to mRNA degradation (4). miRNAs are involved in the onset and progression of cancer as well as in many diseases (5). It is also suggested that miRNAs are modulators of invasion and metastasis of the progression of tumor due to the excess or deficient expression of miRNAs (6,7).

This study aims to look at the expression levels of some miRNAs (miR-302a, miR-302b, miR-520a-3p, miR-520b, miR-520d-3p, miR520e) in the autophagy process in locally advanced stage colorectal cancer patients.

MATERIAL AND METHOD

Tissue samples were collected from the archives of the Kahramanmaras Sutcu Imam University Hospital – Pathology Department; from tumor and neighboring normal tissues of 50 local advanced stage (T3 and T4) colorectal cancer patients. The age of the patients varied from 26 to 86. Patients underwent surgical resection without preoperative neo-adjuvant therapy between 2009-2014. Patients have been monitored for a mean of 31 months (min: 2, max 66). Seven patients died within the first-month post-operation and were removed from the survival tests. The approval of the research protocols was obtained from Clinical Research Ethical Committee of Kahramanmaras Sutcu Imam University.

Total RNA isolation and cDNA conversion

Both tumoral and adjacent normal colorectal specimen tissues of 50 patients were processed by miRNeasy FFPE Kit (Qiagen GmbH, Hilden, Germany) to obtain total RNA for miRNA expression. miScript II RT Kit (Qiagen GmbH, Hilden, Germany) was used to obtain cDNA for qRT-PCR analysis. Rotor-Gene Q (Qiagen Germany) Thermal Cycler was used to reveal expression of miRNAs. Real-time PCR was performed using Rotor-Gene 6000 Real-Time PCR Machine (Qiagen GmbH, Hilden, Germany) with miScript SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany) for miRNA expression.

Statistical Analysis

SPSS 20.0 software (SPSS, USA) was used for statistical analysis. Relative quantification RT-PCR was performed in triplicate. $2^{-\Delta Ct.}$ method was used to calculate relative changes. To calculate fold changes in miR-302a**,** miR302b, miR520a-3p, miR520b, miR520d-3p and miR520e expressions between tumor and adjacent normal tissues, RT^2 Profiler PCR Array Data Analysis version 3.5 (Qiagen GmbH, Hilden, Germany) was used. This analysis program was based on $2^{-\Delta\Delta Ct.}$ method for fold change calculations. Beta Actin expression level was used for normalization.

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Statistical Procedures

Due to the irregular distribution of miRNA fold change values in tumor and normal tissue samples (with Kolmogorov-Smirnov test); student t-test was performed after log2 transformation. Mann-Whitney U test was used to evaluate the relationship between miRNA fold change values with clinicopathological data. The relationship between miRNA and survival was determined by Cox Regression test. The correlation between different miRNAs was evaluated using Pearson Correlation test. The analysis was performed with SPSS (Statistical Package for Social Sciences, version 20.0, ABD) for Windows program. $P<0.05$ was considered to be statistically significant.

RESULTS

*The relationship between miR-302a, miR-302b, miR-520a-3p, miR-520b, miR-520d-3p, miR-520e expressions in colorectal cancer tissues and normal neighboring tissues*CT values were obtained with RT-PCR and fold change values were calculated using $2^{-\Delta Ct}$ formula that was normalized. The endogen control was selected to be RNU-6. Fold change values were transformed into log2 to obtain a normal distribution. The mean and standard error values for miR-302a, miR-302b, miR-520a-3p, miR-520b, miR-520d-3p, miR-520e were revealed to be 0.02±0.37, -0.60±0.38, 0.35±0.33, -1.70±0.30, -1.46±0.33 and -0.66±0.29 respectively. There was a significant decrease in expression levels of miR-520b (p: 0.001), miR-520d-3p (p: 0.001), miR-520e (p: 0.031) in tumor tissues compared to normal tissues (Figure 1). No valuable correlation was observed between miRNAs and clinicopathologic factors (patient age, gender, tumor size, tumor stage, histologic grade and tumor).

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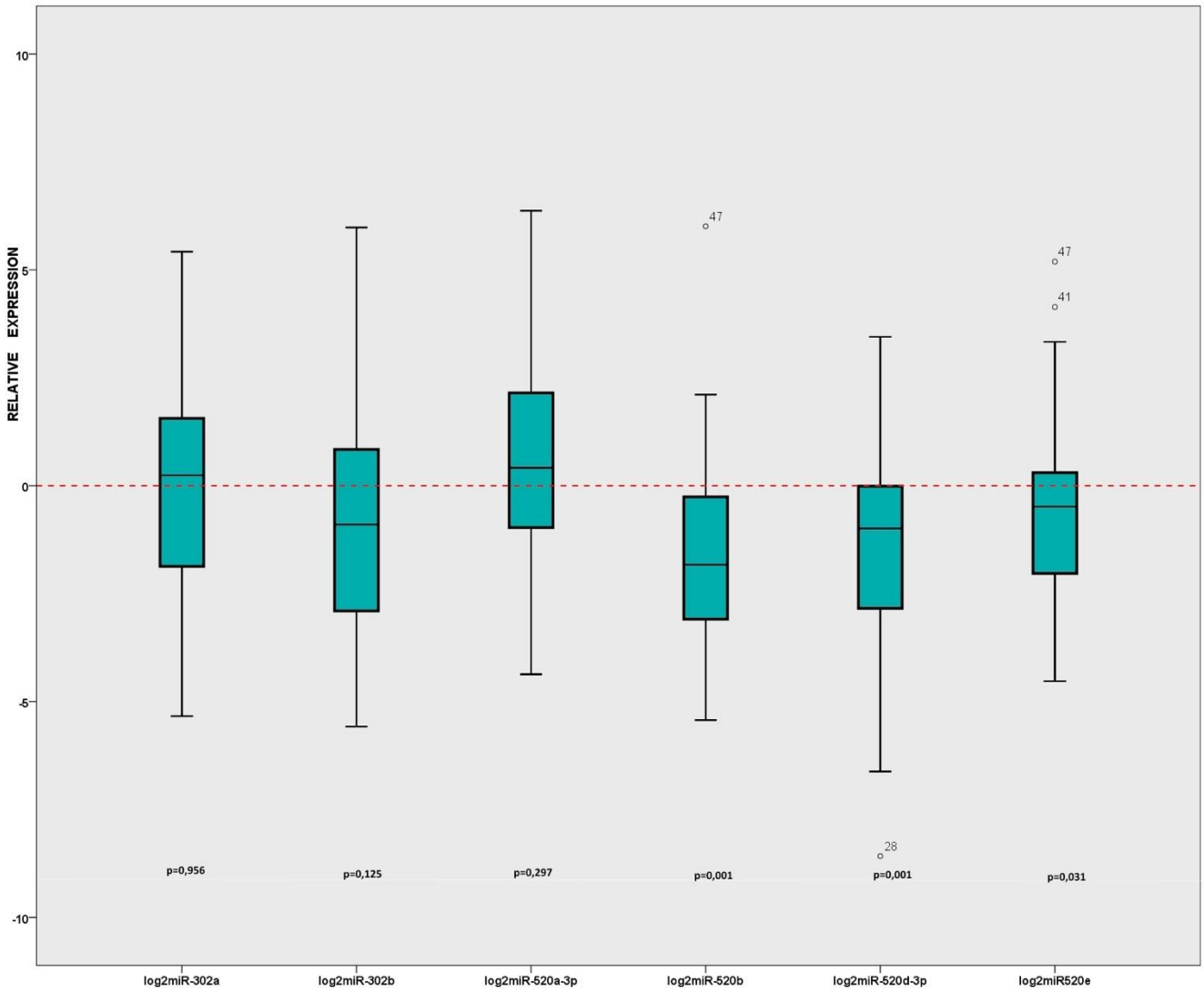

FIGURE 1: Real-time PCR analysis of miRNA expressions in colorectal carcinoma and adjacent normal tissue by using relative quantification method. The results were normalized with endogenous control RNU6, and the fold change was calculated by equation $2^{-\Delta\Delta Ct}$. Fold change values were transformed into log2 to obtain a normal distribution. Statistical significance was determined by student t-test. miR520b, miR520d-3p, miR520e significantly downregulated in colorectal tissues compared with noncancerous tissue($P<0.05$)

Although there was no relationship between survival and expression of miR-302a, miR-302b, miR-520a-3p, miR-520d-3p and miR-520e, there was a statistically high accordance between miR-520b and survival (p=0.048).

A significant -moderate- positive correlation was found between miR-520b, miR-520d-3p, miR-520e expressions (Pearson Point-Biserial correlation test, r=[0,483]-[0,560]). While no accordance was observed between miR-302a and miR-520b, there was meaningful relationship between miRNAs in other

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matchings (r=[0,288]-[0,603]). Contrary, there was a significant -strong- negative correlation between miR-520a-3p and other miRNAs (r=[-0,715]-[-0,359]). (Table 1 and Figure 2)

Correlations

		log2miR302a	log2miR302b	log2miR520a-3p	log2miR520b	log2miR520d-3p	log2miR520e
log2miR302a	Pearson Correlation	1	,430**	-,359*	,239	,508**	,603**
	Sig. (2-tailed)		,002	,011	,095	,000	,000
	N	50	50	50	50	50	50
log2miR302b	Pearson Correlation	,430**	1	-,394**	,340*	,515**	,288*
	Sig. (2-tailed)	,002		,005	,016	,000	,043
	N	50	50	50	50	50	50
log2miR520a-3p	Pearson Correlation	-,359*	-,394**	1	-,464**	-,715**	-,560**
	Sig. (2-tailed)	,011	,005		,001	,000	,000
	N	50	50	50	50	50	50
log2miR520b	Pearson Correlation	,239	,340*	-,464**	1	,483**	,560**
	Sig. (2-tailed)	,095	,016	,001		,000	,000
	N	50	50	50	50	50	50
log2miR520e	Pearson Correlation	,603**	,288*	-,560**	,560**	,558**	1
	Sig. (2-tailed)	,000	,043	,000	,000	,000	
	N	50	50	50	50	50	50
log2miR520d-3p	Pearson Correlation	,508**	,515**	-,715**	,483**	1	,558**
	Sig. (2-tailed)	,000	,000	,000	,000		,000
	N	50	50	50	50	50	50

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

TABLE 1: Bivariate analysis of miRNA expressions (Pearson Correlation Tests

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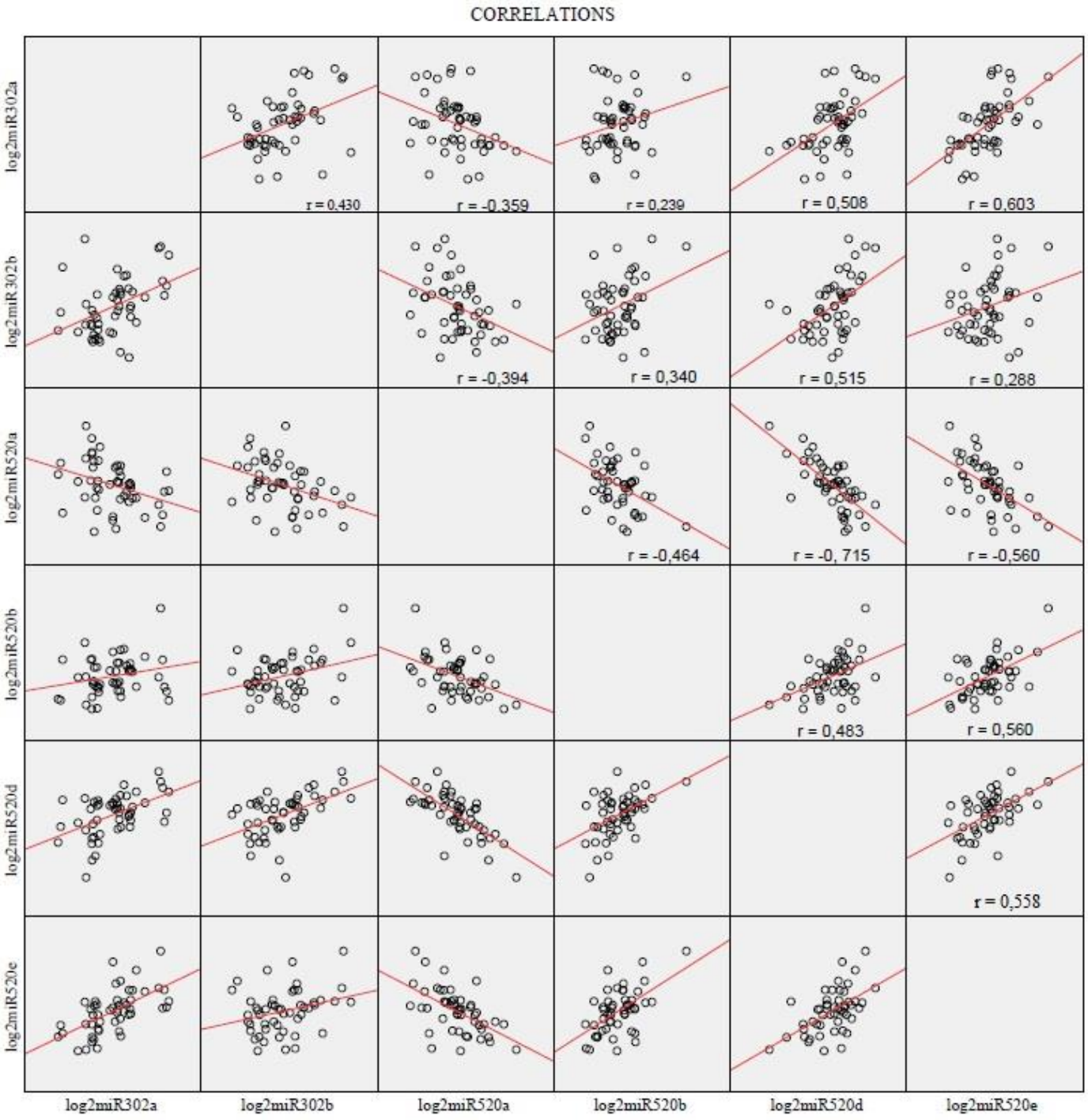

FIGURE 2: Bivariate analysis of miRNA expressions with each other (Pearson Correlation Tests-Scatterplot Matrix).

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DISCUSSION

The study group included patients with locally advanced stage T3/T4 (with or without lymph node involvement) and stage 2/3/4 colorectal carcinoma. Radiotherapy and chemotherapy in addition to surgery were added to the treatment of patients with stage 2/3/4 or locally advanced stage tumors as there was an increased risk of advanced local recurrence or relapse. Current studies on these patient groups focus on the new chemotherapeutic agents and models that will increase the efficiency of chemotherapy.

miRNA's a key role in many biological processes including cell proliferation and apoptosis. The possibility of identifying the expression differences between cancer and normal tumor cells highlighted the importance of the role of miRNAs in cancer pathogenesis. miRNAs that have decreased the level of expression in cancer are classified as tumor suppressors (8). The results illustrated significant downregulation of miR-520b, miR-520d-3p and miR-520e in colorectal cancer tissues compared to normal tissues. These miRNAs might have a tumor suppressor role in colorectal carcinogenesis. In literally, Fang et al. demonstrate that the plasma levels of miR-302a were reduced in patients with colorectal carcinoma compared with healthy controls and they suggest that microRNA-320a in a potential biomarker for colorectal carcinoma (9). In additionally, miR-302a expression was found downregulated in ovarian cancer (10), metastatic breast carcinoma cell lines (11), and prostatic carcinoma (12). In our study, we were not observed a significant difference between colorectal carcinoma and normal tissue sample in term of miR-302a expression levels.

Wang H et al. presented that miR-302b expression was downregulated in both Colorectal carcinoma tissues and cell lines also miR-320b suppresses cell proliferation by targeting c-Myc (13). Other reports have shown miR-302b downregulation in several kinds of tumors including esophageal squamous cell carcinoma (14), ovarian epithelial cancer(15), and hepatocellular carcinoma(16). In Student's t-test, we observed downregulation in mean and standard error values of miR-302b expression (0.60±0.38), but there were not statistically significant.

There is one study in cancer research literature about miR-520a-3p. Yu J et al. found that miR-520a-3p expression was decreased in Non-Small Cell Lung Cancer tissues compared with their normal counterparts (17). Unlike this study, in our results miR-520a-3p expression was increased and there is a strong negative correlation between miR-520a-3p and other miRNAs (r=[-0,715]-[-0,483]).

There is still no report showing miR-520b, miR-520d-3p, and miR-520e expression in colorectal carcinoma. There are some reports that present downregulation of these miRNA's in different types of cancer for instance miR-520b in hepatocellular carcinoma (18,19), breast carcinoma (20,21), and glioblastoma (22) miR-520d-3p in gastric cancer (23), and ovarian cancer (24); miR-520e in hepatocellular cancer (25), and non-small cell lung cancer (26); In contrary, miR-520b was reported as overexpressed in urothelial bladder cancer (27).

In our study, we found that miR-520b, miR-520d-3p, and miR-520e were significantly underexpressed in colorectal carcinoma when compared with normal colorectal tissues. We also found strong positive correlation between these miRNA's (r=[0,483]-[0,560]). In a recent study, Chen D et al. reported that miR-93, miR-302a, miR-302b, miR-302c, miR-302d, miR-372, miR-373, miR-520e, miR-520d, miR-520b and miR-520a were closely associated with the occurrence and development of breast cancer(28). We also found a correlation in bivariate analysis between miR-520b, miR-520e, miR-520d, miR-520a, miR-302a, miR-302a and therefore it may be a relation in colorectal carcinoma and these miRNA's.Table 2 summarizes miRNAs included in this study and their expression profile in various cancer types. In these studies, miRNAs were associated with different pathways in various cancer types. However, as can be seen, almost in all studies, miRNAs were reported to be downregulated.

	DOWNREGULATED	UPREGULATED
miR-302a	Colorectal Carcinoma/Plasma Sample (Fang et al. [9]) Ovarian Cancer/Tissue Sample (Guo et al.[10]) Metastatic Breast Carcinoma/Cell Lines (Liang et al. [11]) Prostatic Carcinoma/Tissue Sample (Zhang GM et al.[12])	
miR-302b	Esophageal SCC*/Tissue Sample (Zhang M. et al. [13]) Colorectal Carcinoma/Tissue Sample & Cell Lines (Wang et al. [14]) Epithelial Ovarian Cancer/Tissue Sample (Ge et al. [15]) HCC** /Tissue Sample (Wang et al. [16])	
miR-520a-3p	NSCLC*** / Tissue Sample (Yu et al.)[17]	
miR-520b	HCC/Tissue Sample (Zhang W. et al. [18,19]) Breast Carcinoma/Tissue Sample & Cell Lines (Cui et al.[20], Hu et al..[21]) Glioblastoma (Liu, X et al.)[22]	Urothelial Bladder Cancer (Kriebel et al.)[27]
miR-520d-3p	Gastric Cancer/Cell Lines (Li et al. [23]) Ovarian Cancer/Tissue Sample (Nishimura et al. [24])	
miR-520e	NSCLC/Tissue Sample (Ma et al. [25]) HCC/Tissue Sample&Cell Lines (Zhang et al. [26])	

TABLE 2: Summary of studies about miR-302a, miR-302b, miR-520b, miR-520a-3p, miR-520d-3p and miR-520e in literature.

*SCC = Squamous Cell Carcinom, **HCC =Hepatocellular Carcinoma, ***NSCLC=Non Small Cell Lung Carcinoma

Although not statistically meaningful, there was an overexpression tendency of miR-520a-3p and in contrary downregulation tendency of other miRNAs. This tendency was especially explicit in bivariate analysis (Pearson correlation test). There was a medium-significant positive correlation between the expressions of miR-302a, miR-302b, miR-520b, miR-520d-3p and miR-520e. On the other hand, there was a high-medium significant negative correlation in miR-520a-3p (Figure 2). (No meaningful correlation was recorded between miR-302a and miR-520b).

In conclusion, this study demonstrates a significant downregulation of miR-520b, miR-520d-3p, miR-520e in local advanced stage colorectal cancer. miR-520b might be a promising biomarker for colorectal cancer diagnosis and treatment. Similarly, although not as strong as miR-520b, downregulation of miR-520d-3p and miR-520e is striking. However, further studies are required to illuminate the importance of miR-520b, miR-520d-3p and miR-520e in colorectal cancer.

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