OTJHS
Online Turkish Journal of Health Sciences

e-ISSN: 2459-1467

OTSBD
Online Türk Sağlık Bilimleri Dergisi

Online Turkish Journal of Health Sciences 2023;8(1):16-23 *Online Türk Sağlık Bilimleri Dergisi 2023;8(1):16-23*

Ten-Year Recurrence Outcomes in Early Stage and Early Onset Colon Cancer and the Expression Profiles of MicroRNAs and Cancer Stem Cell Markers in These Tumors

Erken Evre Kolon Kanserinde On Yıllık Nüks Sonuçları ve Bu Tümörlerde MikroRNA'ların ve Kanser Kök Hücre Belirteçlerinin Ekspresyon Profilleri

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ABSTRACT

Objective: We hypothesized that microRNAs (miRNAs) might be involved in tumor development by critically regulating cancer stem cell (CSC) markers in the early stages of colon cancer (eCC). This study aimed to determine the expression profiles of miRNAs in CSC-positive eCC patients and examine their associations with recurrence.

Materials and Methods: We analyzed CD133, LGR5 and SOX2 expression profiles to determine CSC status in 30 eCC specimens. Then, using the results of RT2 miRNA PCR custom arrays, we evaluated the expression profiles of 38 miRNAs in CSC-positive eCC patients.

Results: Recurrence occurred in 5 patients within ten years after surgery. We determined down-regulation of miR-125b and up-regulation of miR-135b were significant in CSC-positive eCC patients (p=0.021, p=0.001, respectively). We found that low expression of miR-125b was associated with recurrence in eCC (p=0.0022).

Conclusions: We suggest that recurrence might be prevented by increasing the expression of miR-125b in eCC.

Keywords: Cancer stem cell, colon cancer, early-onset, early-stage, microRNA

ÖZ

Amaç: MikroRNA'ların (miRNA'lar), kolon kanserinin erken evrelerinde (eCC) kanser kök hücre (CSC) belirteçlerini kritik olarak düzenleyerek tümör gelişiminde rol oynayabileceğini varsaydık. Bu çalışmanın amacı, CSC pozitif eCC hastalarında miRNA'ların ekspresyon profillerini belirlemek ve nüks ile ilişkilerini incelemektir.

Materyal ve Metot: 30 eCC örneğinde CSC durumunu belirlemek için CD133, LGR5 ve SOX2 ekspresyon profillerini analiz ettik. Ardından, RT2 miRNA PCR özel dizilerinin sonuçlarını kullanarak, CSC pozitif eCC hastalarında 38 miRNA'nın ekspresyon profillerini değerlendirdik.

Bulgular: Ameliyattan sonraki 10 yıl içinde 5 hastada nüks meydana geldi. CSC pozitif eCC hastalarında miR-125b'nin aşağı regülasyonunun ve miR-135b'nin yukarı regülasyonunun anlamlı olduğunu belirledik (sırasıyla p=0,21, p=0,001). miR-125b'nin düşük ifadesinin eCC'de tekrarlama ile ilişkili olduğunu bulduk (p=0,0022).

Sonuç: eCC'de miR-125b ekspresyonunun arttırılmasıyla nüksün önlenebileceğini düşünüyoruz.

Anahtar Kelimeler: Erken başlangıçlı, erken evre, kanser kök hücresi, kolon kanseri, mikroRNA

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Yayın Bilgisi / Article Info:
Gönderi Tarihi/ Received: 28/02/2022
Kabul Tarihi/ Accepted: 13/02/2023
Online Yayın Tarihi/ Published: 05/03/2023

Atıf / Cited: Ak Aksoy S and et al. Ten-Year Recurrence Outcomes in Early Stage and Early Onset Colon Cancer and the Expression Profiles of MicroRNAs and Cancer Stem Cell Markers in These Tumors. *Online Türk Sağlık Bilimleri Dergisi* 2023;8(1):16-23. doi: 10.26453/otjhs.1074644

INTRODUCTION

Colon cancer (CC) is the third leading cause of cancer-related death worldwide. Recurrence following surgery is a major problem in CC. Approximately 10 –20% of early-stage CC (eCC) patients (T1-T3N0M0) develop recurrence after curative resection.[1] Recent studies suggested that CC tumors with poor prognosis originated from a unique and rare subset of cancer cells having self-renewal capacity and the potential to differentiate into several cell lineages.[2] These cancer cells with stem cell-like properties, referred to as cancer stem cells (CSCs), are responsible for cancer initiation, progression, and metastasis.[3]

Many surface proteins, such as CD44, CD133, CD24, SSEA3, LGR5 and SOX2, have been identified as CSC markers. CD133 (prominin 1, AC133) is a transmembrane and cell surface protein. Overexpression of CD133 correlated with survival and recurrence in cancer.[4] It has been shown to be a characteristic of CSCs in CC. LGR5 is a G-protein-couple receptor considered a CSC biomarker in CC. It is rarely expressed in normal tissue.[4] SOX2, involved in the induction of pluripotent stem cells, is associated with poor prognosis in cancer tissue.[8] Emerging evidence suggests that CD133, LGR5 and SOX2 may be involved in tumor maintenance, therapy resistance, tumor progression, and recurrence in colorectal cancer. Despite their potential clinical significance, how intrinsic CSC properties are regulated at the molecular level needs to be better understood.[6] Therefore, there is an urgent need to understand the molecular mechanisms controlling CSC populations and functions to develop effective therapies to eradicate recurrences, which are a real threat to complete cancer cures.

MicroRNAs (miRNAs) are small noncoding RNAs that function as negative regulators of mRNAs. Functional studies indicate that miRNAs regulate molecular pathways in cancer via targeting various oncogenes and/or tumor suppressors.[5] Recent evidence suggests that miRNAs may also be involved in tumor development by critically regulating CSCs.[9,10]

This study aimed to determine the expression profiles of miRNAs in CSC-positive and early-stage CC patients and to examine their associations with recurrence formation. The identification of patients with a high risk of dissemination in this subset may optimize the use of adjuvant therapies. In addition, there are no known molecules specific to CSCs among current cancer treatment modalities. Therefore, we aimed to contribute to developing biomarkers for miRNA-based therapies, mainly targeting CSCs.

MATERIALS AND METHODS

Ethics Committee Approval: The study was approved by Bursa Uludag University Faculty of Medicine Clinical Research Ethics Committee (Date: 13.01.2015, decision no: 2015-1/35) and was by the ethical standards of the Declaration of Helsinki.

Patient Population and Clinical Specimens: The eCC archive database of the Uludag University Medical Faculty and the Department of General Surgery was used to collect clinical information and follow-up data for these patients. Basic clinical and tumor characteristics, such as age, gender, location, and pathological stage (assessed by the tumor-node-metastases classification), were analyzed. All patients were considered sporadic cases and microsatellite stable. Only CC patients under the age of 50 were included. Any patient receiving preoperative chemotherapy and/or radiation was excluded to avoid a confounding influence on tumor composition and clinical outcome.

Total RNA Extraction and Quality Control: Colon tumors and surgical margins (normal colon tissue) from 30 patients were formalin-fixed and paraffin-embedded (FFPE). Total RNA from microdissected cells was isolated using the RNeasy FFPE Kit (Qiagen, Germantown, Maryland, USA). According to the manufacturer's protocol, miRNAs were also extracted from specimens using a miRNeasy Mini Kit (Qiagen). All RNA samples were assessed for RNA quantity and quality using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA samples with 1.8–2.0 for 260:280 ratios, >1.8 for 260:230 ratios and with a total concentration ranging from 200 to 400 ng/µl were selected for complementary DNA synthesis.

RT–PCR Assay: Complementary DNA was synthesized from 5 ng total RNA using the RT2 mRNA First Strand Kit (Qiagen), followed by analysis by Light Cycler 480II (Roche Diagnostics, Indianapolis, USA) to profile CD133, LGR5 and SOX2 expression levels in samples. Accession numbers; ACTB: NM_001101.2, SOX2: NM_003106, LGR5: NM_003667 and CD133: NM_00356.

RT-PCR-based miRNA Expression Profiling: Complementary DNA was synthesized from 5 ng small RNA-enriched total RNA using the RT2 miRNA First Strand Kit (Qiagen). Samples were analyzed for the presence and differential expression of 38 miRNAs related to CRC formation using custom RT2 miRNA PCR arrays (SABiosciences, Frederick Md, USA) according to the manufacturer's instructions using a Light Cycler 480II (Roche Diagnostics). One 384-well plate containing 48 assays in six replicates (42-miRNA panel consisting of four duplicated miRNAs and 38 individual miRNAs) was used for six samples. The accession numbers of pri-

mers are listed in Table 1. The RNA input was normalized to endogenous controls including SNORD 44, SNORD 47 and SNORD 48 for miRNAs and the TATA-binding protein for protein-encoding genes.

Statistical analysis: Significant differences among the various pathological and clinical characteristics depending on mRNA expression levels in the study group were calculated using the Chi-squared test (χ^2)

Table 1. Accession numbers of miRNA primers.

miRNA Sanger ID	miRNA accession number	miRNA Sanger ID	miRNA accession number	miRNA Sanger ID	miRNA accession number
hsa-miR-21	MIMAT0000076	hsa-miR-16	MIMAT0000069	hsa-miR-221	MIMAT0000278
hsa-miR-143	MIMAT0000435	hsa-miR-181b	MIMAT0000257	hsa-miR-223	MIMAT0000280
hsa-miR-145	MIMAT0000437	hsa-miR-200c	MIMAT0000617	hsa-miR-148a	MIMAT0000243
hsa-miR-19a	MIMAT0000073	hsa-miR-139-3p	MIMAT0004552	hsa-miR-200a*	MIMAT0001620
hsa-let-7a	MIMAT0000062	hsa-miR-26a	MIMAT0000082	hsa-miR-15b	MIMAT0000417
hsa-let-7b	MIMAT0000063	hsa-miR-27a	MIMAT0000084	hsa-miR-15a	MIMAT0000068
hsa-let-7c	MIMAT0000064	hsa-miR-30a	MIMAT0000087	hsa-miR-20a	MIMAT0000075
hsa-miR-17	MIMAT0000070	hsa-miR-34a	MIMAT0000255	hsa-miR-99a	MIMAT0000097
hsa-miR-155	MIMAT0000646	hsa-miR-96	MIMAT0000095	hsa-miR-498	MIMAT0002824
hsa-miR-29b	MIMAT0000100	hsa-miR-133b	MIMAT0000770	hsa-miR-320	MIMAT0000510
hsa-miR-106a	MIMAT0000103	hsa-miR-135b	MIMAT0000758	hsa-miR-183	MIMAT0000261
hsa-miR-139-5p	MIMAT0000250	hsa-miR-125b	MIMAT0000423	hsa-miR-124	MIMAT0000422
hsa-miR-191	MIMAT0000440	hsa-miR-137	MIMAT0000429		

and Fisher's exact test. The samples were analyzed for the presence and differential expression of a panel of 38 miRNAs, depending on LGR5 and SOX2 expression status, using RT^2 Profiler PCR Array Data Analysis (http://www.sabiosciences.com/pcr/arrayanalysis.php) to compare the PCR array analysis results and the characteristics of the tumors and patients. Progression-free survival curves were plotted using the Kaplan-Meier method. The log-rank test was used to assess the survival differences between groups. Overall survival was defined as the interval between sampling and the last follow-up. The chi-squared test (χ^2) and Fisher's exact test were performed using SPSS 16.00 software for Windows (Chicago, IL, USA), and the Kaplan-Meier analysis and log-rank test were performed using MedCalc 12.4.0 statistical software (Ostend, Belgium). The 95% confidence intervals were calculated using associated estimated standard errors. A p-value < 0.05 was considered significant.

RESULTS

The present study included 30 unrelated patients who were diagnosed with early-onset eCC. The median age at diagnosis was 36.39±1.15 years (range: 18–47 years), and the study included 16 males and 14 females. Primary tumors were localized in the right colon in 15 patients and the left in 15 patients. Recurrence was observed in 5 patients within ten years of surgery. The expression profiles of CD133, LGR5 and SOX2 were evaluated among different patients and tumor characteristics to determine prognostic predictions. The rate of positive expression of CD133, SOX2 and LGR5 in our eCC tumor samples were 57% (17/30), 24% (7/30) and 77% (23/30), respectively. Twenty-three of 30 tumors had high expression levels for at least two of the CD133, LGR5 and SOX2 genes, and they were identified as aggressive tumors due to CSC marker positivity. There was no correlation between the expression of these genes and age, gender, or stage (p>0.05). CSC marker-positive status was associated with right localization and recurrence over ten years of follow-up (p<0.05). CSC-positivity was observed in 86% of patients with recurrences. The expression profiles of LGR5 were 3.24-fold (Figure 1A), the expression profiles of CD133 were 2.17-fold (Figure 1B), and the expression level of SOX2 was 2.05-fold upregulated (Figure 1C) in recurrent CC compared with non-recurrent CC (Figure 1, p<0.05).

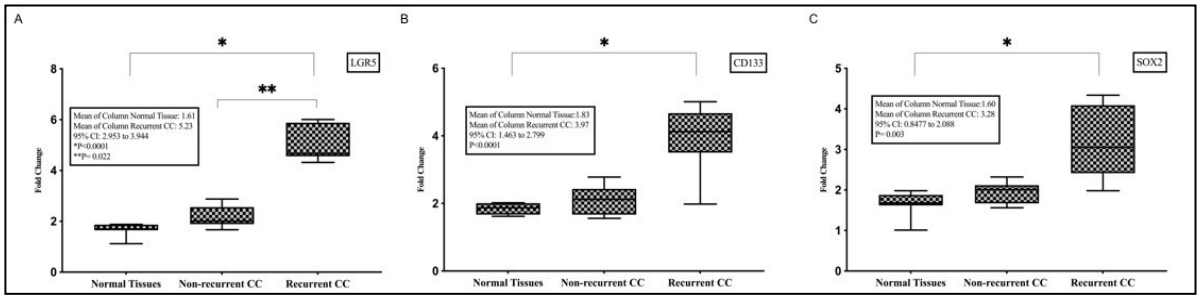

Figure 1. CSC marker status in non-recurrent, recurrent tumors and normal tissues.

Using RT^2 miRNA PCR custom arrays, we determined the expression profiles of 38 different miRNAs in the 30 colon tumor samples. The expression profiles of these tumor cells were compared with 10 normal colon mucosa samples. Among the 38 miRNAs, low expression levels of miR-125b, miR-143, miR-145, miR-125b and high expression levels of miR-106a, miR-135b were found to be significant in early-onset eCC (Table 2).

Table 2. Clinical and bio-pathological features of the CRC patients included in this study, depending on the presence of CSCs markers.

	Characteristics	Number of cases (%)	CSC markers negative (%)	CSC markers positive (%)	p value
Total number		30	7	23	
Patients Median age (year)	18-29	3 (10.00)	0 (00.00)	3 (13.04)	
	30-39	17 (56.67)	4 (57.14)	14 (60.86)	> 0.05
	40-47	10 (33.33)	3 (42.86)	6 (26.08)	
Gender	Male	16	3 (42.86)	13 (56.52)	> 0.05
	Female	14	4 (57.14)	10 (43.48)	
Tumor side	Right Colon	15 (50)	2 (28.57)	13 (56.53)	< 0.05
	Left Colon	15 (50)	5 (71.43)	10 (43.48)	
Stage (T)	T1	9 (26.67)	2 (28.57)	7 (30.43)	
	T2	7 (26.67)	2 (28.57)	5 (21.74)	> 0.05
	T3	14(46.66)	3 (42.85)	7(30.43)	
Recurrence status	No	14 (46.67)	3 (50.00)	11 (57.89)	< 0.05
	Yes	11 (36.67)	3 (50.00)	8 (42.10)	
Overall survival	Five years and over	26 (86.67)	7 (100.00)	19 (82.60)	> 0.05
	Less than five years	4 (13.33)	0 (00.00)	4 (17.39)	
Disease free survival	Five years and over	11 (36.67)	4 (57.14)	12 (52.17)	< 0.05
	Less than five years	14 (46.67)	3 (42.86)	11 (47.83)	

Then, miRNA expression differences between normal colon mucosa and the tumors with different CD133, LGR5 and SOX2 expression statuses as their CSC statuses were evaluated. When sorting the 38 miRNAs based on the fold-change of expression levels (more than 1.5-fold) between these two groups, miR-125b was the top miRNA-exhibiting expression level loss, and miR-135b was the maximum miRNA-exhibiting expression level gain in colon tumors with CSC markers compared to colon tumors without CSC markers. The expression level of miR-135b exhibited approximately 2.01-fold gain, and the expression level of miR-125b exhibited approximately 2.12-fold loss in colon tumors with CSC markers compared to colon tumors without CSC markers (Figure 2A and Figure 2B).

Expression profiles were particularly evaluated for T1-3 CC tumors to determine the true prognostic values of CSC status and characteristic features. The expression level of miR-125b was lower, and that of miR-135b was higher in colon tumors with CSC markers than in colon tumors without CSC markers. Expression levels of these miRNAs were evaluated in different subgroups of tumor tissues to clarify the role of these miRNAs in terms of clinicopathological features. There were no correlations between the

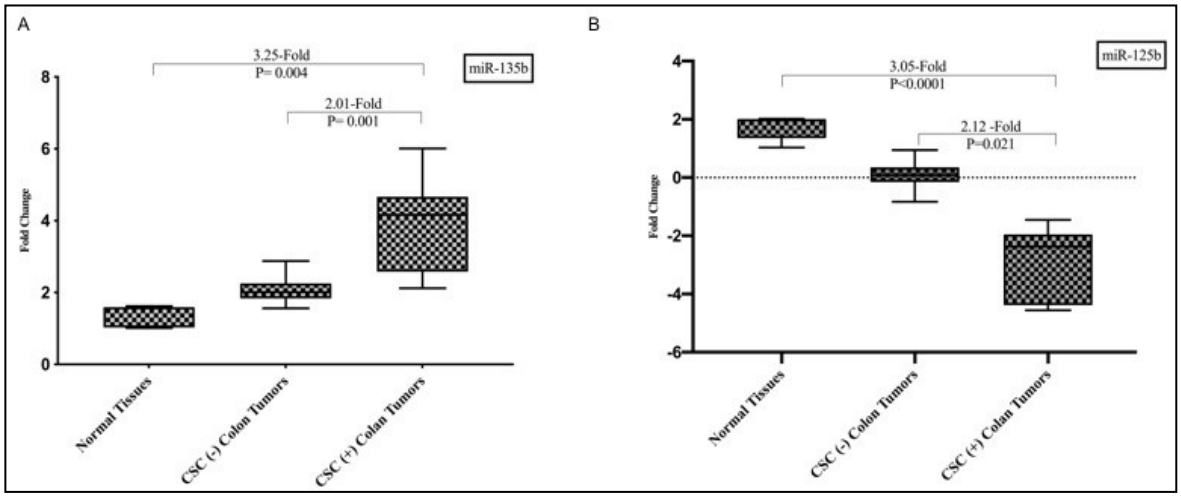

Figure 2. The differential expression of miR-135b (A) and miR-125b (B) depending on CSC marker expression status

expression of these miRNAs and tumor size, gender, or age in CSC-positive eCC tissues. However, a low expression level of miR-125b was associated with recurrence in CSCs (4.12-fold; p=0.002). There were no associations between miR-135b expression status, recurrence status and any histopathological characteristic in CSC-positive eCC tumors.

Follow-up was carried out for ten years in surviving patients. According to Kaplan-Meier analysis and log-rank test, DFS increases in patients with high expression levels of miR-135b expression were not significant (log-rank, p value >0.05). However, DFS was higher in patients with low expression levels of miR-125b than in patients with the opposite expression levels of these miRNAs (Figure 3A and Figure 3B, log-rank, p value <0.05). Therefore, these results suggest that the downregulation of miR-125b would be associated with DFS in colon tumors having CSCs if the case numbers were larger.

DISCUSSION AND CONCLUSION

CSC has been identified in almost all major cancer types, including breast cancer, leukemia and CC. Importantly, CSCs are resistant to traditional anti-cancer therapies, such as chemo-radiotherapy, and these cells also have the ability to sustain systemic/ local relapse. Thus, understanding the biology of CSC in CC is of great clinical significance.[6]

Recent findings indicate that the role of the abnormal expression of miRNAs is related to the regulation of CSC properties, such as asymmetric cell division, tumorigenicity, and drug resistance.[7,8] CSCs usually share specific stemness-related markers, such as CD44 and CD133, but they can differ depending on the cancer type. Early studies showed that miR-34a suppressed prostate CSCs and metastasis by directly repressing CD44.[10] However, there has been no study focusing on miRNAs specifically involved in the regulation of CSCs in early-onset eCC tumors. In our study, we evaluated 38 individual miRNAs in CSC-positive early-onset eCC patients who were at an early stage and had sporadic tumors.

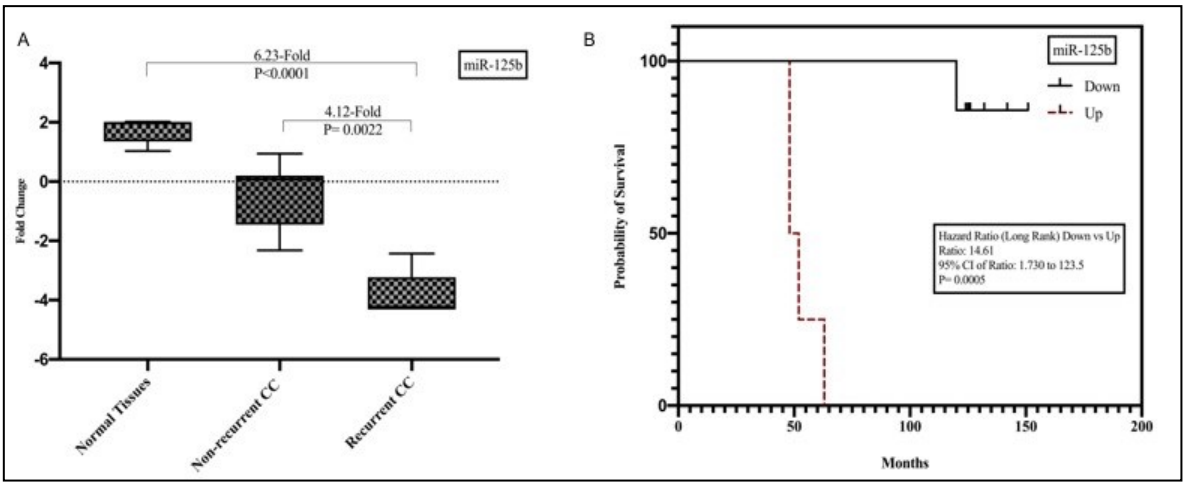

Figure 3. Low expression of miR-125b was associated with recurrence in CSC-positive CC tumors (A). Kaplan-Meier survival curves for CC patients according to miR-125b (B)

First, we determined CSC-positive tumors (n= 23), and we evaluated the association of CSC status with tumor size, stage, gender, OS and DFS to determine the predictive potential of these features for the aggressiveness of tumors. Two studies showed reduced OS in colorectal cancer patients with CD133-positivity. CSCs play a role in cancer metastasis, and the ability of CSC markers to predict disease progression and patients' survival is being intensely investigated. Recent studies reported that recorded expression of LGR5 mRNA in ~60% of circulating tumor cells in CRC patients reported a high correlation between LGR5 expression and metastasis development.[11,12] In a recent study, LGR5+ stem cells were reported to be responsible for recurrence and therapeutic resistance in Stage II-III colon cancer.[13] In our study, CD133, SOX2 and LGR5 were used to determine CSC status. High expression of at least two of them was accepted as a criterion for CSC-positive tumors. There was no significant association between gender, tumor stage and OS (p>0.05). On the other hand, CSC-positivity was associated with right colon localization. Furthermore, we found that patients with CSCs had recurrence over five years and had reduced DSF. Although Stotz et al. examined variants of CD44 in stage II and stage III with poor prognosis, there are no studies regarding the expression status of CSCs in eCC.[14]

In the present study, the down-regulation of miR-

125b and up-regulation of miR-135b were significant in CSC-positive eCC. Recent studies also found that miRNAs played an important role in regulating the expression of colon CSCs.[15-17] Zhang et al. showed that overexpression of miR-125b inhibited proliferation, promoted apoptosis in the SW480 colon cancer cell line, and was accompanied by upregulated Bax and downregulated Bcl-2 expression.[18] Yu et al. found that miRNAs, specifically miR-21 and miR-145, played important roles in regulating colon CSCs. They found the expression of miR-21 to be much higher and miR-145 to be lower in colon cancer cells that were highly enriched in CSCs, suggesting a role for these miRNAs in regulating CSCs.[19]

When we evaluated associations between prognosis and expression of miR-135b and miR-125b, there were no associations between miR-135b and any clinicopathological features in CSC-positive eCC tumors. Our results suggested that miR-135b played a role in cancer initiation, but our data have shown that this miRNA was ineffective in recurrence. Similarly, data from colorectal tissues suggested that miR-135b upregulation was an early event in tumor transformation.[20] According to the result from siRNA silencing, miR-135b regulation appeared to be independent of c-Myc activation; thus, these two factors may control collateral transcriptional programs orchestrating the tumor initiation process.[20] The miR-135b has been documented as a tumor-promoting factor and to play a role in migration and metastasis in CRC.[21,22.]

In the present study, we found that low expression levels of miR-125b were associated with recurrence in eCC. We previously determined miRNA expression profiles in early-onset colorectal cancer. In that study, the expression of miR-106a was upregulated, and levels of miR-143 and miR-125b were downregulated in different stages of colon and rectal tumor compared with normal tissues.[23] In our study, we selected only young patients and those with early-onset colon tumors. Although some reports highlighted the oncogenic aspects of miR-125b, many suggested that miR-125b worked as a tumor-suppressive microRNA in various cancers. miR-125a was significantly downregulated in breast and gastric cancer, and miR-125a substantially inhibited cancer cells' proliferation, migration, and invasion activities.[27-29] There is only one study showing an association between miR-125b and CSCs. Zhoug et al. determined that miR-125b negatively correlated with CD133 expression in hepatocellular carcinoma patients.[30]

Although this study included a homogeneous and carefully selected group of patients, its limitation was the relatively small group of patients analyzed. We evaluated only 30 patients at early onset and early stage.

In conclusion, currently, the reasons underlying the recurrence of eCC remain unclear, and the role of stem cells and microRNAs in tumor biological processes has not yet been fully elucidated. In the present study, we showed that miR-125b was significantly downregulated in early-onset eCC tumors. The expression of miR-125b increased tumor aggressiveness and recurrence potential. We suggest that our findings will inspire researchers to create new strategies for confidently diagnosing eCC patients with recurrence potential, offering promise for discovering new targets related to this mechanism, and contributing to future targeted cancer therapy studies based on this new direction. Further studies and validations are required; miR-125b may constitute a novel molecular target for CSC-positive CC treatment. Collectively, a better understanding of miRNA functions associated with CSC properties could provide new insight into cancer therapeutics.

Ethics Committee Approval: The study was approved by Bursa Uludag University Faculty of Medicine Clinical Research Ethics Committee (Date: 13.01.2015, decision no: 2015-1/35) and was by the ethical standards of the Declaration of Helsinki.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – SAA, BT; Supervision – SAA, BT, TY, OI, EO; Materials –SAA, ME, CT, BG, NU, OY; Data Collection and/or Processing – SAA, BT, ME, CT, GC, UE; Analysis and/or Interpretation – SAA, BT, TY, OI, EO.

Peer-review: Externally peer-reviewed.

Financial Support: This work was supported by the Scientific Research Projects Foundation (BAP) of Bursa Uludag University in Türkiye [Project No: OUAP(T)2015/2].

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