Original Article

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Serum expression levels of miR-17, miR-21, and miR-92 as potential biomarkers for recurrence after adjuvant chemotherapy in colon cancer patients

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Summary **The present study examined whether miR-17, miR-21, miR-29a, and miR-92 that are dysregulated in colon cancer (CC) can serve as potential predictive markers for relapse of disease after radical surgery and adjuvant chemotherapy. Real-time reverse transcription quantitative polymerase chain reaction was used to measure the expression levels of the miRNAs in serum samples from 37 patients with CC and 7 healthy individuals, tested as a control group. The area under the receiver operating characteristic curve (AUC) was then used to evaluate the predictive performance of the four miRNAs alone or in combination and compare it with carcinoembryonic antigen. The expression of miR-17, miR-21 and miR-92 were significantly higher in serum of patients with disease relapse. The AUCs for miR-17, miR-21, miR-92 for Nx patients were 0.844, 0.948, and 0.935, respectively ($p < 0.05$). Combining the four miRNAs for stage III patients increased the diagnostic performance, yielding an AUC of 0.881, with a sensitivity of 83.3% and a specificity of 85.7% ($p < 0.05$). Our study suggests that the expression levels of serum miR-21, miR-17, and miR-92 in patients with CC who underwent radical surgery and adjuvant chemotherapy may have diagnostic value for differentiating between recurred and non-recurred patients.**

Keywords: miRNA, marker, relapse, Nx

1. Introduction

Colorectal cancer (CRC) is the most commonly diagnosed gastrointestinal cancer worldwide with more than 1.2 million new cases and 600,000 deaths annually (*1*). Oncological management of colon cancer patients is based on the initial clinical staging of the disease. For patients without metastatic disease (M0), surgery is the first option, used with curative intention. Unfortunately, for 20-30% of the patients the prognosis is poor due to recurrence of the disease (*2*). 5 Fluorouracil (5-FU) based chemotherapy is the standard adjuvant treatment. In stage III patients, the benefit from additional adjuvant chemotherapy is confirmed in several large-scale trials and they have about 40% lower risk of recurrence, compared to patients who were stated on observation (*3,4*). In stage II patients, adjuvant treatment remains controversial (*5,6*). Survival rate is usually complicated by the side effects of chemotherapy. This is mainly ascribed to the lack of reliable markers that could predict development of disease relapse. Therefore, identification of such markers would be greatly beneficial for the individualized chemotherapy of patients with a high risk for recurrence of the disease.

Disease relapses after surgery alone or combined

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with adjuvant treatment for colon cancer are a function of both degree of bowel wall penetration of the primary lesion and nodal status (*7*). Nx category includes patients with unknown nodal status. Last Surveillance, Epidemiology, and End Results (SEER) population-based colon cancer analysis includes 130,762 patients with colon cancer, among which 14% (n = 18,312) were defined as Nx (*7*). Despite the large number of Nx cases, the potential benefit of chemotherapy is not known (*8*) and there are only a few biomarkers that could predict recurrence of the disease in this group of patients (*9*).

miRNAs are a class of short (18-23 nucleotides in length), endogenous, non-protein-coding RNAs that play critical roles in diverse biological processes through negative post-transcriptional regulation (*10,11*). miRNAs can function as oncogenes or tumor suppressors by repressing cancer-related genes (*11,12*). Alterations of miRNA expression have been observed in a variety of human tumors, including colon cancer, and it was discovered that miRNAs are stably present in circulating blood at sufficient levels for use as blood-based biomarkers (*13,14*). Thus, identification of novel serum-based miRNAs as biomarkers for response to therapy or tumor recurrence could improve the outcome of the disease.

In this study, we investigated the serum levels of four miRNAs involved in tumor cell proliferation (miR-17, miR-92), survival (miR-21), and metastasis (miR-29a) in colon cancer patients (*15*). Blood samples were collected immediately after completion of adjuvant chemotherapy. We aimed to monitor the tumor biological behavior and the prediction capacity of the serum levels of miR 17, miR-92, miR-21 and miR-29a for recurrence of the disease in one year period.

2. Materials and Methods

2.1. *Ethics statement*

All procedures were approved by the Scientific Research Ethics Committee of Medical University "Prof. Dr. Paraskev Stoyanov", Varna. Blood samples were collected from 37 colon cancer patients and 7 healthy volunteers at the Medical Oncology Clinic, University Hospital "St. Marina", Varna after obtaining informed consent form (ICF) from all study participants. All patients with indications for adjuvant chemotherapy were discussed for participation in our trial and only subjects who agreed to participate and signed ICF were included.

2.2. *Patient selection*

All subjects in the study were of Caucasian race. Controls were matched to colon cancer patients by age. The characteristics of patients and controls are represented in Table 1. We included patients with colon cancer stage II (n = 6) and III (n = 13) as per American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 7th ed., who have undergone radical surgery in our hospital; there was no residual disease or compromised edges post-surgery and patients have completed 5-FU based adjuvant chemotherapy. 18 patients had Nx lymph node status because the involvement of lymph nodes could not be determined (less than 12 lymph nodes examined). We obtained serum after last cycle of adjuvant chemotherapy and patients started follow-up regularly (every 3 months) with computed tomography (CT) or positron emission tomography PET-CT until progression for 1 year of follow-up.

2.3. *RNA isolation and real-time PCR*

Total RNA was extracted from 200 μL serum, using the miRNeasy mini kit (Qiagen) following the manufacturer's instructions. 5.6×10^8 copies cel-miR-39 synthetic RNA per sample were used as spike-in control (Qiagen). The RNA was eluted in 14 μL nuclease-free water supplied with the kit. Small RNAs were reverse transcribed with miScript II RT kit (Qiagen), using 5 μL of the eluted RNA and HiSpec buffer. Before use, cDNA was diluted as recommended. Quantitative real-time PCR was done on the StepOne

Table 1. Demographic and clinical features of patients and healthy subjects

Patients	
Sex %	
Male	43.2 (n = 16)
Female	56.8 (n = 21)
Age at diagnosis (yrs., mean, S.D.)	
Male	63.50 (7.694)
Female	64.86 (7.009)
Total	64.27 (7.241) Min. 44, Max. 77
Tumor localization (N, %)	
Colon ascendens	8 (21.6)
Colon descendens	6 (16.2)
Sigma	23 (62.2)
Stage (N, %)	
II	6 (16.2)
III	13 (35.1)
Nx (Stage N/A)	18 (48.6)
Grade (N, %)	
Gr 1 + Gr 2	32 (86.5)
Gr 3	5 (13.5)
Recurrence (N, %)	
Non recurrence	24 (64.9)
Recurrence	13 (35.1)
Recurrence on stage (N, %)	
II (n = 6)	0 (0)
III (n = 13)	6 (46.2)
Nx (n = 18)	7 (38.9)
Controls	
Sex %	
Male	42.9 (n = 3)
Female	57.1 (n = 4)
Age (yrs., mean, S.D.)	
	56.29 (10.704) Min. 38, Max. 69

Plus (Applied Biosystems) with miScript Sybr Green PCR kit and miScript Primer Assays (Qiagen). Relative quantities (RQ) of target miRNAs were calculated using method by the StepOne Software v2.0. The results were normalized using the spike-in control cel-miR-39 as a reference target and were expressed as relative quantity to a single reference sample (*16*). The relative levels of miRNA were normalized to cel-miR-39 and were calculated using the equation $2^{-\Delta\Delta Ct}$. RQ values for miRNA-17, miRNA-21, miRNA-29a or miRNA-92 were then normalized to the average expression of each of these miRNAs in healthy individual (*17*). Greater than the median RQ values of miRNAs we denoted as high expression levels, and values below or equal to the median RQ we denoted as low expression levels.

2.4. *Clinical and pathological features*

We collected demographical data (sex and age at initial staging), date and extent of surgery, as well as tumor characteristics: localization, TNM classification, histology of tumor, total number of histologically examined lymph nodes, and grade of differentiation. Associations of serum miR-17, miR-21, miR-29a, and miR-92 expression with clinicopathological parameters are represented in Table S1 (*http://www.biosciencetrends.com/docindex.php?year=2015&kanno=6*).

2.5. *Statistical analysis*

Statistical analysis was carried out with SPSS Statistics v.23 using descriptive statistics. Categorical features were summarized with frequencies and percentages. The Mann-Whitney *U* test, Pearson correlation, and χ^2 test or Fisher's exact test were used for comparison and estimation of correlations between miRNAs expression levels and clinicopathological characteristics such as tumor stage and grade, and age. Specificity and sensitivity of serum miRNAs expression levels for discriminating patients with recurrent disease (recurred patients, RP) from patients with non-recurrent disease (non-recurred patients, NP) at 1 year of follow-up were evaluated with receiver operating curve (ROC) analysis. Diagnostic accuracy of biomarkers was also determined by obtaining the largest possible area under the curve (AUC) in ROC analysis. The best linear combination of miRNA markers in the sense that the area under the ROC curve of this combination is maximized among all possible linear combinations was calculated according to the method of Su and Liu (*18*). Kaplan-Meier survival curves and the log-rank test were used to compare the survival differences between groups. Although our study was not powered enough to compare different subgroups, hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were calculated by Cox regression models. Two-tailed *p*-values (< 0.05) were considered as significant.

3. Results

3.1. *Expression of miRNAs in NP, RP and control groups*

Relative miRNA expression levels in serum samples of colon cancer patients (n = 37) who underwent curative surgery were obtained by using cel-miR-39 as reference genes for normalization. Relative quantification (RQ) values, calculated by the $2^{-\Delta\Delta Ct}$ method were used for evaluation of expression in patients. The Mann-Whitney test showed that there were no significant differences of RQ values of all 4 miRNAs between the control and NP group. Both miR-21 and miR-92 showed significant expression level differences between RP group with control and NP group (Figures 1B and 1D). In contrast, miR-17 showed significant difference only between RP group and NP group, but not between RP group and control group (Figure 1A). Expression level of miR-29a did not differ between groups (Figure 1C).

3.2. *Expression of miR-21 differs between and within stages*

There were no significant differences of RQ values of all miRNAs in patients with stage II, stage III and Nx except for miR-21, which differed between patients with stage II and III (Figure 2). Within stage III patients, none of the 4 miRNAs expression levels were different between RP and NP groups. However, within the Nx patients all miRNAs except miR-29a had significant differences in expression levels between RP *vs.* NP groups (Figures 3A, 3B and 3D).

3.3. *miR-21, miR-17 and miR-92 alone discriminate RP and NP groups*

After 1 year follow-up post adjuvant chemotherapy none of patients with stage II disease experienced recurrence. Six patients with stage III disease and 7 patients with Nx experienced recurrence after 1 year of follow-up. ROC analysis was performed to explore the potential value of analyzed miRNAs expression levels as noninvasive diagnostic biomarkers for recurrence after adjuvant chemotherapy (Figure 4). miR-21 allowed most accurate discrimination (AUC = 0.901, 95% CI: 0.788-1, $p < 0.001$) between RP and NP groups. At the optimal cutoff values of RQ, the sensitivity was 84.6% and specificity was 79.2% (Figure 4B). miR-92 and miR-17 could also discriminate RP and NP groups with the following AUC = 0.885 (95% CI: 0.778-0.991, $p < 0.001$) and with 84.6% sensitivity and 70.8% specificity (Figure 4D) and AUC = 0.814 (95% CI: 0.658-0.971, $p = 0.002$) and with 84.6% sensitivity

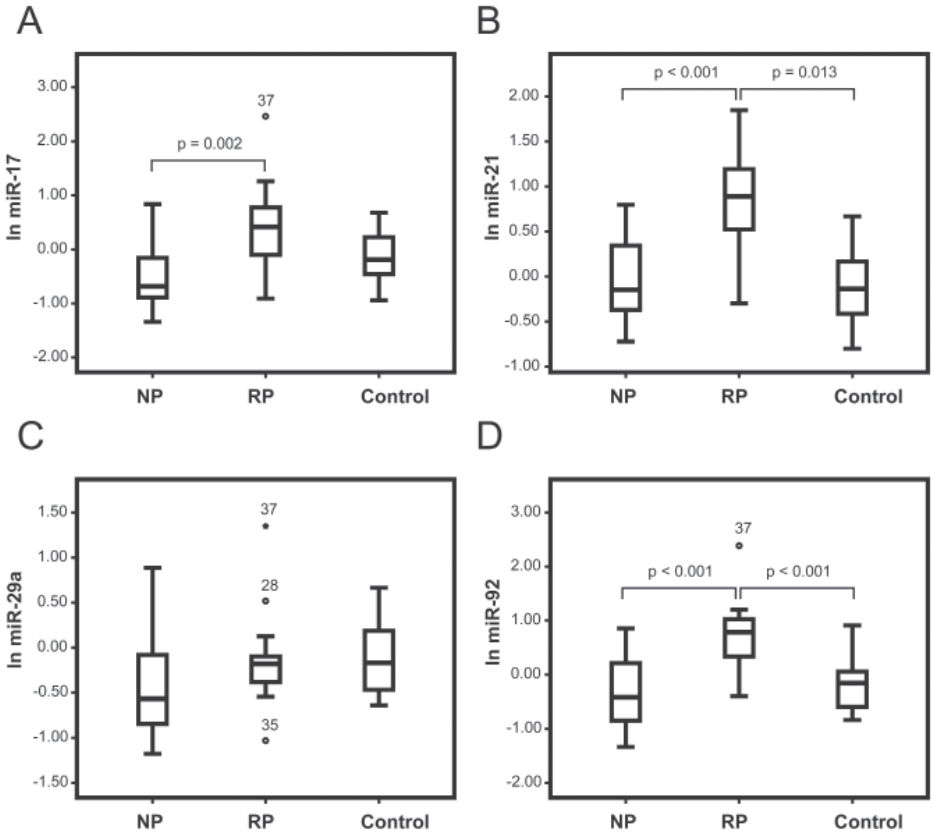

Figure 1. Box plots, representing serum miRNAs expression levels in NP (non-recurred patients), RP (recurred patients) and controls. Expression levels of miRNAs (scale of y axis: ln). The Mann-Whitney test was used to detect significant differences of RQ (relative quantities) values of all 4 miRNAs among control, NP and RP group. Two-tailed *p*-values (< 0.05) were considered as significant. **A**, miR-17; **B**, miR-21; **C**, miR-29a; **D**, miR-92.

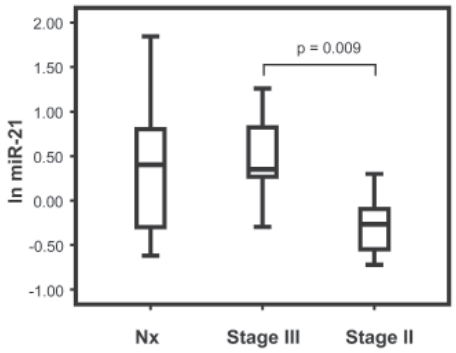

Figure 2. Box plots, representing serum miR-21 expression levels in patients with Nx, Stage III and II (scale of y axis: ln). The Mann-Whitney test was used to detect significant differences of RQ (relative quantities) values of miR-21 between Nx, Stage III and II. Two-tailed *p*-values (< 0.05) were considered as significant.

and 66.7% specificity (Figure 4A) at the optimal cutoff values of RQ, respectively. miR-29a discriminates RP and NP groups with the following AUC = 0.670 (95% CI: 0.493-0.846, $p = 0.092$) and with 53.8% sensitivity and 70.8% specificity at the optimal cutoff values of RQ, but the result did not reach statistical significance (Figure 4C). For comparison, carcinoembryonic antigen (CEA) after adjuvant chemotherapy has lower power to discriminate RP and NP groups AUC= 0.655 (95% CI: 0.472-0.839, $p = 0.123$) with 61.5% sensitivity and 62.5% specificity, but the result did not reach statistical significance (Figure 4E).

3.4. *miR-21, miR-17 and miR-92 alone or in combination discriminate RP and NP groups within stage III and Nx patients*

The potential value of analyzed miRNAs as biomarkers for recurrence after adjuvant chemotherapy within stage III and Nx patients is shown in Table 2. Because none of the studied biomarkers could discriminate accurately in stage III between RP and NP groups, we constructed multimarker ROC analysis. The combination of all 4 biomarkers discriminates RP and NP groups with the following AUC = 0.881 (95% CI: 0.655-1, $p = 0.022$) and with 83.3% sensitivity and 85.7% specificity at the optimal cutoff values of RQ (Figure 4F). In patients with Nx disease only expression levels of miR-29a were not good enough to discriminate between patients with recurrence and no recurrence of the disease (Table 2). CEA after adjuvant chemotherapy has similar power to discriminate recurrence in stage III and Nx patients (Table 2).

3.5. *No correlation of miRNA expression levels with disease free survival, age and CEA*

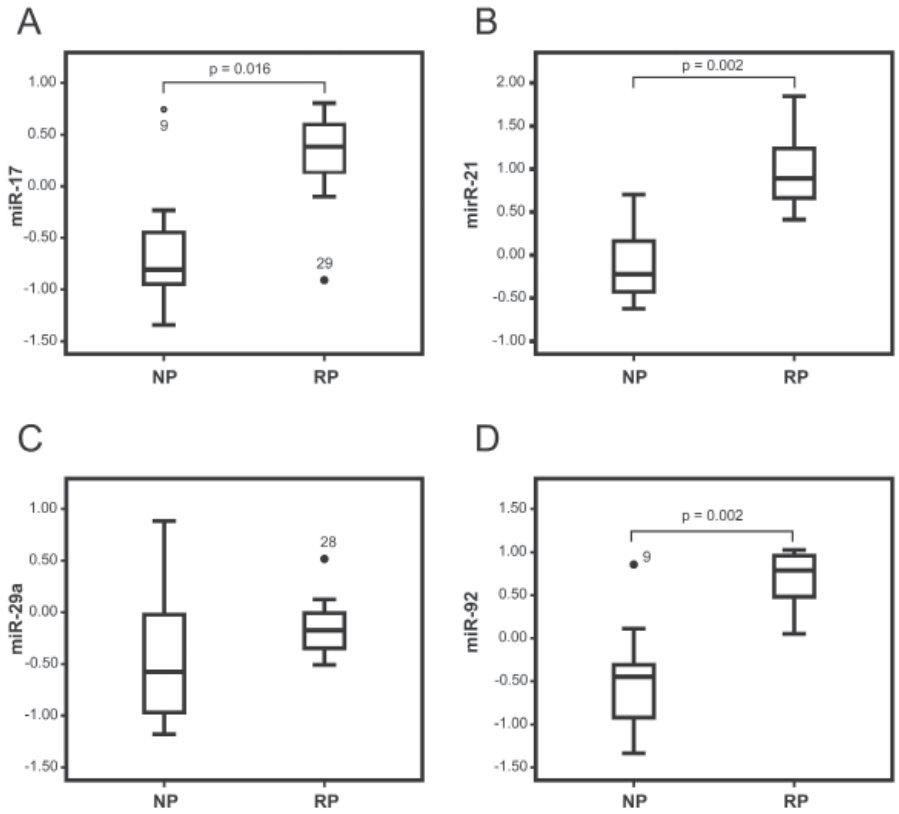

Figure 3. Box plots, representing serum miRNAs expression levels in Nx group in RP (recurred patients) and NP (non-recurred patients). Expression levels of miRNAs (scale of y axis: ln). The Mann-Whitney test was used to detect significant differences of RQ (relative quantities) values of all 4 miRNAs between NP and RP group. Two-tailed *p*-values (< 0.05) were considered as significant. **A**, miR-17; **B**, miR-21; **C**, miR-29a; **D**, miR-92.

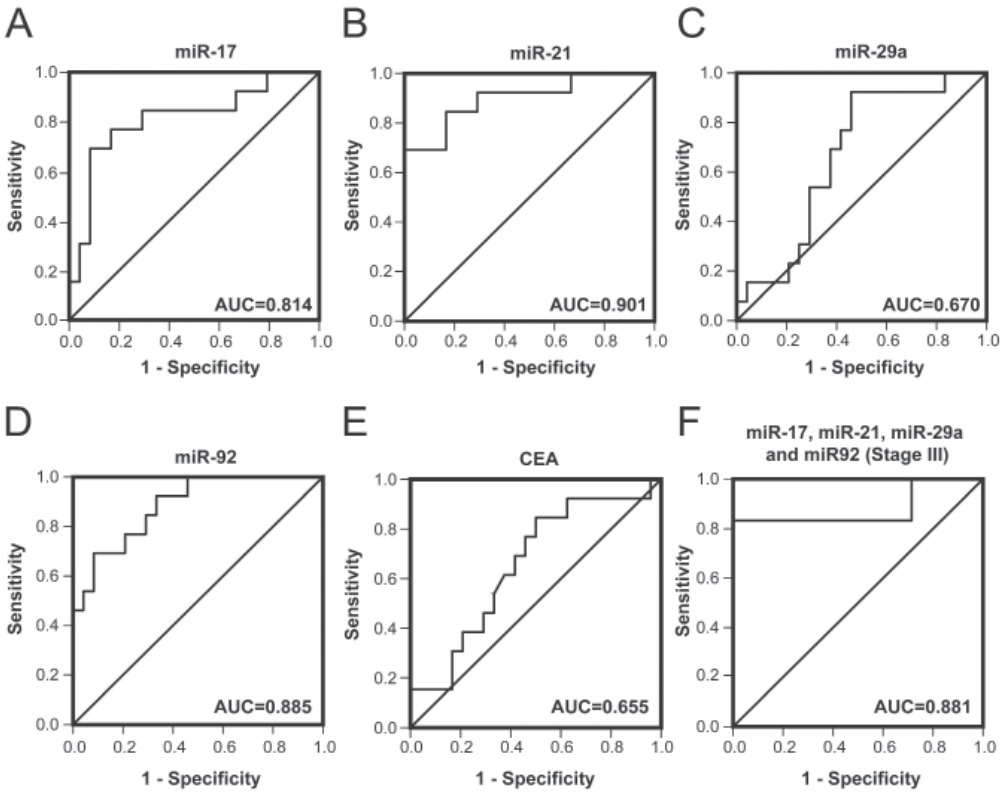

Figure 4. Receiver operating curve (ROC) curve analysis, using four miRNAs and CEA (carcinoembryonic antigen) to differentiate RP (recurred patients) from NP (non-recurred patients). Diagnostic accuracy of biomarkers was determined by obtaining the largest possible area under the curve (AUC) in ROC analysis. **A**, miR-17; **B**, miR-21; **C**, miR-29a; **D**, miR-92; **E**, CEA; **F**, Multimarker ROC analysis, using four miRNAs in stage III patients.

Table 2. Result from Receiver operating curve (ROC) analysis according to data by using four miRNAs and CEA (carcinoembryonic antigen) to differentiate RP (recurred patients) from NP (non-recurred patients)

Patients	Biomarkers	AUC (95% CI)	*p*-value	Sensitivity (%)	Specificity (%)
Stage III	miR-17	0.738	0.153	66.7	85.7
	miR-21	0.738	0.153	66.7	85.7
	miR-29a	0.452	0.775	50.0	42.9
	miR-92	0.786	0.086	66.7	85.8
	CEA	0.643	0.391	66.7	57.1
Nx	miR-17	0.844	0.016	85.7	81.8
	miR-21	0.948	0.002	85.7	81.8
	miR-29a	0.701	0.16	85.7	63.6
	miR-92	0.935	0.002	85.7	90.9
	CEA	0.662	0.258	71.4	59.5

Disease free survival (DFS) was calculated as the time from the date of surgery to the date of documented recurrence of the disease. Colon cancer patients with high expression levels of any miRNAs had no significant differences in DFS compared with those with low expression levels of miRNAs (Figure S1, *http://www.biosciencetrends.com/docindex.php?year=2015&kanno=6*). In univariate analysis, older age, stage of disease, sex, histologic grade and expression levels of miRNAs were not associated with poor 1 year DFS (Table S2, *http://www.biosciencetrends.com/docindex.php?year=2015&kanno=6*).

Pearson correlation tests did not detect significant correlation between the DFS and the serum levels of the miRNAs studied. We also did not observe correlation between age and expression levels of the studied miRNAs. Furthermore, none of the 4 miRNAs correlated with serum level of CEA after adjuvant chemotherapy. However, strong correlation was observed between miR-17 and miR-92 ($r = 0.971$, $p < 0.001$, Figure S2), moderate correlation between miR-21 and miR-92 ($r = 0.492$, $p = 0.002$, Figure S2, *http://www.biosciencetrends.com/docindex.php?year=2015&kanno=6*) and weak correlation between miR-17 and miR-21 ($r = 0.360$, $p = 0.029$, Figure S2).

4. Discussion

Previous studies have identified multiple circulating miRNAs that are differently expressed and dysregulated in colon cancer patients compared with control healthy people (*11,19,20*). Several lines of evidence have suggested that miRNAs might serve as potential biomarkers for the diagnosis and prognosis of patients with colon cancer (*19,21-23*).

The goal of our study was to identify potential biomarkers that could predict early recurrence after adjuvant treatment. Additionally we included in our analysis patients with unknown lymph node status (Nx patients). Usually dysregulated miRNAs are associated with wide variety of cancers (*24*). To increase the sensitivity and specificity of our assay we used miR-21, miR-17, miR-29a and miR-92 which have been shown to be upregulated in colon cancer patients (*25*). ROC analysis in our study showed that upregulation of any of miR-21, miR-92 and miR-17 in serum is an accurate biomarker and can distinguish colon cancer patients with recurrence from patients without recurrence with high sensitivity and specificity (Figure 4). In stage III patients, only the combination of all miRNAs reached statistically significant result in distinguishing RP from NP, which might be as a result of the small sample size. All miRNAs except miR-29a outperformed CEA serum levels in predicting recurrence. The sensitivity and specificity of ROC curve for prediction of recurrence on the basis of CEA only was low, which is consistent with other research (*26*).

Only a few studies have evaluated the expression levels of miRNAs after curative surgical resection of the primary tumor (*21,27,28*) and have looked for a diagnostic correlation between serum levels of miRNAs and post-adjuvant levels of CEA. In the present study the expression levels and the diagnostic significance of 4 circulating miRNAs (miR-17, miR-21, miR-29a and miR-92) from blood samples of colon cancer patients, who underwent radical surgery of the primary tumor and adjuvant chemotherapy were correlated with the serum levels of CEA (measured after end of adjuvant chemotherapy) and the stage of the disease.

Previous studies have found higher levels of circulating miR-17, miR-21, miR-29a and miR-92 as compared to healthy individuals and some of those miRNAs were associated with poor survival, independent of other clinicopathological factors (*28-31*). Among the most important target genes of miR-17 and miR-92 are the E2F family of transcription factors (Figure 5) that drive the progression from the G1 into the S phase of the cell cycle (*15*). Thus, increased levels of miR-17 and miR-92 promote tumor cell proliferation. Crucial target of miR-21 is PTEN (phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase) which negatively regulates the PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) pathway (*15*). As a result, increased level of miR-21 promotes tumor cell survival (Figure 5). In one study it was shown that the serum levels of miR-21 statistically

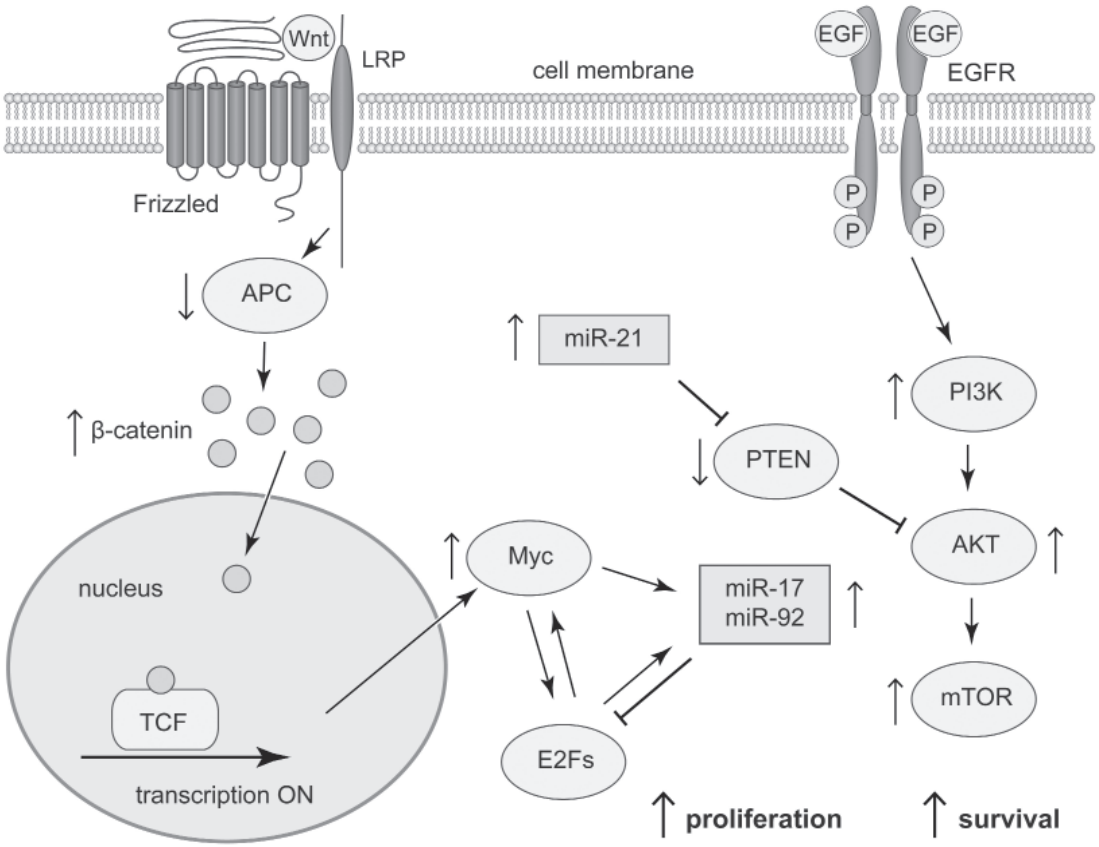

Figure 5. Targets of the studied microRNAs: miR-17 and miR-92 promote cell proliferation through regulation of E2Fs, whereas miR-21 promotes cell survival through regulation of PTEN. AKT, protein kinase B; APC, adenomatous polyposis coli; E2Fs, E2F family of transcription factors; EGF, epidermal growth factor; EGFR, EGF receptor; LRP, low-density lipoprotein receptor-related protein; mTOR, mammalian target of rapamycin; P, phosphorylated site; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; TCF, T cell factor; Wnt, wingless/integrase-1.

significantly plummeted after surgery in the subset of patients with potentially curative surgeries (*21*). Others demonstrated that stromal miR-21 expression was related to the expression of E-cadherin and metastasis-associated protein 1 in colorectal cancer and high miR-21 expression in stage III patients was not associated with poor DFS (*32*). Stage II colorectal cancer patients with high levels of miR-21 are at higher risk for tumor recurrence and should be considered for more intensive treatment (*32*). We found from our panel of miRNAs that only miR-21 had significantly altered expression levels in patients with III *vs.* II stage disease, which may reflect the tumor burden or may indicate noncurative surgeries. Patients with recurrence of the disease had significantly higher expression levels in Nx group for all miRNAs except miR-29a which again may reflect the tumor burden or may indicate noncurative surgeries.

There is considerable sample-to-sample variability in both protein and lipid content of serum samples, which could affect efficiency of RNA extraction (*33*). To ensure the accuracy of our assay, including variability in polymerase chain reaction amplification efficiencies, we used the novel method for normalization of experimental miRNA data using spiked-in synthetic, nonhuman mature miRNA from *Caenorhabditis elegans* (*33*). Although our approach of quantifying relative expression of serum miRNAs is widely used, absolute quantitation of serum miRNAs expression levels may further improve the translation of these data into a clinically viable diagnostic test for early detection of disease relapse.

Inadequate examination of lymph nodes may lead to tumor understaging and subsequent treatment failure (*34*). Many factors affect the number of lymph nodes examined, including extent of surgical resection, patient age, tumor location, and pathology techniques. Population-based data suggest that only 37% of colon cancer patients have adequate lymph node evaluation (*35*).

Colon cancer patients may benefit from discovering prognostic markers that can identify those individuals that are more likely to recur by selecting patients that are suitable for adjuvant therapy. Several miRNAs (miR-192, miR-215, miR-140, miR-129, let-7, miR-21, miR-181b, miR-200 s) were found to be associated with chemoresistance by regulating key cell death pathways (*24*). High levels of miR-21 may partly be responsible for poor response to 5-FU; therefore, patients with high miR-21 expression are at high-risk for disease recurrence (*36*). In colon cancer cell lines it was demonstrated that increased miR-21 expression reduced apoptosis and G2/

M arrest due to damage by 5-FU (*37*).

In summary, the most important finding in our study is that miR-17, miR-21 and miR-92 alone in Nx or in combination in stage III patients may help detecting early recurrence of colon cancer after radical surgery and adjuvant chemotherapy with high accuracy in clinical practice. Our results suggest that patients with elevated serum levels of miR-17, miR-21 and miR-92 should undergo more intensive follow-up in order to increase the possibility for early relapse detection and potential subsequent curative interventions. To our knowledge our study, despite its small sample size, is one of the few, testing potential biomarkers that could predict disease recurrence in patients with Nx colon cancer. Future studies may further evaluate the potential use of miR-17, miR-21 and miR-92 in post-operative surveillance, which may provide an opportunity for early detection of recurrent disease.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69-90.
2. O'Connell MJ, Campbell ME, Goldberg RM, Grothey A, Seitz JF, Benedetti JK, Andre T, Haller DG, Sargent DJ. Survival following recurrence in stage II and III colon cancer: Findings from the ACCENT data set. J Clin Oncol. 2008; 26:2336-2341.
3. Andre T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, Bonetti A, Clingan P, Bridgewater J, Rivera F, de Gramont A. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J Clin Oncol. 2009; 27:3109-3116.
4. Sargent D, Shi Q, Yothers G, Van Cutsem E, Cassidy J, Saltz L, Wolmark N, Bot B, Grothey A, Buyse M, de Gramont A, Adjuvant Colon Cancer End-points G. Two or three year disease-free survival (DFS) as a primary end-point in stage III adjuvant colon cancer trials with fluoropyrimidines with or without oxaliplatin or irinotecan: Data from 12,676 patients from MOSAIC, X-ACT, PETACC-3, C-06, C-07 and C89803. Eur J Cancer. 2011; 47:990-996.
5. Chau I, Cunningham D. Adjuvant therapy in colon cancer – what, when and how? Ann Oncol. 2006; 17:1347-1359.
6. Quasar Collaborative G, Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ. Adjuvant chemotherapy versus observation in patients with colorectal cancer: A randomised study. Lancet. 2007; 370:2020-2029.
7. Gunderson LL, Jessup JM, Sargent DJ, Greene FL, Stewart AK. Revised TN categorization for colon cancer based on national survival outcomes data. J Clin Oncol. 2010; 28:264-271.
8. Wolpin BM, Meyerhardt JA, Mamon HJ, Mayer RJ. Adjuvant treatment of colorectal cancer. CA Cancer J Clin. 2007; 57:168-185.
9. Drebber U, Lay M, Wedemeyer I, Vallbohmer D, Bollschweiler E, Brabender J, Monig SP, Holscher AH, Dienes HP, Odenthal M. Altered levels of the onco-microRNA 21 and the tumor-supressor microRNAs 143 and 145 in advanced rectal cancer indicate successful neoadjuvant chemoradiotherapy. Int J Oncol. 2011; 39:409-415.
10. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell. 2004; 116:281-297.
11. Slack FJ, Weidhaas JB. MicroRNA in cancer prognosis. N Engl J Med. 2008; 359:2720-2722.
12. Jansson MD, Lund AH. MicroRNA and cancer. Mol Oncol. 2012; 6:590-610.
13. Chen X, Ba Y, Ma L, *et al*. Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008; 18:997-1006.
14. Mitchell PS, Parkin RK, Kroh EM, *et al*. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008; 105:10513-10518.
15. Slaby O, Svoboda M, Michalek J, Vyzula R. MicroRNAs in colorectal cancer. In: MicroRNAs in Cancer Translational Research (Cho WCS, ed. Springer, Dordrecht, Netherlands, 2011; pp. 107-133.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001; 25:402-408.
17. Subramani A, Alsidawi S, Jagannathan S, Sumita K, Sasaki AT, Aronow B, Warnick RE, Lawler S, Driscoll JJ. The brain microenvironment negatively regulates miRNA-768-3p to promote K-ras expression and lung cancer metastasis. Sci Rep. 2013; 3:2392.
18. Su JQ, Liu JS. Linear combinations of multiple diagnostic markers. J Am Stat Assoc. 1993; 88:1350-1355.
19. Ogata-Kawata H, Izumiya M, Kurioka D, Honma Y, Yamada Y, Furuta K, Gunji T, Ohta H, Okamoto H, Sonoda H, Watanabe M, Nakagama H, Yokota J, Kohno T, Tsuchiya N. Circulating exosomal microRNAs as biomarkers of colon cancer. PLoS One. 2014; 9:e92921.
20. Wang CJ, Zhou ZG, Wang L, Yang L, Zhou B, Gu J, Chen HY, Sun XF. Clinicopathological significance of microRNA-31, -143 and -145 expression in colorectal cancer. Dis Markers. 2009; 26:27-34.
21. Toiyama Y, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, Kusunoki M, Boland CR, Goel A. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. J Natl Cancer Inst. 2013; 105:849-859.
22. Corte H, Manceau G, Blons H, Laurent-Puig P. MicroRNA and colorectal cancer. Dig Liver Dis. 2012; 44:195-200.
23. Schetter AJ, Okayama H, Harris CC. The role of microRNAs in colorectal cancer. Cancer J. 2012; 18:244-252.
24. Mishra PJ. MicroRNAs as promising biomarkers in cancer diagnostics. Biomark Res. 2014; 2:19.
25. Dong Y, Wu WKK, Wu CW, Sung JJY, Yu J, Ng SSM. MicroRNA dysregulation in colorectal cancer: A clinical perspective. Br J Cancer. 2011; 104:893-898.
26. Peng Y, Zhai Z, Li Z, Wang L, Gu J. Role of blood tumor markers in predicting metastasis and local recurrence after curative resection of colon cancer. Int J Clin Exp Med. 2015; 8:982-990.
27. Luo X, Burwinkel B, Tao S, Brenner H. MicroRNA signatures: Novel biomarker for colorectal cancer? Cancer Epidemiol Biomarkers Prev. 2011; 20:1272-1286.
28. Li J, Liu Y, Wang C, Deng T, Liang H, Wang Y, Huang D,

Fan Q, Wang X, Ning T, Liu R, Zhang CY, Zen K, Chen X, Ba Y. Serum miRNA expression profile as a prognostic biomarker of stage II/III colorectal adenocarcinoma. Sci Rep. 2015; 5:12921.
29. Brunet Vega A, Pericay C, Moya I, Ferrer A, Dotor E, Pisa A, Casalots A, Serra-Aracil X, Oliva JC, Ruiz A, Saigi E. microRNA expression profile in stage III colorectal cancer: Circulating miR-18a and miR-29a as promising biomarkers. Oncol Rep. 2013; 30:320-326.
30. Rothschild SI. microRNA therapies in cancer. Mol Cell Ther. 2014; 2:7.
31. Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer. 2010; 127:118-126.
32. Kang WK, Lee JK, Oh ST, Lee SH, Jung CK. Stromal expression of miR-21 in T3-4a colorectal cancer is an independent predictor of early tumor relapse. BMC Gastroenterol. 2015; 15:2.
33. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods. 2010; 50:298-301.
34. Wolpin BM, Mayer RJ. Systemic treatment of colorectal cancer. Gastroenterology. 2008; 134:1296-1310.
35. Baxter NN, Virnig DJ, Rothenberger DA, Morris AM, Jessurun J, Virnig BA. Lymph node evaluation in colorectal cancer patients: A population-based study. J Natl Cancer Inst. 2005; 97:219-225.
36. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA. 2008; 299:425-436.
37. Valeri N, Gasparini P, Braconi C, Paone A, Lovat F, Fabbri M, Sumani KM, Alder H, Amadori D, Patel T, Nuovo GJ, Fishel R, Croce CM. MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2). Proc Natl Acad Sci U S A. 2010; 107:21098-21103.

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