Int J Clin Exp Pathol 2017;10(1):105-116
www.ijcep.com /ISSN:1936-2625/IJCEP0042090

Original Article

Serum miRNA-203 expression is associated with chemo-response to standard FOLFOX treatment of patients with colorectal cancer

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Received October 17, 2016; Accepted October 26, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: Prognosis of chemo-response of patients with colorectal cancer (CRC) is generally poor because of the lack of simple, convenient, and noninvasive tools for CRC detection at the early stage. The discovery of microRNAs (miRNAs) and their different expression profiles among different kinds of patients has opened a new avenue for tumor prediction. The aim of our study is to identify serum miRNA expression in CRC patients with different responding status to FOLFOX treatment and explore their roles in the diagnosis and prognosis of CRC. Genome-wide miRNA analysis by Miseq sequencing followed by two phases of reverse transcription quantitative real-time PCR (RT-qPCR) assays were performed on serum from 164 CRC patients showing response and 164 showing no response to FOLFOX (oxaliplatin combining 5-fluorouracil and leucovorin) treatment. Our date showed that miR-203, miR-155, miR-218 and miR-34 were differently expressed between responding and non-responding CRC patients. Moreover, the receiver operating characteristic (ROC) curve analysis showed that the corresponding area under the curves (AUCs) of miR-203, miR-155, miR-218 and miR-34 were 0.796, 0.715, 0.709, and 0.698, respectively. In addition, low serum miR-203 expression was associated with poor response to FOLFOX treatment. Kaplan-Meier analysis showed that patients with CRC with low miR-203 level had worse recurrence-free survival. In multivariate Cox regression analysis, miR-203 was independently associated with tumor recurrence of CRC patients receiving FOLFOX treatment. Our systematic approach demonstrated that serum miR-203 can be used as a noninvasive biomarker for the diagnosis and prognosis of chemo-response in CRC.

Keywords: Colorectal cancer, FOLFOX, chemo-response, miR-203, prognosis

Introduction

Colorectal cancer (CRC) is one of the most frequent cancers, and the second leading cause of cancer-related mortality worldwide [1]. Survival rates of patients with CRC have increased in the past few years, possibly as a result of earlier diagnosis and improved treatment regimens; nonetheless, approximately 30%-50% of patients who undergo curative resection subsequently experience local and systemic recurrence [2]. Currently, standard FOLFOX (oxaliplatin combining 5-fluorouracil and leucovorin) chemotherapy after surgery resection is one of the most frequently used therapeutic strategies [3]. However, large proportion of patients receiving chemotherapy finally becomes chemo-resistant, and this has been a key barrier to the efficacy of CRC treatment [4]. Thus, finding new therapeutic and prognostic targets will be indispensable for developing effective therapy for CRC patients.

MicroRNAs (miRNAs) are a class of short, endogenous, single-stranded RNAs that regulate gene expression. They are increasingly recognized to be key regulators of gene function in several biological systems, including cancer [5, 6]. Altered expression of miRNAs is observed in CRC and some of these miRNAs (e.g., miR-429, miR-218, miR-203) have prognostic relevance [7-9]. These suggest that deregulation of miRNA may play vital roles in the carcinogenesis of CRC. Moreover, miRNAs sometime exert as a role of oncogenes or tumor suppressor genes through affecting the response to various therapeutic regimens. In recent years, studies have highlighted the association be-

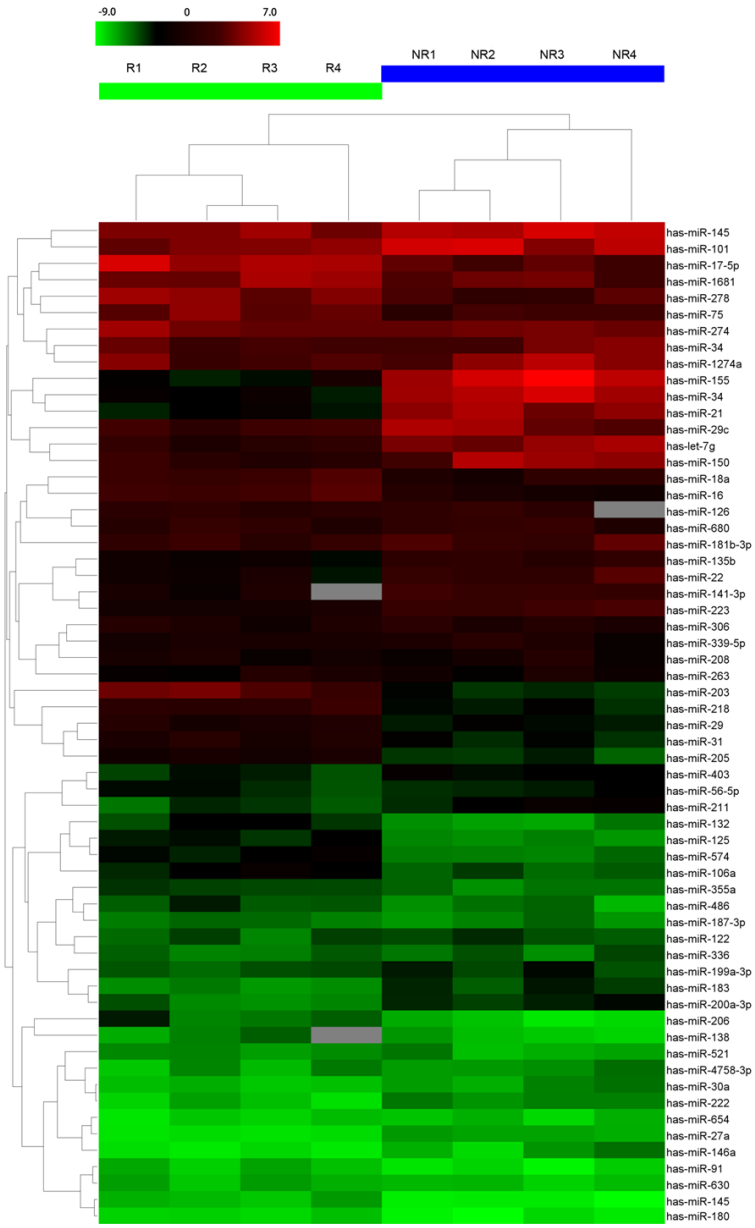

Figure 1. Serum samples pooled from four patient showing response and four patients showing no response to FOLFOX treatment were subjected to Miseq sequencing to identify miRNAs that were significantly differentially expressed. Among the 237 serum miRNAs that were scanned by Miseq sequencing (≥1 copy), 61 miRNAs were detectable (≥10 copies) in both responding and non-responding patients.

tween miRNAs and response to some tumors [10]. These suggest that miRNA can act as important regulators during chemoresistance among different cancers.

Early detection and removal of cancerous or precancerous lesions is thought to be of critical importance for reducing the incidence and improve the prognosis of CRC [11]. The development of noninvasive and convenient method which can complement and improve on current CRC screening strategies has become a major challenge. Despite of high concentration of ribonucleases in peripheral blood, studies indicated that it is feasible to detect circulating RNA in serum of tumor patients [12]. At present, more and more researchers focus on circulating biomarkers, and a specific marker is urgently needed for increasing the early detection rate and decreasing the mortality rate in CRC [13]. On the other hand, predictive biomarkers are better blood-based, as blood is easily available and provides the chance to monitor chemotherapy response. Thus, it is important to identify cell-free markers that predict a patient's responsiveness to chemotherapy, which may allow for the development of targeted therapies for overcoming chemoresistance. The previous studies have shown that miRNAs exist stably in human serum and have potential role in the diagnosis and prognosis of various cancers, such as breast cancer, prostate cancer, gastric cancer [14-16]. However, unique serum miRNA that could predict tumor response to FOLFOX treatment of CRC have not been identified.

In current study, we conducted high-throughput Miseq sequencing followed by two phases of reverse transcription quantitative real-time PCR (RT-qPCR) assays to test the hypothesis that specific miRNAs exist in serum and can be useful in predicting chemo-response with the hope that such findings may guide therapeutic choice. Our systematic approach demonstrated that serum miR-203 was significantly down-regulated in non-responding patients and may

Table 1. Candidate altered miRNAs in serum of responding group and nonresponding group determined by Miseq sequencing

Deregulated miRNAs	Fold change (NR vs. R)	*P* value (NR vs. R)
has-miR-155	4.67	<0.05
has-miR-34	3.49	<0.05
has-miR-21	3.28	<0.05
has-let-7g	2.15	<0.05
has-miR-203	0.23	<0.05
has-miR-16	0.42	<0.05
has-miR-135b	2.08	<0.05
has-miR-218	0.33	<0.05
has-miR-205	0.31	<0.05
has-miR-31	0.46	<0.05
has-miR-211	2.26	<0.05
has-miR-206	4.37	<0.05
has-miR-200a-3p	2.41	<0.05

NR: Non-response; R: Response.

prove useful as serum biomarker for early diagnosis prognosis of CRC. In addition, the correlation between the miR-203 and the CRC recurrence was further assessed.

Materials and methods

Patients and samples

A multiphase, case-control study was designed to identify serum miRNAs as potential biomarkers for differentiating chemo-response to standard FOLFOX therapy in CRC. Tumor response status was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 criteria and was assigned to patients with complete or partial response (CR and PR, respectively) and stable or progressive disease (SD and PD, respectively) in tumor measurements confirmed by repeat studies performed no less than four weeks after the criteria for response was first met. Briefly, 328 patients diagnosed with CRC but without other diseases were recruited from Zhejiang Provincial People's hospital between January 2009 and February 2012. Among the 324 patients, there are 164 patients showing response (CR and PR) to FOLFOX treatment while the other 164 patients showing no response (SD and PD). All these participants were allocated to three phases. In the discovery phase, serum samples pooled from four patient showing response and four patients showing no response were subjected to Miseq sequencing to identify miRNAs that were significantly differentially expressed. In the training phase, the candidate miRNAs were tested with RT-qPCR in an independent cohort from 70 CRC patients responding to FOLFOX treatment and 70 patients showing no response to treatment. In the validation phase, serum samples from another cohort of 90 CRC patients with response and 90 patients without response to FOLFOX treatment were entered into the study to further investigate the diagnostic and prognostic accuracy of the candidate miRNAs for chemo-response in CRC. All the patients were pathologically confirmed and the clinical samples were collected before chemotherapy was started. They were classified according to the WHO criteria and staged according to the tumor-node-metastasis (TNM) classification. Overall survival (OS) was updated on 1 February 2012 and was defined as the time from inclusion to death for any reason. Recurrence free survival (RFS) was defined as the time from inclusion to recurrence or metastasis progression. Written informed consent was obtained from all patients according to local ethical regulations of the Ethics Committee of the Zhejiang Provincial People's Hospital.

Miseq sequencing

For Miseq sequencing, equal volumes of serum from four patients showing chemo-response, four patients showing no response with similar age and sex distributions were pooled, respectively. Total RNA was extracted and purified using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the protocol provided by the manufacturer. Briefly, a pair of adaptors was ligated sequentially to the 3' and 5' ends of miRNA, and the ligated miRNA molecules were amplified by RT-qPCR to construct a cDNA library. Quality of the library was measured by the KAPA RT-qPCR kit and cDNAs with concentrations of higher than 1 nM and no dimmer contamination were used directly for sequencing analysis (Miseq sequencer, Illumina, San Diego, CA, USA). The final reads of miRNA were identified by normalization with the total reads of all called miRNAs in the sample. Bioinformatics analysis was conducted by searching against the miRBase 17.0 to determine known mature miRNAs.

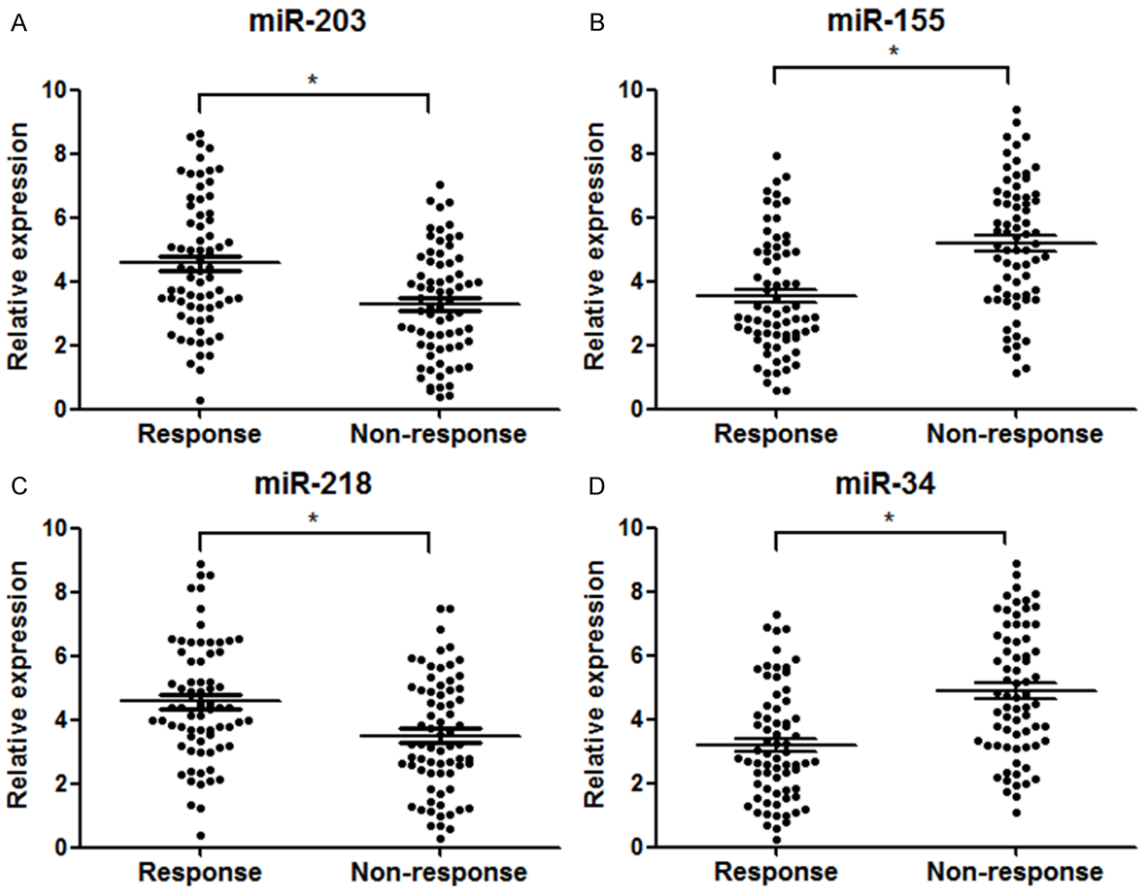

Figure 2. Concentrations of four miRNAs in CRC patients showing response (n=70) and showing non-response (n=70) using RT-qPCR assay in training set (A-D). *, P<0.01.

RNA extraction

Total RNA was isolated from serum samples from patients with CRC with or without chemo-response to FOLFOX treatment by using Trizol and acid phenol according to the manufacturer's instructions. The extracted total RNA was eluted in 20 μl nuclease-free water and the RNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The samples with A260 nm/A280 nm ratios between 1.8 and 2.0 were used for further experiments.

Quantitative real-time PCR (RT-qPCR)

For serum miRNA detection, we treated the total RNA with a reverse transcription kit (Bioteck, Beijing, China) according to the protocol to obtain cDNA and then RT-qPCR was performed by using 7500 RealTime PCR system (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantification of gene expression levels and U6 was used as a housekeeping gene. All reactions were performed in triplicate.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, California, USA). The differences between two groups were analyzed by the Mann-Whitney U-test. Receiver operating characteristic (ROC) curves were established to discriminate the patients with response from non-response. Area under the receiver operating characteristic curve (AUC) was employed as an accuracy index for evaluating the diagnostic performance of the selected miRNA. A log-rank test was used to analyze the statistical differences in survival as deduced from Kaplan-Meier curves. Cox proportional-hazard regression analysis was performed to calculate HR and 95% CI

Table 2. Characteristics of responding patients in training set and validation set

Variable	Training set (n=70)	Validation set (n=90)	*P*-value
Age (years)	61 (53-78)	63 (49-77)	0.48
Sex			0.87
Male	44 (62.86%)	58 (64.44%)	
Female	26 (37.14%)	32 (35.56%)	
Tumor location			0.75
Colon	33 (47.14%)	45 (50.00%)	
Rectum	37 (52.86%)	45 (50.00%)	
Differentiation			0.18
Well	18 (25.71%)	21 (23.33%)	
Moderate	31 (44.29%)	41 (45.56%)	
Poor	21 (30.00%)	28 (31.11%)	
Lymph node metastasis			0.61
Negative	21 (30.00%)	31 (34.44%)	
Positive	49 (70.00%)	59 (65.56%)	
Tumor stage			0.54
II	21 (30.00%)	27 (30.00%)	
III	31 (44.29%)	44 (48.89%)	
IV	18 (25.71%)	19 (21.11%)	
Histology subtype			0.99
Adenocarcinoma	52 (74.29%)	67 (74.44%)	
Mucous adenocarcinoma	10 (14.29%)	13 (14.44%)	
Anaplastic carcinoma	8 (11.43%)	10 (11.12%)	

for each covariable. All differences were regarded as statistically significant when P<0.05.

Results

High-throughput sequencing of serum miRNAs from CRC patients responding or non-responding to FOLFOX treatment

To identify novel serum miRNAs involved in response to FOLFOX therapy, we determined the miRNA expression profile in 4 CRC patients showing response (CR and PR) and 4 showing non-response (SD and PD) to FOLFOX treatment through small RNA deep sequencing. Among the 237 serum miRNAs that were scanned by Miseq sequencing (≥1 copy), 92 and 108 miRNAs were detectable (≥10 copies) in responding group and non-responding group, respectively. Besides, 61 miRNAs were detectable in both responding and non-responding patients (**Figure 1**). Expression of a miRNA was considered altered only if at least 50 copies were detected by Miseq sequencing together with significant deregulations larger than two-fold change in non-responding group vs. responding group comparison. Based on these criteria, 13 miRNAs were selected as differentially expressed in which 8 miRNAs were up-regulated and 5 miRNAs were down-regulated in non-responding patients compared with responding patients (**Table 1**). Thus, 13 miRNAs were selected as candidates for further testing via RT-qPCR.

Evaluation of miRNA expression by RT-qPCR

The 13 candidate miRNAs were first tested with RT-qPCR using an independent cohort of 70 responding patients and 70 non-responding patients. MiRNAs with a Cq value of >35 and detection rate of <75% in either responding group or non-responding group were excluded from further analysis. Two miRNAs (miR-155 and miR-34) showed significant increased expression levels while two miRNAs (miR-203 and miR-218) showed significant down-regulated expression levels in non-responding patients when compared with responding patients (all at P<0.01, **Figure 2**). The concentrations of the four miRNAs were further measured using the validation cohort consisted of 90 responding patients and 90 non-responding patients. There was no significant difference in the distribution of age, sex and tumor characteristics between the training and the validation sets for the responding group (**Table 2**) and non-responding group (**Table 3**). Alterations in miRNA expression pattern of validation set were consistent with those of training set (**Figure 3**).

Correlation between the four miRNAs and clinicopathological characteristics in CRC

After having found the deregulations of the four miRNAs, we then sought to evaluate the correlation between miRNAs expression levels and clinical pathological characteristics. The data summarized in **Table 4** showed the relationship between the four miRNAs and the clinicopathological characteristics of the patients with CRC in the validation set. Higher levels of serum miR-155 and lower levels of miR-203

Table 3. Characteristics of non-responding patients in training set and validation set

Variables	Training set (n=70)	Validation set (n=90)	*P*-value
Age (years)	64 (51-76)	66 (54-81)	0.62
Sex			0.61
Male	46 (65.71%)	63 (70.00%)	
Female	24 (34.29%)	27 (30.00%)	
Tumor location			0.99
Colon	39 (55.71%)	51 (56.67%)	
Rectum	31 (44.29%)	39 (43.33%)	
Differentiation			0.41
Well	16 (22.86%)	24 (26.67%)	
Moderate	32 (45.71%)	41 (45.56%)	
Poor	22 (31.43%)	25 (27.78%)	
Lymph node metastasis			0.72
Negative	23 (32.86%)	32 (35.56%)	
Positive	47 (67.14%)	58 (64.44%)	
Tumor stage			0.38
II	22 (31.42%)	27 (30.00%)	
III	31 (44.29%)	44 (48.89%)	
IV	17 (24.29%)	19 (21.11%)	
Histology subtype			0.41
Adenocarcinoma	54 (77.14%)	69 (76.67%)	
Mucous adenocarcinoma	8 (11.43%)	15 (16.67%)	
Anaplastic carcinoma	8 (11.43%)	6 (6.67%)	

significantly correlated with advanced tumor stage (P<0.05). Lower levels of miR-203 and miR-218 correlated with positive lymph nodes metastasis (P=0.02 and 0.01, respectively). However, no significant associations were found between the four miRNAs with age, sex, tumor location and tumor differentiation (all at P>0.05).

Investigation of the diagnostic value of four candidate miRNAs in differentiating responding from non-responding patients

We then sought to evaluate the potential diagnostic role of the four miRNAs in differentiating patients between the responding and non-responding group by using the validation set. By performing the receiver operating characteristic (ROC) curve analysis, we found that the corresponding area under the curves (AUCs) of miR-203, miR-155, miR-218 and miR-34 were 0.796, 0.715, 0.709, and 0.698, respectively (**Figure 4**). MiR-203 showed the largest AUC but no statistical significance was found (P=0.062). Besides, their diagnostic sensitivity and specificity were 71.4% and 72.0%, 69.3% and 64.1%, 69.2% and 61.5%, 64.0% and 68.0%, respectively. Thus, miR-203 yielded the highest diagnostic value in differentiating responding status in CRC patients receiving standard FOLFOX treatment.

Serum miR-203 expression is associated with response to standard FOLFOX treatment in CRC

Based on the above results, we further investigated the correlation between the four miRNAs expression and chemo-response to FOLFOX treatment. When we stratified patients into a high (n=90) and a low (n=90) miR-203 expressing group with median value as optimal cut-offs on the validation set, the proportion of patients not responding to chemotherapy was significantly higher in the high miR-203 expressing group than in the low group (P<0.01, **Figure 5A**). However, no statistical significance was found in the other three miRNAs (all at P>0.05, **Figure 5B-D**). Thus, we focused on miR-203. By performing the Kaplan-Meier survival analysis, we revealed that patients with high level of circulating miR-203 level had a significant better survival than did those with a low miR-203 expression level (P<0.0001, **Figure 5E**). The 5-year survival rate of the CRC patients whose serum expressed low levels of miR-203 was 34.4% (31/90), which was significantly lower than that of the patients whose tumors expressed high levels of miR-203 (68.8%, 62/90); this difference was statistically significant (P<0.001). To conclude, our date reveals that circulating miR-203 level is significantly correlated with chemo-response to FOLFOX treatment in CRC patients.

Serum miR-203 expression is associated with tumor recurrence and metastasis progression in patients with CRC

miR-203 was found significantly correlated to OS in our cohort of patients treated with FOLFOX. We considered the possibility that miR-

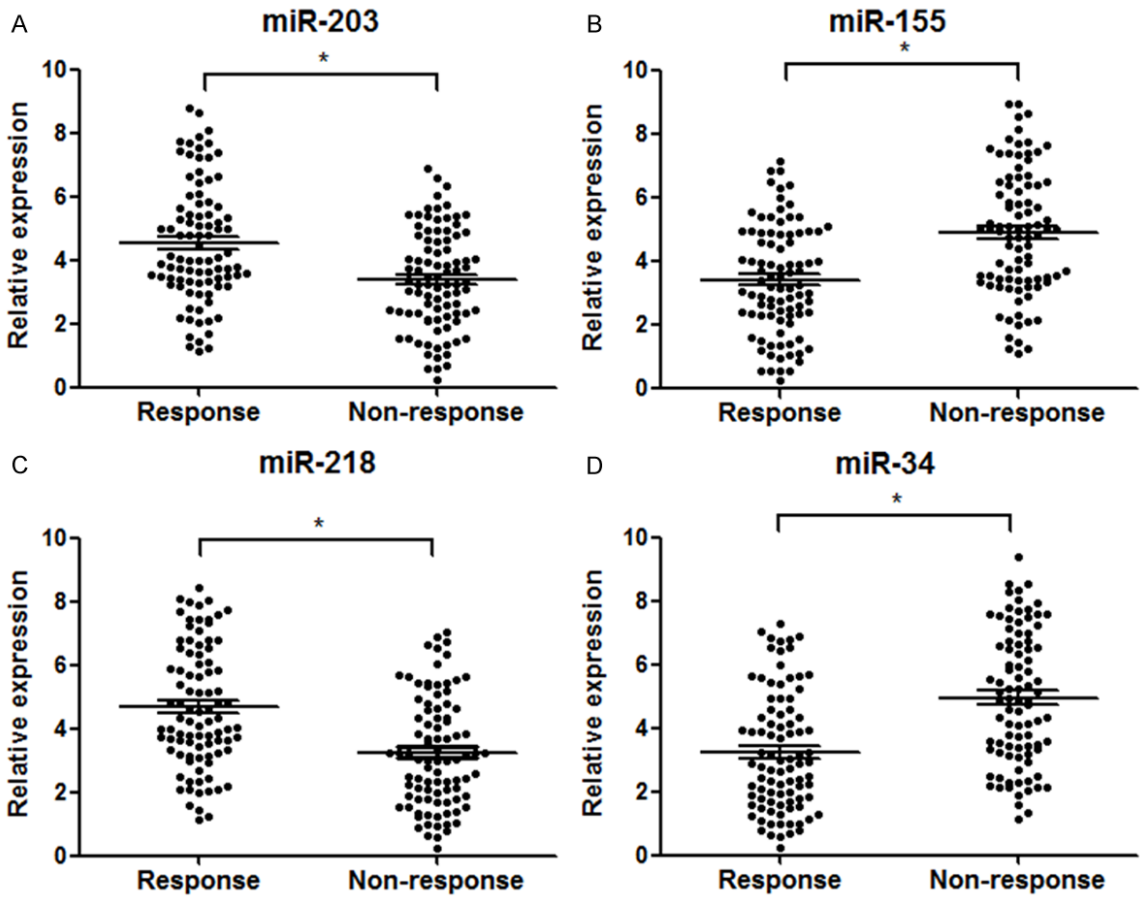

Figure 3. Concentrations of four miRNAs in CRC patients showing response (n=90) and showing non-response (n=90) using RT-qPCR assay in validation set (A-D). *, P<0.01.

203 might be prognostic in addition to predictive. To address this possibility, we examined whether serum miR-203 level was associated with metastatic recurrence of disease curatively intended surgery in validation cohort. Kaplan-Meier survival analysis revealed that patients with low miR-203 levels had a markedly lower recurrence-free survival (RFS) rather than those with high miR-203 levels (P<0.001, **Figure 6A**). On the other hand, serum miR-203 expression was significantly suppressed in recurrence patients compared with non-recurrence patients (P<0.01, **Figure 6B**). Univariate Cox proportional hazards regression model analysis revealed a statistically significant correlation between RFS of CRC patients and miR-203 level (P=0.016), lymph node metastasis (P=0.024) and tumor stage (P=0.012). Parameters significantly related to recurrence in the univariate analysis were put into the multivariate analysis to identify the independent factors for prognoses. The results showed that miR-203 level and tumor stage maintained their significance as independent prognostic factors for RFS of CRC patients receiving FOLFOX treatment (P=0.011 and 0.015, respectively) (**Table 5**).

Discussion

Currently, the major challenge to improve the clinical outcome of patients with CRC is the chemotherapy failure caused by drug resistance. Identification of noninvasive and invasive phenotypes is vital to rational clinical management. Yet, little was known about noninvasive miRNA biomarkers that can effectively accomplish this task. In the present study, our analysis revealed that miR-203, miR-155, miR-218 and miR-34 were differently expressed between CRC patients responding and non-responding to FOLFOX treatment in a specific manner. Moreover, serum miR-203 demonstrated high accuracy for diagnosis and progno-

Table 4. Correlations between miRNA concentrations and clinicopathological characteristics of CRC patients in validation set [median (interquartile range)]

Parameters	Total cases	miR-203	P	miR-155	P	miR-218	P	miR-34	P
Age			0.44		0.82		0.10		0.76
<65	97	3.87 (1.08-7.94)		4.51 (1.14-8.45)		4.41 (1.30-7.65)		4.10 (0.36-7.77)	
≥65	83	4.31 (1.25-8.06)		4.86 (1.13-9.27)		4.03 (1.27-8.85)		3.83 (1.30-7.09)	
Sex			0.35		0.51		0.57		0.58
Male	121	4.04 (1.18-7.94)		4.86 (1.14-8.06)		4.06 (1.30-7.81)		4.08 (1.30-7.89)	
Female	59	4.30 (1.50-6.57)		4.40 (1.04-7.83)		4.33 (1.26-7.46)		3.68 (0.40-7.35)	
Tumor location			0.89		0.96		0.99		0.21
Colon	96	4.32 (1.09-6.89)		4.47 (1.23-7.68)		4.21 (1.23-8.32)		4.23 (1.21-6.78)	
Rectum	84	4.16 (1.12-7.78)		4.69 (2.01-8.36)		4.09 (0.99-7.87)		3.86 (1.00-7.31)	
Lymph node metastasis			0.02		0.36		0.01		0.81
Negative	63	3.56 (1.12-6.57)		5.09 (1.14-8.94)		4.79 (2.33-8.95)		4.23 (1.33-8.09)	
Positive	117	4.90 (1.39-8.48)		4.57 (1.00-7.44)		3.32 (0.21-6.46)		4.02 (1.29-7.69)	
Differentiation			0.07		0.26		0.43		0.93
Well	45	4.56 (1.29-7.94)		4.34 (1.14-7.80)		4.46 (1.31-7.74)		4.16 (1.29-7.92)	
Moderate	82	3.74 (1.14-2.97)		4.68 (1.13-7.85)		4.11 (1.24-7.78)		4.27 (1.36-7.99)	
Poor	53	3.92 (1.22-3.02)		5.03 (2.12-8.77)		3.86 (0.67-8.78)		4.36 (1.39-8.03)	
Tumor stage			0.01		0.02		0.09		0.06
II	54	5.76 (2.74-9.08)		3.17 (0.32-6.55)		4.78 (1.79-8.46)		3.84 (0.35-6.01)	
III	88	4.23 (1.03-3.34)		4.60 (1.20-7.41)		4.26 (1.90-8.61)		4.36 (1.25-7.65)	
IV	38	3.74 (1.21-2.90)		4.75 (2.30-8.83)		4.06 (1.14-7.29)		4.53 (1.38-8.83)	

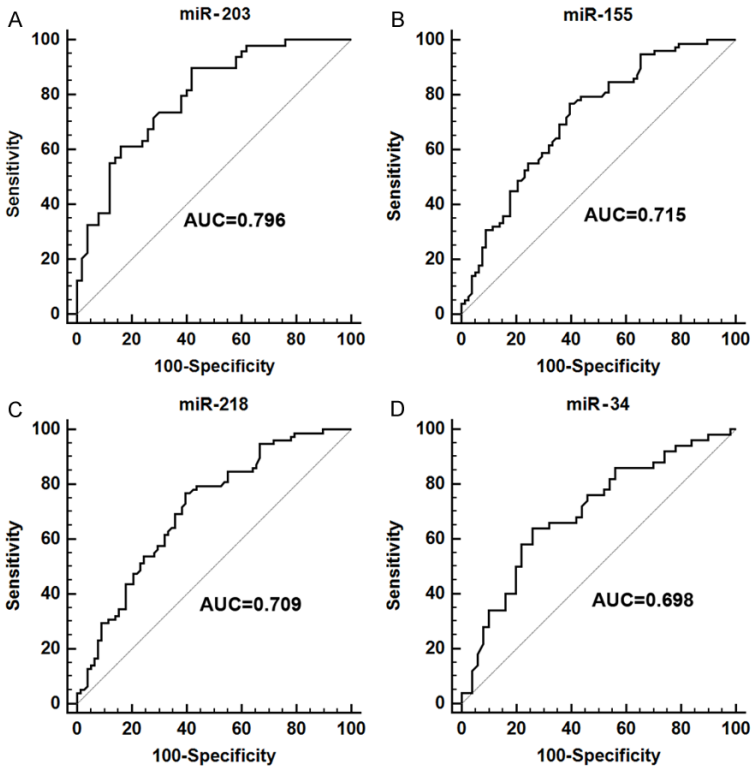

Figure 4. ROC curves analysis for detection of chemo-response status using (A) miR-203, (B) miR-155, (C) miR-218, (D) miR-34 in CRC patients showing chemo-response to FOLFOX treatment (n=90) and showing non-response to FOLFOX treatment (n=90) in validation set.

sis of CRC chemo-response. To our knowledge, this is the first study to characterize a serum miRNA expression signature for distinguishing response and non-response to standard FOLFOX treatment in CRC patients by use of the genome-wide Miseq sequencing platform.

Many studies have found that miRNA expression is aberrant in CRC development; however, most of these studies focused on the expression of miRNAs in tumor tissues and cells. Although tissue miRNAs can provide an accurate diagnosis for various types of cancer, the difficulty in collecting tissue samples limits its application for the detection of cancer biomarkers. Acquiring tissue samples is an invasive procedure and depends on surgical sections after initial clinical classification. The search for noninvasive tools

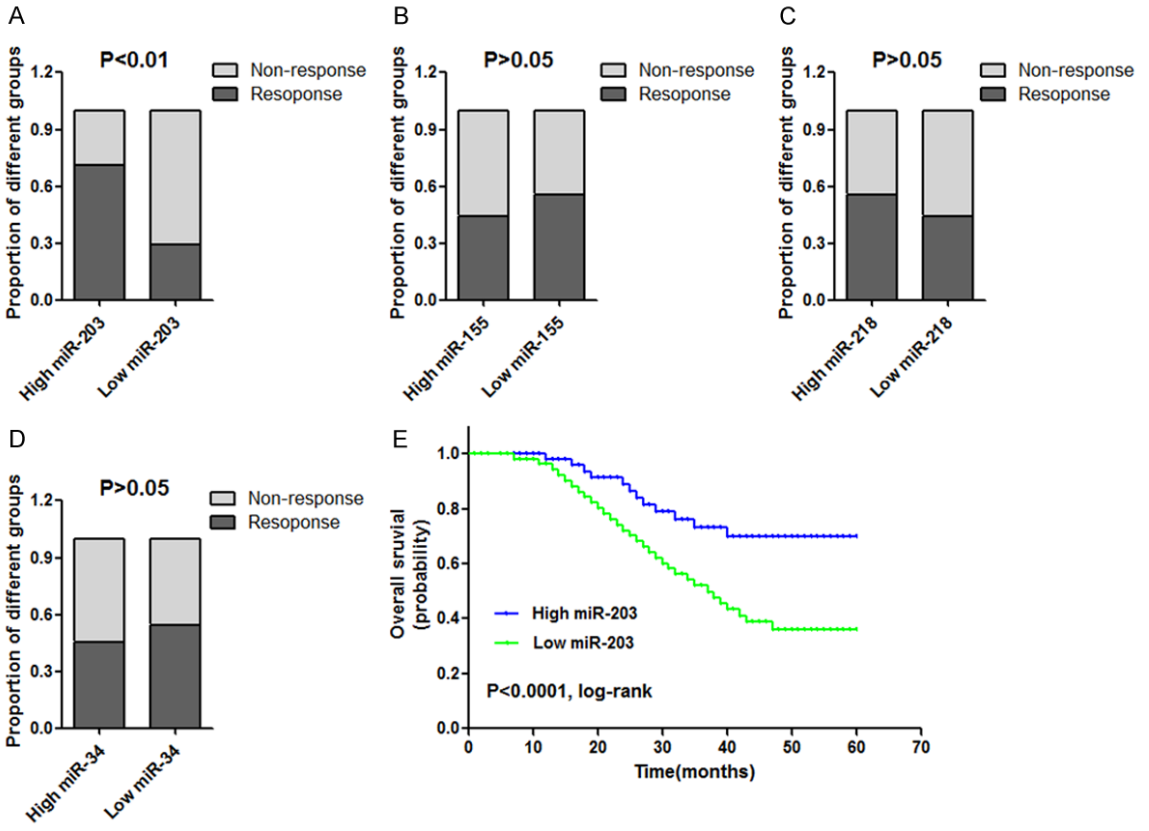

Figure 5. Serum miR-203 expression is associated with response to standard FOLFOX treatment in CRC. (A) The proportion of patients responding to chemotherapy was significantly higher in the high miR-203 expressing group than in the low group (P<0.01). While no statistical significance was found in (B) miR-155, (C) miR-218 and (D) miR-34 (P>0.05). (E) Kaplan-Meier survival analysis revealed that patients with high level of circulating miR-203 level had a significant better survival than did those with a low miR-203 expression level (P<0.0001).

for the diagnosis of cancer has long been a goal of many researchers, and much of the interest has been on the circulation of nucleic acids in plasma and serum. Compared with DNA and mRNA, circulating miRNAs show remarkable stability after prolonged incubation at room temperature and/or multiple freezing-thawing processes [17]. In our study, we screened the whole miRNA profile serum samples from CRC patients via Miseq sequencing, which enabled us to have better chance to identify potential diagnostic biomarkers. Miseq sequencing is a high-throughput assay to initially screen miRNAs and could exclude possible contamination by other small RNA and DNA fragments. However, the Miseq results from pooled serum samples might include inaccurate information owing to the individual variation. For this reason, candidate miRNAs revealed by Miseq sequencing were evaluated by two phases of RT-qPCR assays using a large number of individual samples.

Resistance to chemotherapy is one of the major causes for treatment failure in advanced CRC. Thus, predictive markers are required to increase the efficacy of chemotherapy and may also be helpful in monitoring therapy response in CRC. The standard FOLFOX treatment method is one of the most common used first-line chemotherapy regimens in advanced CRC patients. However, there are currently no available molecular predictive markers for these combination regimens. The identification of cancer-specific miRNAs is critical for understanding the roles of miRNAs in tumorigenesis and may be important for defining novel therapeutic targets. Previous studies identified that a circulating microRNA signature was of much importance for CRC detection [18]. Besides, various reports also showed that miRNAs may play important roles during chemoresistance in several cancers. Zhang et al reported that cell-free miR-155 in urine is up-regulated and plays a potential role in diagnosis and progno-

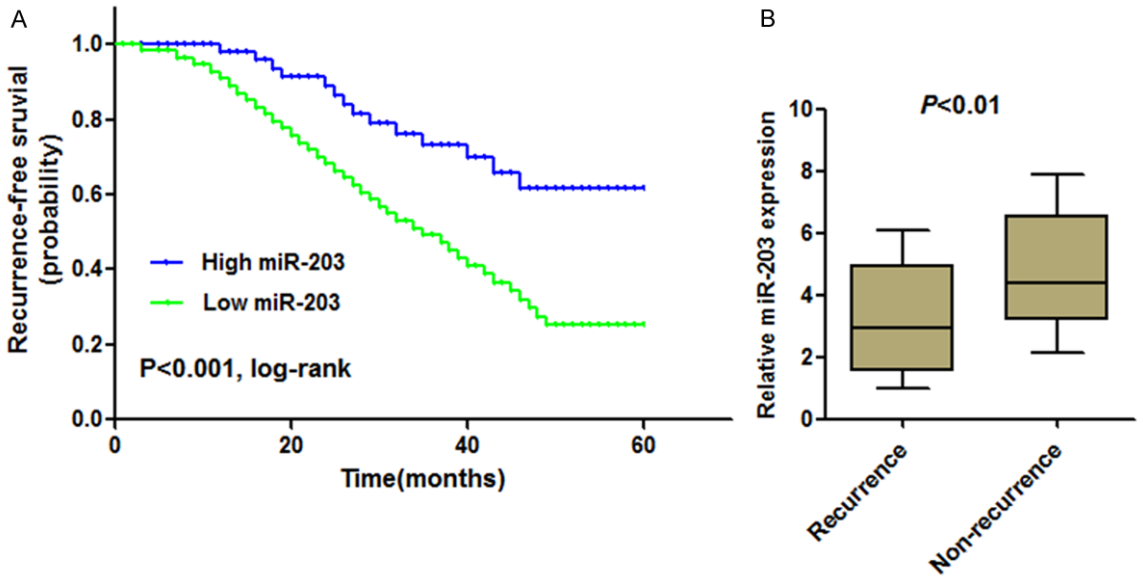

Figure 6. Serum miR-203 expression is associated with tumor recurrence and metastasis progression in patients with CRC. A: Kaplan-Meier survival analysis revealed that patients with low miR-203 levels had a markedly lower recurrence-free survival (RFS) rather than those with high miR-203 levels (P<0.001). B: Serum miR-203 expression was significantly suppressed in recurrence patients compared with non-recurrence patients (P<0.01).

Table 5. Univariate and multivariate Cox proportional hazards regression model analysis of RFS in patients with CRC in validation set

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.132	0.689-3.013	0.398			
Sex	1.408	0.756-3.457	0.231			
Tumor location	0.792	0.417-1.901	0.635			
Differentiation	1.471	0.625-2.867	0.303			
Lymph node status	2.353	1.014-3.726	0.024	2.384	1.213-4.126	0.057
TNM stage	3.582	1.502-7.471	0.012	3.578	1.489-7.492	0.015
MiR-203 expression	2.549	1.215-4.457	0.016	2.553	1.223-4.601	0.011

sis for non-muscle invasive bladder cancer [19]. Li et al demonstrated that miR-218 is a chemotherapeutic factor in CRC and promotes 5-fluorouracil induced cell apoptosis [8]. Similarly, Siemens et al revealed that repression of c-Kit by p53 is mediated by miR-34 and is associated with reduced chemoresistance, migration and stemness [20]. Our date showed that miR-155 and miR-34 expression levels are significantly elevated while miR-218 is down-regulated in non-responding patients. These are consistent with previous conclusion about miR-218, miR-155 and miR-34. As miR-203, there exist contradictory conclusions on its role in CRC chemoresistance. Zhou et al demonstrated that miR-203 induces oxaliplatin resistance in CRC cells [21], while another report by Li showed that miR-203 enhances chemosensitivity to 5-fluorouracil by targeting thymidylate synthase in colorectal cancer [22]. Our date indicated that serum miR-203 level is significantly decreased in non-responding patients compared responding patients. These results revealed a potential clinical value of the four candidate circulating miRNAs in CRC chemotherapy.

After having found the deregulation of the four miRNAs, we then investigated their diagnostic value in distinguishing responding patients from non-responding patients. Our date showed that all the four miRNAs exerts relative high

diagnostic accuracy in the validation set, and the high diagnostic accuracy indicated that the expression profile of the four miRNAs could serve as an accurate biomarker for the detection of chemo-response in CRC patients. Moreover, the miR-203 and miR-155 were significantly correlated with tumor stage and progression. Based on these findings, the serum miRNAs provides a much more sensitive detection of chemo-response. Furthermore, technically speaking, serum test is more convenient and noninvasive, and thus being an ideal for the investigation of a panel containing a small number of miRNAs.

Finally, we focused on the predictive role of serum miRNA in CRC chemoresistance. Our date showed that the proportion of patients not responding to chemotherapy was significantly higher in the high miR-203 expressing group than in the low group, while no significant discrepancy was found in miR-218, miR-155 and miR-34. Moreover, CRC patients expressed high serum miR-203 levels showed a short 5-year survival rate than patients with low serum miR-203 levels. Additionally, the survival analysis indicated that overall survival of CRC patients with high level of miR-203 who received FOLFOX chemotherapy was better than that of patients with low-level serum miR-203. This suggests that serum miR-203 is significantly correlated with chemo-response to FOLFOX treatment in CRC patients. On this basis, we further considered the possibility that miR-203 might be prognostic in addition to predictive. To address this possibility, we examined whether serum miR-203 level was associated with metastatic recurrence of disease curatively intended surgery in validation cohort. Kaplan-Meier survival analysis revealed that patients with high miR-203 levels had a markedly lower recurrence-free survival (RFS) rather than those with low miR-203 levels. These data, together with our Cox proportional hazards regression model analyses, suggest that serum miR-203 is an independent prognostic indicator of survival and metastatic recurrence in CRC patients receiving FOLFOX treatment.

In conclusion, the present work has identified serum miR-203 as an effective biomarker related response to first-line FOLFOX treatment in CRC patients. These findings may provide a foundation for development of a novel noninvasive test to predict chemo-response and determination of innovative therapeutic strategies.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 8530-52288) and the Natural Science Foundation of Zhejiang province (No. H2015LN78431B).

Disclosure of conflict of interest

None.

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References

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.

[2] Lieberman DA, Rex DK, Winawer SJ, Giardiello FM, Johnson DA, Levin TR. Guidelines for colonoscopy surveillance after screening and polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer. Gastroenterology 2012; 143: 844-857.

[3] Alberts SR, Horvath WL, Sternfeld WC, Goldberg RM, Mahoney MR, Dakhil SR, Levitt R, Rowland K, Nair S, Sargent DJ, Donohue JH. Oxaliplatin, fluorouracil, and leucovorin for patients with unresectable liver-only metastases from colorectal cancer: a north central cancer treatment group phase II study. J Clin Oncol 2005; 23: 9243-9249.

[4] Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitot HC, Alberts SR. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004; 22: 23-30.

[5] Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001; 409: 363-366.

[6] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.

[7] Li J, Du L, Yang Y, Wang C, Liu H, Wang L, Zhang X, Li W, Zheng G, Dong Z. MiR-429 is an independent prognostic factor in colorectal cancer and exerts its anti-apoptotic function by targeting SOX2. Cancer Lett 2013; 329: 84-90.

[8] Li PL, Zhang X, Wang LL, Du LT, Yang YM, Li J, Wang CX. MicroRNA-218 is a prognostic indicator in colorectal cancer and enhances 5-fluorouracil-induced apoptosis by targeting BIRC5. Carcinogenesis 2015; 36: 1484-1493.

[9] Hur K, Toiyama Y, Okugawa Y, Ide S, Imaoka H, Boland CR, Goel A. Circulating microRNA-203 predicts prognosis and metastasis in human colorectal cancer. Gut 2015; [Epub ahead of print].

[10] Schetter AJ, Okayama H, Harris CC. The role of microRNAs in colorectal cancer. Cancer J 2012; 18: 244-252.

[11] Edwards BK, Noone AM, Mariotto AB, Simard EP, Boscoe FP, Henley SJ, Jemal A, Cho H, Anderson RN, Kohler BA, Eheman CR, Ward EM. Annual report to the nation on the status of cancer, 1975-2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. Cancer 2014; 120: 1290-1314.

[12] Wang H, Zhang X, Wang L, Zheng G, Du L, Yang Y, Dong Z, Liu Y, Qu A, Wang C. Investigation of cell free BIRC5 mRNA as a serum diagnostic and prognostic biomarker for colorectal cancer. J Surg Oncol 2014; 109: 574-579.

[13] Zhang X, Yang X, Zhang Y, Liu X, Zheng G, Yang Y, Wang L, Du L, Wang C. Direct serum assay for cell-free bmi-1 mRNA and its potential diagnostic and prognostic value for colorectal cancer. Clin Cancer Res 2015; 21: 1225-1233.

[14] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J, Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008; 18: 997-1006.

[15] Chan M, Liaw CS, Ji SM, Tan HH, Wong CY, Thike AA, Tan PH, Ho GH, Lee AS. Identification of circulating microRNA signatures for breast cancer detection. Clin Cancer Res 2013; 19: 4477-4487.

[16] Selth LA, Townley SL, Bert AG, Stricker PD, Sutherland PD, Horvath LG, Goodall GJ, Butler LM, Tilley WD. Circulating microRNAs predict biochemical recurrence in prostate cancer patients. Br J Cancer 2013; 109: 641-650.

[17] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature 2005; 435: 834-838.

[18] Wang J, Huang SK, Zhao M, Yang M, Zhong JL, Gu YY, Peng H, Che YQ, Huang CZ. Identification of a circulating microRNA signature for colorectal cancer detection. PLoS One 2014; 9: e87451.

[19] Zhang X, Zhang Y, Liu X, Fang A, Wang J, Yang Y, Wang L, Du L, Wang C. Direct quantitative detection for cell-free miR-155 in urine: a potential role in diagnosis and prognosis for non-muscle invasive bladder cancer. Oncotarget 2016; 7: 3255-3266.

[20] Siemens H, Jackstadt R, Kaller M, Hermeking H. Repression of c-Kit by p53 is mediated by miR-34 and is associated with reduced chemoresistance, migration and stemness. Oncotarget 2013; 4: 1399-1415.

[21] Zhou Y, Wan G, Spizzo R, Ivan C, Mathur R, Hu X, Ye X, Lu J, Fan F, Xia L, Calin GA, Ellis LM, Lu X. MiR-203 induces oxaliplatin resistance in colorectal cancer cells by negatively regulating ATM kinase. Mol Oncol 2014; 8: 83-92.

[22] Li T, Gao F, Zhang XP. MiR-203 enhances chemosensitivity to 5-fluorouracil by targeting thymidylate synthase in colorectal cancer. Oncol Rep 2015; 33: 607-614.