RESEARCH ARTICLE

Serum miR-19a Predicts Resistance to FOLFOX Chemotherapy in Advanced Colorectal Cancer Cases

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Abstract

Background: Colorectal cancer is the fourth most common cancer worldwide and the second leading cause of cancer-related death. FOLFOX is the most common regimen used in the first-line chemotherapy in advanced colorectal cancer, but only half of the patients respond to this regimen and we have almost no clue in predicting resistance in such first-line application. Methods: To explore the potential molecular biomarkers predicting the resistance of FOLFOX regimen as the first-line treatment in advanced colorectal cancer, we screened microRNAs in serum samples from drug-responsive and drug-resistant patients by microarrays. Then differential microRNA expression was further validated in an independent population by reverse transcription and quantitative real-time PCR. Results: 62 microRNAs expressing differentially with fold-change >2 were screened out by microarray analysis. Among them, 5 (miR-221, miR-222, miR-122, miR-19a, miR-144) were chosen for further validation in an independent population (N=72). Our results indicated serum miR-19a to be significantly up-regulated in resistance-phase serum (p=0.009). The ROC curve analysis showed that the sensitivity of serum miR-19a to discriminate the resistant patients from the response ones was 66.7%, and the specificity was 63.9% when the AUC was 0.679. We additionally observed serum miR-19a had a complementary value for cancer embryonic antigen (CEA). Stratified analysis further revealed that serum miR-19a predicted both intrinsic and acquired drug resistance. Conclusions: Our findings confirmed aberrant expression of serum miR-19a in FOLFOX chemotherapy resistance patients, suggesting serum miR-19a could be a potential molecular biomarker for predicting and monitoring resistance to first-line FOLFOX chemotherapy regimens in advanced colorectal cancer patients.

Keywords: Advanced colorectal cancer - miRNA - FOLFOX resistance - serum biomarker

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Introduction

Colorectal cancer (CRC) is the fourth most common cancer worldwide, with 875, 000 new cases every year, accounting for about 9% of all new cancer cases. And its mortality ranks the second in cancer-related death, responsible for about 208, 000 deaths per year (Gutierrez-Ibarluzea et al., 2008). Due to its huge disease burden, tremendous efforts have been devoted to improving the multidisciplinary treatment strategy of CRC patients. In recent decades, the development in earlier diagnosis through screening and better treatment modalities has obviously decreased the mortality of colorectal cancer (Cheng et al., 2011; Siegel et al., 2011). However, the majority of CRC patients still die from tumor recurrence and metastasis. Although the advent of targeted drugs, such as EGFR inhibitors (cetuximab), TKI inhibitors (erlotinib) and anti-angiogenesis agents (bevacizumab) prolongs patients' overall survival (OS) greatly, the huge costs and the limited suitable population restrict their clinical application. Chemotherapy which composes the comprehensive strategy in tumor treatment was of crucial importance, especially for those who would not benefit from targeted therapy determined by KRAS test and those who could not afford the expensive agents. For the reason that therapeutic effectiveness diminished following the multiple-line chemotherapies because of drug resistance or the decreased tumor response, we make our decision in first-line treatment regimen with cautious consideration. According to the latest NCCN (The National Comprehensive Cancer Network) clinical practice guidelines in Oncology version 1.2013, five chemotherapy regimens including FOLFOX (Tournigand et al., 2004), FOLFIRI (Tournigand et al., 2004; Colucci et al., 2005), CapeOx (Cassidy et al., 2004; Porschen et al., 2007; Cassidy et al., 2008), infusional 5-FU/LV or capecitabine (Petrelli et al., 1987; Jager et al., 1996) and FOLFOXIRI (Souglakos et al., 2006; Falcone et al., 2007) are recommended as the first-line treatment in metastatic CRCs. FOLFOX is the most common regimen. But only half of the patients responds to this regimen and we have almost no clue in predicting the resistance of its first-line application. For this reason, it is of urgent necessity to identify molecular biomarkers to further stratify patients

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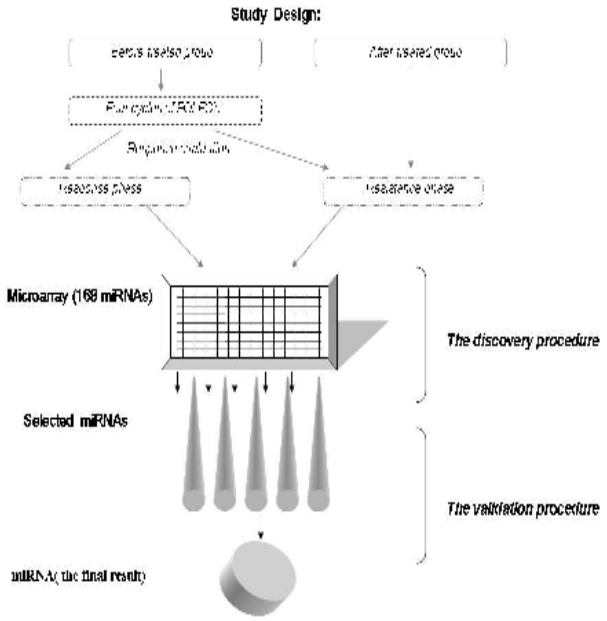

Figure 1. Blood Samples of These Two Groups Were Obtained When Patients Participated in the Study with Written Consent. The study was implemented in two main procedures, a biomarker discovery procedure and a validation procedure. In the biomarker discovery procedure, miRNAs were screened by PCR miRNA microarrays in pooled serum samples. In the validation procedure, expression levels of the selected miRNAs were evaluated in an independent population by RT-qPCR

and predict therapy response or resistance of FOLFOX. Mounting evidences have demonstrated that a newly discovered group of endogenous non-coding small RNAs, termed microRNAs (miRNAs), is associated with a variety of biological processes, such as cellular development, proliferation, differentiation, metabolism, cell death and carcinogenesis (Schmittgen et al., 2008; Li et al., 2011; Shen et al., 2013). However, the vital role of miRNAs involved in drug resistance has just been recognized in the past several years (Song et al., 2009). In addition, due to their small size (18-22 nucleotides), miRNAs present in serum as circulating miRNAs are remarkable steady. So, the miRNA is considered as one of the best choices as the serum biomarker.

Our study aimed to explore promising serum miRNAs as non-invasive biomarkers predicting resistance to FOLFOX chemotherapy as first-line treatment in advanced CRC patients. This present research was implemented in two main procedures, a biomarker discovery procedure and a validation procedure (Figure 1). In the biomarker discovery procedure, miRNAs were screened by PCR miRNA microarrays in pooled serum samples from 16 patients (8 response-phase patients and 8 resistance-phase patients). In the validation procedure, expression levels of the selected miRNAs were evaluated in an independent population of 72 patients.

Materials and Methods

Study Design and Patients

Our study enrolled two groups of patients. Patients with histologically confirmed advanced colorectal cancer, including patients who were diagnosed with IV-stage colorectal cancer and patients whose cancer progressed after the adjuvant therapy, were enrolled in before-treated group. Patients with progressive disease after first-line FOLFOX treatment, but hadn't receive the second-line treatment yet were enrolled in after-treated group. Participants were all enrolled consecutively between March and June in 2012 in the West China Hospital, China. Blood samples of these two groups were obtained when they participated in the study with written consent. According to the tumor response to FOLFOX regimen, the blood samples of the patients were defined as response-phase and resistance-phase. The before-treated group patients then received four cycles of standard first-line FOLFOX regimen chemotherapy (5-fluorouracil/leucovorin/oxaliplatin). We then evaluated the tumor response comprehensively based on the latest Response Evaluation Criteria in Solid Tumors (RECIST1.1). Patients with complete remission (CR), partial remission (PR) or stable disease (SD) continued the previous FOLFOX chemotherapy and we evaluated their tumor response every 2 months regularly until the disease progressed. Their blood samples belonged to response-phase. And patients with progressive disease (PD) received the second-line therapy, and their samples belonged to resistance-phase. The blood samples from after-treated group belonged to resistance-phase (Figure 1).

Serum Sample Collection

5 milliliters of whole blood were collected from each participant using plain tube with no anti-coagulants. Leave the whole blood still in 4 °C refrigerator for a while, and they stratified quickly. Then the clear light yellow supernatant was centrifuged at 1, 200 rpm for 10 min at 4 °C. Cell-free supernatant was re-centrifuged at 12, 000 rpm for a further 10 min at 4 °C to guarantee complete removal of residual cellular components. Every 250 ul of the supernatant serum was stored in an RNase-free eppendorf tube at -80 °C until use. This whole procedure was completed within 2 hours after the venipuncture.

RNA Isolation

For each sample, total RNA with preserved miRNAs was extracted by TRIzol LS Reagent (Invitrogen life technologies, USA) according the provided protocol. 750 ul TRIzol LS reagent was added into 250 ul of the serum sample, pipetted up and down several times, and incubated for 5 minutes at room temperature. As exogenous control, equal amount of ath-miR-159 was added into each sample. 200 ul chloroform was added, shaken vigorously for 15 seconds, and incubated for 2-15 minutes at room temperature. A centrifugation at 12, 000 rpm for 15 minutes at 4 °C was followed. The aqueous phase was transferred into a new Eppendorf tube and 500 ul of 100% isopropanol was added, incubated at room temperature for 10 minutes, and centrifuged at 12, 000 rpm for 10 minutes at 4 °C. The supernatant was removed, and 1 ml of 75% ethanol was added to wash the RNA pellet. The sample was then centrifuged at 7, 500 rpm for 5 minutes at 4 °C. The supernatant was removed and the RNA pellet was

Table 1. Differentially Expressing miRNAs with Fold Change >2

hsa-miR-339-5p	hsa-miR-20a	hsa-miR-19b	hsa-miR-210	hsa-miR-15a
hsa-miR-142-3p	hsa-miR-30e	hsa-miR-21	hsa-miR-21	hsa-miR-451
hsa-miR-502-3p	hsa-miR-106a	hsa-miR-205	hsa-miR-101	hsa-let-7g
hsa-miR-423-3p	hsa-miR-221	hsa-miR-185	hsa-miR-18a	hsa-miR-107
hsa-miR-103_1	hsa-miR-222	hsa-miR-30a	hsa-miR-10b	hsa-miR-181a
hsa-miR-103_2	hsa-miR-144	hsa-miR-122	hsa-miR-20b	hsa-miR-10a
hsa-miR-199a-3p	hsa-miR-192	hsa-miR-32	hsa-miR-29b	hsa-let-7i
hsa-miR-501-3p	hsa-miR-146a	hsa-miR-29c	hsa-miR-26b	hsa-miR-99a
hsa-miR-409-3p	hsa-miR-148a	hsa-miR-19a	hsa-miR-18a*	hsa-miR-720
hsa-miR-331-3p	hsa-miR-660	hsa-miR-125b	hsa-miR-29a	hsa-miR-543
hsa-miR-151-59p	hsa-miR-148b	hsa-miR-652	hsa-miR-22	hsa-miR-34a
hsa-miR-103-2*	hsa-miR-335	hsa-miR-215	hsa-miR-193b	hsa-miR-133b
hsa-miR-324-3p	hsa-miR-365			

The microarrays tested 168 miRNAs. Among them, 3 were with Ct value >35, 11 were undetermined, and 62 were identified differential expression with Fold Change >2. Finally, five miRNAs (miR-222, miR-144, miR-221, miR-19a, miR-122) were selected for further validation by RT-qPCR

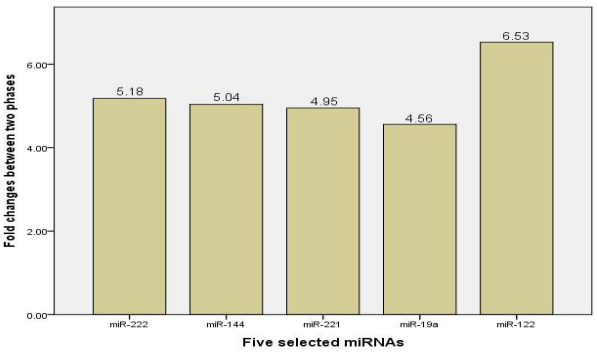

Figure 2. Five miRNAs (miR-222, miR-144, miR-221, miR-19a, miR-122) **Were Selected for Further Validation by RT-qPCR According to the Fold Change** (All were with fold change>4.5) **and Related Literatures**

air dried for 5-10 minutes. 25 ul RNase-free water was pipetted onto the RNA pellet up and down several times, incubated in a water bath at 55-60 °C for 10-15 minutes. The concentration of isolated RNA was quantified by NanoDrop 2000 (Thermo Fisher Scientific, USA).

miRNA Microarray

8 response-phase serum samples and 8 resistance-phase serum samples which were then mixed together separately, were chosen randomly for miRNA microarrays (Serum/Plasma Focus microRNA PCR Panel, V1.M, Exiqon). A total of 2 mixtures were shipped on dry ice to KangChen Bio-tech Inc. (Shanghai, China), which completed the miRNA microarrays according to the manufacturer's instructions. Each microarray had 4 sets of Ready-to-Use Serum/Plasma Focus microRNA PCR Panels. Each set of panels comprised 168 LNA™ microRNA primer sets focusing on serum/plasma relevant human miRNAs and 7 reference miRNAs. Microarrays were scanned using ABI PRISM7900 system (Applied Biosystems) and fold changes in miRNA expression between the two groups were calculated using the $2^{-\Delta Ct}$ method.

Reverse Transcription and Quantitative Real-time PCR

A larger independent population was then enrolled to validate the predictive potential of the selected miRNAs by reverse transcription and real-time PCR.

Reverse transcription was carried out on 120 ng of total RNA in a final volume of 10 ul reaction system. The 10 ul-RT reaction mixture was incubated at 37 °C for 60 minutes, 85 °C for 5 seconds, and then held at 4 °C using the One Step PrimeScript(R)miRNA cDNA Synthesis Kit (Takara, Japan) according to the manufacturers' instructions. Then 90 ul of the RNase-free water was added to dilute the RT product.

Real-time PCR was performed using SsoAdvanced™ SYBR® Green Supermix (BIO-RAD, USA). 3.2 ul diluted RT product was added into a 10-ul PCR reaction, which also contained 5 ul SsoAdvanced SYBR Green supermix, 0.4 ul forward primer (TIANGEN, China) and 0.4 ul Uni-miR qPCR Primer (Takara, Japan) and 1 ul RNase-free water. All PCR reactions, including no-template controls, were run in triplicate using iQ5 Real-time PCR Detection System (BIO-RAD, USA) as follows: 1) 1 cycle of 95 °C for 30 seconds, 2) 45 cycles of 95 °C for 5 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds, 3) 71 cycles of 60 °C for 10 seconds. Finally, the raw data were analyzed with the iQ5 Optical System Software version 2.1.

Statistical Analysis

The Student's t test was used to compare values, and the Chi-squared test was performed on categorical data between two groups. Relative expression levels of miRNA between the two groups were calculated using the $2^{-\Delta Ct}$ method, Mann-Whitney U test was used to evaluate statistical significance. To evaluate the diagnostic sensitivity and specificity, we constructed receiver operating characteristic (ROC) curves and the area under the curve (AUC) was then estimated. $P<0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS 18.0 software.

Results

Profiling of Serum miRNAs by Microarrays

8 serum samples from response-phase (n=8) and 8 from resistance-phase (n=8) were evaluated by microarrays. 168 miRNA primer sets were integrated into the miRNA microarray each. Of these 168 miRNAs, 62 were screened out differentially expressing between response-phase and resistance-phase with fold change >2 (Table 1). Among these, five miRNAs (miR-222, miR-144, miR-221, miR-19a, miR-122) with fold change >4.5 (Figure 2) were selected for further validation in a larger independent population by reverse transcription and quantitative real-time PCR (RT-qPCR) to examine whether they could be regarded as biomarkers to predict resistance to the first-line FOLFOX chemotherapy in advanced CRC patients.

Characteristics of the Independent Validation Population

To validate the predictive potential of the selected miRNAs, we investigated these items in an independent population. 72 participants were enrolled, 36 in the response-phase cohort and 36 in the resistance-phase cohort. The characteristics were presented in Table 2. In general, resistance-phase patients were older than response-phase patients (57.94±14.41 vs 51.08±14.26, $p<0.05$). There were 21 females and 15 males in response-phase group, and 18 females and 18 males were in resistance-phase group ($p>0.05$). CEA was no

Table 2. Characteristics of the Validation Population

	Response-phase	Resistance-phase	*p* value
Number(N=)	36	36	
Age(mean±SD,years)	51.08±14.26	57.94±14.41	<0.05
Gender			
Female	21	18	>0.05
Male	15	18	
CEA (mean±SD, ng/ml)	74.82±161.24	86.55±145.21	>0.05
Resistance pattern (n=)*			
Intrinsic resistance		21	
Acquired resistance		15	

*Patients of resistance-phase group were enrolled in two kinds of ways. Those who experienced progressive disease (PD) in the tumor response evaluation at first time were defined with the intrinsic resistance, and those who developed progressive disease (PD) during the subsequent FOLFOX treatment were defined with the acquired resistance

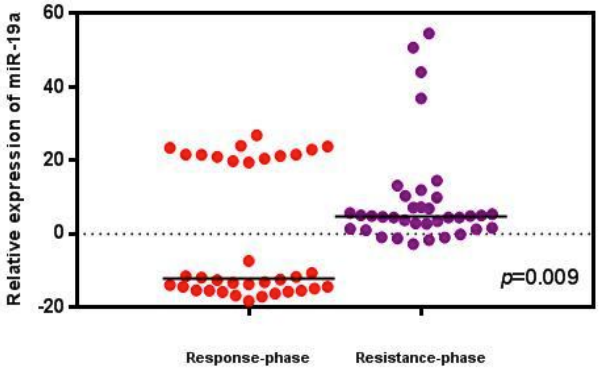

Figure 3. The Relative Expression Levels of Serum miR-19a Between Response-phase and Resistance-phase Evaluated by RT-qPCR. *P* value is based on Mann-Whitney U test

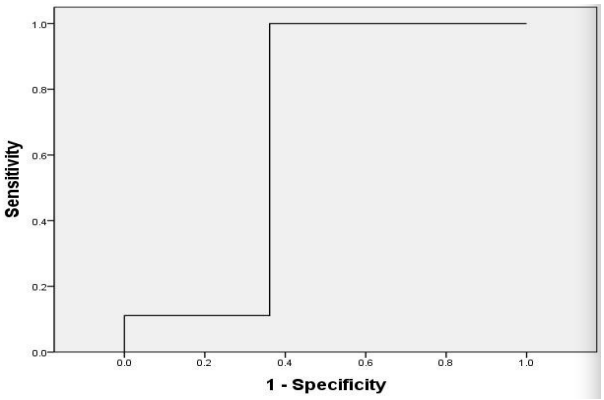

Figure 4. The miR-19a Yields an AUC of 0.679 (95% confidence interval (CI) 0.534-0.824) with Sensitivity of 66.7% and a Specificity of 63.9%

significant difference between two groups (74.82±161.24 vs 86.55±145.21, $p>0.05$). According to our study design, resistance-phase patients were enrolled in two ways. Patients who experienced progressive disease (PD) in the tumor response evaluation at the first time during the first-line FOLFOX chemotherapy were defined with intrinsic resistance (n=21), and those who developed progressive disease (PD) during the subsequent FOLFOX treatment were defined with acquired resistance (n=15).

Validation of Selected Serum miRNAs by RT-qPCR

To validate the predictive potential of serum miRNAs as novel biomarkers, we further investigated the levels of the five selected miRNAs (miR-222, miR-144, miR-221, miR-19a, miR-122) individually in the serum samples from 72 patients (36 response-phase patients and 36 resistance-phase patients) normalized to spike in ath-miR-159. Expression level of miR-19a in serum was significantly up-regulated ($p=0.009$) in resistance-phase patients than in response-phase patients (Figure 3). The other four miRNAs(miR-222, miR-144, miR-221, miR-122) showed no statistical differences ($p>0.05$) between two groups. ROC curve was constructed to evaluate the diagnostic value of serum miR-19a. Based on the ROC curve, the miR-19a had an AUC of 0.679 (95% confidence interval [CI] 0.534-0.824) with a sensitivity of 66.7% and specificity of 63.9% (Figure 4). In addition, we didn't identify significant difference ($p>0.05$) between the two resistance patterns (intrinsic resistance and acquired resistance) in resistance-phase group.

Discussion

Currently, the chemotherapy is still the main skeleton of the first-line treatment in advanced CRC. Herein, the American NCCN guidelines recommend 5 chemotherapy regimens. For lacking of supports from the evidence-based medicine, it's difficult to tell which one is more suitable for a certain individual, especially among the three two-drug combinational regimens. Thus the medicine decisions are made depending more on doctors' personal experience. Starting from this point, our present study explored the related circulating biomarkers to assist the medicine decision, aiming at FOLFOX regimen. FOLFOX is the most common regimen administrated as first-line treatment for advanced CRC, consisting of 5-fluorouracil, leucovorin and oxaliplatin. Despite it significantly improved the survival rate of CRC patients, FOLFOX chemotherapy fails to work in all CRC patients because of intrinsic or acquired drug resistance. Mounting evidences had identified miRNAs as biomarkers to diagnose or predict prognosis in various carcinomas (Mitchell et al., 2008; Skog et al., 2008; Huang et al., 2010). Why so many researches have focused on miRNAs as biomarkers? On one hand, miRNAs are a series of small molecules and they could remain integrity in vivo or in vitro for a long time. The evidence showed that extracellular circulating miRNAs maintained stable for as long as one month at least (Turchinovich et al., 2011). On the other hand, miRNAs were detected in various tissues and it was demonstrated that they participated in a variety of physiological and pathological processes (Schmittgen et al., 2008; Li et al., 2011; Shen et al., 2013). Notably, an increasing number of researches suggested that aberrant miRNA expression could play crucial roles in the response to anticancer drugs as well (Blower et al., 2008) and miRNAs changed during the treatment with anticancer drugs (Meng et al., 2006; Kovalchuk et al., 2008; Xia et al., 2008). These two main points contribute to the value of miRNAs as the best choices for biomarkers. Our work did consider serum miRNAs as biomarkers, and demonstrated that up-regulated serum miR-19a could serve as biomarkers predicting the resistance to first-line

FOLFOX chemotherapy in CRC patients.

In our study, we planned to collect two groups of blood samples, from FOLFOX-response population and FOLFOX-resistance population (including intrinsic resistance and acquired resistance) respectively. From March to June in 2012, 16 participants were enrolled in the first procedure, and 72 participants were in the second procedure. 62 differential expression miRNAs with Fold-change >2 were screened out by microarrays from the 16 serum samples in the first procedure. Among them, 5 miRNAs (miR-221, miR-222, miR-122, miR-19a, miR-144) were selected for further validation procedure. And then the five selected miRNAs were evaluated in the 72 serum samples by RT-qPCR. Our results indicated that serum miR-19a significantly up-regulated in the resistance-phase blood and this was consistent with the standpoint that miRNAs levels changed during the treatment. The ROC curve analysis presented that the sensitivity of serum miR-19a to discriminate the resistant patients from the response ones was 66.7%, and the specificity was 63.9% when the AUC was 0.679. Carcinoembryonic antigen (CEA) is an established tumor biomarker, especially in CRC. The level of CEA changed following the disease development and treatment response. But due to its low sensitivity and specificity, it had not been a valid diagnostic biomarker. Indeed, we didn't detect any significant difference of CEA between the two totally different groups in terms of drug response. Our results just remedied this because miR-19a did recognize the resistance when CEA couldn't and this suggested miR-19a might have a complementary value for CEA. Stratified analysis further revealed that serum miR-19a predicted drug resistance, including both the intrinsic resistance and the acquired resistance. In other words, serum miR-19a could predict intrinsic resistance of FOLFOX before treatment as well as monitor the acquired resistance of FOLFOX during the treatment.

MiR-19a is an important component of the miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) which is located on chromosome 13q31.3 (Ota et al., 2004). The human genomic region which encodes the miR-17-92 cluster, playing a dominant role in promoting tumorigenesis with abundant supports (He et al., 2005), often experiences amplification in various types of lymphoma and other solid cancers (Ota et al., 2004). The oncogenic role of the miR-17-92 cluster in lymphoma was well recognized (Ventura et al., 2007; Mu et al., 2008), and they targeted several tumor suppressor genes, including E2F1 (O'Donnell et al., 2005; Sylvestre et al., 2007), CDKN1A (p21) (Ivanovska et al., 2008), PTEN (Pezzolesi et al., 2008), BCL2L11/BIM (Koralov et al., 2008; Petrocca et al., 2008), and c-Myc (Dews et al., 2006) to regulate the cell cycle, cell apoptosis and angiogenesis in solid tumors. These different intracellular mechanisms contributed to aberrant miR-19a expression at various degrees in cells during the treatment, and consequently, it was reflected by miR-19a in serum in an integrated manner.

To our knowledge, this was the first study to evaluate the resistance of first-line FOLFOX (5-fluorouracil, leucovorin and oxaliplatin) chemotherapy in advanced CRC patients using circulating miRNAs. The other related studies almost focused on either 5-fluorouracil (Song et al., 2009; Boni et al., 2010; Valeri et al., 2010; Nishida et al., 2012) or oxaliplatin (Akao et al., 2011; Chai et al., 2011; Xu et al., 2012; Xu et al., 2013). Compared with the studies about one single agent, our work served the clinical practice better.

In recent several years, various studies on miRNA gave us a concept that miRNAs either from tissues or body fluids carried abundant information about carcinogenesis process, anticancer treatment response, disease prognosis and so on. This group of small RNA molecules deserves great attention as well as histopathologic parameters. Besides, additional molecular biomarkers such as miRNAs are needed to further stratify advanced CRC patients for optimal treatment selection.

In conclusion, our work verified the inspiring value of serum miR-19a as non-invasive biomarker predicting the resistance of firs-line FOLFOX regimen chemotherapy in advanced CRC patients for the first time and it contributes to better medical decisions for clinicians. Serum miR-19a test is non-invasive, convenient, painless and low-cost, and these characteristics attach the great clinical application value to serum miR-19a test. But due to the small size of our work, large-scale prospective trials are urgently needed before its clinical application. Meanwhile, our laboratory would keep on exploring the miRNA-related resistance mechanisms of the first-line FOLFOX chemotherapy in advanced CRC patients.

Acknowledgements

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