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ORIGINAL ARTICLE

A panel of serum exosomal microRNAs as predictive markers for chemoresistance in advanced colorectal cancer

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Abstract

Background Chemoresistance is a common problem for cancer treatment worldwide. Circulating exosomal microRNAs (miRNAs) have been considered as promising biomarkers of cancers. However, few studies have assessed the relationship between serum/plasma exosomal microRNAs and chemoresistance in colorectal cancer (CRC).

Methods Based on previous microarray analysis, we selected 30 miRNAs which are aberrantly expressed during CRC progression and then detected their expression levels in three pairs of oxaliplatin/5-fluorouracil-resistant CRC cell lines and the corresponding secreted exosomes. Six candidate exosomal miRNAs were identified for further evaluating potential value in predicting chemotherapeutic effect in advanced CRC patients. Finally, the molecular mechanisms of these miRNAs in drug resistance were explored by bioinformatics preliminarily.

Results We observed that the expression of 14 miRNAs was significantly higher in three drug-resistant CRC cells comparing with their parental cells. Among these miRNAs, miR-21-5p, miR-1246, miR-1229-5p, miR-135b, miR-425 and miR-96-5p are also up-regulated in exosomes from culture media of resistant cells. Clinical sample analysis confirmed that the expression levels of miR-21-5p, miR-1246, miR-1229-5p and miR-96-5p in serum exosomes were significantly higher in chemoresistant patients in contrast with chemosensitive controls. ROC curve showed that the combination of the four miRNAs had an area of under the curve (AUC) of 0.804 ($P < 0.05$). In addition, GO analysis and KEGG pathway analysis revealed that these miRNAs were enriched in PI3K-Akt signaling pathway, FoxO signaling pathway and autophagy pathway.

Conclusions Our study demonstrates that a panel of serum exosomal miRNAs containing miR-21-5p, miR-1246, miR-1229-5p and miR-96-5p could significantly distinguish the chemotherapy-resistant group from advanced colorectal cancer patients. Targeting these miRNAs may promote chemosensitivity to oxaliplatin and 5-fluorouracil, and might be promising strategy for CRC treatment.

Keywords Colorectal cancer · Chemoresistance · Exosome · MicroRNAs

Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies; it is the third and fifth leading cause of cancer-related death in the United States and China, respectively [1, 2]. In clinic, surgery, chemotherapy, and radiotherapy are the most common performed procedures

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for human cancers, including CRC. As clinical first-line drugs treated for CRC, 5-fluorouracil (5-FU) and oxaliplatin (LOHP) are two important and common chemotherapeutic drugs used extensively in the patient of stage I–IV [3]. However, drug resistance is still one of the main reasons to reduce the effectiveness of chemotherapy and cause the progressive disease states and poor prognosis.

Human has a large amount of body fluids, including breast milk, blood, malignant pleural effusions, and urine, which may be used to reflect physical or pathological status. Although carcinoembryonic antigen (CEA) and carbohydrate antigen19-9 (CA19-9) in blood are acknowledged markers to predict treatment effect and prognosis of gastrointestinal carcinomas, they are easily affected by other physiological factors and provide too little information to evaluate tumor recurrence rate and patients' disease status [4]. Moreover, there are no valid biomarkers for prediction of response to chemotherapy. Therefore, it is highly desirable to clarify the precise molecular mechanism behind drug resistance and identify more valid biomarkers to improve the accuracy of assessment or prediction for chemoresistance in CRC.

Exosomes, 50–150 nm in diameter, are small spherical membrane vesicles and have been found in amount of body fluids [5]. Exosomes contain various proteins, bioactive lipids, coding and non-coding RNAs [6]. Stably existing in many body fluids, exosomes transport cellular materials from donor cells to recipient cells, and thus undertake the crucial communication of information to regulate normal and pathological microenvironment.

MicroRNAs (miRNAs) are 17–24 nucleotides, non-coding and highly conserved RNAs that can negatively regulate target genes through binding to their 3′UTR [7]. In recent years, it is reported that miRNAs are promising tumor markers [8]. MiRNAs can be either circulated in biological fluids in free format or via exosomes, but the exosomal microRNAs are the research object of interest because of the following reasons: (1) free circulating miRNAs are considered to be passively leaked from broken cells during tumorigenesis or tissue injury, whereas exosomal miRNAs are specifically packaged and reflect human physiological/pathological status and, therefore, potentially provide useful information for prognosis; (2) exosomes offer protection against RNase activity and make exosomal miRNAs more stable than their free forms; (3) exosomal miRNAs may possess the unique features of both the donor cells and the recipient cells that regulate physiological properties in disease process.

In this study, we first revealed that a panel of serum exosomal miRNAs (miR-21-5p, miR-96-5p, miR-1246 and miR-1229-5p), may distinguish chemoresistant CRC patients from chemosensitive patients. These four miRNAs are upregulated both in the exosomes of chemoresistant CRC cells and serum of late stage CRC patients comparing with their corresponding controls. Furthermore, the related signaling pathways involved in the candidate target genes of these four miRNAs were provided by KEGG database and GO analysis. Consequently, our study reveals a panel of new predictive markers for chemoresistance in advanced CRC patients.

Materials and methods

Clinical sample collection

Serum samples from 43 CRC patients in stage III–IV were collected from Affiliated Hospital of Jiangnan University. According to the Institutional Review Board of Ethical Committee approved protocol, we got the informed consent from all patients in this study. To exclude the operation, we recruited late-stage (III–IV) CRC patients who cannot be operated. Patients (whom) already underwent molecular target therapy such as bevacizumab were also screened out. All the blood samples were collected before chemotherapy. After the blood samples collected, patients received conventional chemotherapy with 5-FU, LOHP and leucovorin, and the outcomes were evaluated by imaging diagnosis every two periods. In addition, their clinicopathological characteristics, follow-up information and assessment of chemotherapies were gathered. Patients with new distant metastases, enlarged tumors volume or abnormally elevated tumor biomarkers (CEA and CA19-9) after chemotherapy compared with the values prior to treatment were regarded as chemoresistant ($n = 25$), whereas patients with obvious tumors shrinking in imaging diagnosis after drug application were identified as chemosensitive group ($n = 18$). All the radiological diagnoses were independently evaluated by three radiologists and two doctors. These patients had no other malignant disorders, and the serum samples were non-hemolytic, which obtained by centrifuging 2 ml peripheral blood at 2000 rpm for 10 min and 500 µl of supernatants were stored at – 80 °C for further use.

Cell lines and culture

Human CRC cell lines (HCT116, SW480, and HCT-8) were purchased from ATCC during 2008 to 2014 and cultured under the conditions recommended by ATCC as we described previously [9]. Cells were confirmed to be mycoplasma-free, and characterized by Genewiz Inc. (China) using short tandem repeat (STR) markers (latest tested in 2017). To establish LOHP- and 5-FU-resistant CRC cell lines, HCT116, SW480 and HCT-8 cells were cultured with gradually increasing concentrations of LOHP or 5-FU, respectively (HCT116-LOHP, SW480-LOHP and HCT-8-5FU). The 5-FU-resistant HCT-8 cells were established from HCT-8 cells treated with progressively increased

concentrations of 5-FU (0, 0.01, 0.1, 0.5, 2, and 50 μg/ml) over 9 months. Similarly, the oxaliplatin-resistant HCT116 cells and SW480 cells were fostered and stabilized in 10 μg/ml and 5 μg/ml oxaliplatin-containing medium, respectively. HCT-8 cells and HCT116 cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (Biological Industries) and penicillin–streptomycin (Beyotime) at 37 °C with 5% CO_2. SW480 cells were maintained in RPMI-1640 medium (Hyclone) in the same way. When the cells grow to 70% confluency (about 7.0×10^6 per/10 cm dish), the media were replaced with serum-free media and the cells were kept growing for 72 h. Then, the conditioned media (CM) were gathered and centrifuged for 2000 g for 30 min to remove the dead cells and other debris. And all supernatants were stored at − 80 °C until exosome extraction.

Transmission electron microscopy (TEM)

The sample of exosomes was diluted to 0.5 mg/ml with PBS. Then the exosomes were spotted onto a glow-discharged copper grid on the filter paper which dried for 10 min under the infrared lamp. Finally, exosomes were stained with a drop of 1% phosphotungstic acid for 1 min then were dried with the infrared lamp. Exosomes were observed under transmission electron microscopy (JEM-1010 microscope, Japan) at 80 kV.

Isolation of exosomes and extraction of RNA

For cell conditioned media (CM), we adopted the ultracentrifuge methods. Briefly, thawed the supernatants sample in a 25 °C water bath, and then centrifuged them at 10,000 g for 1 h, the supernatant was centrifuged at 110,000*g* for 70 min (all of these steps were performed at 4 °C). The pellets were vortexed with phosphate-buffered saline (PBS) for TEM. For CRC patient serum sample, exosomes were extracted from 500 μl serum using a Total Exosome Isolation Kit (Invitrogen, Carlsbad, CA, USA) based on the manufacturers' protocol. Then, exosomes were at the bottom of the tube and suspended in 100 μl PBS fully and then mixed with 1 ml of RNAiso reagent (Takara, Japan), the following extraction was performed as previously reported [10]. Finally, total RNAs were dissolved in 20 μl RNase-free water. The quality of RNA was determined using a NanoDrop 2000 (Thermo, USA) by $OD_{260/280}$ and stored at – 80 °C for further analysis.

Assessment of cells drug sensitivity

CRC cells were treated with 5-FU or LOHP and cell viability was then assessed by a CCK-8 assay (Beyotime). HCT116, SW480 and HCT-8 cells or their corresponding drug resistant cells were seeded at a density of 3000 cells/well, respectively into 96-well plates. 24 h after seeding, CRC cells were treated with varied concentrations of 5-FU or LOHP (LOHP concentrations in HCT116: 0, 2, 4, 6, 8, 10, 50, 80, 120 μg/ml; LOHP concentrations in SW480: 0, 2, 4, 6, 8, 10, 12, 20, 50 μg/ml; 5FU concentrations in HCT-8: 0, 2, 4, 8, 16, 32, 64, 150, 300, 500 μg/ml). 2 days after drug treatment, 10 μl of CCK-8 solution was added to each well and incubated at 37 °C for 2 h, and the absorbance of OD_{450} was then measured. The half-maximal inhibitory concentration (IC50) was calculated.

Western blot

The protein of exosomes was extracted with RIPA buffer (CWBIO) and centrifuged at 13,000 rpm for 15 min at 4 °C. Then a small amount of supernatant was quantified by the bicinchoninic acid assay (BCA) method, and the proteins were separated by SDS-PAGE and transferred onto a PVDF membrane according to the standard process. After blocked with 5% non-fat milk for 2 h, the membrane continually incubated with primary antibodies for CD63 (BOSTER, 1:1000), Flotillin-2 (sc-28320, 1:500), PTEN (proteintech, 1:2000) and β-actin (Beyotime, 1:1000) at 4 °C overnight. After washing primary antibodies, the membrane was incubated with secondary HRP-conjugated anti-mouse or anti-rabbit antibodies (CWBIO), and the antigen–antibody reaction was detected by an chemiluminescence HRP substrate kit (Millipore, WBKLS0500).

Quantitative RT-PCR (qRT-PCR)

Complementary DNA (cDNA) of miRNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa). And then stem-loop qRT-PCR assays using TaqMan miRNA probes (Applied Biosystems) were conducted to quantitate the levels of the mature miRNAs. For CM exosomes, the relative levels of miRNAs were normalized to the levels of RNU6B (U6), and for serum exosomes, microRNA-16 was used as an internal control for qRT-PCR analysis. qRT-PCR reactions were performed on the ViiA7 real-time PCR system in 384-well plates at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and finally added a melting curve analysis at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Each sample was analyzed in duplicate. $2^{-\Delta\Delta CT}$ was calculated to determine the relative expression of miRNAs in exosomes as previously reported [9].

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathways

The candidate miRNAs were loaded into TargetScan for target gene prediction [11]. And then the R package named cluster Profiler V3.8.0 was used to analyze these target gene

clusters and visualize the GO terms and KEGG pathways. GO database (http://geneontology.org/) is used to analyze the gene function, including three terms of biological pathway, molecular function, and cell component [12]. KEGG pathway analysis (www.genome.jp/kegg/) was performed to analyze the possible pathways enriched with the different target genes. And p value < 0.05 was considered as significant enrichment between the compared groups.

Statistical analysis

The results are expressed as the mean ± SEM. Student t tests, the Mann–Whitney U test and χ^2 test were used to compare the differences among different groups. Receiver operator characteristic (ROC) curve and the area under curve (AUC) were performed to estimate the diagnostic accuracy. A value of $P < 0.05$ was considered to be statistically significant. All statistical analyses were carried out using SPSS22.0 and R software (version 3.5.1).

Results

Characteristics of LOHP/5-FU-resistant CRC cell lines and cultural media exosomes

It has been known that LOHP and 5-FU are the first-line drugs to treat CRC. LOHP and 5-FU-resistant CRC cell lines were established, and CCK-8 assays were performed to assess the drug resistance of these cells. Compared with control cells, HCT116-LOHP, SW480-LOHP and HCT-8-5FU cells showed higher resistance to LOHP/5FU, with the IC50 of 25.01 μg/ml, 11.05 μg/ml and 115.50 μg/ml, respectively (Fig. 1a). When these cells acquired resistance to LOHP or 5-FU, we changed the culture media to serum-free media and got cellular exosomes by ultracentrifuge method. These exosomes were verified by TEM, as shown in Fig. 1b, several round microvesicles with diameters of 50–150 nm are in the field. Western blot was also performed to validate these exosomes by detecting the exosomal marker proteins CD63 and Flotillin-2 (Fig. 1c) [13]. Taken together,

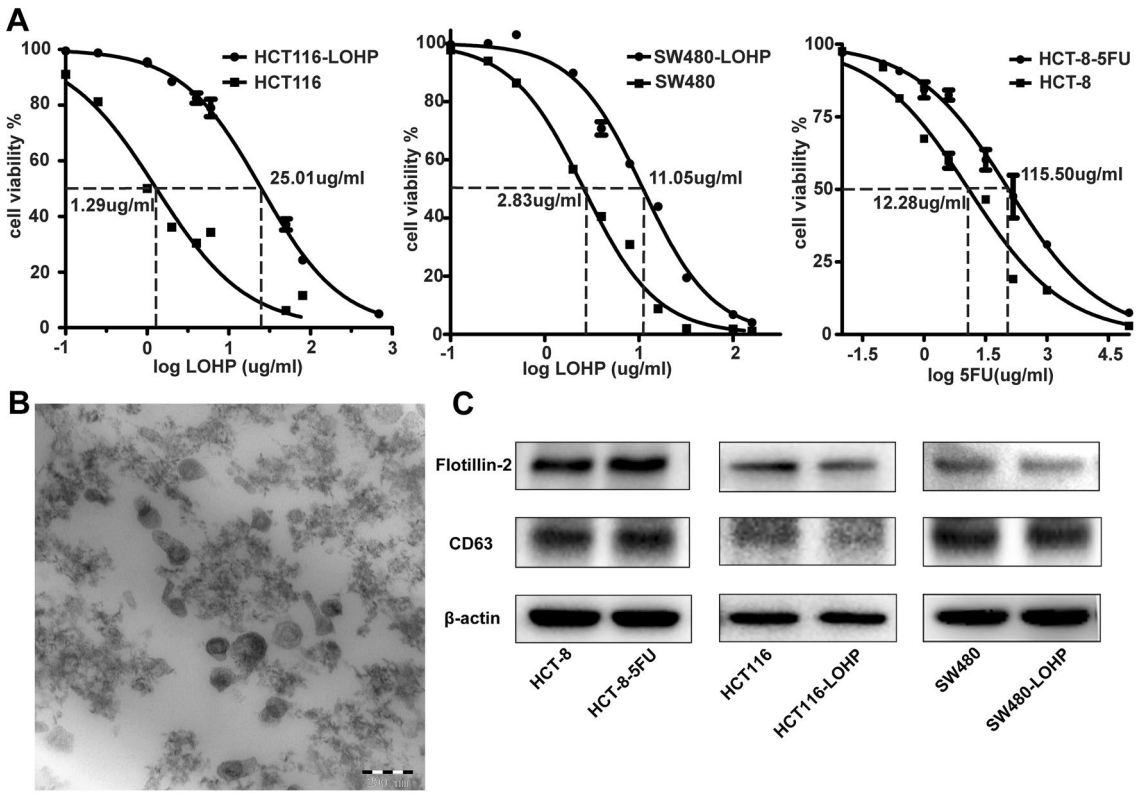


Fig. 1 Identification of LOHP/5-FU-resistant cells from HCT116, SW480 and HCT-8 cells and exosomes secreted by these cells. **a** CCK-8 assay was used to determine the cell growth rate under LOHP/5-FU treatment. The data represent the mean ± SD from three independent experiments. **b** Exosomes isolated from cells underwent a transmission electron microscopy (TEM) to ensure exosomal morphology and size distribution. **c** Exosomes were lysed and used to western blot analysis with the indicated antibodies for exosomal markers

we successfully got the LOHP and 5-FU resistant cell lines and their exosomes.

Detection of miRNAs signature in three pairs of drug-resistant cells and exosomes

In our previous study, miRNA expression profiles in paired CRC tissues and NCTs were analyzed using a microarray to screen differentially expressed miRNAs in CRC. Of the 866 human miRNAs analyzed, 67 exhibited significantly differential expression in CRC tissues. Gene function and bioinformatics analyses revealed that among them, 30 miRNAs are related with cell apoptosis, DNA damage and repair, suggesting that these miRNAs may be involved in chemoresistance [14, 15]. To verify the hypothesis, we first detected the expression of these 30 candidate miRNAs in HCT116-LOHP, SW480-LOHP, HCT-8-5-FU and their parental control cells by qRT-PCR assay. The results indicated that 14 miRNAs were significantly increased in LOHP/5-FU resistant cells comparing with control parental cells (Fig. 2a and Supplementary Table 1). The expression levels of these 30 miRNAs in cellular exosomes were also measured in the same way. By comparing the expression levels of miRNAs in the three cell exosomal groups, the top eight up-regulated exosomal miRNAs were picked out (miR-1229-5p, miR-96-5p, miR-135b, miR-425, miR-1246, miR-21-5p, miR-19a-3p and miR-223) (Fig. 2b and Supplementary Table 2). Collectively, combining the expression of miRNAs in cells with cellular exosomes, we selected six overlapping candidate miRNAs (miR-21-5p, miR-96-5p, miR-1246, miR-1229-5p, and miR-135b, miR-425) for following study (Fig. 2c).

Expression and clinical significance of serum exosomal miRNAs in chemoresistant CRC patients

To exclude the influence of operation on the levels of exosomal miRNAs, we recruited late-stage (III–IV) CRC patients without suitable operation chances. Patients already underwent molecular target therapy such as bevacizumab were also screened out. Furthermore, the young patients (< 40 years old) and the old patients (> 70 years old) were excluded as well. The clinical characteristics of 43 selected patients are shown in Table 1, and there are no statistical differences in age, gender and others listed in Table 1. qRT-PCR analysis was performed to detect the expression of miR-21-5p, miR-96-5p, miR-1246, miR-1229-5p, miR-135b and miR-425 in serum exosomes. miR-16 was served as internal normalization gene, as recommended by other studies. Among these six miRNAs, the expression level of miR-425 is too low in qRT-PCR assay with Ct values around 36 in these patients samples (Supplementary Table 3). The unsupervised hierarchical clustering analysis showed that miR-135b expression alteration is inconsistent with the other four miRNAs (Fig. 3a). The Youden index of miR-21-5p, miR-96-5p, miR-1246, miR-1229-5p and miR-135b expression levels in 43 patients were set as the cutoff for analysis as showed in Fig. 3b and Supplementary Table 2. Univariate analysis revealed that miR-1229-5p, miR-1246, miR-21-5p and miR-96-5p were frequently upregulated in chemoresistant patients compared with the chemosensitive group (Fig. 3c), whereas miR-135b had no statistical significance between chemotherapy-resistant group and sensitive group (Supplementary Fig. 1a). Interestingly, by reviewing the results showed in Fig. 3a, b, we found that the chemosensitive group and chemoresistant group were relatively separated according to the expression levels of serum exosomal miR-1229-5p, miR-1246, miR-21-5p and miR-96-5p. It suggested that these four serum exosomal miRNAs may be valuable predictive markers of chemoresistance. Then we used ROC curves to assess the potential predictive value of these exosomal miRNAs. As shown in Supplementary Fig. 1b, the area under the ROC curve (AUC) of miR-1229-5p, miR-1246, miR-21-5p and miR-96-5p was 0.701, 0.749, 0.696, 0.689, respectively ($P < 0.05$) (Supplementary Fig. 1b). By combining ROC analysis of these four miRNAs, the AUC increased to 0.804 ($P < 0.01$), providing a sensitivity of 78.00% and a specificity of 88.90% and demonstrating their important value in distinguishing chemoresistant CRC patients from chemosensitive group.

GO/KEGG pathway analysis and validation of downstream target genes

To explore the potential biological roles of these four miRNAs in chemoresistance, gene ontology analysis was performed with three items: biological processes (BPs), cellular component (CCs) and molecular function (MFs). As shown in Fig. 4a, the candidate downstream target genes of the four miRNAs were mainly enriched in Ras protein signal transduction, endomembrane system organization and regulation of MAP kinase activity for biological processes terms ($P < 0.001$). In cellular component terms, these genes gathered in postsynapse and endosome membrane ($P < 0.001$), which may reveal that the miRNAs are connected with delivering information or phagocytosis and exocytosis. The top three molecular functions (MFs) contained cell adhesion molecule binding, ubiquitin-like protein transferase activity and proximal promoter sequence-specific DNA binding. Furthermore, KEGG database provided nine signaling pathways in which the downstream target genes of the four miRNAs were enriched ($P < 0.05$) (Fig. 4b). We selected three pathways for the following analysis, "PI3K-Akt signaling pathway" (hsa04151), "FoxO signaling pathway" (hsa04068) and "Autophagy-animal" (hsa04140). Cancer stem cells (CSCs) are cells with differentiation capacity,

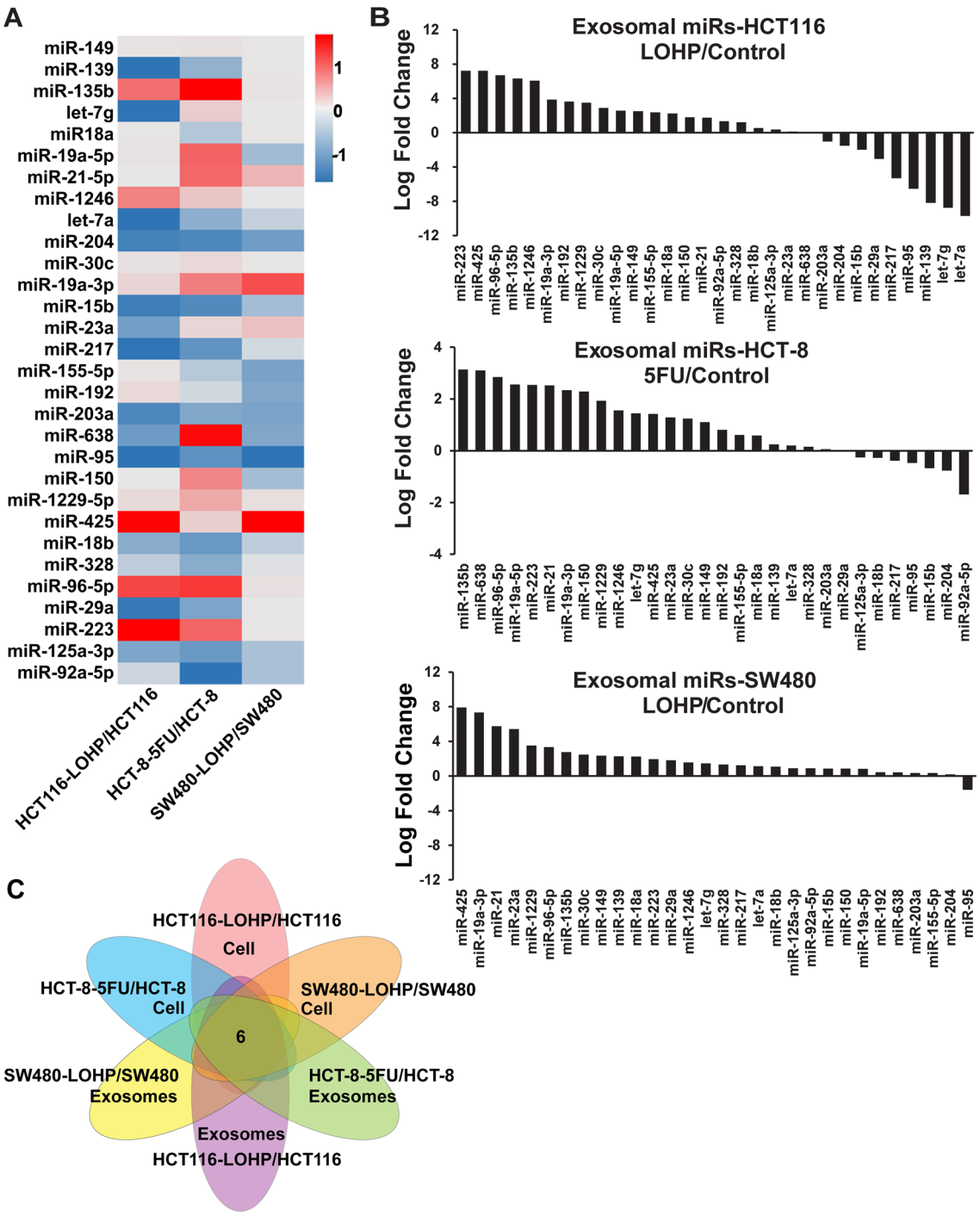


Fig. 2 Differentially expressed miRNAs in three pairs of drug-resistant cells and exosomes. **a** The heatmap describes the miRNA expression profiles in three pairs of drug-resistant CRC cells measured by quantitative real-time RT-PCR ($P < 0.01$). **b** Differentially expressed miRNAs in three pairs of CRC drug-resistant cells derived exosomes measured by quantitative real-time RT-PCR (upregulate or downregulated, fold change > 2, $P < 0.01$). **c** Selection of miRNAs significantly altered both in drug-resistant cell strains and drug-resistant cells exosomes

which reported to be associated with chemoresistance, cancer onset and progression [16]. It is well known that the PI3K-Akt signaling pathway played a critical role in CSCs [17]. After overlap these three signaling pathway, we got 11 genes that co-regulated by the 4 miRNAs (Fig. 4c and Supplementary Table 5). Among these target genes, PTEN had already proven to be related with chemotherapy resistance in cancer [18]. To confirm the validity of these target

Table 1 Correlation between the state of progressive disease and clinicopathologic variables in colorectal cancer patients

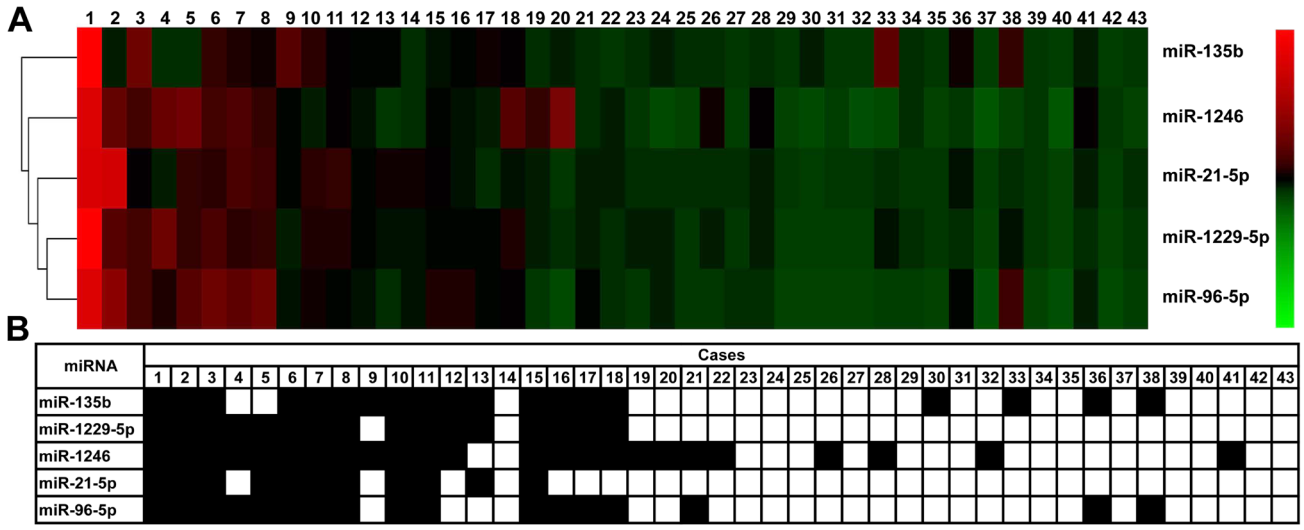
Variables	Progressive disease		*P* value
	Yes	No	
Age			0.09
< 55 years	3	6	
≥ 55 years	22	12	
Gender			0.22
Female	10	4	
Male	15	14	
Localization			0.407
Rectum-left	15	13	
Colon-right	10	5	
Tumor size			0.359
< 5 cm	9	9	
≥ 5 cm	16	9	
Histological type			0.553
Well	13	11	
Not well	12	7	
Depth of invasion			0.359
pT2	3	0	
> pT2	22	18	
Lymphatic invasion			0.567
Absent	4	1	
Present	21	17	
Chemotherapy program			0.456
FOLFOX	9	8	
FLOFIRI	6	2	
XELOX	10	8	
Chemotherapy courses			0.519
≤ 6	17	13	
> 6	8	5	
New metastases, or primary or metastatic tumors became larger, or CEA and CA199 were continuously increasing after chemotherapy			0
Yes	25	0	
No	0	18	

genes, we further detected the PTEN protein levels in three pairs of chemoresistant cells. When the four miRNAs were higher expressed in drug resistant cells comparing to their parental cells, the PTEN protein levels were in inverse as showed in Fig. 4d.

Discussion

Although clinical studies and treatments about CRC are making progress all the time, the doctor and patient are still facing the problem which chemotherapy resistance could occur when treatment is invalid at the beginning or after several courses of chemotherapy. Once the patients develop resistance to chemotherapy, their prognosis will be pretty poor, especially in advanced patients. These facts highlight the need for more careful evaluation and exploration of circular markers to provide a robust basis for a noninvasive diagnosis. Recently, circulating miRNAs from plasma and serum have been a research hotspot to analyze the molecular mechanisms in tumor development and progress or serve as diagnostic markers and predictive markers [19, 20]. Here, we focus on miRNAs from exosomes secreted by cells both in vivo and in vitro. The aim of our study was to find specific serum exosomal miRNAs, which can serve as biomarkers to enhance predictive ability for response to chemotherapeutic drugs in advanced CRC.

Comprehensive analysis of our previous microarray provided 30 miRNAs that were expressed with significant fluctuation. So we detected these miRNAs in three pairs of LOHP/5-FU resistant cell lines and in exosomes separated from each cell culture medium, respectively. By



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Group	miR-1229-5p levels		Total	P value
	high	low		
Resistant	14	11	25	
Sensitive	1	17	18	0.001
Total	15	28	43	

Group	miR-1246 levels		Total	P value
	high	low		
Resistant	19	6	25	
Sensitive	5	13	18	0.003
Total	24	19	43	

Group	miR-21-5p levels		Total	P value
	high	low		
Resistant	11	14	25	
Sensitive	0	18	18	0.001
Total	11	32	43	

Group	miR-96-5p levels		Total	P value
	high	low		
Resistant	14	11	25	
Sensitive	3	15	18	0.009
Total	17	26	43	

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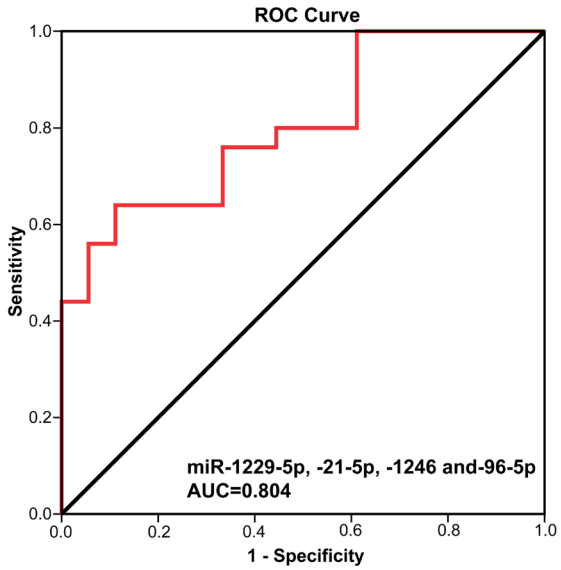

Fig. 3 Clinical significance of serum exosomal miRNA candidates in late stage CRC patients. **a** Unsupervised hierarchical clustering of miRNA expression profiles in 43 late stage CRC serum exosomes measured by microarray analysis. **b** Analysis of four serum exosomal miRNAs in 43 late stage CRC patients. The Youden index of each miRNA expression levels was set as cut-off value, black boxes indicate that miRNAs levels are over the cut-off value of miRNA levels while white boxes below the cut-off value of miRNA levels. **c** 43 enrolled CRC patients were divided into chemoresistant group and chemosensitive group and the four miRNAs levels were detected, the Youden index of each miRNA expression levels were set as cut-off value. **d** Receiver operating characteristics (ROC) curve analysis illustrated the combination of four serum exosomal miRNAs levels may distinguish the chemoresistance in the late stage CRC patients

overlapping the two results, six miRNAs (miR-21-5p, miR-96-5p, miR-1246, miR-1229-5p, miR-135b and miR-425) were picked out to be significantly higher in resistant cell lines and exosomes comparing to the corresponding groups. The serum exosomal miR-21-5p, miR-96-5p, miR-1246 and miR-1229-5p were also found to be statistical significant in chemoresistant patients when compared to sensitive controls except miR-135b. Though there were studies reported that miR-135b could strengthen oxaliplatin resistance and reduce cell apoptosis in CRC cells and also increase cisplatin resistance in gastric cancer [21, 22], there was no report that miR-135b was involved in pathways of 5-FU-resistant cells yet. Moreover, Liu et al. found that miR-135b can enhance doxorubincin sensitivity in breast cancer. In another study, miR-135b was reported to reverse chemoresistance in non-small cell lung cancer cells by targeting FZD1 [23, 24]. MiR-135b

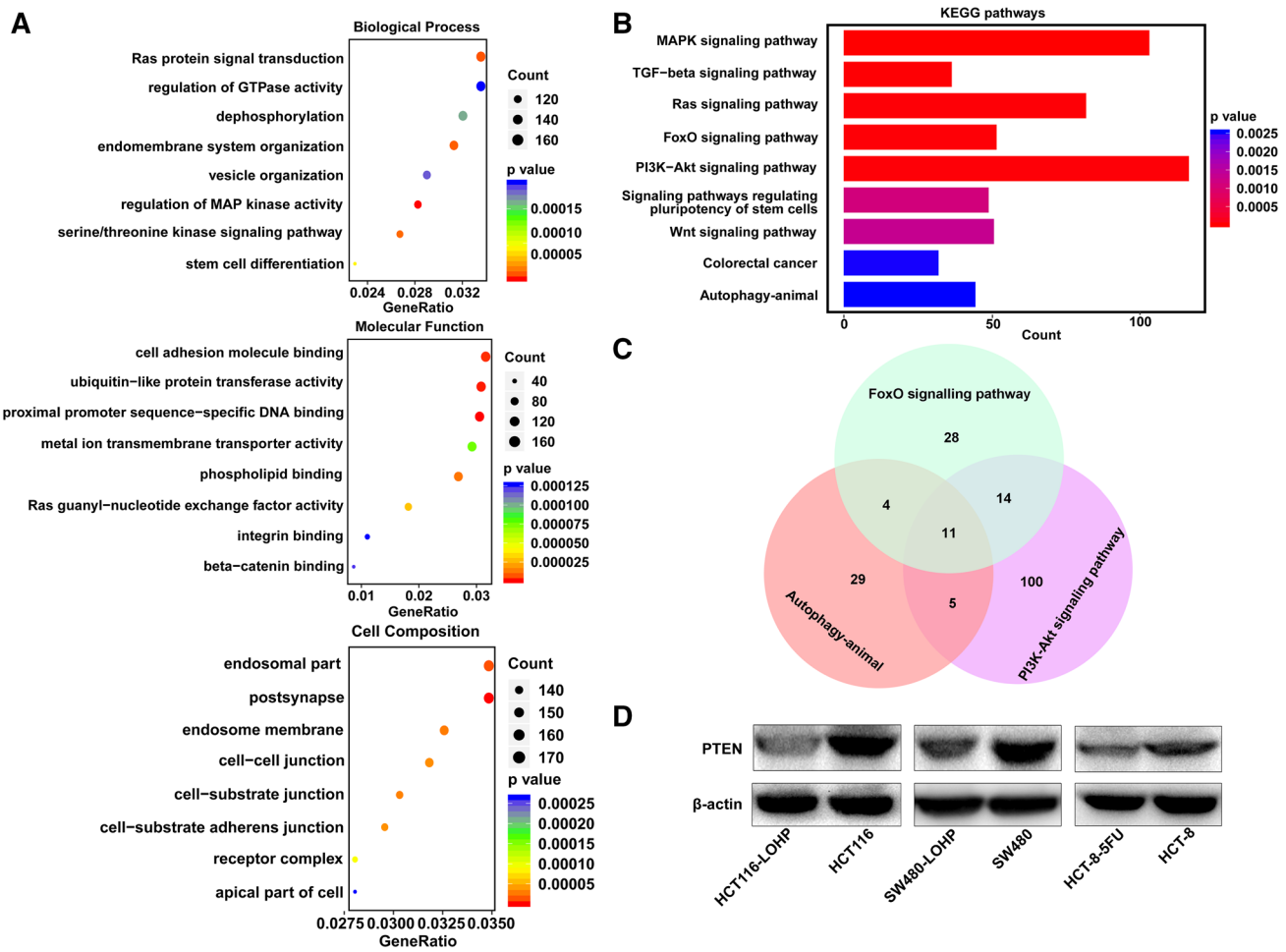

Fig. 4 GO/KEGG pathway analysis and validation of downstream target genes. **a** GO analysis in three terms: biological processes, cellular component and molecule function. **b** The top nine KEGG pathways enriched in the downstream target genes of these four miRNAs. **c** Analysis of three enriched signaling pathway connected with chemoresistance. **d** The selected target gene PTEN was validated by Western blot

plays different roles according to tumor microenvironment in various tumors. Therefore, further studies are required to reveal the exact role of miR-135b in chemosensitivity. Following ROC curve analysis indicated that though a single miRNAs has relative lower AUC value, synergistic effect of the four miRNAs was observed (AUC = 0.804, $P < 0.01$). In general, a panel of biomarkers could enhance the power of diagnosis comparing to single one [25].

There were numerous researches demonstrated that miR-21-5p, miR-1246 or several other miRNAs in serum/plasma, which not packaged in vesicles, were diagnostic biomarkers in CRC [19, 20, 26]. However, microvesicles were reported to show better stability to treatment, meaning that exosomal miRNAs are more preferable as biological specimens because of their stability in serum/plasma [27]. Exosomes offer protection against RNase activity and make exosomal miRNAs stable. On the other hand, Zhanyao Tan et al. showed that miR-1229-5p increases cells proliferation and oncogenicity in breast cancer [28]. In addition, up-regulation of miR-96-5p in glioblastoma cells confers radioresistance [29]. Several reports have suggested miR-1246 enhance chemoresistance in different cancers [30–32], and miR-21-5p plays a role in multi-drug resistance in renal carcinoma [33]. Our study, for the first time, demonstrated that serum exosomal miR-1246, miR-21-5p, miR-96-5p and miR-1229-5p may be suitable diagnostic biomarkers to identify chemotherapy resistant group in advanced CRC patients. In all, these results supported that these four serum exosomal miRNAs could predict drug resistance in CRC patients. Meanwhile it will provide more accurate clinical information for the treatment options in late stage CRC.

Finally, we focus on these miRNAs and study the molecular mechanism to understand the connection between chemotherapy resistance and the four miRNAs. Bioinformatics tools as GO analysis and KEGG database help us to get the genes enrichment signaling pathways (PI3K-Akt, FoxO

and Autophagy-animal) and target gene (PTEN). PI3K-AKT signaling pathway plays important roles in various biological processes including cell growth, metabolism, cancer stem cells and chemoresistance [34], whereas PTEN could negatively regulate the PI3K signal [35]. It is reported that PI3K/Akt axis induce tumor chemoresistance, and PTEN loss is connected with resistance to cetuximab [36, 37]. It was also demonstrated that miR-543 enhanced 5-FU resistance in CRC by regulating PTEN [38]. In addition, we got other ten genes that may co-regulated by the four miRNAs, which were mainly involved in PI3K-Akt pathway (PIK3R1, PIK3CA, PIK3R3, PRKAA2, IGF1R, IRS1, PDPK1, KRAS, MAPK1 and MAP2K1). For example, Huang et al. showed that PIK3R1 can increase cisplatin resistance in gastric cancer via sponging miR-198 [39]. It was found that mutations of PIK3CA and KRAS genes were oncogenic and may be related to chemotherapy resistance [40, 41]. In addition, MAPK1 and MAP2K1 were also been reported to regulate drug resistance [42, 43].

Up to now, there are little biomarkers to predict chemotherapy resistance in CRC. Our study, for the first time, provided evidences that a panel of serum exosomes-derived miRNAs is novel promising markers to distinguish the drug-resistant group from late stage CRC patients. It will help doctors to identify potential chemotherapy-resistant patients as early as possible, and transfer to other drug such as beacizumab and cetuximab. Furthermore, miR-1246, miR-21-5p, miR-96-5p and miR-1229-5p involved in chemotherapy resistance may be new therapeutic targets, downregulating these miRNAs may promote CRC cell sensitivity to chemotherapeutic drugs. Certainly, more studies are required in further investigation.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest were disclosed.

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