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Serum miR-199a as a potential diagnostic biomarker for detection of colorectal cancer

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) play an essential role in the development and progression of colorectal cancer (CRC). Aberrant expression of miR-199a was associated with cancer development in many cancers. However, little was known about the clinical value of miR-199a in CRC. This study aimed to investigate the potential relationship between miR-199a expression and CRC prognosis.

PATIENTS AND METHODS: Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out to detect miR-199a expression level in serum from 107 CRC patients and 60 healthy controls.

RESULTS: Serum miR-199a levels were significantly lower in CRC patients, particularly in advanced stage CRC subjects. MiR-199a expression in patients with distant metastasis and lymph node metastasis was markedly reduced compared to those without. Moreover, low serum miR-199a expression was associated with various CRC clinicopathological parameters. The serum miR-199a levels in CRC patients were increased significantly after their treatment. Furthermore, the patients in low serum miR-199a expression group had both worse overall survival and disease-free survival. In the multivariate analysis, serum miR-199a was identified as an independent prognostic marker.

CONCLUSIONS: Reduced serum miR-199a was associated with poor prognosis in CRC and it might be useful as a marker for diagnosis and prognosis in CRC.

Key Words
Colorectal Cancer, MiR-199a, Serum, Prognosis.

Introduction

In China, colorectal cancer (CRC) is the fourth most common cancer in men and the third in women, accounting for 6-8% of all cancer deaths in 2013[1, 2]. Early-stage diagnosis of CRC can significantly improve patient survival and decrease the mortality of this malignancy, but less than 40% of patients rtain limitations. For instance, fecal test shows poor performance to detect precancerous lesions, and colonoscopy is an invasive procedure at high costs[6-8]. Thus, there is an urgent need to identify non-invasive, highly sensitive biomarkers to detect early CRC. MicroRNAs (miRNAs) are a class of small noncoding RNAs (19-24 nucleotides) and regulate gene expression at post-transcription level[9,10]. The association between miRNA and cancer was reported for the first time in 2002[11]. After that, more and more evidence revealed that aberrant expressed miRNAs could affect cancer development and act as cancer suppressors or oncogenes. In 2008, Mitchell et al[12] reported serum or plasma miRNAs could be used as biomarkers for the blood-based detection of human cancer. MiR-199a is located on human chromosome 19 within intron 14 of the dynamin-2 gene, and miR-199a-5p and miR-199a-3p are expressed from the miR-199a precursors[13,14]. Recent studies demonstrated that miR-199a is involved in the initiation and progression of various cancer types, such as hepatocellular carcinoma[20], cutaneous squamous cell carcinoma[22], renal cell carcinoma[23], testicular cancer[24], ovarian cancer[26, 27] and gastric cancer[28, 29]. Further studies had reported that miR-199a could act as a potential diagnostic and prognostic marker in diffuse large B-cell lymphoma[19] and ovarian cancer[25]. However, the clinical significance of miR-199a in CRC remained poorly known. In this study, we aimed to explore the diagnostic and prognostic role of miR-199a in CRC.

Patients and Methods

Patients and Blood Collection

A total of 107 CRC cases including 65 men and 42 women were enrolled, and no patient had received any chemotherapy or radiotherapy before surgery. Tumor stage was determined according to the fifth edition of the American Joint Commission on Cancer Tumor-Node-Metastasis (TNM) classification system. Patient characteristics were

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shown in Table I. Moreover, sixty healthy individuals between 29 to 73 years old were recruited as controls. Our study was approved by the Ethics Committee of Renmin Hospital of Wuhan University. Written informed consent was obtained from each patient, and all specimens were handled and made anonymous according to the Ethical and Legal standards. Up to 5 ml of whole blood were withdrawn from all participants early in the morning. All blood samples were centrifuged at 3,000 rpm for 20 min and at 12,000 rpm for 10 min at 4°C, then the supernatant was collected and stored at -80°C for further analysis.

RNA Isolation and Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA containing small RNA was extracted from plasma using a miRVana PARIS Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. RNA purity and concentration were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, miR-199a levels were quantified in triplicate by qRT-PCR using the Maxima SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 7900 HT qRT-PCR instrument (Applied Biosystems, Foster City, CA, USA). The relative expression level of miR-199a in serum was calculated by the equation $2^{-\Delta\Delta Ct}$, and cer-miR-39 was used as an internal control.

Statistical Analysis

The statistical significance was determined with the Mann-Whitney U test or Kruskal-Wallis test between groups. Receiver-operating characteristic (ROC) curves were generated, and the area under ROC curve (AUC) was calculated to determine the diagnostic accuracy of serum miR-199a for CRC. The Pearson's chi-squared analysis was performed to assess association between serum miR-199a expression and clinical variables. The association between survival and serum miR-199a was analyzed by Kaplan-Meier method and Log-rank test. Multivariate cox proportional hazards regression analysis was performed to identify independent predictive factors of survival. Overall sur-

Table I. Correlation between serum miR-199a levels and clinical parameters in CRC patients.

Features	N	High miR-199a (n=49)	Low miR-199a (n=58)	*p*-value
Age				NS
< 60	52	28	24	
≥ 60	55	21	34	
Gender				NS
Male	65	27	38	
Female	42	22	20	
Preoperative CEA level				NS
< 5 ng/mL	50	24	26	
≥ 5 ng/mL	57	25	32	
Distant metastasis				0.0262
Negative	76	40	36	
Positive	31	9	22	
Histological grade				NS
Well	34	19	15	
Moderate	52	23	29	
Poor	21	7	14	
Lymph node metastasis				0.0189
Negative	48	28	20	
Positive	59	21	38	
Size				NS
< 5 cm	55	29	26	
≥ 5 cm	52	20	32	
TNM stage				0.0019
I/II	43	27	16	
III	41	18	23	
IV	23	4	19	

*TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; NS, not statistically significant.

vival (OS) was measured as the time from the date of initially surgery to the date of death or last follow-up. Disease-free survival (DFS) was measured as the time from the date of initially surgery to the date of relapse or death or last follow-up. Statistical analysis was performed using MedCalc 16.4.3 (MedCalc Software, Ostend, Belgium). A p-value less than 0.05 was statistically significant.

Results

The Expression Level of Serum miR-199a in CRC Patients

Serum miR-199a levels in 107 CRC subjects and 60 healthy controls were detected by qRT-PCR. The data showed that serum miR-199a levels were significantly decreased in CRC patients compared to healthy controls ($p<0.01$, Figure 1A). Furthermore, when all CRC cases were grouped based upon TNM stage, serum miR-199a levels in patients with stage I/II were greatly higher than those in stage III or IV subjects (both $p<0.01$, Figure 1A). In addition, a significant decrease in serum miR-199a levels was observed in patients (n=31) with distant metastasis in comparison with those (n=76) without ($p<0.01$, Figure 1B). Likewise, patients with lymph node metastasis (n=59) had significantly lower serum miR-199a expression than those (n=48) without ($p<0.01$, Figure 1C). Next, ROC curve analysis was performed to evaluate the diagnostic value of serum miR-199a. As presented in Figure 2A, the serum level of miR-199a had a specificity of 83.3% and a sensitivity of 77.6% to discriminate the CRC patients from the healthy controls, with an AUC of 0.864. Moreover, serum miR-199a level could well distinguish advanced CRC patients (stage III/IV) from healthy subjects with an AUC val-

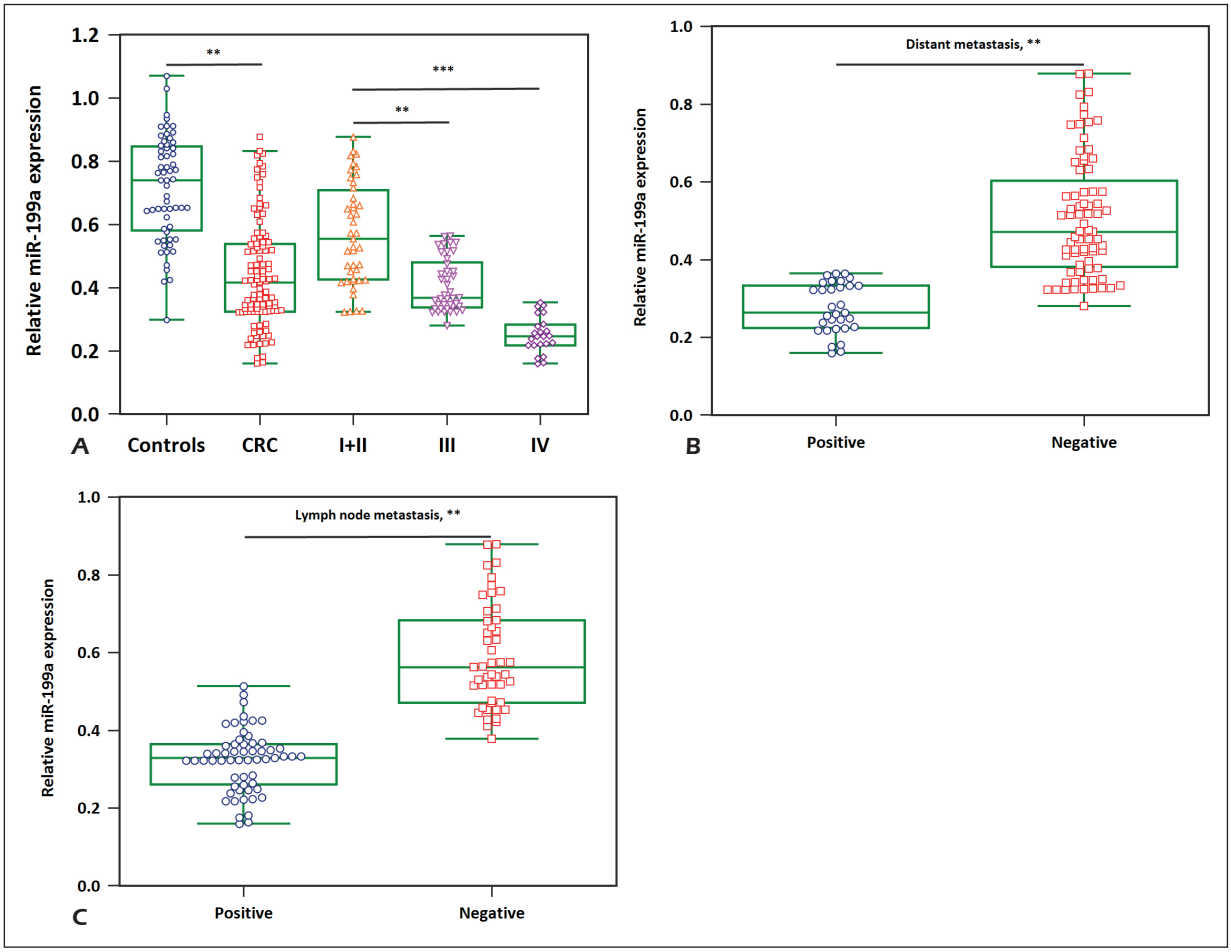

Figure 1. A, Significant under expression of miR-199a in CRC patients compared to controls. **B**, MiR-199a expression in CRC patients with distant metastasis was significantly lower than those without metastasis. **C**, MiR-199a expression in CRC patients with lymph node metastasis was significantly lower than those without metastasis.

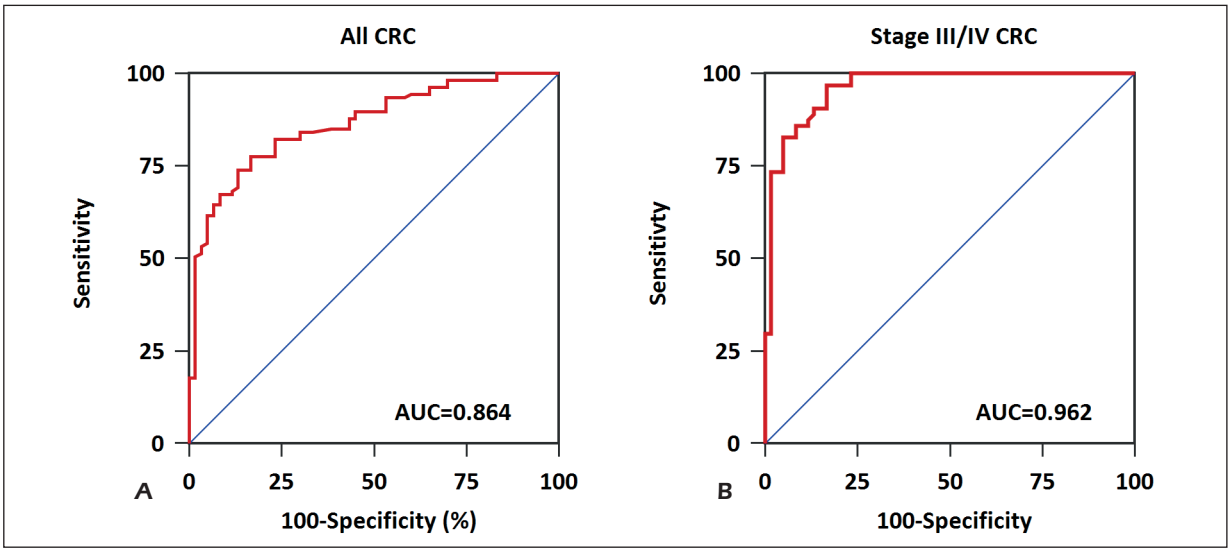

Figure 2. A, ROC curve analysis illustrated that the serum miR-199a was a potential biomarker for screening CRC patients from healthy controls. **B**, ROC curve analysis illustrated that the serum miR-199a could well screen advanced CRC patients from healthy controls.

ue of 0.962, and the specificity and sensitivity were 83.3% and 96.9%, respectively (Figure 2B). To determine whether serum miR-199a levels were associated with treatment response, blood samples were collected from all CRC cases two weeks after treatment and detected by qRT-PCR. We found serum miR-199a levels in post-operative samples were significantly higher than those in pre-operative serum samples ($p < 0.01$, Figure 3).

The Association Between Serum miR-199a Expression and Clinical Parameters of CRC

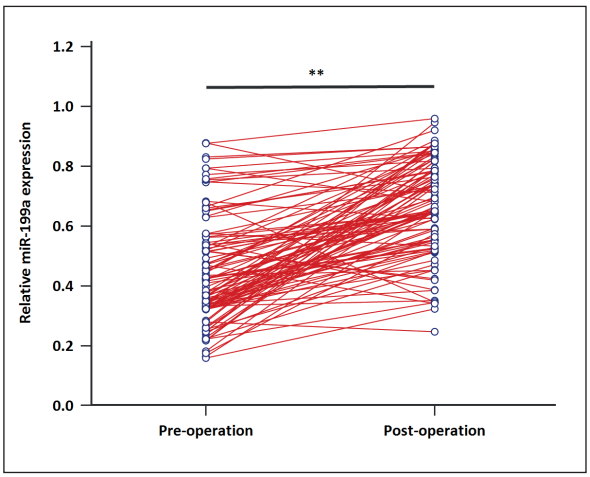

Figure 3. The association between serum miR-199a levels and treatment response.

The correlation between serum miR-199a expression levels and clinicopathological factors was statistically analyzed in Table I. All CRC subjects were divided into miR-199a high and miR-199a low groups according to the median fold-change values. Low serum miR-199a expression was closely associated with distant metastasis (p=0.0262), lymph node metastasis (p=0.0189) and TNM stage (p=0.0019). Whereas, there were no significant changes between serum miR-199a expression and other parameters including age, gender, preoperative carcinoembryonic antigen (CEA) level, histological grade, and size (all p>0.05).

CRC Patients With Low Serum miR-199a Expression Had Shorter OS and DFS

The correlation between serum miR-199a levels and survival of CRC patients was further evaluated. A Kaplan-Meier survival analysis showed that low expression of serum miR-199a was significantly correlated with worse overall survival, as well as poorer disease free survival (p=0.013, Figure 4A; p=0.007, Figure 4B).

Serum miR-199a Was an independent Risk Factor for CRC

As shown in Table II, a multivariate Cox regression analysis revealed that miR-199a expression was an independent prognostic marker in CRC patients (OS: RR=2.86, 95% CI=1.43-4.38, p=0.009; DFS: RR=3.29, 95% CI=1.56-5.13, p=0.005). Furthermore, the other factors

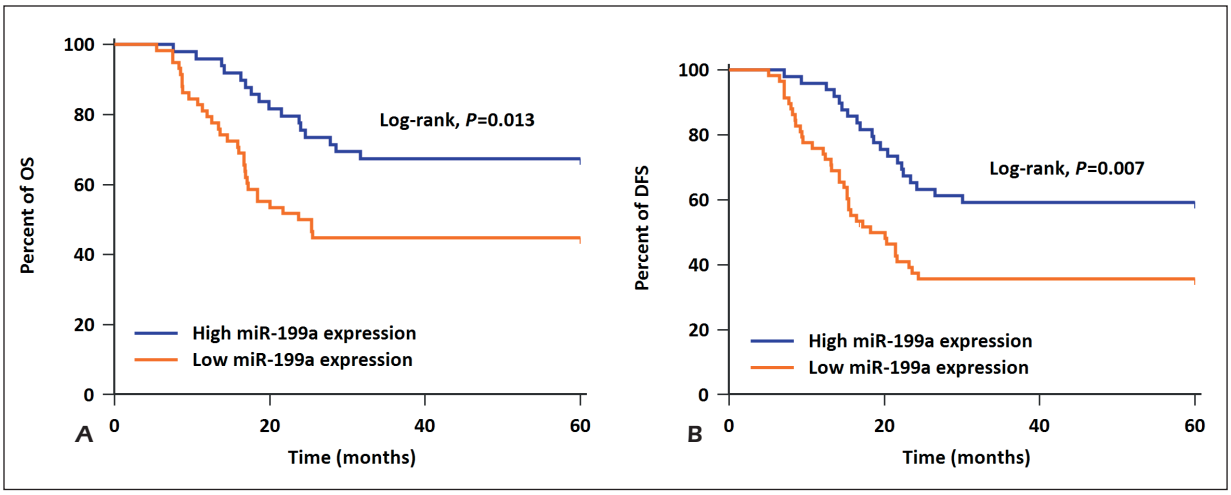

Figure 4. A, Kaplan-Meier curve of the overall survival of 107 CRC patients. **B**, Kaplan-Meier curve of the disease-free survival of 107 CRC patients.

responsible for CRC prognosis included lymph node metastasis (OS: RR=1.92, 95% CI=1.15-2.83, p=0.018; DFS: RR=2.25, 95% CI=1.26-3.37, p=0.011), distant metastasis (OS: RR=1.71, 95% CI=1.02-2.58, p=0.021; DFS: RR=2.13, 95% CI=1.24-3.16, p=0.012) and TNM stage (OS: RR=3.47, 95% CI=1.64-5.57, p=0.003; DFS: RR=3.95, 95% CI=1.82-6.26, p<0.001).

Discussion

We explored the potential clinical utility of serum miR-199a to serve as a noninvasive biomarker in CRC patients. Firstly, serum miR-199a levels were significantly lower in CRC patients than those in controls. Secondly, miR-199a levels in CRC patients with lymph node metastasis or distant metastasis were lower than those without metastasis. Thirdly, serum miR-199a showed good performance to differentiate CRC subjects or stage III/IV CRC from healthy volunteers. Fourthly, low miR-199a expression was strongly associated with aggressive clinical variables. Finally, CRC patients with low miR-199a expression tended to have poorer OS and DFS, and serum miR-199a was considered as an independent prognostic factor for worse prognosis. Our data demonstrated that low miR-199a expression occurred more frequently in CRC, and the findings were in agreement with previous studies. Ye et al[15] noted that miR-199a was distinctly reduced both in CRC and metastasis tissues, and re-expression of miR-199a decreased HIF-1α and VEGF expression and inhibited cancer cell migration and invasive capabilities. Kong et al[16] found that miR-199a-5p expression was greatly lower in CRC tissues than normal tissues. CAC1 knockdown or miR-199a-5p upregulation reduced the tumor cell drug resistance and dramatically inhibited cancer cell growth, invasive and migratory

Table II. Multivariate Cox regression analyses for overall survival and disease-free survival.

	Overall Survival		Disease Free Survival	
	RR (95% CI)	*p*-value	RR (95% CI)	*p*-value
Lymph node metastasis (Positive *vs.* Negative)	1.92 (1.15-2.83)	0.018	2.25 (1.26-3.37)	0.011
Distant metastasis (Positive *vs.* Negative)	1.71 (1.02-2.58)	0.021	2.13 (1.24-3.16)	0.012
TNM stage (III/IV *vs.* I/II)	3.47 (1.64-5.57)	0.003	3.95 (1.82-6.26)	<0.001
Serum miR-199a (Low vs. High)	2.86 (1.43-4.38)	0.009	3.29 (1.56-5.13)	0.005

phenotypes. Similarly, *in vitro* evidence demonstrated ectopic miR-199a-5p expression would result in inhibition of cancer development, and miR-199a-5p upregulation led to decrease DDR1[17] or FZD6 expression[18]. To date, a growing number of researches have reported miR-199a played a critical role in tumorigenesis of many cancers. Troppan et al[19] reported that diffuse large B-cell lymphoma (DLBCL) patients with high miR-199a expression levels were associated with longer overall survival, and miR-199a upregulation significantly enhanced the chemosensitivity of cancer cells and reduced cell viability after exposure to doxorubicin, rituximab and vincristine. In hepatocellular carcinoma (HCC), Duan et al[20] found that miR-199a/214 expression was significantly markedly lower in cancer tissues and cell lines. NFκB was inversely correlated with miR-199a/214 expression and NFκB inhibition could restrain tumor growth. Likewise, Song et al[21] reported miR-199a was frequently decreased in cancerous tissues and cells. Low miR-199a expression was a good prognostic indicator for HCC patients, and *in vitro and in vivo* evidence showed miR-199a overexpression resulted in suppression of the carcinogenesis by degrading FZD7. Wang et al[22] revealed that expression of miR-199a was greatly reduced in cutaneous squamous cell carcinoma (cSCC) tissues when compared to the normal tissues. High miR-199a expression significantly suppressed cSCC cell proliferation and invasion through directly targeting CD44, and *vice versa*. Zhang et al[23] found miR-199a was frequently underexpressed in renal cell carcinoma (RCC) tissues and negatively related to poor prognosis. High miR-199a expression significantly inhibited the tumorigenesis by downregulating ROCK1 expression. Cheung et al[24] revealed that restoration of miR-199a in testicular cancer led to decreases in cell growth, migration, invasion and metastasis *in vitro* and in tumor cell proliferation *in vivo,* PODXL was its downstream target. In ovarian cancer, miR-199a downregulation was observed in cancerous tissues compared with corresponding normal specimens and strongly associated with shortened survival time. Moreover, *in vitro* analysis showed high miR-199a expression could reverse cisplatin resistance and enhanced cisplatin-induced apoptosis by negatively regulating HIF1α or mTOR[25-27]. In contrast, some researches had reported higher miR-199a expression in gastric cancer (GC). A previous study showed miR-199a directly targeted Smad4 and enhanced GC cells growth and survival, indicating miR-199a promoted the tumorigenesis of GC[28]. Similarly, Song et al[29] recently reported that miR-199a was dramatically upregulated in GC tissues and metastatic tissues, and the increased miR-199a expression induced GC cell proliferation, migration and invasion *via* regulating mitogen-activated protein kinase kinase kinase 11, which was known as a tumor suppressor.

Conclusions

We firstly provided the evidence that serum miR-199a levels were highly decreased in CRC patients in comparison with normal controls, and its lower expression was closely correlated with poor prognosis of CRC, suggesting miR-199a might perform a key role in CRC oncogenesis. Thus, serum miR-199a was useful as a potential diagnostic and prognostic marker for CRC. There were still some limitations to this study. For instance, the sample size was relatively small, and the patients with colorectal polyps should be enrolled for comparison. Therefore, the role of miR-199a in CRC needed further investigation.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Zheng R, Zeng H, Zhang S, Chen W. Estimates of cancer incidence and mortality in China, 2013. Chin J Cancer 2017; 36: 66.

2) Zhang JS, Yang LQ, Du BR, Gao H. Higher RABEX-5 mRNA predicts unfavorable survival in patients with colorectal cancer. Eur Rev Med Pharmacol Sci 2017; 21: 2372-2376.

3) Hill DA, Furman WL, Billups CA, Riedley SE, Cain AM, Rao BN, Pratt CB, Spunt SL. Colorectal carcinoma in childhood and adolescence: a clinicopathologic review. J Clin Oncol 2007; 25: 5808-5814.

4) Feng LM, Zhao DW, Li SJ, Huang J. Association of the upregulation of LncRNA00673 with poor prognosis for colorectal cancer. Eur Rev Med Pharmacol Sci 2018; 22: 687-694.

5) Gonzalez-Pons M, Cruz-Correa M. Colorectal cancer biomarkers: where are we now? Biomed Res Int 2015; 2015: 149014.

6) Weizman AV, Nguyen GC. Colon cancer screening in 2010: an up-date. Minerva Gastroenterol Dietol 2010; 56:181-188.

7) Kim DH, Pickhardt PJ, Taylor AJ, Leung WK, Winter TC, Hinshaw JL, Gopal DV, Reichelderfer M, Hsu RH, Pfau PR. CT colonography versus colonoscopy for the detection of advanced neoplasia. N Engl J Med 2007; 357: 1403-1412.

8) Mandel JS. Screening for colorectal cancer. Gastroenterol Clin North Am 2008; 37: 97-115.

9) Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell 2006; 11: 441-450.

10) Stefani G, Slack FJ. Small noncoding RNAs in animal development. Nat Rev Mol Cell Biol 2008; 9: 219-230.

11) Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2002; 99: 15524-15529.

12) Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008; 105: 10513-10518.

13) Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW, Kim S. MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 2008; 14: 2690-2695.

14) Suzuki T, Mizutani K, Minami A, Nobutani K, Kurita S, Nagino M, Shimono Y, Takai Y. Suppression of the TGF-β1-induced protein expression of SNAI1 and N-cadherin by miR99a. Genes Cells 2014; 19: 667-675.

15) Ye H, Pang L, Wu Q, Zhu Y, Guo C, Deng Y, Zheng X. A critical role of mir-199a in the cell biological behaviors of colorectal cancer. Diagn Pathol 2015; 10: 65.

16) Kong Y, Bai PS, Sun H, Nan KJ, Chen NZ, Qi XG. The deoxycholic acid targets miRNA-dependent CAC1 gene expression in multidrug resistance of human colorectal cancer. Int J Biochem Cell Biol 2012; 44: 2321-2332.

17) Hu Y, Liu J, Jiang B, Chen J, Fu Z, Bai F, Jiang J, Tang Z. MiR-199a-5p loss up-regulated DDR1 aggravated colorectal cancer by activating epithelial-to-mesenchymal transition related signaling. Dig Dis Sci 2014; 59: 2163-2172.

18) Kim BK, Yoo HI, Kim I, Park J, Kim Yoon S. FZD6 expression is negatively regulated by miR-199a-5p in human colorectal cancer. BMB Rep 2015; 48: 360-366.

19) Troppan K, Wenzl K, Pichler M, Pursche B, Schwarzenbacher D, Feichtinger J, Thallinger GG, Beham-Schmid C, Neumeister P, Deutsch A. MiR-199a and miR-497 are associated with better overall survival due to increased chemosensitivity in diffuse large B-cell lymphoma patients. Int J Mol Sci 2015; 16: 18077-18095.

20) Duan Q, Wang X, Gong W, Ni L, Chen C, He X, Chen F, Yang L, Wang P, Wang DW. ER stress negatively modulates the expression of the miR-199a/214 cluster to regulates tumor survival and progression in human hepatocellular cancer. PLoS One 2012; 7: e31518.

21) Song J, Gao L, Yang G, Tang S, Xie H, Wang Y, Wang J, Zhang Y, Jin J, Gou Y, Yang Z, Chen Z, Wu K, Liu J, Fan D. MiR-199a regulates cell proliferation and survival by targeting FZD7. PLoS One 2014; 9: e110074.

22) Wang SH, Zhou JD, He QY, Yin ZQ, Cao K, Luo CQ. MiR-199a inhibits the ability of proliferation and migration by regulating CD44-Ezrin signaling in cutaneous squamous cell carcinoma cells. Int J Clin Exp Pathol 2014; 7: 7131-7141.

23) Zhang X, Li P, Ding Z, Wang H, Wang J, Han L, Ding S. The putative tumor suppressor, miR-199a, regulated by Snail, modulates clear cell renal cell carcinoma aggressiveness by repressing ROCK1. Onco Targets Ther 2017; 11: 103-112.

24) Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, Rennert OM, Chan WY. Methylation of an intronic region regulates miR-199a in testicular tumor malignancy. Oncogene 2011; 30: 3404-3415.

25) Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW, Kim S. MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 2008; 14: 2690-2695.

26) Feng X, Liu N, Deng S, Zhang D, Wang K, Lu M. MiR-199a modulates cisplatin resistance in ovarian cancer by targeting Hif1α. Onco Targets Ther 2017; 10: 5899-5906.

27) Wang Z, Ting Z, Li Y, Chen G, Lu Y, Hao X. MicroRNA-199a is able to reverse cisplatin resistance in human ovarian cancer cells through the inhibition of mammalian target of rapamycin. Oncol Lett 2013; 6: 789-794.

28) Zhang Y, Fan KJ, Sun Q, Chen AZ, Shen WL, Zhao ZH, Zheng XF, Yang X. Functional screening for miRNAs targeting Smad4 identified miR-199a as a negative regulator of TGF-β signalling pathway. Nucleic Acids Res 2012; 40: 9286-9297.

29) Song G, Zeng H, Li J, Xiao L, He Y, Tang Y, Li Y. MiR-199a regulates the tumor suppressor mitogen-activated protein kinase kinase kinase 11 in gastric cancer. Biol Pharm Bull 2010; 33: 1822-1827.