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# Reduced serum miR-98 predicts unfavorable clinical outcome of colorectal cancer

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**Abstract. – OBJECTIVE: Circulating microRNAs (miRNAs) are considered to be promising biomarkers for the diagnosis and prognosis prediction of cancers. However, the potential clinical significance of the serum miR-98 in colorectal cancer (CRC) remained unclear. This study aimed to examine the serum miR-98 levels in CRC patients and explore its potential value for CRC.**

**PATIENTS AND METHODS: A total of 115 CRC cases and 50 healthy volunteers were enrolled in this study. Quantitative reverse-transcription PCR (qRT-PCR) was performed to detect serum miR-98 expression in all the participants.**

**RESULTS: The results revealed that serum miR-98 levels were frequently downregulated in CRC patients compared with controls. In addition, low serum miR-98 levels were closely associated with aggressive clinical features and shorter survival. Receiver operating characteristic (ROC) curve analysis demonstrated that serum miR-98 could well differentiate CRC patients from healthy controls with relatively high accuracy. Multivariate analysis further demonstrated that serum miR-98 was an independent prognostic factor for both overall survival and disease-free survival. Mechanistically, MYC, IL-6, and HIST1H2BH were identified as direct downstream targets of miR-98 in CRC cells.**

**CONCLUSIONS: Collectively, serum miR-98 might be useful as an indicator for predicting the clinical outcome of CRC patients.**

*Key Words:*
Colorectal cancer, MiR-98, Serum, Biomarker.

## Introduction

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-associated deaths around the world[1,2]. Each year, an estimated 1.3 million people are diagnosed with CRC, and more than 0.6 million cases die of this disease worldwide[3-5]. Despite what has been achieved so far in terms of diagnosing and treating CRC, patients with CRC still display a poor prognosis because most patients are diagnosed in an advanced stage[6,7]. Since early detection is important for improving outcomes and reducing mortality of this fatal malignancy, novel, and reliable biomarkers for CRC diagnosis and prognosis prediction are urgently needed.

MicroRNAs (miRNAs) are small, highly conserved, non-coding RNAs (19-24 nucleotides in length) that negatively regulate target genes expression at the post-transcriptional level[8,9]. It is well known that miRNAs play crucial roles in multiple biological processes, including cell growth, proliferation, differentiation, and tumorigenesis[10]. Scholars[11,12] have highlighted that tumor-derived miRNAs could be detectable in remarkably stable forms in the serum or plasma. To date, some serum miRNAs in CRC patients have been reported by previous studies. For instance, serum exosomal miR-23a, serum exosomal miR-301a, and serum miR-103 expression in CRC patients was significantly increased[13,14]. Whereas, low serum miR-101 expression was significantly downregulated in CRC patients and correlated with poor prognosis[15].

MiR-98 is the member of the let-7 miRNA family, and might act as an oncogene or tumor suppressor gene in various human cancers. It has been reported that miR-98 played a tumor suppressive role in the invasion and metastasis of CRC. Ectopic expression of miR-98 restrained CRC cell proliferation, migration, and invasion, as well as stimulated cell apoptosis by directly targeting CLDN1[16]. However, to the best of our knowledge, the potential clinical significance of serum miR-98 as a noninvasive biomarker in CRC was unknown. In this study, we aimed to investigate whether serum miR-98 expression was aberrantly expressed in CRC, and its potential correlation with the clinical outcome of CRC patients

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## Patients and Methods

### Patients and Samples

The current investigation was approved by the Ethics Committee of Shanxi Provincial People's Hospital. Prior informed consent was collected from all the participants. All specimens were handled and made anonymous according to the ethical and legal standards. A total of 115 CRC patients, including 72 men and 43 women, were enrolled. According to the Tumor Node Metastasis (TNM) staging system from the American Joint Committee on Cancer, 75 cases were with I/II stage and 40 cases were with III/IV stage. Table I illustrated the demographics and clinical features of the CRC patients. Moreover, 50 healthy volunteers (25 men and 25 women) were recruited as controls. From each subject, up to 5 mL of whole blood was withdrawn, centrifuged, and the serum was separated. All serum samples were transferred to RNase/DNase-free tubes and stored at –80 °C for RNA extraction.

### Cell Culture and Transfection

Two CRC cell lines HCT116 and LoVo were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin. Cell culture was conducted in an incubator with 5% $CO_2$ at 37°C along with a 95% saturated humidity. The miR-98 mimic and miRNA control were transfected into CRC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

### RNA Isolation and Quantitative Reverse-Transcription PCR (qRT-PCR)

Total RNA was extracted from serum samples using a QIAamp RNA Blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA quality was assessed on NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The complementary DNA was synthesized by Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The real-time PCR for cDNA amplification was carried out on ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All qRT-PCR reactions were performed in triplicate. The *Caenorhabditis elegans*

**Table I.** Clinicopathological variables of 115 patients with CRC and expression of serum miR-98.

| Factors | Total (N=115) | miR-98 Low (n=52) | High (n=63) miR-98 | *p*-value |
|---|---|---|---|---|
| Age | | | | 0.097 |
| < 60 | 54 | 20 | 34 | |
| ≥ 60 | 61 | 32 | 29 | |
| Gender | | | | 0.576 |
| Male | 72 | 34 | 38 | |
| Female | 43 | 18 | 25 | |
| Smoking status | | | | 0.488 |
| Current | 37 | 15 | 22 | |
| Past/never | 78 | 37 | 41 | |
| Drinking status | | | | 0.760 |
| Current | 46 | 20 | 26 | |
| Past/never | 69 | 32 | 37 | |
| TNM stage | | | | <0.0001 |
| I-II | 75 | 23 | 52 | |
| III-IV | 40 | 29 | 11 | |
| Tumor size | | | | 0.137 |
| < 5 cm | 81 | 33 | 48 | |
| ≥ 5 cm | 34 | 19 | 15 | |
| Lymph node metastasis | | | | 0.042 |
| Absent | 99 | 41 | 58 | |
| Present | 16 | 11 | 5 | |
| Histologic grading | | | | 0.003 |
| Well | 48 | 14 | 34 | |
| Moderately/Poorly | 67 | 38 | 29 | |

miRNA cel-miR-39 (5′-UCACCGGGUGUAAAUCAGCUUG) was used as a synthetic spike-in control RNA oligonucleotide expression levels of serum miR-98 were normalized to those of cel-miR-39 and determined by the $2^{-\Delta\Delta Ct}$ method. The following primers were used:

miR-98 forward: 5′-ATCCAGTGCGTGTCGTG-3′; miR-98 reverse: 5′-TGCTTGAGGTAGTAAGTTG-3′.

### Luciferase Reporter

The cancer cells were seeded in 24-well plates with the density of $1 \times 10^5$ cells/well. Co-transfection was performed with reporter plasmids of pGL3-MYC-WT or pGL3-IL6-WT or pGL3-HIST1H2BH-WT and miR-98 mimic or miRNA control in 293t cells. The luciferase activities were determined with a Dual-Luciferase reporter assay system (Promega, Madison WI, USA) after co-transfection for 48 h. All transfection experiments were repeated three times.

### Statistical Analysis

CRC patients were divided into high serum miR-98 group (n=52) and low serum miR-98 group (n=52) based on the median value of serum miR-98 levels in all CRC patients. Overall survival (OS) was defined as the time from inclusion in the study until death for any reason or last follow-up. Disease free survival (DFS) was defined as the time from inclusion in the study until relapse or last follow-up. Statistical difference in serum miR-98 expression between unpaired groups was determined using Mann-Whitney U test. The correlation between serum miR-98 expression and clinical variables was analyzed using the Chi-square test. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to evaluate the diagnostic performance of serum miR-98 expression in discriminating CRC cases from the healthy controls. The survival curves of CRC patients were established by the Kaplan-Meier method, and the difference was assessed using log-rank testing. Multivariate logistic regressions were performed to determine independent prognostic factors for OS/DFS. The

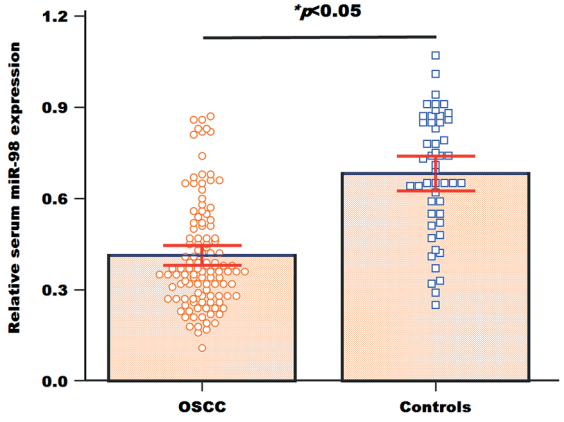


**Figure 1.** The expression levels of serum miR-98 in CRC patients and controls.

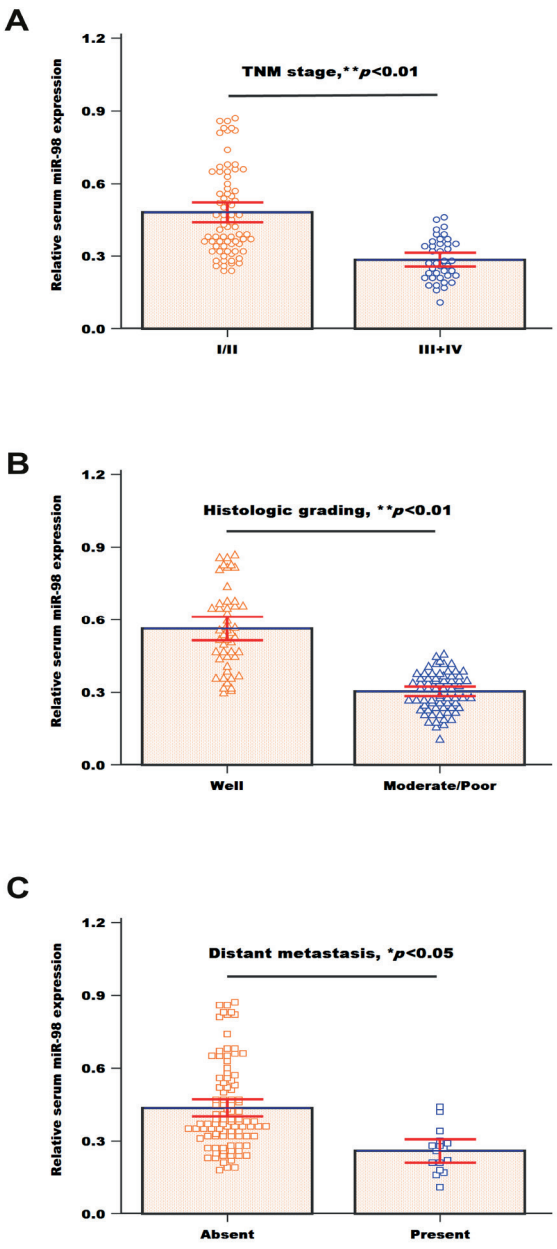


**Figure 2.** The serum miR-98 expression levels were lower in patients with advanced clinical stage (**A**), moderate/poor differentiation (**B**) or lymph node metastasis (**C**).

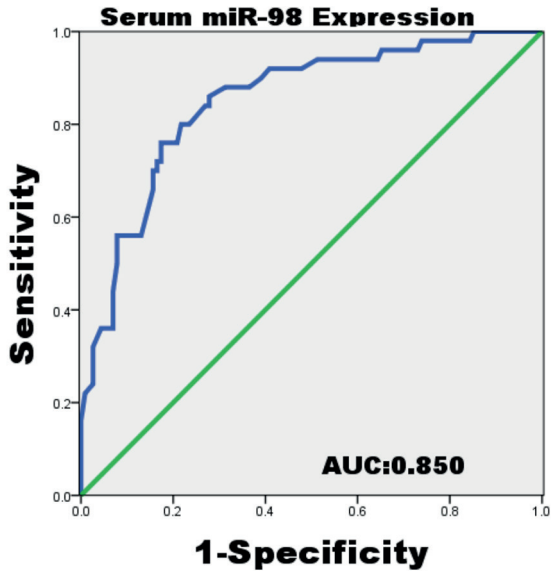


**Figure 3.** ROC analysis of serum miR-98 for the differentiation of CRC patients from healthy controls.

gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were performed to analyze the downstream targets of miR-98. Protein-protein interaction (PPI) analysis was performed to identify the number of interactions of the downstream targets of miR-98. All statistical analyses were performed using Medcalc version 12.3.0 (MedCalc, Mariakerke, Belgium) and SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). $p$-value < 0.05 was considered significant.

## Results

### *Down-regulation of Serum MiR-98 in Patients with CRC*

We evaluated the serum miR-98 levels in a total of 115 CRC patients and 50 healthy controls by qRT-PCR. As shown in Figure 1, the relative serum miR-98 expression levels were greatly lower in CRC patients than in controls ($p$<0.0001). Next, all CRC subjects were stratified by TNM stage, histologic grading, and lymph node metastasis. The results showed that the serum miR-98 levels were lower in CRC patients at the advanced stage than those at the early stage ($p$<0.0001, Figure 2A). Similarly, CRC patients with moderately/poorly differentiated tumors or positive lymph node metastasis had lower serum miR-98 levels than those with well-differentiated tumors or negative lymph node metastasis, respectively ($p$<0.0001, Figure 2B-2C). Next, the diagnostic value of serum miR-98 for CRC was assessed by ROC curve analysis. Figure 3 showed the diagnostic sensitivity and specificity was 82.6% and 76.0% respectively, and the AUC value was 0.850. These results illustrated that serum miR-98 expression was a promising biomarker for screening CRC patients from healthy controls.

### *Correlation of Serum MiR-98 Levels with the Clinicopathological Features of CRC*

The association between serum miR-98 expression and clinicopathological characteristics of CRC was summarized in Table I. Low serum miR-98 expression was found to be significantly associated

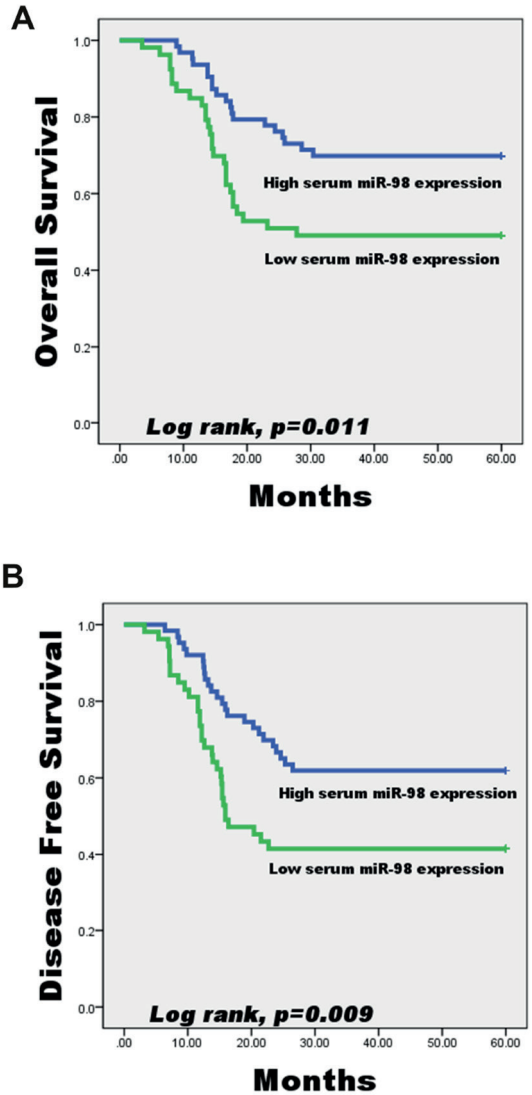


**Figure 4**. The OS (**A**) and DFS (**B**) rate of CRC patients in the high or low serum miR-98 expression group.

**Table II.** Multivariate analysis of the impact of variables on overall survival and disease-free survival in CRC patients.

| | Multivariate analysis (OS) | | | Multivariate analysis (DFS) | | |
|---|---|---|---|---|---|---|
| **Parameters** | **RR** | **95% CI** | ***p*** | **RR** | **95% CI** | ***p*** |
| TNM stage | | | | | | |
| III/IV vs. I/II | 4.93 | 1.65-8.57 | 0.003 | 5.37 | 1.78-9.24 | 0.001 |
| Histologic grading | | | | | | |
| Moderately/Poorly vs. Well | 3.87 | 1.21-6.68 | 0.016 | 4.35 | 1.38-7.42 | 0.009 |
| Lymph node metastasis | | | | | | |
| Present vs. Absent | 2.75 | 0.95-4.72 | 0.027 | 3.12 | 1.08-5.26 | 0.022 |
| Serum miR-98 | | | | | | |
| Low *vs.* High | 4.13 | 1.34-7.13 | 0.012 | 4.76 | 1.57-8.18 | 0.006 |

RR, relative risk; CI, confidence interval.

with advanced TNM stage ($p<0.0001$), moderately or poorly histologic grading ($p=0.003$), and positive lymph node metastasis ($p=0.042$). There was no significant association of serum miR-98 level with other clinicopathological parameters such as age ($p=0.097$), gender ($p=0.576$), smoking status ($p=0.488$), drinking status ($p=0.760$), and tumor size ($p=0.137$).

### Correlation of Serum MiR-98 Levels with Clinical Outcome of CRC Patients

Follow-up data were available for all CRC cases. The Kaplan-Meier curves for OS and DFS stratified according to the median value of serum miR-98 expression in CRC cases were constructed. The CRC patients in the low serum mR-98 expression group had significantly shorter OS ($p=0.011$, Figure 4A) and DFS ($p=0.009$, Figure 4B) than those in low serum miR-98 expression group. As shown in Table II, multivariate analysis of indicators associated with OS revealed a significantly worse survival in the patients with advanced TNM stage (RR, 4.93, 95% CI, 1.65-8.57, $p=0.003$), moderately or poorly histologic grading (RR, 3.87, 95% CI, 1.21-6.68, $p=0.016$), lymph node metastasis (RR, 2.75, 95% CI, 0.95-4.72, $p=0.027$), and lower miR-98 expression (RR, 4.13, 95% CI, 1.34-7.13, $p=0.012$). On the other hand, the variables that were correlated with shorter DFS on multivariate analysis were also advanced TNM stage (RR, 5.37, 95% CI, 1.78-9.24, $p=0.001$), moderately or poorly histologic grading (RR, 4.35, 95% CI, 1.38-7.42, $p=0.009$), lymph node metastasis (RR, 3.12, 95% CI, 1.08-5.26, $p=0.022$), and lower miR-98 expression (RR, 4.76, 95% CI, 1.57-8.18, $p=0.006$).

### GO and KEGG Pathway Analysis of the Downstream Targets of MiR-98

GO:0006334-nucleosome assembly, GO:0006366-transcription from RNA polymerase II promoter, GO:0000082-G1/S transition of mitotic cell cycle, GO:0006977-DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest, and GO:0006915-apoptotic process were the top biological processes (Figure 5A). GO:0005654-nucleoplasm, GO:0000788-nuclear nucleosome, GO:0000786-nucleosome, GO:0005634-nucleus, and GO:0016020-membrane were the top enriched cellular components (Figure 5B). GO:0005515-protein binding, GO:0044822-poly(A) RNA binding, GO:0003677-DNA binding, GO:0046982-protein heterodimerization activity, and GO:0004386-helicase activity were the top molecular functions (Figure 5C). hsa04066:HIF-1 signaling pathway, hsa04110:Cell cycle, hsa04068:FoxO signaling pathway, hsa05200:Pathways in cancer, hsa05219:Bladder cancer, hsa04910:Insulin signaling pathway, hsa05205:Proteoglycans in cancer, hsa05206:MicroRNAs in cancer, hsa04012:ErbB signaling pathway, and hsa04350:TGF-beta signaling pathway were the top enriched pathways (Figure 5D).

### Analysis and Validation of the Downstream Targets of MiR-98 in CRC Cells

Figure 6A-6B showed that the top candidate genes with most interactions based on PPI analysis. The luciferase reporter assay showed that the luciferase activities of pGL3-MYC-WT + miR-98 mimic group were significantly downregulated

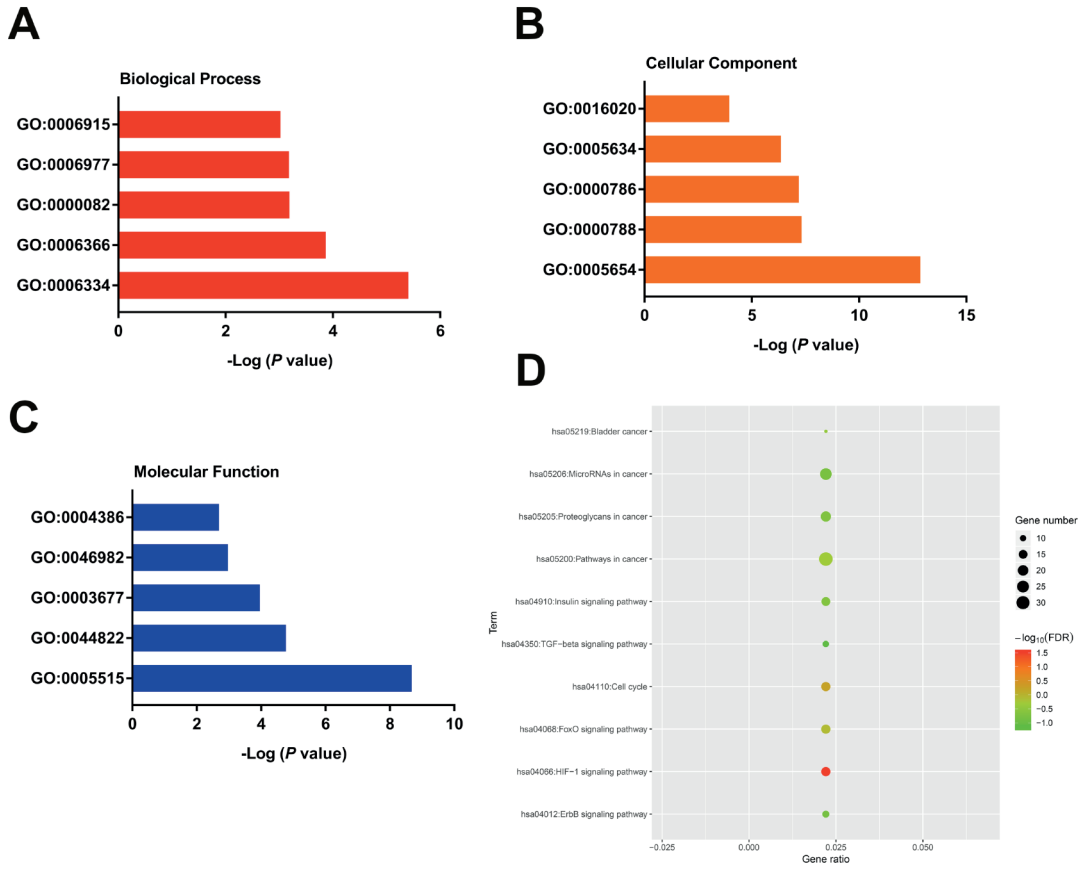


**Figure 5.** GO and KEGG analysis of the downstream targets of miR-98.

compared with pGL3-MYC-WT + miRNA control group (Figure 6C). Similar results were observed for IL6 and HIST1H2BH. In addition, the expression levels of MYC, IL-6, and HIST1H2BH were remarkably lower in CRC cells transfected with miR-98 mimic than those transfected with miRNA control (Figure 6D-6E).

## Discussion

To date, the diagnostic and prognostic value of serum miR-98 in CRC remained largely elusive. In this context, the present study was performed to analyze the possible correlation between serum miR-98 expression and the clinical features in CRC. There were five significant findings according to our results: serum miR-98 levels were frequently reduced in patients with CRC, compared to healthy controls; loss of miR-98 was closely associated with advanced TNM stage, moderately or poorly histologic grading, lymph node metastasis of CRC cases; miR-98 expression was found to be a promising candidate biomarker for CRC at diagnosis; the survival analysis demonstrated CRC patients with low serum miR-98 expression had significantly shorter OS and DFS. Multivariate analysis revealed that low serum miR-98 expression was an independent prognostic predictor for OS and DFS. Mechanistically, MYC, IL-6, and HIST1H2BH were identified as direct downstream targets of miR-98 in CRC cells. These results suggested that serum miR-98 might serve as a valuable diagnostic and prognostic biomarker for CRC.

MiR-98 was proposed as a tumor suppressor because its expression was frequently downregulated in many cancers. For instance, Tan et al[17] showed that miR-98 was reduced in squamous cell carcinoma of the head and neck (SCCHN) tissues, and miR-98 overexpression greatly suppressed the tumorigenesis by targeting MTDH. Moreover, the downregulation of miR-98 level was associated with aggressive clinical parameters and shortened overall survival in SCCHN. In hepatocellular carcinoma (HCC), Zhou et al[18]

revealed that the low miR-98 expression was closely correlated with shorter survival time. Ectopic expression of miR-98 or SALL4 inhibition resulted in decreased HCC cell proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) *in vitro*, and tumor growth *in vivo*. In glioma, miR-98 expression was dramatically downregulated in cell lines and cancerous tissues. Enforced miR-98 expression significantly induced cell apoptosis by suppressing IKBKE/NF- kB pathway as well as inhibited cell migration and invasion through silencing HMGA2 and IKKε[19-21]. Moreover, miR-98 was frequently decreased in tissues and cells of non-small cell carcinoma lung cancer (NSCLC). In addition, miR-98 inhibited the expression of PAK1 or integrin β3, leading to reduced cell proliferation, migration, invasion of NSCLC[22,23]. Li et al[24] reported that miR-98 levels were decreased in melanoma tissues. Upregulation of miR-98 significantly attenuated cellular migration *in vitro* and the metastatic tumor size *in vivo* by regulating IL-6 expression, and *vice versa*[24]. In esophageal squamous cell carcinoma, *in vivo* evidence showed that miR-98 overexpression increased the radiosensitivity of cancer cells, induced cell apoptosis, and suppressed cell viability *via* targeting the Bcl-2 pathway[25]. In salivary adenoid cystic carcinomas, Liu et al[26] revealed that miR-98 was markedly decreased in tumor tissues and cell lines, restoration of miR-98 markedly inhibited the carcinogenesis *in vitro* by silencing neurobla-

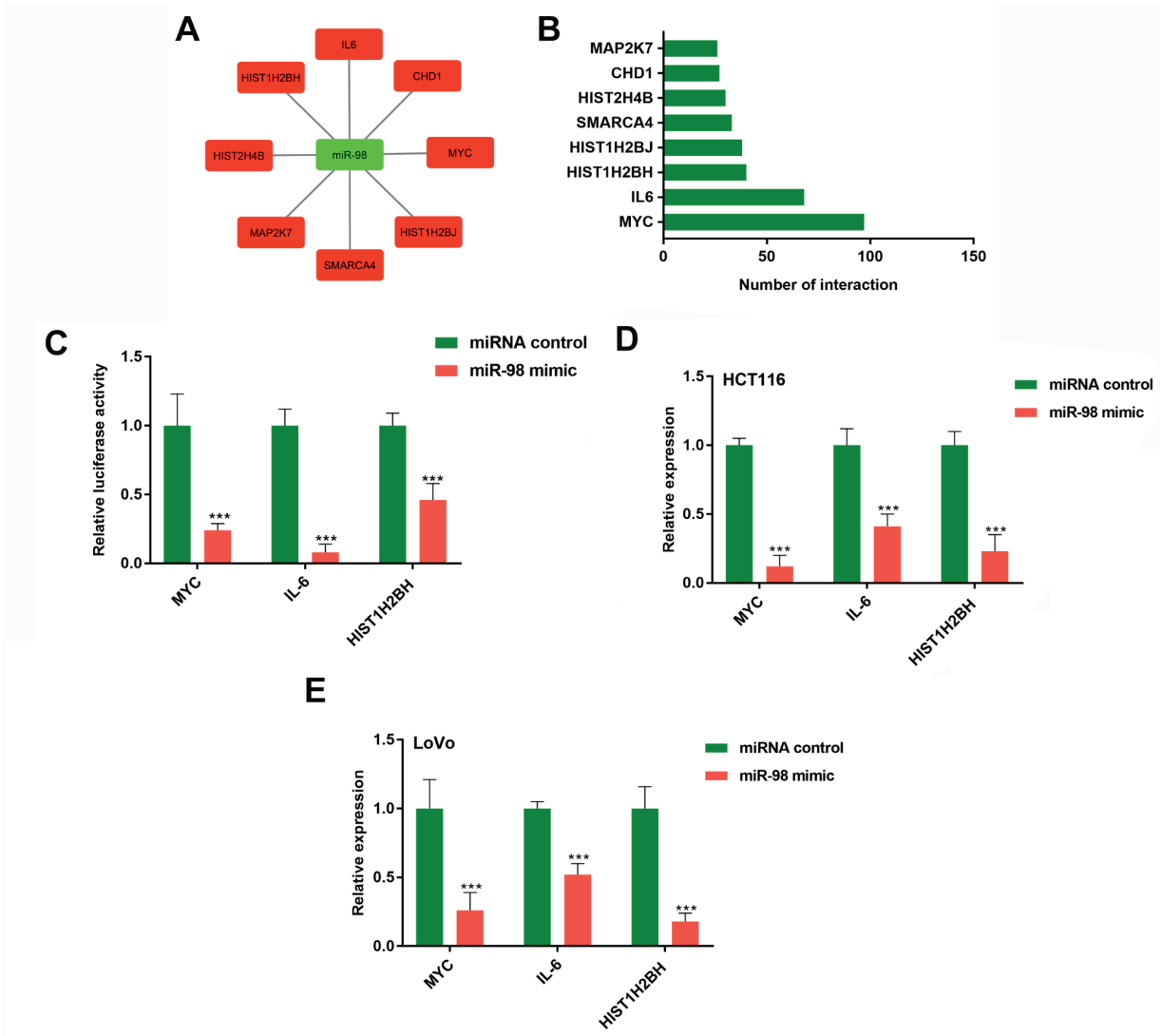


**Figure 6.** MYC, IL-6 and HIST1H2BH were identified as direct downstream targets of miR-98 in CRC cells.

stoma RAS expression. Enforced miR-98 expression significantly decreased oral squamous cell carcinoma cell proliferation, colony formation, migration, and invasion by regulating IGF1R, and its expression was markedly downregulated in both cancer tissues and cell lines[27].

Though miR-98 functioned as a tumor-suppressive gene in most types of cancers, it might play a completely different role in certain cancer type, such as breast cancer (BC). MiR-98 was confirmed to be significantly higher in BC tissues than in cancer free tissues. In addition, miR-98 expression showed good performance to identify BC patients from normal controls and could serve as a marker for BC diagnosis[28]. Therefore, the concrete role miR-98 in tumorigenesis varied with different types of cancer.

## Conclusions

Our data offered convincing evidence that decreased miR-98 expression was a common event in CRC patients and strongly correlated with aggressive parameters of this malignancy. Moreover, low miR-98 expression was an independent prognostic indicator. Collectively, miR-98 might serve as a novel diagnostic and prognostic biomarker for CRC.

### Conflicts of interest

The authors declare no conflicts of interest.

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