

## ORIGINAL ARTICLE

# Serum miR-1301-3p, miR-335-5p, miR-28-5p, and their target B7-H3 may serve as novel biomarkers for colorectal cancer

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## Summary

**Purpose:** B7-H3, a member of the B7 family of immune regulatory ligands, plays a critical role in the T cell-mediated immune response. It is broadly expressed in several human cancers and leads to poor prognosis. Nevertheless, the clinical significance of B7-H3 expression in colorectal cancer (CRC) remains unclear.

**Methods:** The serum B7-H3, B7-H1, cancer-associated carbohydrate antigen-50 (CA-50) and carcinoembryonic antigen (CEA) expressions in patients with CRC, benign gastrointestinal diseases, and healthy controls were measured by ELISA. The miRNAs that target B7-H3 were predicted by using the miRTarBase. Real-time PCR was performed to examine their expressions in patients with CRC.

**Results:** B7-H3, B7-H1, and CA-50 expressions were higher in patients with CRC than those in healthy controls and

the patients with benign gastrointestinal diseases. B7-H3 expression was correlated with TNM stage and metastasis. It was predicted that B7-H3 was a target of miR-1301-3p, miR-335-5p, and miR-28-5p and its expression was negatively related to these three miRNAs expressions. Serum miR-1301-3p, miR-335-5p, and miR-28-5p expressions were also correlated with the TNM stage and metastasis.

**Conclusion:** Our results indicated that serum miR-1301-3p, miR-28-5p, miR-335-5p, and B7-H3 expressions were correlated with pathological stages of CRC and metastasis and may therefore serve as novel biomarkers for CRC diagnosis and treatment.

**Key words:** colorectal cancer, B7-H3, biomarker, microRNA

## Introduction

Colorectal cancer (CRC) is the fourth most common malignant tumor worldwide and one of the most common cancers in the digestive tract [1]. The incidence of CRC increases each year, with more than 1 million cases and 600,000 deaths occurring each year [2,3]. The average 5-year overall survival rates of colon cancer and rectal cancer were once 57% and 56%, respectively [4,5]. With the improvement of diagnosis and treatment methods, the prognosis of early CRC patients has improved significantly. The 5-year overall survival rates of patients with stage I, II and III disease have risen up to 44-93% [6]; however, it drops to 8.1% in stage

IV disease and 25% in patients with metastasis [7]. Conventional methods for treating CRC include surgery, chemotherapy, and radiotherapy, but more than half of the CRC patients develop tumor metastasis, drug resistance, or recurrence after treatment [8]. Therefore, it is of great significance for the diagnosis and treatment of CRC to explore the complex mechanism of the CRC development and to find out the genes that may help identify early diagnosis, targeted therapy, and prognosis.

Biomarkers that can be used for the early detection of cancer and precisely identify early-stage solid tumors after surgery at high risk for

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recurrence could reduce mortality. Serum tumor markers including CEA, CA-50, CA19-9, CA72-4, and CA-15-3 are mainly used for CRC patients for auxiliary diagnosis, response to treatment, and disease monitoring [9-12]. However, none of these serological markers have brought out high specificity and sensitivity in order to detect early stage CRC in clinical tests [13]. CEA is the most commonly used tumor marker in CRC, but its sensitivity in early diagnosis is less than 36% [14], suggesting that it should not be solely used as an early diagnostic indicator of CRC.

MicroRNA (miRNA) - a small non-coding RNA molecule - consists of 18-22 nucleotides, recognizes and binds to the 3'UTR of target mRNA, degrading target mRNA or inhibiting the translation process of target protein [15]. miRNAs are considered as potential biomarkers because of their involvement in the occurrence, development, diagnosis, and metastasis of tumors [16]. In addition, circulating miRNAs is a class of miRNAs with good stability in serum, plasma or whole blood. The sequences of most circulating miRNAs is conserved among different species, the expression of some circulating miRNAs is specific to tissues or biological stages, and the level of circulating miRNAs can be easily assessed by various methods [17,18], suggesting that serum miRNA level may serve as a biomarker in cancer diagnosis and prognosis. Let-7g, miR-21, miR-203, miR-181b, miR-92a, and miR-31 were dysregulated in CRC serum samples and associated with TNM staging [19]. miR-194 and miR-29b expressions were down-regulated in the serum of CRC patients compared with age- and gender-matched healthy controls, serving as potential biomarkers for diagnosis and prognosis of CRC [20]. However, the relative stability of miRNAs is a prerequisite for their functions as diagnostic markers.

The B7 family members B7-H1 and B7-H3 are highly expressed in many tumors and participate in immune escape of tumor cells. B7-H1 and B7-H3 expressions were up-regulated in gallbladder carcinoma, breast cancer, gastric cancer, and non-small cell lung cancer and associated with postoperative survival, metastasis, and recurrence [21,22]. miR-155/miR-143 targeting B7-H3 inhibited the immune escape mechanism of tumor cells in CRC [23]. Increased expression of B7-H3 was observed in CRC tissues and related to advanced tumor grades and lower recurrence-free survival in CRC patients with stage I disease [24,25]. However, the clinical significance of circulating B7-H3 expression as well as its regulatory miRNAs still needs further investigation.

In this study we tried to explore the expressions of B7-H3, B7-H1 and CA-50 and the clinical

significance of miR-335p, miR-1301-3p, miR-28-5p and their target B7-H3 expression in CRC.

## Methods

### *Patient and serum samples*

The clinicopathological characteristics of 113 patients with CRC are shown in Table 1. In addition, this study involved 73 patients with benign gastrointestinal diseases including 29 females and 44 males, with a median age of 63 years (range, 33 to 91). Gastrointestinal diseases included polyps (n=42), inflammations (n=18), and others (n=13). A total of 59 healthy controls included 25 females and 34 males, with a median age of 63 years (range, 17 to 86). Serum samples collected from these patients and healthy controls were stored at -80°C until enzyme-linked immunosorbent assay (ELISA). A total of 186 patients, who underwent blood collection at Shanghai No.8 People's Hospital, Xuhui Branch of Shanghai No.6 People's Hospital between October 2016 and June 2017, were included in our study. Written informed consent was obtained from each participant and complied with the guidelines of the ethics committee. The study was approved by the Research Ethics Committee in Shanghai No.8 People's Hospital, Xuhui Branch of Shanghai No.6 People's Hospital.

**Table 1.** Clinicopathological characteristics of 113 patients with colorectal cancer

Characteristics	Number of patients (%)
Age, years	
≥64	58 (51.3)
<64	55 (48.7)
Gender	
Female	47 (41.6)
Male	66 (58.4)
Invasion depth	
I	7 (6.2)
II	19 (16.8)
III	27 (23.9)
IV	60 (53.1)
TNM stages	
I	17 (15.0)
II	27 (23.9)
III	27 (23.9)
IV	42 (37.2)
Metastasis	
Yes	23 (20.4)
No	90 (79.6)
Tumor location	
Colon	70 (61.9)
Rectum	43 (38.1)

### ELISA and other tests

Serum B7-H3 and B7-H1 levels in patients with CRC, gastrointestinal disease, and healthy controls were measured by commercial sandwich ELISA kits (R&D Systems, USA) following the manufacturer's instructions. CA19-9, CA72-4, and CEA levels were measured by Roche Cobas e602 instrument (Roche Diagnostics, Mannheim, Germany). CA-50 level was measured by Autolumo A2000 Plus (Zhengzhou, China). Absorbance was recorded at 540 nm using spectrophotometer (Tianjin Precise Instrument, Tianjin, China).

### Analysis of predicted putative miRNAs

The putative miRNAs targeting B7-H3 were predicted using miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>), an experimentally validated miRNA-target interaction database.

### Real-time PCR

Total RNA was extracted using miRNeasy kit (QIAGEN) and stored at  $-80^{\circ}\text{C}$  in RNA secure RNase Inactivation Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Complementary DNA was synthesized by TaqMan MicroRNA RT Kit (Applied Biosystems) and PrimeScript reagent kit of reverse reaction (DRR037A; TaKaRa). Real-time PCR amplification was performed using the SYBR Green qRT-PCR kit (Promega, USA) on an ABI 7500 system according to manufacturer's instruction. Expression levels are given as ratios to U6 RNA. The primer sequences for qRT-PCR are listed as follows: hsa-miR-1301-3p, sense: 5'-ACACTCCAGCTGGGUUGCAGCUGCCUGGGAGU-3', antisense: 5'-TGGTGTCTGTTGGAGTCG-3'; hsa-miR-335-5p, sense: 5'-ACACTCCAGCTGGGUCAAGAGCAAUAACGAA-3', antisense: 5'-TGGTGTCTGTTGGAGTCG-3'; hsa-miR-28-5p, sense: 5'-ACACTCCAGCTGGGAAGGAGCUCACAGUCU-3', antisense: 5'-TGGTGTCTGTTGGAGTCG-3'; U6, sense: 5'-GCTTCGGCTGGACATATACTAAAAT-3', antisense:

5'-GAACGCTTCACGATTTTGCG-3'. The relative expression of miRNAs was calculated through the  $2^{-\Delta\Delta\text{CT}}$  method.

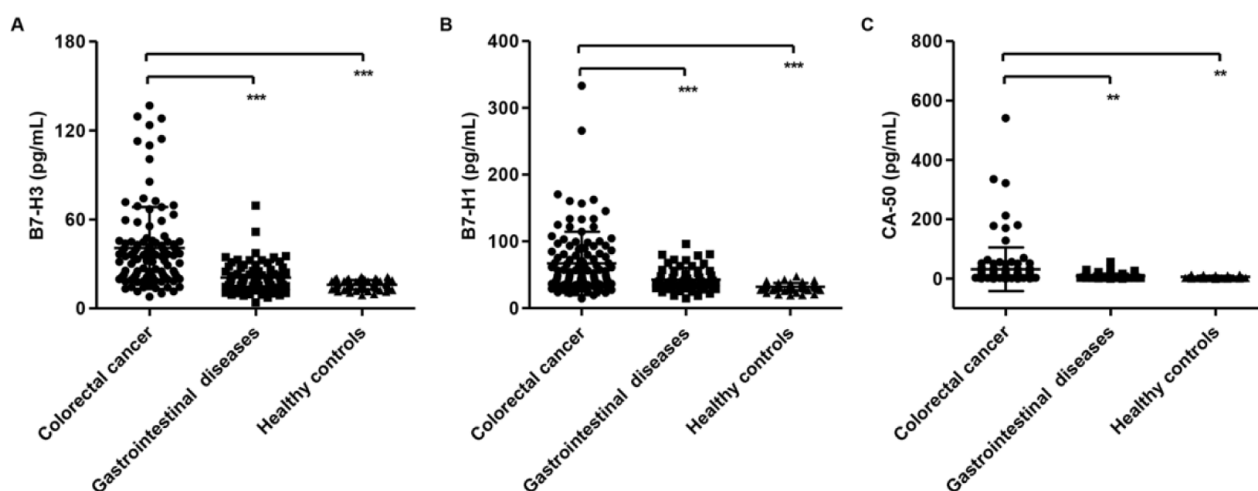
### Statistics

All the results are presented as mean  $\pm$  SD, and each test was repeated at least three times. Statistical analyses were carried out with the GraphPad Prism 5.0 software using one-way ANOVA followed by Tukey's *post hoc* test and unpaired T test.  $P < 0.05$  suggested that the difference between groups was statistically significant.

## Results

### Serum B7-H3, B7-H1, and CA-50 expressions

In order to investigate the function of B7 family members and tumor markers in CRC, the serum expression of B7 family members-B7-H3 and B7-H1, and tumor markers CA-50, CEA, CA19-9, and CA72-4 in patients with CRC, gastrointestinal disease, and healthy controls were measured. As shown in Figure 1A-C, B7-H3, B7-H1 and CA-50 expressions were increased in patients with CRC compared to patients with gastrointestinal diseases and healthy controls but the differences were not statistically significant between patients with gastrointestinal disease and healthy controls. Meanwhile, the serum CEA, CA19-9, and CA72-4 expressions among patients with CRC, gastrointestinal diseases and healthy controls were not significantly different (data not shown). These results suggest that serum B7-H3, B7-H1 and CA-50 expressions may correlate with the occurrence of CRC.



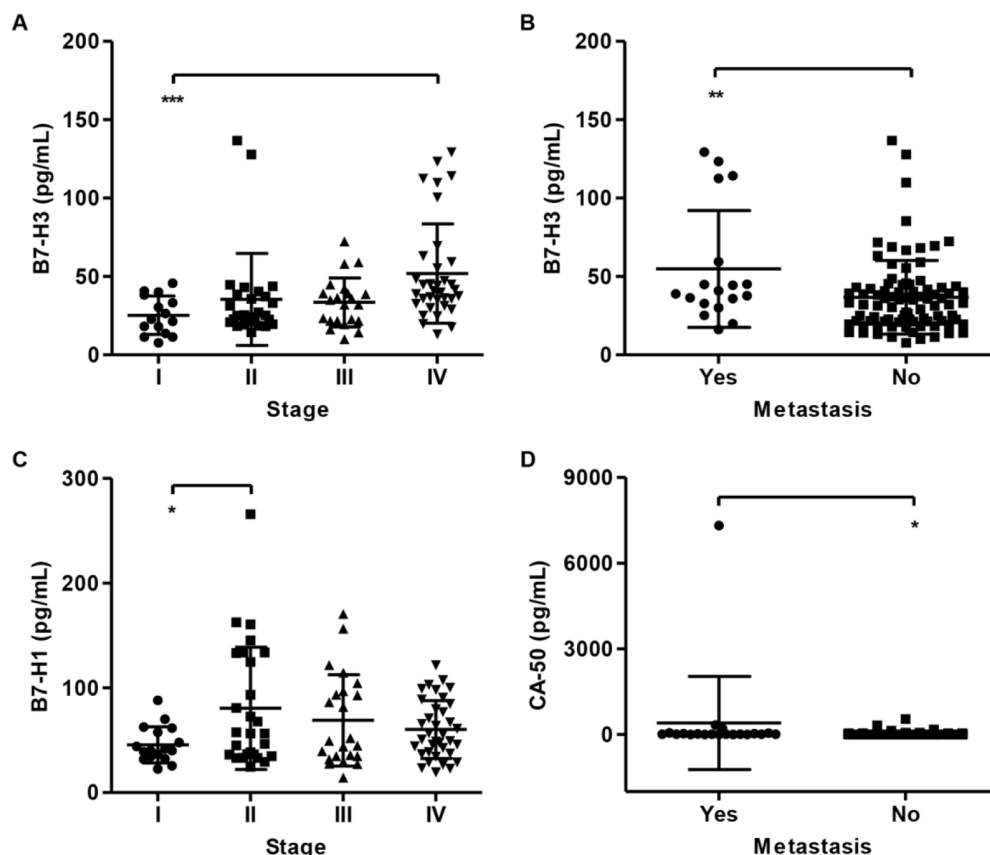
**Figure 1.** Serum B7-H3 (A), B7-H1 (B) and CA-50 (C) expression in patients with CRC, benign gastrointestinal diseases, and healthy controls were measured by ELISA. B7-H3, B7-H1 and CA-50 expressions were increased in patients with CRC compared to patients with benign gastrointestinal diseases and healthy controls but the differences were not statistically significant between patients with benign gastrointestinal diseases and healthy controls. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Serum B7-H3, B7-H1, and CA-50 expressions are correlated with TNM stages and/or metastasis

In view of the correlation between CRC occurrence and B7-H3, B7-H1 and CA-50 expressions, the correlation of the expressions of serum B7-H3, B7-H1 and CA-50 with clinicopathological features of CRC patients, including age, gender, TNM stages, depth of invasion, metastasis and tumor localization, were further investigated. We demonstrated that serum B7-H3 expression was increased in stage IV disease compared to stage I; Serum B7-H1 expression was increased in stage II disease compared to stage I, suggesting that serum B7-H3 and B7-H1 expressions were positively related to advanced TNM stages (Figure 2A and C). Additionally, it was found that serum B7-H3 and CA-50 expressions were both increased in CRC patients with metastasis, indicating that serum B7-H3 and CA-50 expressions were positively related to the tumor metastasis. However, other clinicopathological features were not correlated to the serum B7-H3, B7-H1 and CA-50 expressions (data not shown). Taken together, our findings indicated that increased serum B7-H3, B7-H1 and CA-50 expressions promoted CRC progression.

B7-H3 is predicted to be a target of miR-1301-3p, miR-28-5p and miR-335-5p

In order to extend the B7-H3 function in the development of CRC, the putative miRNAs that target B7-H3 were predicted. According to the miR-TarBase [26], 39 miRNAs are predicted to target B7-H3 based on the validation methods, including Reporter assay, western blot, qPCR, stable isotope labeling with amino acids in cell culture (SILAC), next-generation sequencing (NGS) and microarray, among which miR-28-5p, miR-1301-3p, and miR-335-5p have been demonstrated to be potential biomarker candidates in small intestinal biopsies for celiac diseases [27]. Moreover, miR-1301-3p positively regulates the p53 pathway in CRC [28] and miR-28-5p was down-regulated in CRC tissues and inhibited the cell growth, migration, and invasion of CRC [29]. These three miRNAs were therefore analyzed in our experiments. As shown in Figure 3A, the target sites of B7-H3 by miR-1301-3p, miR-335-5p, and miR-28-5p were at position 1-23/44-67/70-94, 573-597/1017-1039/1374-1396, and 1371-1395/96-118/21-42, respectively. To confirm the correlation of B7-H3 with miR-28-5p, miR-1301-3p, and miR-335-5p in CRC, the serum



**Figure 2.** Correlation between serum B7-H3, B7-H1 and CA-50 expression and clinicopathological characteristics of patients with colorectal cancer. Serum B7-H3 (A,B), B7-H1 (C) and CA-50 (D) expression were correlated with TNM stages and/or metastasis. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .



expressions of these three miRNAs were measured by real-time PCR and the data demonstrated that serum B7-H3 expression was negatively related to these three miRNAs expressions (Figure 3B-C).

*Serum miR-1301-3p, miR-28-5p and miR-335-5p expressions are correlated with TNM stage and metastasis*

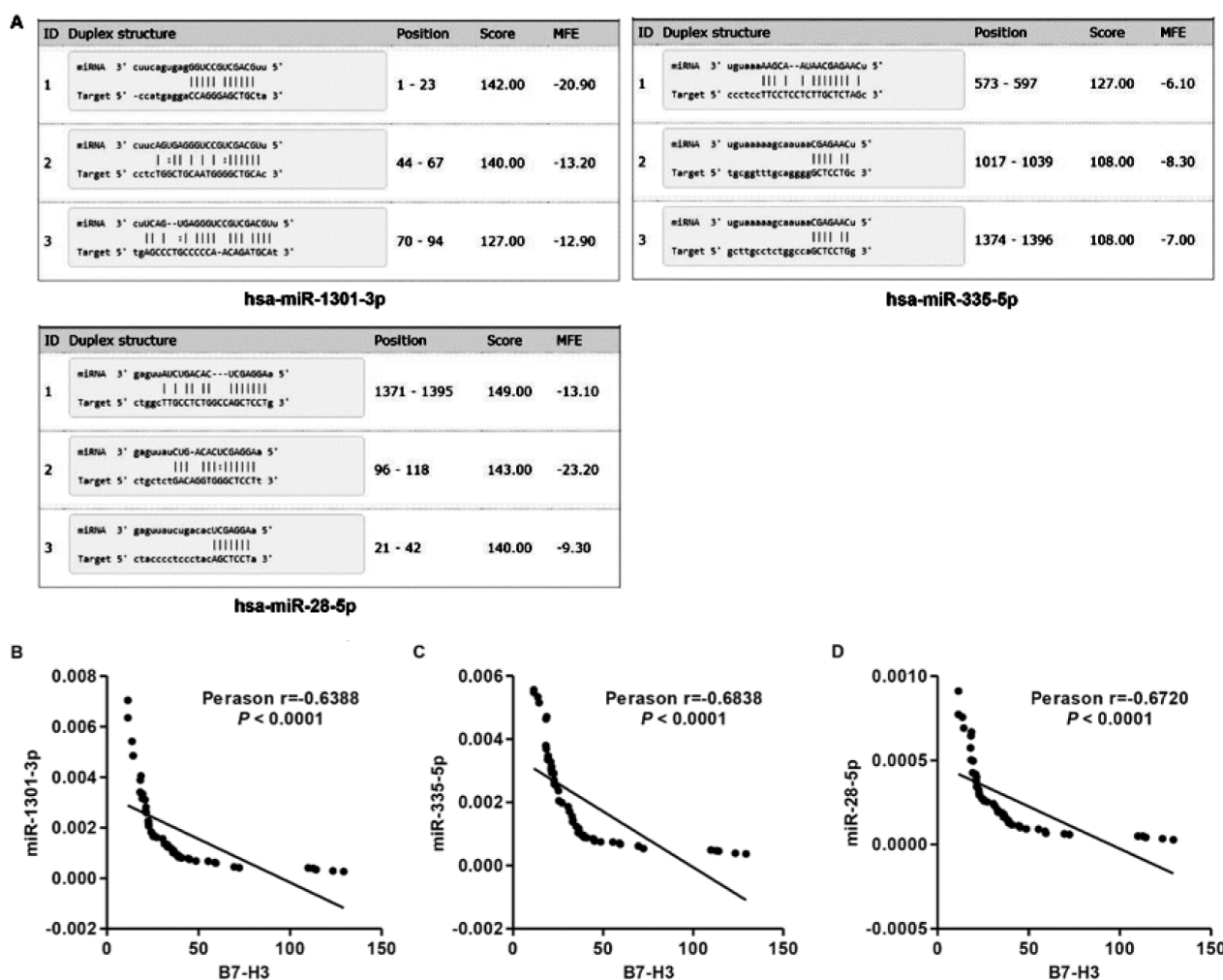
In view of the correlation of B7-H3 with miR-28-5p, miR-1301-3p and miR-335-5p expressions in CRC patients, we hypothesize that these three miRNAs expressions may also be associated with CRC development.

According to the serum B7-H3 expression in CRC patients, 50 out of 113 patients with lower B7-H3 expression (n=25) and higher B7-H3 expression (n=25) were randomly selected and the expression was measured by real-time PCR. We found that serum miR-28-5p, miR-1301-3p and miR-335-5p expressions were decreased in stage

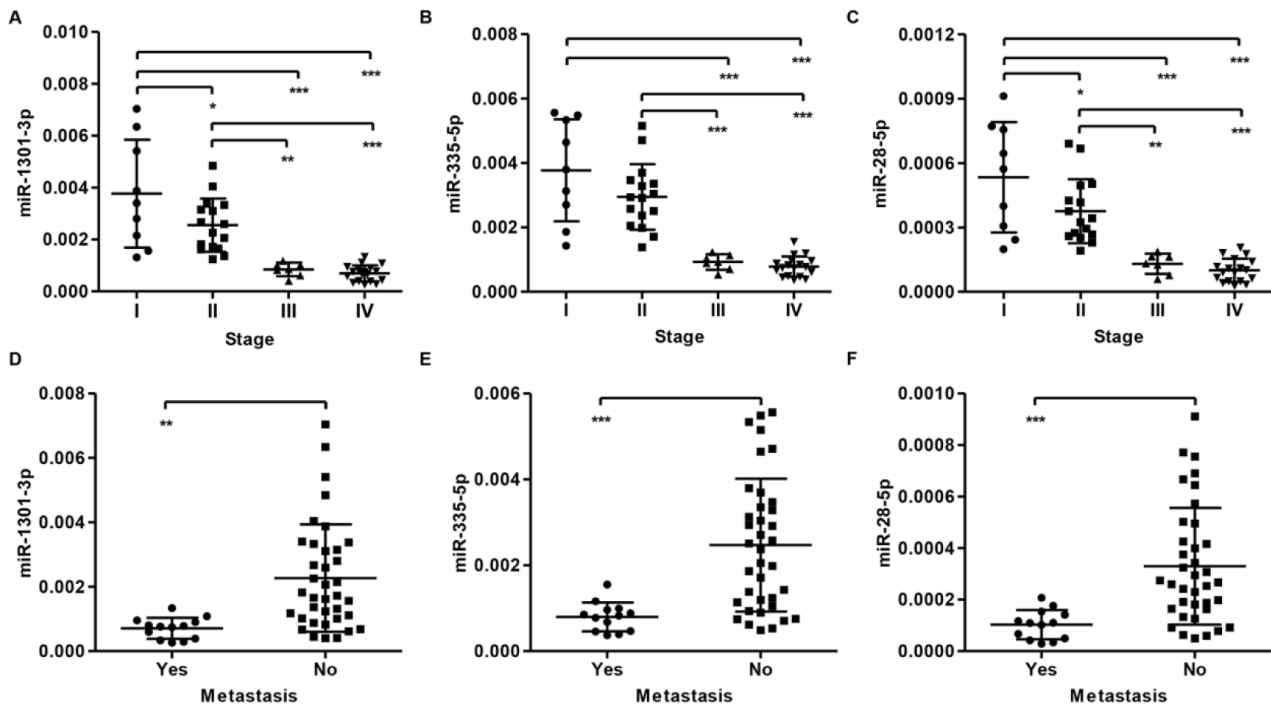
III and IV disease compared to stage I and II, suggesting that these miRNAs were negatively related to the advanced TNM stages (Figure 4A-C). Furthermore, serum miR-335-5p, miR-28-5p and miR-1301-3p expressions were also decreased in the CRC patients with metastasis, indicating that these miRNAs were negatively related to tumor metastasis (Figure 4D-F). Taken together, our data demonstrated that decreased serum miR-1301-3p, miR-335-5p and miR-28-5p expressions inhibited CRC progression.

## Discussion

The occurrence of CRC is influenced by genetic and environmental factors. Early diagnosis and early treatment are the most effective methods to improve prognosis. Detection of glycoproteins such as CEA, CA19-9, and CA72-4 can provide reference for diagnosis and is of great importance



**Figure 3.** B7-H3 was predicted to be a target of miR-1301-3p, miR-335-5p and miR-28-5p. **A:** The target sequence of miR-1301-3p miR-28-5p and miR-335-5p in the B7-H3 3'-UTR was predicted by using the miRTarBase. Linear regression showed that serum B7-H3 expression was negatively related to miR-1301-3p (**B**), miR-335-5p (**C**) and miR-28-5p (**D**) expression in patients with colorectal cancer.



**Figure 4.** Correlation between serum miR-1301-3p, miR-335-5p and miR-28-5p expression and clinicopathological characteristics of patients with colorectal cancer. Serum miR-1301-3p (**A,D**), miR-335-5p (**B,E**) and miR-28-5p (**C,F**) expressions were correlated with TNM stages and metastasis. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

in the follow-up of gastrointestinal cancers, but there is lack of positive clinical evidence in early diagnosis [10,11,13]. Surgery, radiotherapy, and chemotherapy are still the mainstay of CRC treatment. The survival rate of patients is improved to a certain extent, but the toxic and side effects are obvious and the long-term survival is still unsatisfying. Therefore, searching for sensitive and specific early screening markers is important for early CRC diagnosis. In this study, our data showed that serum B7-H3, B7-H1, and CA-50 expressions were up-regulated in CRC patients. B7-H3 was predicted to be a target of miR-1301-3p, miR-335-5p and miR-28-5p and negatively related to these miRNAs expressions. miR-1301-3p, miR-28-5p, miR-335-5p and their target-B7-H3 were associated with CRC TNM stages and metastasis.

Serum CA 19-9 and CA-50 expressions were markedly up-regulated in pancreatic cancer patients compared to healthy controls [30] and associated with pathological stages and lymphatic invasion in gastric cancer [31]. CEA, CA72-4 and CA19-9 are commonly used for monitoring treatment effect and postoperative surveillance for gastric cancer, pancreatic cancer and CRC [11,32,33] and are associated with the TNM stages, abdominal lymph node metastasis, tumor invasion and differentiation, but not with age, gender, pathological type and localization of CRC [11]. Partly similar to these findings, our results showed that

serum CA-50 expression was up-regulated in CRC patients, but CA19-9 and CA72-4 expressions between CRC patients and gastrointestinal diseases or healthy controls were not significantly different. Moreover, among the three tumor markers, only CA-50 expression was associated with tumor metastasis. The differences may reflect patient population, methods of analysis, and study design.

B7-H1 and B7-H3 expressions were increased in CRC tissues and resulted in advanced tumor grades, poor prognosis and reduced recurrence-free survival in CRC patients with stage I disease [24,25,34]. Similar to previous studies, our data demonstrated that serum B7-H1 and B7-H3 expressions were up-regulated in CRC patients compared to gastrointestinal diseases and healthy controls and associated with advanced TNM stages. Importantly, serum B7-H3 expression was simply increased in stage IV disease compared to stage I and serum B7-H1 expression was simply increased in stage II disease compared to stage I, suggesting that serum B7-H3 expression may serve as a more meaningful biomarker than B7-H1 in CRC progression. Serum B7-H3 expression was also related to tumor metastasis, which was in line with the findings in a previous study [25]. However, a previous study demonstrated that B7-H3 expression in CRC tissues was not associated with nodal and distant metastasis [24]. The differences may reflect differ-

ent sources of B7-H3. Therefore, B7-H1 and B7-H3 may be potential targets for early diagnosis and prevention of CRC.

Increasing evidence has suggested that the detection of circulating miRNA has broad application prospects in early diagnosis, therapeutic monitoring, prognosis evaluation and medication guidance for malignant tumors [17,18]. In view of the clinical significance of B7-H3 expression in CRC, we further predicted some miRNAs that may target B7-H3 using miRTarBase. Among 39 putative miRNAs, miR-1301-3p, miR-335-5p and miR-28-5p were investigated in CRC patients. We found that miR-28-5p, miR-335-5p and miR-1301-3p were negatively related to the serum B7-H3 expressions and related to the TNM stages and metastasis. In line with our findings, miR-28-5p expression was down-regulated in colorectal liver metastases in comparison with corresponding primary tumors and non-tumor colorectal tissues [35]. miR-1301-

3p restrained the invasion and migration of colon cancer cells through increasing p53 tumor suppressor expression [28], which further supported the findings in CRC patients *in vivo*. However, the direct interaction between miR-1301-3p, miR-335-5p and miR-28-5p and their target B7-H3 in CRC needs further investigation.

In conclusion, our study showed that serum B7-H3, B7-H1 and CA-50 expressions were up-regulated in CRC patients. B7-H3 was predicted to be a target of miR-1301-3p, miR-335-5p and miR-28-5p and negatively related to these miRNAs expressions. miR-28-5p, miR-335-5p, miR-1301-3p and their target B7-H3 were associated with CRC TNM stages and metastasis and may therefore serve as novel biomarkers for CRC.

### Conflict of interests

The authors declare no conflict of interests.

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