Serum microRNA-592 serves as a novel potential biomarker for early diagnosis of colorectal cancer

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Abstract. Colorectal cancer (CRC) is the second leading cause of cancer-associated mortality worldwide. Currently, available diagnostic biomarkers are neither sensitive nor specific. Thus, the present study aimed to identify novel circulating microRNAs (miRNAs) as biomarkers for the early diagnosis of CRC. All samples were provided by The Second Affiliated Hospital of Nanjing Medical University (Nanjing, China). Analysis of the GSE108153 and GSE55139 datasets, downloaded from the Gene Expression Omnibus (GEO) database was performed using the online tool, GEO2R. Reverse transcription-quantitative PCR was performed to determine miR-592 expression in CRC tissues, cells and serums of patients. Subsequently, the diagnostic value of serum miR-592 was assessed via receiver operating characteristic (ROC) curve analysis. Both the assessment of clinical samples and bioinformatics analysis demonstrated that miR-592 expression levels were significantly upregulated in the tissues and serum of patients with CRC, suggesting that elevated serum miR-592 may be tumor-derived. ROC analysis indicated that serum miR-592 levels may differentiate patients with early stage CRC and advanced adenoma from healthy individuals, with area under the curve values of 0.801 and 0.747, respectively. Taken together, the results of the present study suggest that serum miR-592 may be implicated as a potential biomarker for the early diagnosis of CRC.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and the second leading cause of cancer-associated mortality worldwide accounting for 9.2% of total cases in 2018 (1). The prognosis of patients with CRC varies according to tumor stage at the time of diagnosis, whereby ~90% of mortalities are preventable if patients are diagnosed at an early stage (2). However, the currently available fecal occult blood test and serum tumor biomarkers, such as carcinoembryonic antigen (CEA), are neither highly sensitive nor specific for early diagnosis of CRC (3). Colonoscopy and tissue biopsy remain the gold standard for detecting and diagnosing CRC; however, the invasiveness of colonoscopy limits its use in scanning patients with CRC (4). Thus, novel promising diagnostic biomarkers for CRC are required.

microRNAs (miRNAs/miR) are short single-stranded non-coding RNAs that degrade target mRNA or inhibit its translation by directly binding to the 3′-untranslated region of targets (5). Dysregulated miRNAs have been implicated in several types of cancer, including CRC, and are associated with tumor development and progression (6-9). Increasing evidence indicates that cancer cells secrete intracellular miRNAs into the peripheral blood of patients and the circulating miRNAs may persist in serum when protected by particles, such as exosomes (10,11), which makes circulating miRNAs novel promising diagnostic molecules of different types of cancer (12-14). For example, Abu-Duhier *et al* (15) reported that plasma miR-21 expression is notably upregulated in patients with lung cancer compared with healthy controls, thus confirming circulating miR-21 as an efficient non-invasive biomarker for the screening of patients with lung cancer. Furthermore, Imaoka *et al* (16) demonstrated that elevated circulating miR-1290 may be developed as a novel diagnostic and prognostic biomarker in human CRC, suggesting that tumor-derived miRNAs used for diagnosis may improve the specificity of biomarkers.

Overall, the present study aimed to identify novel circulating miRNAs that differentiate between patients with CRC and advanced colorectal adenomas (AAs) from healthy individuals, with notable diagnostic precision.

Materials and methods

Study design. The present study consisted of three phases (Fig. 1). The discovery phase used the GSE108153 (17) and GSE55139 (18) datasets, downloaded from the Gene Expression

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Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov), in order to identify CRC tissues and pre-operation serum samples with upregulated miR-592 expression, which decreased following surgical excision of the tumor. The GSE108153 dataset consisted of 21-paired CRC tissues and adjacent normal tissues (ANTs), while the GSE55139 dataset included 10 paired pre- and post-operative serum samples. Dysregulated miRNAs were analyzed by using online tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) in GEO database according to the instructions. In the training phase, miR-592 expression was identified in 15 paired CRC tissues and ANTs, as well as CRC cell lines and fetal human colon (FHC) cells. Furthermore, 12 paired pre- and post-operative serum samples of patients with CRC (7 males and 5 females) whose mean age was 63 (range from 45 to 79) were implemented to validate the source of serum miR-592. Subsequently, 30 serum samples collected from 15 healthy individuals (8 males and 7 females) and 15 patients with CRC (9 males and 6 females) were used to measure serum miR-592 expression and its diagnostic value. The mean age of healthy individuals and CRC patients were 59 (range from 43 to 78) and 61 (range from 44 to 80), respectively. In the validation phase, another independent cohort with a larger number of serum samples collected from; 50 healthy controls (HCs) (34 males and 26 females), 84 patients with stages I-II CRC (51 males and 33 females), 50 patients with stages III-IV CRC (37 males and 13 females) and 50 patients with advanced colorectal adenomas (32 males and 18 females) was implemented to confirm the diagnostic value of serum miR-592. The mean age of HCs, patients with CRC and patients with advanced colorectal adenomas were 59 (range from 41 to 79), 62 (range from 43 to 81) and 60 (range from 41 to 78), respectively.

Study population. The present study was approved by the Research and Ethical Committee at the Second Affiliated Hospital of Nanjing Medical University (Nanjing, China) and written informed consent was provided by all patients prior to the study start. Diagnosis of CRC and AAs was histologically confirmed by two independent pathologists from Department of Pathology of the Second Affiliated Hospital of Nanjing Medical University via analysis of resected tumors following surgery and colonoscopy examination, and tumor stage was determined according to the tumor-node-metastasis (TNM) system (19). Patients with any anti-tumor treatment before specimen collection, such as chemoradiotherapy, were excluded. The detailed characteristics of patients with CRC were downloaded from the medical record system of the Second Affiliated Hospital of Nanjing Medical University and are presented in Table I. The post-operative serum samples of patients with CRC were collected one week after surgical excision of the tumors. HCs were collected from age-matched volunteers who participated in the routine physical examination. There was no difference in age and sex among patients with CRC, AAs and the HCs.

Sample processing. The CRC cell lines (HCT8, HT-29, HCT116, SW480 and SW620) and normal FHC cells were purchased from Shanghai Institute of Biological Sciences. Cells were cultured in DMEM supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. Following histological confirmation, tissue samples were immediately stored in liquid nitrogen (-196˚C), while serum samples collected from venous blood were centrifuged at 3,500 x g at 4˚C for 10 min and stored at -80˚C for subsequent experimentation. All cell lines were authenticated via the Short Tandem Repeat profiling method.

Cell transfection. miR-592 inhibitor (5'-ACATCATCG CATATTGACACAA-3') and corresponding non-targeting sequence (5'-TTCTCCGAACGTGTCACGTTTC-3') were obtained from Shanghai GenePharma Co., Ltd. HCT116 and SW480 cells at $5x10^5$ density were transfected with miR-592 inhibitor or corresponding non-targeting sequence at a final concentration of 100 μM by using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instruction. CRC cells that were transfected with miR-592 inhibitor and corresponding non-targeting sequence were classified as the inhibitor group and negative control (NC) group, respectively. After transfection, the expression of miR-592 in the media of CRC cells were assessed by RT-qPCR every 12 h. The differential expression of miR-592 between these two groups was analyzed 48 h after transfection.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from CRC tissues, sera and media using TRIzol® LS reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. *Caenorhabditis elegans* miR-39 (Shanghai GenePharma Co., Ltd.) was added to each serum sample at a final concentration of $1x10^{-4}$ pmol/μl, which served as the external reference. RT-qPCR was performed using the Hairpin-it™ miRNA RT-PCR Quantitation kit (Shanghai GenePharma Co., Ltd.), according to the manufacturer's protocol. The primer sequences were as follows: miR-592, forward: 5'-ACGTTGTGTCAATATGCGATGA-3' and reverse: 5'-GTGCAGGGTCCGAGGT-3'; miR-39, forward: 5'-ATATCATCTCACCGGGTGTAATC-3', and reverse: 5'-TATGGTTTTGACGACTGTGTGAT-3'. The following thermocycling conditions used for qPCR were as follows: Initial denaturation at 95˚C for 3 min, 40 cycles of denaturation at 95˚C for 15 sec, annealing and elongation at 62˚C for 34 sec. Relative miR-592 expression was measured using the $2^{-\Delta\Delta Cq}$ method (20) and normalized to the internal reference gene, U6 small nuclear RNA. The primer sequences of U6 were as follows: Forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Statistical analysis. Statistical analysis was performed using SPSS (version 22.0; SPSS, Inc.) and GraphPad Prism (version 8; GraphPad Software, Inc.) software. The association between miR-592 expression and clinicopathological characteristics was assessed using the χ^2 test. Differential expression of miR-592 was determined using Student's paired or unpaired t-test. The comparisons among multiple groups were analyzed using Tukey's post hoc test. The receiver operating characteristic (ROC) curve and area under the curve (AUC) were established to determine the diagnostic value of serum miR-592. Cut-off values of serum miR-592 were determined using Youden's index. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Association between serum miR-592 expression and clinicopathological characteristics in patients with colorectal cancer (n=134).

Characteristic	Patient, n	miR-592 expression		P-value
		High	Low	
Age, years				
<65	53	24	29	
≥65	81	43	38	0.480
Sex				
Male	88	46	42	
Female	46	21	25	0.590
Location				
Colon	98	51	47	
Rectum	36	16	20	0.560
Tumor size, cm				
<5	60	23	37	
≥5	74	44	30	0.024
Differentiation				
High/middle	65	27	38	
Low	69	40	29	0.110
TNM stage				
I-II	84	35	49	
III-IV	50	32	18	0.020
Lymphatic metastasis				
No	65	29	36	
Yes	69	38	31	0.300
Distant metastasis				
No	63	25	38	
Yes	71	42	29	0.027

miR, microRNA; TNM, tumor-node-metastasis.

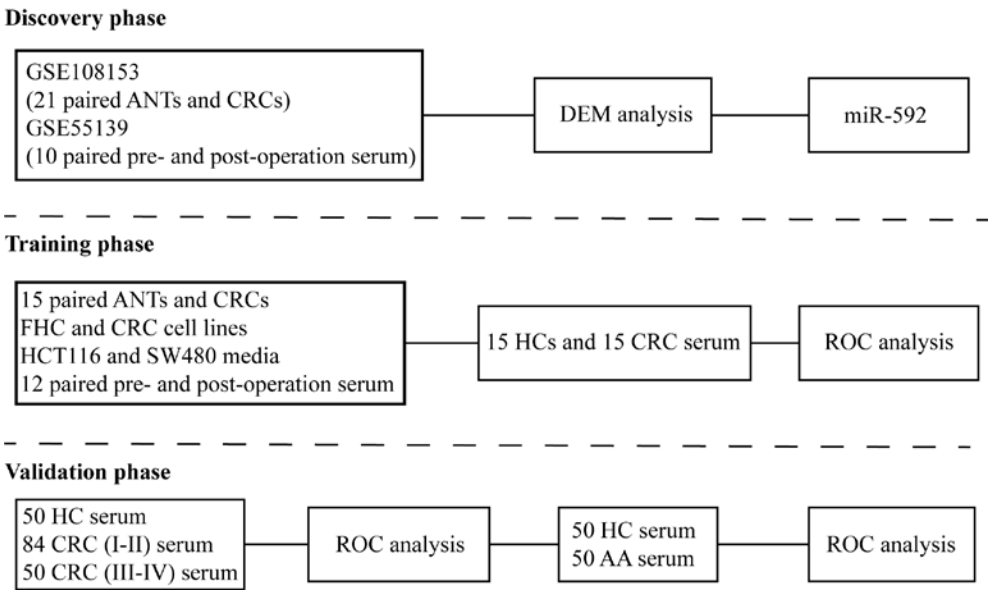

Figure 1. Flow diagram of the study design. ANT, adjacent normal tumor; CRC, colorectal cancer; FHC, fetal human colon; DEM, differentially expressed miRNA; miR, microRNA; HC, healthy control; ROC, receiver operating characteristic; AA, advanced colorectal adenoma.

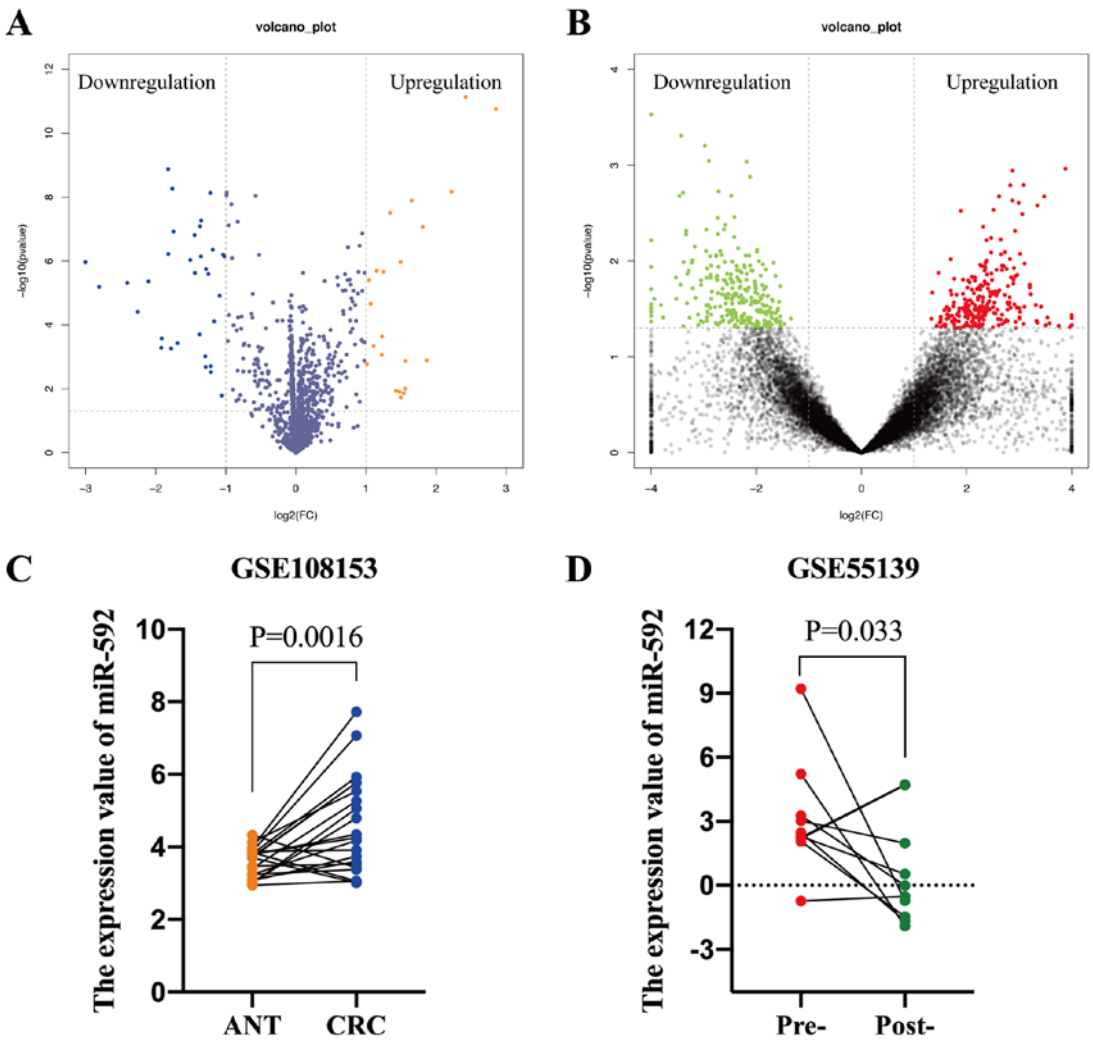

Figure 2. Integrated analysis of the Gene Expression Omnibus datasets, with miR-592 as the selected candidate. (A) Dysregulated miRNAs in CRC tissues based on the GSE108153 dataset. Blue and orange represent downregulation and upregulation, respectively. (B) Dysregulated miRNAs in the serum of patients with CRC following surgical excision of the tumor, based on the GSE55139 dataset. Green and red represent downregulation and upregulation, respectively. miR-592 expression in CRC tissues compared with ANTs in the (C) GSE108153 dataset and (D) GSE55139 dataset. miR/miRNA, microRNA; CRC, colorectal cancer; ANT, adjacent normal tumor; FC, fold change.

Results

Dysregulated serum miR-592 expression may be a tumor-derived miRNA in patients with CRC. The GEO database was searched using keywords, such as 'miRNA' and 'colorectal cancer', in order to identify notably differentially expressed miRNAs (DEMs) in CRC, of which two datasets were acquired. The GSE108153 dataset [Agilent-046064 Unrestricted_Human_miRNA_V19.0_ Microarray (GPL19730) platform] (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108153) contained 21-paired CRC tissues and ANTs. The GEO2R online tool was used to analyze the DEMs, which identified 22 upregulated and 33 downregulated miRNAs in CRC tissues compared with ANTs, respectively (Fig. 2A). The GSE55139 dataset [Agilent-021827 Human miRNA Microarray G4470C (GPL14767) platform] (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi), which consisted of 10-paired pre- and post-operative serum samples of patients with CRC, was used to determine whether the elevated miRNAs were secreted into the peripheral blood by tumor cells. Notably, 248 miRNAs were downregulated following surgical resection of the tumor tissues (Fig. 2B). Furthermore, miR-592 expression levels were significantly upregulated in CRC tissues (P=0.0016) and pre-operative serum samples (P=0.033), which decreased following surgical excision (Fig. 2C and D). Taken together, these results suggest that dysregulated serum miR-592 may be a tumor-derived miRNA in patients with CRC.

Tumor-derived miR-592 is notably elevated in the serum of patients with CRC. miR-592 expression was determined across all CRC tissues and cell lines. RT-qPCR analysis demonstrated that miR-592 expression was significantly upregulated in both CRC tissues and cell lines compared with ANTs and FHC cells, respectively (all P<0.05) (Fig. 3A and B). Subsequently, miR-592 expression was assessed in the 10 paired pre- and post-operative serum samples of patients with CRC (P<0.001), which demonstrated that miR-592 expression significantly decreased following surgical excision of the tumors (Fig. 3C). miR-592 expression was also analyzed in cultured media of CRC cells (HCT116 and SW480 cells). Increased expression of miR-592 in media was dependent on time in culture, and

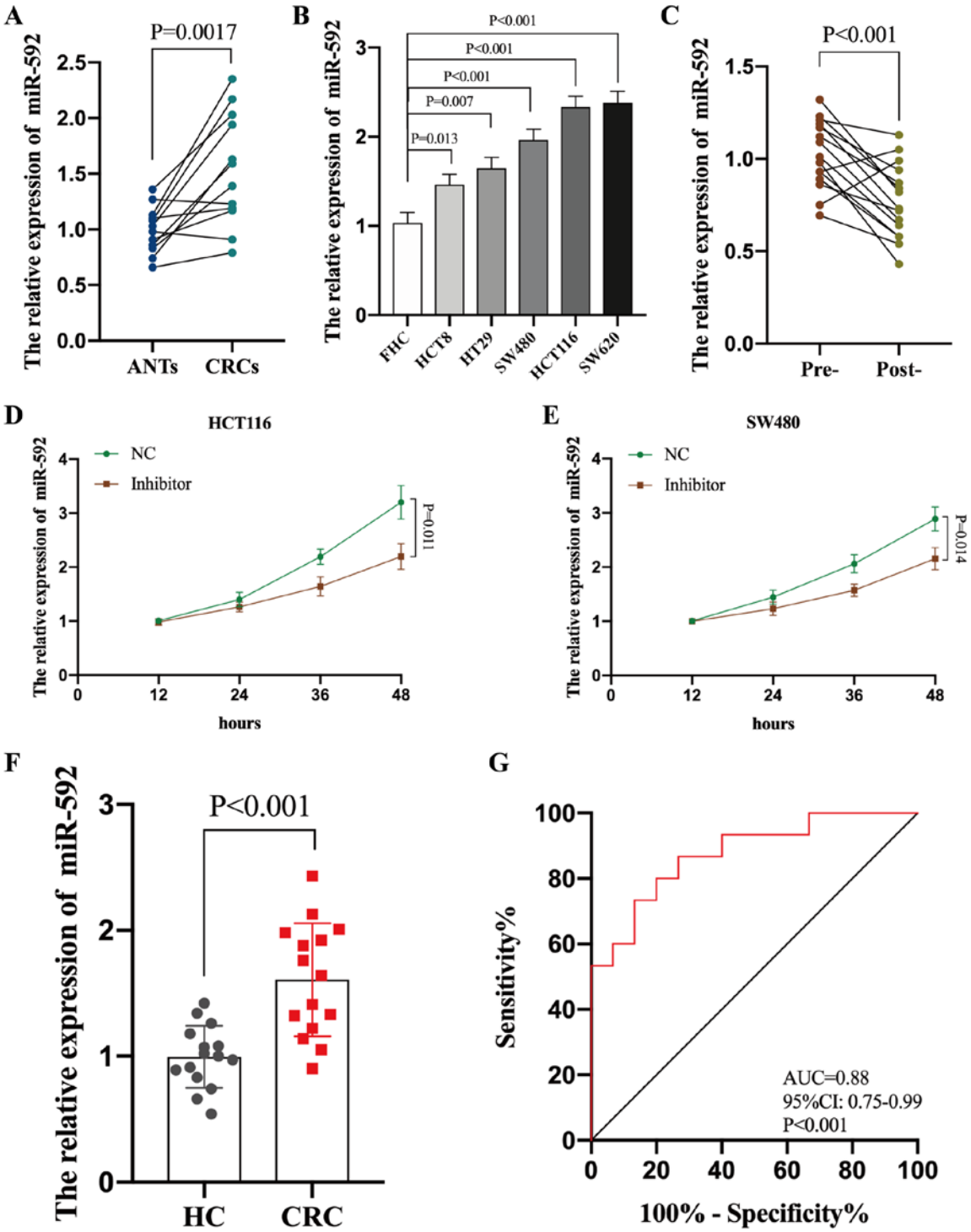

Figure 3. Upregulated serum miR-592 expression is tumor-derived in CRC. (A) miR-592 expression was significantly upregulated in CRC tissues compared with ANTs. (B) miR-592 expression was significantly upregulated in CRC cell lines compared with FHC cells. (C) miR-592 expression was significantly downregulated following surgical excision of the tumor. miR-592 expression levels in (D) HCT116 and (E) SW480 cells secreted intracellular miR-592 into the culture media. (F) miR-592 expression was significantly upregulated in the serum of patients with CRC compared with HCs. (G) Receiver operating characteristic curve of serum miR-592, differentiating patients with CRC from healthy controls. miR, microRNA; CRC, colorectal cancer; ANT, adjacent normal tumor; FHC, fetal human colon; HC, healthy control; AUC, area under the curve; NC, negative control.

CRC cells released less miR-592 into media after intracellular suppression with miR-592 inhibitor (P<0.05, Fig. 3D and E).

Increasing evidence suggests that several tumor-derived miRNAs are significantly dysregulated in the peripheral blood of patients which can be used to differentiate patients from healthy individuals, with a high diagnostic value (21-23). A total of 30 serum samples collected from 15 healthy individuals and 15 patients with CRC were analyzed to determine whether serum miR-592 expression may be used to diagnose patients with CRC. RT-qPCR analysis indicated that serum miR-592 expression was significantly upregulated in patients with CRC compared with HCs (P<0.001) (Fig. 3F). Furthermore, ROC analysis

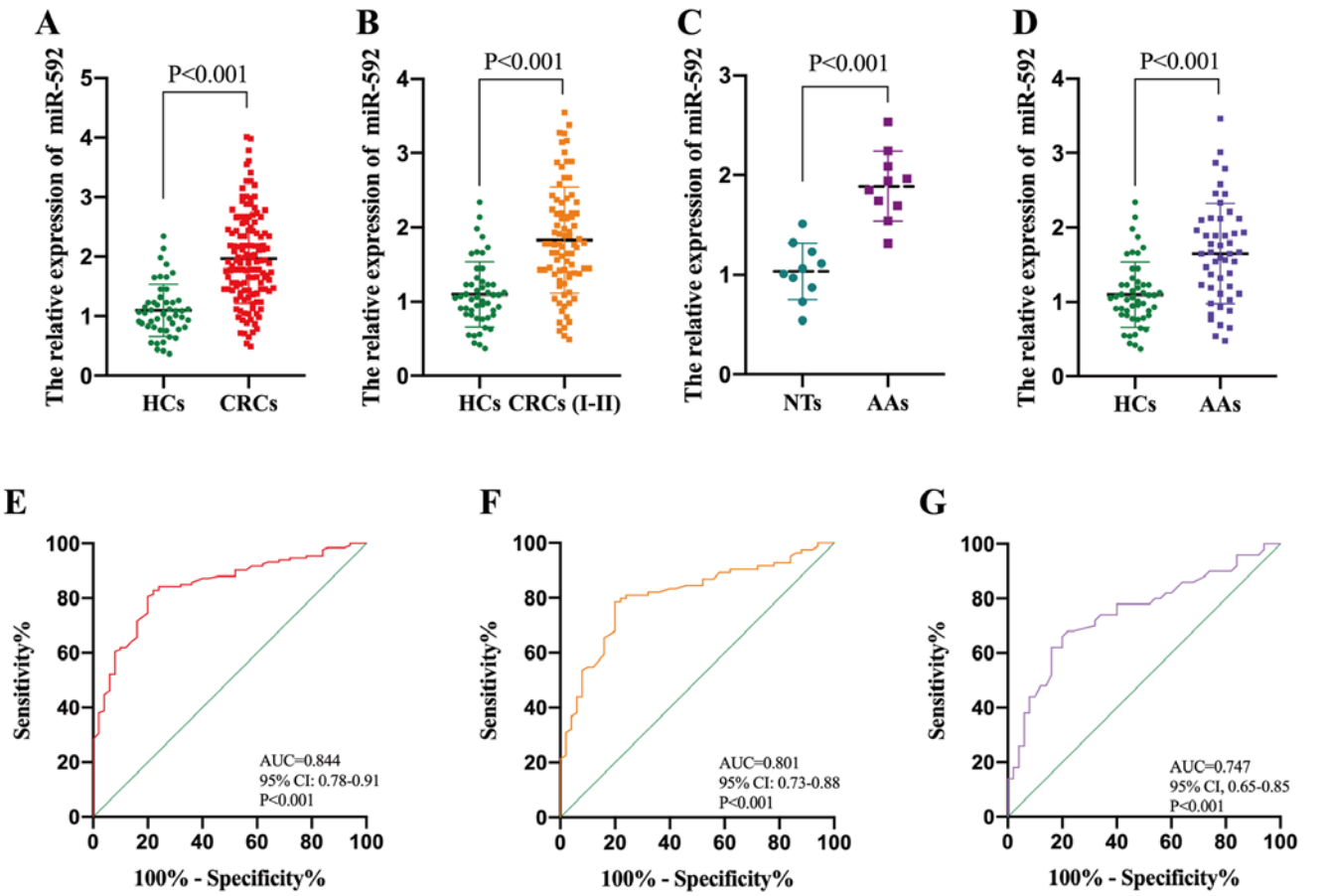

Figure 4. Upregulated serum miR-592 expression is a novel diagnostic biomarker for CRC. Serum miR-592 expression levels were significantly upregulated in (A) CRCs compared with HCs and (B) during early stages of CRC. Relative miR-592 expression levels were significantly upregulated in (C) AA tissues and (D) in the serum of patients with AA. Receiver operating characteristics curve analysis was performed on serum miR-592 to differentiate HCs from patients with CRC at (E) TNM stage I-IV, (F) TNM stage I-II and (G) patients with advanced adenoma. miR, microRNA; CRC, colorectal cancer; HC, healthy control; AA, advanced colorectal adenoma; NT, normal tissue.

demonstrated that serum miR-592 expression may be used to differentiate patients with CRC from HCs, with high sensitivity (86.6%) and specificity (73.4%), with an AUC value of 0.88 (95% CI, 0.75-0.99; P<0.001) (Fig. 3G). Taken together, these results suggest that elevated serum miR-592 expression may be a novel and potential diagnostic biomarker for patients with CRC.

Serum miR-592 is a novel potential biomarker for early diagnosis of CRC. In order to validate the diagnostic value of serum miR-592 in CRC, another independent cohort containing 134 patients with CRC and 50 HCs was assessed. Consistently, serum miR-592 expression was significantly upregulated in patients with CRC compared with HCs (Fig. 4A). ROC analysis demonstrated that serum miR-592 expression may be used to differentiate patients with CRC from HCs, with high sensitivity (82.8%) and specificity (78.0%), and an AUC value of 0.844 (95% CI, 0.78-0.91; P<0.001) (Fig. 4E). In addition, patients with CRC were classified into high group and low groups, according to the median value of miR-592 expression (1.91). The association between serum miR-592 expression and clinicopathological characteristics of patients with CRC indicated that elevated serum miR-592 expression was significantly associated with large tumor size, advanced TNM stage and distant metastasis (Table I). It has been reported that ~90% of CRC-associated mortalities are preventable if patients are diagnosed at an early stage (24). Thus, miR-592 expression levels in the serum of HCs and patients with stages I-II of CRC were analyzed, in order to determine the value of serum miR-592 as an early diagnostic biomarker for CRC. The results demonstrated that miR-592 expression increased in the peripheral blood of patients with stages I-II of CRC (Fig. 4B), which may be used to differentiate patients at an early stage of CRC from HCs, with high sensitivity (78.6%) and specificity (80.0%), and an AUC value of 0.801 (95% CI, 0.73-0.88; P<0.001) (Fig. 4F).

CRC typically develops in a progressive manner, from normal colon epithelial cells, to adenomas and ultimately to malignant cancer lesions (25). This led to investigating the association between serum miR-592 expression and patients with AAs. RT-qPCR analysis demonstrated that miR-592 expression was significantly upregulated in AA tissues compared with normal tissues (NTs) (Fig. 4C). Furthermore, serum miR-592 expression was significantly upregulated in patients with AA compared with HCs (Fig. 4D). ROC analysis indicated that serum miR-592 expression may be used to differentiate patients with AA from HCs, with high sensitivity (68.6%) and specificity (78.1%), and an AUC value of 0.747 (95% CI, 0.65-0.85; P<0.001) (Fig. 4G). There was no difference in age, sex and drinking status among the patients with AA and CRC patients. Taken together, these results suggest that serum miR-592 is a potential biomarker for early diagnosis of CRC.

Discussion

The present study identified serum miR-592 as a tumor-derived miRNA, which was significantly upregulated in patients with CRC and AA. The results of the present study suggest that circulating miR-592 may be used to differentiate patients with CRC and AA from healthy individuals, with high value. Thus, serum miR-592 is implicated as a novel potential biomarker for early diagnosis of patients with CRC.

The biological impact of miR-592 has been reported across several malignancies, including breast, gastric and non-small cell lung cancer (26-28); however, whether miR-592 takes on the role of an oncogene or tumor suppressor is dependent on the tumor context. For example, miR-592 has been reported to be significantly downregulated in glioma, suppressing the development of glioma by regulating Rho-associated protein kinase (29). However, He *et al* (27) demonstrated that miR-592 is upregulated in gastric cancer (GC), promoting GC cell proliferation, migration and invasion, while inducing endothelial-to-mesenchymal transition via the phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathways. miR-592 has been reported to function as an oncogene in CRC (30), whereby upregulation of miR-592 is associated with poor prognosis in patients with CRC (31). Consistent with the results of the present study, Liu *et al* (31) also reported that miR-592 expression is upregulated in clinical CRC serum samples. To the best of our knowledge, the role of serum miR-592 as a novel diagnostic biomarker for CRC has not been previously investigated. Using independent cohorts, the present study demonstrated that serum miR-592 may be used to differentiate patients at early stages of CRC and patients with AA from HCs, with high diagnostic value. Furthermore, the sensitivity and specificity of serum CEA (55 and 66%), CA19-9 (36 and 71%) and CA72-4 (25 and 66%) (32) are lower than those for serum miR-592, respectively. Since dysregulated miR-592 in CRC tissues was associated with poor prognosis of patients with CRC and elevated serum miR-592 was demonstrated to be tumor-derived (30), it is hypothesized that serum miR-592 may have the ability to predict the prognosis of patients with CRC in a non-invasive manner.

The present study posed several limitations. First, the number of clinical samples was small. Prospective studies with larger sample sizes are required to verify the function of circulating miR-592 as a novel diagnostic biomarker for CRC. Furthermore, the clinical data of patients with CRC, particularly regarding the carcinoembryonic antigen, CA19-9 and CA72-4 were limited. It is speculated that the combination of currently available tumor biomarkers with miR-592 may improve the diagnostic value or sensitivity and specificity for patients with CRC. Previous studies have reported that tumor cells secrete miRNAs into circulation via exosomes (33-35); however, this phenomenon was not investigated in the present study. Thus, future studies will aim to determine whether CRC cells have the ability to release miR-592.

In conclusion, the results of the present study suggest that serum miR-592 may be implicated as a novel potential biomarker for the early and non-invasive detection of CRC.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available in the GEO repository, [http://www.ncbi.nlm.nih.gov/geo].

Authors' contributions

LM designed the present study and drafted the initial manuscript, while ZP acquired the clinical samples and performed RT-qPCR. Both LM and ZP performed statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research and Ethical Committee at Second Affiliated Hospital of Nanjing Medical University (approval no. 2015-KY-040, Nanjing, China) and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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