Circulating MiR-1290 as a Potential Diagnostic and Disease Monitoring Biomarker of Human Gastrointestinal Tumors

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Abstract

Background

Gastrointestinal tumors are a leading cause of mortality worldwide. Our previous research demonstrated that miR-1290 is overexpressed in colorectal cancer (CRC) and promotes tumor progression. We therefore aimed to explore the potential of circulating miR-1290 as a biomarker for gastrointestinal cancer.

Methods

Serum miRNA sequence analysis was performed. Then, circulating miRNA detection technologies were established. miR-1290 expression levels in gastrointestinal tumor cell lines and cellular supernatants were analyzed. Expression levels of circulating miR-1290 in clinical samples were examined. Associations between miR-1290 expression and clinicopathologic characteristics were analyzed. Xenograft models were conducted to assess the fluctuation of serum miR-1290 during disease progression.

Results

Through miRNA sequencing, we identified that miR-1290 was overexpressed in CRC serum. We confirmed that human gastrointestinal tumor cells express and secret miR-1290. Circulating miR-1290 was up-regulated in pancreatic cancer (PC) ($p < 0.01$), CRC ($p < 0.05$), and gastric cancer (GC) ($p < 0.01$). High expression levels of miR-1290 were associated with tumor size, lymphatic invasion, vascular invasion, distant metastasis, tumor differentiation and AJCC stage in PC and CRC. The area under the curve (AUC) was 0.8857 in PC, with 60.9% sensitivity and 90.0% specificity. The AUC was 0.7852 in CRC, with 42.0% sensitivity and 90.0% specificity. In GC, the AUC was 0.6576, with 26.0% sensitivity and 90.0% specificity. The *in vivo* model verified that circulating miR-1290 levels significantly increased after tumor formation and decreased after drug treatment.

Conclusions

Our findings demonstrate that circulating miR-1290 is a potential diagnostic and disease monitoring biomarker in gastrointestinal cancer.

Background

Gastrointestinal tumors are the most common cancers worldwide[1, 2]. Colorectal cancer (CRC) and pancreatic cancer (PC) have the highest incidence and mortality among gastrointestinal tumors in the United States[1]. Compared to western countries, China has a similar incidence of CRC but a significantly higher incidence of liver cancer and gastric cancer (GC). In 2018, the gastrointestinal cancer-related deaths (stomach, liver, and esophagus cancer) accounted for 36.4% of tumor-related deaths in China, while digestive cancer deaths made up less than 5% of total cancer deaths in western countries[3]. This may be related to the low early detection rate in China and the lack of uniformity of clinical treatment strategies in different regions. Therefore, population-based tumor screening can significantly increase the tumor

detection rate, reduce tumor-related mortality, and improve patient prognosis. An effective screening method with high population coverage and compliance could have great clinical significance.

MicroRNAs (miRNAs) are non-coding, single-stranded, small RNAs of approximately 19-23 nucleotides, which widely exist in various organisms. miRNAs directly affect the cellular stability of messenger RNA (mRNA), thereby regulating gene expression at the post-transcriptional level and forming complex regulatory networks in cell proliferation, differentiation, apoptosis, homeostasis and stress response[4]. Extracellular/circulating miRNAs exist in various biological fluids, such as serum, plasma, saliva, urine, cerebrospinal fluid, and breast milk. They are delivered to target cells and act as autocrine, paracrine, and/or endocrine modifiers to affect cell activity[5]. Research has shown that circulating miRNAs are potential diagnostic and prognostic biomarkers in various diseases[6].

An appropriate detection method is an essential step for liquid biopsy based on cell-free miRNA. Extraction efficiency, stability of internal controls and methodological accuracy all need to be considered before clinical application[7]. Although numerous studies have been conducted to screen and identify circulating miRNAs as efficient biomarkers for specific types of tumors, it is of great importance to evaluate the diagnostic value of a certain biomarker in different tumors, considering the broad spectrum of tumor markers.

Our previous research demonstrated that miR-1290 is overexpressed in clinical tumor tissues of colon cancer and promotes tumor progression. However, whether miR-1290 can serve as a marker for early diagnosis and disease monitoring is unclear. In this study, the miR-1290 expression in serum of gastrointestinal tumors was analyzed through miRNA sequencing, and a circulating miRNA detection method was built and evaluated. The diagnostic value of miR-1290 was verified in different tumors (including PC, CRC, and GC), and clinical characteristics of patients were collected to study the correlation between miR-1290 expression and clinicopathologic features. Meanwhile, a xenograft tumor model was established to explore the role of serum miR-1290 in disease surveillance. Therefore, we aimed to explore the potential of circulating miR-1290 as a biomarker for gastrointestinal cancer.

Methods

Patients and Clinical Specimens

In this study, blood samples from 46 PC patients, 50 CRC patients, 50 GC patients, and 50 healthy individuals were obtained from patients at the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China, between January 2017 and December, 2019. Tumor tissues and adjacent non-tumor tissues were collected from ten CRC patients. All gastrointestinal patients enrolled were at an initial diagnosis of tumors and were confirmed by pathological diagnosis later.

Sample Processing

Blood samples were obtained by venipuncture. Serum samples were collected from whole blood in coagulation tubes (BD, New Jersey, USA), and plasma samples were processed from whole blood collected

in an EDTA anticoagulation tubes (BD, New Jersey, USA). Blood samples were stored at 4 ℃ and processed within 4 h. The sample was centrifuged at 1900 g (3000 rpm) at 4 ℃ for 10 mins, and the supernatant was collected in a new centrifuge tube and centrifuged again at 16000 g at 4 ℃ for 10 mins to remove the residual nucleic acid attached to the cell debris. Serum/plasma samples that could be examined on the same day were stored at 2-8 ℃, and samples requiring long-term storage were stored at -80 ℃.

miRNA Sequencing

Three pairs of serum samples of CRC and healthy controls, with matched clinical characteristics such as age, gender, and past history were selected for miRNA sequencing. Differentially expressed miRNAs based on normalized deep-sequencing counts were analyzed by Student's t test. The screening criteria were fold change > 2 and $p < 0.01$. The volcano map, heatmap and cluster analysis were conducted through online analysis tools (https://www.omicstudio.cn/tool).

Cell Culture

The PC cells AsPC-1, BxPC-3, and SW1990; the CRC cells HT-29, HCT116, RKO, SW620, and SW480; and the GC cells SGC7901 and BGC823 were purchased from the cell bank of the Shanghai Branch of the Chinese Academy of Sciences. Primary CRC P4 cell was established in our laboratory from primary colorectal cancer tissues. AsPC-1, BxPC-3, SGC7901, and BGC823 cells were cultured in RPMI-1640 medium (Corning, New York, USA); SW1990, SW620, and SW480 cells were cultured in Leibovitz's L-15 medium (Corning, New York, USA); and HT-29 and HCT116 cells were cultured in McCoy's 5A medium (Corning, New York, USA). RKO was cultured in Minimum Essential Medium (MEM) (Corning, New York, USA). All cell culture media were supplemented with penicillin G (100U/ml), streptomycin (100ug/ml) and 10% fetal bovine serum (FBS) and grown 37 ℃ with 5% CO2.

Internal and Exogenous Controls

hsa-miR-16-5p (*homo sapiens*; 5'-UAGCAGCACGUAAAUAUUGGCG-3') and cel-miR-39 (*caenorhabditis elegans*; 5'-UCACCGGGUGUAAAUCAGCUUG-3') (RIBOBIO, Guangzhou, China) were used as internal and exogenous controls, respectively. Then, 1nmol of cel-miR-39 standard was dissolved in 50μl nuclease-free water to obtain a 20 μM stock solution. The stock solution was diluted to 10 nM, and 5 μl of 10 nM cel-miR-39 standard was added to each 200 μl volume of sample during circulating miRNA extraction.

Isolation of Cell-Free miRNA and Total RNA

Cell-free miRNA (including circulating miRNA and cell-free miRNA in the cell supernatant) was isolated from 200 μl of serum or plasma using five different commercially available extraction kits or reagents (Table S1) following the manufacturer's protocol. Total RNA was extracted from tissue samples and cultured cells using TRIzol reagent (Invitrogen, CA, USA).

Reverse Transcription

Total miRNA pools were used as the template for cDNA synthesis using the miRNA First Strand cDNA Synthesis Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The following conditions were used: 16 ℃ for 30 mins, 37 ℃ for 30 mins, and 85 ℃ for 5 mins. The reverse transcription primer for hsa-miR-1290 was as follows: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCCTG-3' (Sangon Biotech, Shanghai, China); the reverse transcription primer for hsa-miR-16-5p was as follows: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3' (Sangon Biotech, Shanghai, China); the reverse transcription primer for cel-miR-39 was as follows: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGCTGA-3' (Sangon Biotech, Shanghai, China); the reverse transcription primer for U6 was as follows: 5'- CGCTTCACGAATTTGCGTGTCAT-3' (Sangon Biotech, Shanghai, China).

Quantitative Real-Time PCR

Quantitative real-time PCR analyses were performed on a CFX connect system (Bio-Rad Laboratories) with Taq PCR Mix (Sangon Biotech, Shanghai, China). The following cycling conditions were used: 95 ℃ for 150 secs; 40 cycles of 95 ℃ for 15 secs, 60 ℃ for 30 secs, and 72 ℃ for 60 secs; 72 ℃ for 10 mins. Three biological replicates were completed for all samples. The primers for hsa-miR-1290 were as follows: forward, 5'-GCGCGTGGATTTTTGGAT-3'; reverse, 5'-AGTGCAGGGTCCGAGGTATT-3'; probe, 5'-FAM-CGCACTGGATACGACTCCCT-TAMRA-N-3' (Sangon Biotech, Shanghai, China). The primers for hsa-miR-16-5p were as follows: forward, 5'- CGCGTAGCAGCACGTAAATA-3'; reverse, 5'- AGTGCAGGGTCCGAGGTATT -3'; probe, 5'-FAM-CGCACTGGATACGACCGCC-TAMRA-N-3' (Sangon Biotech, Shanghai, China). The primers for cel-miR-39 were as follows: forward, 5'-GGCGTCACCGGGTGTAAA-3'; reverse, 5'-AGTGCAGGGTCCGAGGTATT-3'; probe, 5'-FAM-CAGCTTGGTCGTATCCAGTGCG-TAMRA-N-3' (Sangon Biotech, Shanghai, China). The primers for U6 were as follows: forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; reverse, 5'- CGCTTCACGAATTTGCGTGTCAT-3'; probe, 5'-FAM-CAGAGAAGATTAGCATGGCCCCTG-N-3' (Sangon Biotech, Shanghai, China). Finally, the expression level of miR-1290 was analyzed using the 2-ΔΔCt method, using hsa-miR-16-5p or cel-miR-39 as an internal or exogenous control.

In Vivo Xenograft Mouse Model

Female Balb/c nude mice at 4-6 weeks of age and weighing 18-20 g were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. The Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine approved the animal experiments. HT29 cells in the logarithmic growth phase were collected, adjusted to a cell density of 5×10^7/ml, and injected into the subcutaneous tissue of nude mice at 0.1 ml/each. Overall, 15 mice were randomly divided into three groups with 5 mice in per group (the control group, the tumorigenic group without 5-Fu treatment, and the tumorigenic group with 5-Fu treatment) and raised in a specific-pathogen-free (SPF) environment. In addition to the control group, the other two groups were inoculated with tumor cells. After 2-3 weeks, when planted tumors had grown to a certain size (approximately 100 mm^3), one group was intravenously injected with 5-Fu ($100mg\cdot kg^{-1}\cdot d^{-1}$) every 3 days, while the other group was treated with the same amount

of saline. The length and width of the tumors were measured every 3 days by a researcher not aware of the group allocation until the experiment was ended. Tumor volume (V) was calculated according to the formula: V=length/2×width^2. The mice were placed in a 10 L chamber with 99% CO2 (3 L/min) for 5-10 min, and vital signs (including breath, heartbeat, and muscular tension) were closely monitored until the death was confirmed. Serum samples were collected, and expression of circulating miR-1290 was verified by RT-qPCR.

Statistical Analysis

Descriptive statistics were performed to summarize the clinical features of patients. Quantitative data are presented as the means ± standard deviation. The Chi-squared test was used to analyze the association between miR-1290 expression and clinicopathologic characteristics. Differences between two independent groups were assessed by Student's t test. Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA). The charting and statistical analyses were carried out by GraphPad Prism (version 8.0.2) and SPSS (version 23.0). A p value of 0.05 or less was defined as statistically significant. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Results

Up-regulation of miR-1290 in tumor tissues and serum of CRC patients.

Our early research confirmed that miR-1290 expression is highly increased in tumor tissues of CRC patients, and up-regulation of miR-1290 impairs cytokinesis and affects the reprogramming of colon cancer cells. Therefore, miR-1290 plays an important role in CRC progression. To further verify the expression level of miR-1290 in CRC, we collected 10 pairs of tumor tissues and adjacent tissues, and found that miR-1290 was significantly overexpressed in tumor tissues (Figure 1A).

In order to evaluate circulating miR-1290 expression levels, three pairs of CRC and control serum specimens matched for clinical characteristics, such as age, gender, and past history, were selected for miRNA sequencing analysis. The data suggested that compared to healthy controls, there were 656 differentially expressed miRNAs in CRC serum, of which 261 miRNAs were up-regulated and 395 miRNAs down-regulated ($p < 0.01$). miRNAs with very low expression levels were excluded, and volcano graph analysis was performed. Compared with the control group, 94 miRNAs were up-regulated and 104 miRNAs were down-regulated in CRC ($|log2FC| > 1$, $p < 0.01$) (Figure 1B). Based on the miRNA expression profiles, differentially expressed miRNAs with high expression levels were chosen for cluster analysis. As shown in the heatmap, hsa-miR-1290 was significantly overexpressed in CRC serum (Figure 1C), suggesting that miR-1290 might be a potential biomarker for gastrointestinal tumors.

Establishment and evaluation of circulating miRNA detection technologies for clinical identification.

A reliable methodology was needed to analyze circulating miR-1290 expression levels in clinical samples. First, we used different methods of circulating miRNA extraction, including three extraction kits from QIAGEN and two TRIzol reagents from Invitrogen, each following different extraction principles, as shown

in Table S1. We examined the serum miR-1290 expression levels of 20 healthy individuals using cel-miR-39 or miR-16-5p as reference miRNAs (Table S2). We obtained different results when choosing different reference miRNAs, indicating the importance of an appropriate control. The extraction efficiencies of the miRNeasy Serum/Plasma Kit, miRNeasy Serum/Plasma Advanced Kit and TRIzol LS were better than the other two methods. Considering that the miRNeasy Serum/Plasma Advanced Kit and miRNeasy Serum/Plasma Advanced Kit belong to QIAGEN and miRNeasy Serum/Plasma Advanced Kit was an improvement over the miRNeasy Serum/Plasma Kit, we further compared the extraction efficiency of the miRNeasy Serum/Plasma Advanced Kit and TRIzol LS for cell-free miRNA.

The influence of reference miRNA was eliminated by adding the same amount of cel-miR-39 (10μM, 5μl) to the same volume of serum. The results showed that for the same amount of circulating cel-miR-39, the extraction efficiency of the miRNeasy Serum/Plasma Advanced Kit was apparently higher than that of TRIzol LS (figure 2A, $p < 0.0001$). Therefore, the miRNeasy Serum/Plasma Advanced Kit was chosen as the main method for subsequent experiments.

To further evaluate the recovery efficiency of the entire circulating miRNA detection system, we tested miR-1290 standards with different concentrations (100 nM, 10 nM, 1 nM, 10 pM, 1 pM, 0.1 pM) and drew a standard curve ($y = -4.173x + 58.96$, $R^2 = 0.9998$) (Figure 2B). Then, standard solutions of low, middle, and high concentrations were added to the sample solutions and analyzed in the same way. miRNA concentration in the sample was calculated according to the standard curve, and the recovery rate was evaluated. The recoveries of the three groups with low, middle, and high concentration standards were 103%, 116%, and 87.8%, respectively (Table S3), which were within the acceptable range. The influence of interference factors in the serum was also evaluated. We collected three samples with high bilirubin, triglyceride or rheumatoid factor, and analyzed the recovery efficiency as described above. The results showed that the recovery efficiencies of the three groups were 119%, 93.0%, and 107%, respectively (Table S3), which were within the acceptable range of error. Therefore, serum bilirubin, triglycerides and rheumatoid factor levels did not interfere with the miRNA detection method.

miR-16-5p is a commonly used internal control for circulating miRNA detection. Previous studies have shown that miR-16-5p is overexpressed in breast cancer, chronic lymphocytic leukemia, rheumatoid arthritis, and other diseases. In order to verify the reliability of miR-16-5p as an internal control, we collected serum samples from patients with breast cancer and rheumatoid arthritis and evaluated the miR-16-5p expression in different diseases using cel-miR-39 as the exogenous control (Figure 2C). The expression levels of miR-16-5p in the serum of breast cancer patients and rheumatoid arthritis patients were significantly higher than in healthy controls ($p < 0.001$). We also analyzed the Cq values of miR-16-5p and cel-miR-39 in PC patients, GC patients and healthy controls. The results were shown in Figure 2D, Cq values of miR-16-5p in patients with PC and GC patients were much higher than those in healthy controls, while there were no significant differences in Cq values of cel-miR-39, indicating that miR-16-5p expression fluctuates in gastrointestinal tumors and is not suitable as an internal control.

In the final part of the methodological evaluation, we collected plasma and serum samples from 20 healthy individuals to evaluate the differences between miR-1290 expression in serum and plasma sample

from the same individual (Figure 2E-F). We found that miR-1290 expression levels in plasma were higher than those in serum when cel-miR-39 was used as a reference miRNA. In contrast, in some individuals, the expression levels of miR-1290 were higher in the serum than in plasma when miR-16-5p was used as a reference miRNA, which also indicated that miR-16-5p is not a reliable internal control.

Human gastrointestinal tumor cells express and secret miR-1290.

To clarify the source of circulating miR-1290, we examined expression levels of miR-1290 in human gastrointestinal tumor cell lines. As shown in Figure 3A, five CRC cell lines including HT29, HCT116, P4, SW480, and SW620 cells, three PC cell lines including ASPC-1, BXPC-3, and SW1990 cells, and two GC cell lines including SGC7901 and BGC823 cells were analyzed. The expression levels of miR-1290 in GC cell lines were lower than in CRC and PC cell lines. miR-1290 expression was the lowest in BGC823 cells, while miR-1290 expression in HT29, HCT116, P4, ASPC-1, and BXPC-3 cells was relatively high. We further examined miR-1290 expression levels in cellular supernatants of five cell lines (HT-29, HCT116, ASPC-1, BXPC-3 and SGC7901 cells) when cultured at different cell densities for 24 h, 48 h, and 72 h. As shown in Figure 3B-F, gastrointestinal tumor cells expressed and secreted miR-1290 into the cell supernatant, and miR-1290 expression levels in the cell supernatant increased with cell amounts and culture time. Therefore, circulating miR-1290 might derive from tumor cell secretion as a potential tumor biomarker.

Circulating miR-1290 serves as a diagnostic biomarker in gastrointestinal tumors.

In the subsequent validation phase, circulating miR-1290 expression levels were detected in 46 PC patients, 50 CRC patients, 50 GC patients, and 50 healthy controls. The clinical characteristics of participants are summarized in Table 1. There was no statistical difference in gender, BMI, smoking history, or alcohol consumption between the PC group and control group. Patients in the PC group had a higher average age ($p < 0.05$) and a higher proportion of hypertension ($p < 0.05$) and diabetes ($p < 0.05$). Furthermore, the CEA ($p < 0.001$), CA199 ($p < 0.001$), CA125 ($p < 0.001$), CA242 ($p < 0.001$), and CA211 ($p <0.01$) levels in the PC group were significantly higher than those in the control group. There was no significant variation in age, gender, BMI, alcohol consumption or diabetes history between the CRC group and the control group, but the ratio of smoking ($p < 0.05$) and hypertension ($p < 0.05$) was much higher in the CRC group than in the control group. The CEA level ($p < 0.001$) in the CRC group was apparently higher than that in the control group, while expression levels of the other biomarkers were similar to the controls. In addition, there were no statistical differences in age, gender, BMI, smoking history, alcohol consumption, hypertension history, or diabetes history between the GC group and control group. The CEA expression level was higher in the GC group ($p < 0.01$) while the remaining biomarkers were not significantly different from the control group.

As shown in Figure 4A-C, the expression level of circulating miR-1290 was considerably increased in PC ($p < 0.01$), CRC ($p < 0.05$), and GC ($p < 0.01$). Correlations between serum miR-1290 expression levels and clinicopathological features in gastrointestinal cancer patients were explored. In PC patients (Table 2), there was no statistical correlation between circulating miR-1290 expression and age, gender, BMI, smoking history, alcohol consumption, hypertension history, diabetes history, or tumor site. High expression levels of miR-1290 were associated with tumor size ($p < 0.01$), lymphatic invasion ($p < 0.01$), vascular

invasion ($p < 0.05$), distant metastasis ($p < 0.05$), tumor differentiation ($p < 0.05$), and tumor AJCC stage ($p < 0.05$). However, there was no significant relationship between circulating miR-1290 expression level and the other tumor biomarkers including CEA, CA199, CA125, CA242, and CA211. In CRC patients (Table 3), there was no clear association between circulating miR-1290 expression levels and age, gender, BMI, smoking history, alcohol consumption, hypertension history, diabetes history, or tumor site. Circulating miR-1290 expression was significantly higher in CRC patients with larger tumor size ($p < 0.05$), lymphatic invasion ($p < 0.05$), vascular invasion ($p < 0.05$), distant metastasis ($p < 0.05$), poorer tumor differentiation ($p < 0.05$), and more advanced AJCC stage ($p < 0.05$). miR-1290 expression levels were related with CEA ($p < 0.05$), while there was no statistical correlation with levels of CA199, CA125, CA242, or CA211. As for GC patients (Table 4), there was no significant relationship between circulating miR-1290 expression level and age, gender, BMI, smoking, drinking, diabetes, or tumor location. The size of tumor, lymphoid invasion, vascular invasion, distant metastasis, tumor differentiation degree, and AJCC stage, as well as common tumor markers, also showed no remarkable correlation with circulating miR-1290 expression.

To evaluate the potential diagnostic value of circulating miR-1290, receiver operating characteristic (ROC) curve analysis was performed. The area under the curve (AUC) was 0.8857 ($p < 0.0001$) in PC, with 60.9% sensitivity and 90.0% specificity. The AUC was 0.7852 ($p < 0.0001$) for circulating miR-1290 in CRC, with a sensitivity of 42.0% and specificity of 90.0%. In GC, the AUC of miR-1290 was 0.6576 ($p < 0.01$), with a sensitivity of 26.0%, and specificity of 90.0%. Moreover, as shown in Figure 4G-I, combination of circulating miR-1290 and traditional biomarkers had higher diagnostic value. In PC patients, sensitivity and specificity of miR-1290 combined with CA199 were 80.4% and 90.0%, with the AUC value of 0.9626 ($p < 0.0001$). In CRC patients, sensitivity and specificity of miR-1290 combined with CEA were 58.0% and 90.0%, with the AUC value of 0.8348 ($p < 0.0001$). Sensitivity and specificity of miR-1290 combined with CA211 were 38.0% and 90.0% in GC patients, with the AUC value of 0.7788 ($p < 0.0001$). As a result, miR-1290 may serve as a potential diagnostic biomarker for gastrointestinal cancers, with different diagnostic efficiency in different tumors.

miR-1290 is a potential biomarker for gastrointestinal tumor surveillance.

To assess the monitoring value of circulating miR-1290, we explored the changes of miR-1290 expression levels after surgery in 10 CRC patients. As shown in Figure 5A, miR-1290 expression decreased greatly after surgery ($p < 0.05$). Meanwhile, a subcutaneous xenograft model of CRC in nude mice was constructed to evaluate the fluctuation of circulating miR-1290 during disease progression and drug treatment. Circulating miR-1290 significantly increased after tumor formation (Figure 5B). After treatment with 5-Fu, the tumor tissue structure was destroyed, and tumor cells were largely necrotic, with a remarkable reduction of circulating miR-1290 (Figure 5B). Therefore, miR-1290 is a promising biomarker for gastrointestinal tumor surveillance.

Discussion

In this study, we demonstrated that circulating miR-1290 is an effective biomarker for early diagnosis and disease monitoring of gastrointestinal tumors. Based on previous studies, we further confirmed that miR-

1290 is up-regulated in the tumor tissues and serum of CRC patients. miR-1290 was highly expressed in various cell lines of gastrointestinal cancers (PC, CRC, and GC) and could be released into the cellular supernatant. The diagnostic efficiency of circulating miR-1290 was evaluated in PC, CRC, and GC by establishing a reliable detection method of cell-free miRNA. The results indicated that circulating miR-1290 is a potential biomarker in gastrointestinal tumors, and high expression levels are significantly correlated with disease progression.

Valuable early screening and monitoring indicators can improve patient prognosis and reduce the risk of over-treatment resulting from overdiagnosis[3]. Compared with cellular RNA species, circulating miRNAs are highly stable over long-term storage; multiple freeze-thaw cycles; as well as extreme conditions, including boiling, multiple freeze-thaw, treatments with RNase, and high or low pH[8], making it an essential part of liquid biopsy[9]. Our previous research found that miR-1290 is up-regulated in CRC tissues and plays a crucial role in cancer progression[10]. It has been reported that miR-1290 has a tumor-promoting effect and is involved in the occurrence and development of a variety of tumors, such as CRC[11] and esophageal cancer[12]. Furthermore, miRNA sequencing indicated that miR-1290 is highly expressed in the serum of CRC patients, which might be a potential tumor marker.

Cell-free miRNA detection technology is maturing, but many problems still need to be solved before clinical application. For example, a high extraction efficiency should be guaranteed due to the relatively low concentration of circulating miRNA, the integrity of miRNA and the stability of detection. Especially when a single miRNA is chosen as a clinical biomarker, verification of pre-analysis steps affecting miRNA quantification is critically important. Although highly up-regulated or down-regulated miRNAs have advantages as biomarkers, the clinical value for most markers is dependent on solving the above problems. However, many researches have not performed methodological verification[13]. Considering the difference between miRNA expression and molecular mechanism, a detection method applicable to one study is not necessarily suitable for another. Consequently, it is essential to verify and improve the detection methodology (reliability, repeatability, accuracy) for a specific miRNA[14].

To ensure a high extraction efficiency, we selected the extraction kit with the highest extraction efficiency (miRNeasy Serum/Plasma Advanced Kit) as the main detection method for subsequent research among a variety of kits. The main extraction principles of this kit are protein precipitation and silica technology, which reduce the inhibitory effects of interfering substances in the blood on the reverse transcription and PCR procedure. Then, we established a standard curve to analyze the recovery rate, which was within the acceptable error range and not affected by bilirubin, rheumatoid factor or triglyceride in the serum. Thus, our method is reliable. In addition, there still lacks standard normalization methods for circulating miRNA analysis[15]. miR-16-5p is a widely used endogenous control for circulating miRNA normalization[16]. Research has demonstrated that miR-16-5p is up-regulated in numerous diseases, such as rheumatoid arthritis[17], breast cancer[18], and esophageal cancer[19], and serves as a potential disease marker. In our study, we also confirmed that miR-16-5p is overexpressed in the serum of breast cancer and rheumatoid arthritis patients, the expression levels of circulating miR-16-5p fluctuate with changes of disease activity

in gastrointestinal patients, and normalization with miR-16-5p might decrease variability, as discovered in previous research[7]. Therefore, an external control (cel-miR-39) is preferred.

Our study found that the expression level of miR-1290 in the plasma was higher than that in the serum of the same individual. Contamination of blood cells and PCR amplification processes are the main reasons affecting the results[20]. miRNA contamination of blood cells exists during coagulation or the separation of plasma, and hemolysis leads to the release of miRNA in red blood cells[21]. In addition, the anticoagulants and blood stabilizers used in plasma collection also affect the quantitation of circulating miRNA[22]. The plasma collection process affects the recovery rate and accuracy, regardless of the miRNA expression level. It is worth mentioning that high concentration RNA can reduce the interference of plasma components on PCR quantification, indicating that, when cel-miR-39 is used as an exogenous control, the added amount should be kept appropriate and consistent. Catherine Foye *et al* also discovered that the detection results of endogenous and exogenous controls varied in the serum and plasma, with higher expression levels in plasma[23]. Therefore, it is crucial to select the appropriate samples, and standardized blood sample processing procedures reduce the detection errors in clinical analysis.

Many commonly used biomarkers for digestive tract malignant tumors have a broad spectrum. To comprehensively understand the diagnostic value of miR-1290 as a biomarker, we compared its expression level and diagnostic efficiency in PC, CRC, and GC. It was found that miR-1290 expression was statistically higher in PC, CRC, and GC patients than control, with the highest diagnostic efficiency in PC. Previous studies had confirmed the diagnostic value of circulating miR-1290 in gastrointestinal tumors, for example, PC[16, 24], CRC[25, 26], and ESCC[27]. However, these studies were all conducted in one specific tumor, lacking evaluation of the role of miR-1290 in different types of tumors. In our study, high miR-1290 expression in the serum was associated with tumor size, lymphatic invasion, venous invasion, distant metastasis, differentiation, and TNM stage (AJCC) in PC and CRC, while this relationship was not observed in GC. Circulating miR-1290 might be a potential marker for invasiveness and disease progression in PC and CRC, while the correlation with aggressiveness of GC was not high. Patients with high miR-1290 expression levels also had relatively higher CEA levels in CRC, while there was no association between miR-1290 expression and other tumor markers. In PC patients, there was no correlation between miR-1290 expression and other common biomarkers. These results suggested that miR-1290 in serum/plasma is an independent biomarker with a crucial reference value in tumor diagnosis and judgement of a patient's condition. We also explored the role of miR-1290 in disease monitoring. Combining clinical patients and xenograft mouse models, it was concluded that circulating miR-1290 fluctuated during tumor development and medication, playing an important role in real-time tumor progression monitoring. Although there have been similar studies in the past, our study focused on the establishment and verification of the methodology and compared the diagnostic efficiency of miR-1290 in various gastrointestinal tumors. In addition, clinical samples and animal models were combined to ensure the reliability of the results and the clinical significance of research conclusions.

There are several limitations in this study. To begin with, the sample size was small. A large sample from a multicenter study is needed to further confirm the conclusion. Secondly, it will be more convincing to

evaluate the role of circulating miR-1290 in other gastrointestinal tumors. Thirdly, we have not explored the correlation between miR-1290 expression and patient prognosis. We plan to conduct a long-term clinical follow-up to determine whether circulating miR-1290 can serve as a prognostic biomarker. In addition, previous studies have suggested that miR-1290 is involved in the regulation of chemotherapy resistance in a variety of tumors[28]. Ling Ye *et al* discovered that tissue miR-1290 expression levels were positively correlated with dMMR status and predicted the prognosis of CRC patients receiving 5-FU treatment[29]. Circulating miR-1290 was also a potential biomarker for response to 5-FU-based chemoradiotherapy of patients with advanced oral squamous cell cancer[30]. Therefore, the relationship between circulating miR-1290 expression and chemotherapy resistance in gastrointestinal tumors is worthy of study. Finally, the combined diagnosis of multiple different markers also has great research value.

Conclusions

we have established a reliable method for the detection of circulating miRNA, and circulating miR-1290 is a potential diagnostic and disease monitoring biomarker in human gastrointestinal tumors.

Abbreviations

CRC: colorectal cancer; PC: pancreatic cancer; GC: gastric cancer; ROC curve: receiver operating characteristic curve; AUC: area under the curve.

Declarations

Ethics approval and consent to participate

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (No.2015-023). Written informed consent was obtained from all individuals included in this study. The research related to animal use has been complied with the guidelines of Institutional Animal Care and Use Committee (IACUC). The maximum tumor size must not exceed 20 mm (2.0 cm) and this size was not exceeded at any point during the duration of our study. The Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine approved the animal experiments.

Consent for publication

Consent to publish was obtained from all individuals included in this study.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission. CX collected clinical samples, XLY designed the research study, performed the research and wrote the paper, ZYL and CJT revised the manuscript.

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Tables

Table 1. Clinical characteristics of patients included in the study.

Groups	Control	Group 1 (n=46)		Group 2 (n=50)		Group 3 (n=50)	
	(n=50)	Pancreatic cancer	p value	Colorectal cancer	p value	Gastric cancer	p value
Age (year)			**0.016***		0.102		0.151
< 65 y	34	20		26		27	
≥ 65 y	16	26		24		23	
Gender			0.084		0.105		0.841
Male	25	31		33		26	
Female	25	15		17		24	
BMI (kg/m^2)			0.524		0.832		0.663
< 25	34	34		33		36	
≥ 25	16	12		17		14	
Smoke			0.167		**0.021***		0.817
Yes	12	17		23		13	
No	38	29		27		37	
Alcohol			0.478		0.817		1.000
Yes	12	14		13		12	
No	38	32		37		38	
Hypertension			**0.013***		**0.047***		0.727
Yes	10	20		19		12	
No	40	26		31		38	
Diabetes			**0.022***		1.000		0.629
Yes	5	13		5		4	
No	45	33		45		46	
CEA (ng/ml)			**<0.0001*****		**<0.0001*****		**<0.006****
< 5	50	30		33		43	
≥ 5	0	16		17		7	
CA199 (U/ml)			**<0.0001*****		0.617		<0.617
< 37	39	6		41		41	

≥ 37	11	40		9		9	
CA125 (U/ml)			**<0.0001*** **		0.169		<0.137
< 35	46	24		49		41	
≥ 35	4	22		1		9	
CA242 (U/ml)			**<0.0001*** **		0.558		<0.169
< 20	49	16		48		46	
≥ 20	1	30		2		4	
CA211 (ng/ml)			**0.001** **		0.307		0.092
< 5	49	34		47		45	
≥ 5	1	12		3		5	

*** p < 0.05, ** p <0.01, *** p <0.001**

Table 2. Association between circulating miR-1290 expression and clinicopathologic characteristics in 46 pancreatic cancer patients.

Variable	Total (n=46)	miR-1290 expression		p value
		Low (n=13)	High (n=33)	
Age (year)	65.48±1.77	69.15±3.53	64.03±2.01	0.195
Gender				0.975
Male	32	9	23	
Female	14	4	10	
BMI (kg/m^2)	22.01 (19.69-25.40)	23.26 (20.00-26.88)	21.97 (19.16-25.13)	0.518
Smoke				0.057
Yes	17	2	15	
No	29	11	18	
Alcohol				0.164
Yes	14	2	12	
No	32	11	21	
Hypertension				0.818
Yes	20	6	14	
No	26	7	19	
Diabetes				0.813
Yes	13	4	9	
No	33	9	24	
Location				0.894
Head	29	8	21	
Body and tail	17	5	12	
Tumor size (cm)	3.55 (2.32-4.53)	2.30 (1.80-2.80)	4.00 (2.87-4.60)	**0.002****
Lymphatic invasion				**0.003****
Positive	35	6	29	
Negative	11	7	4	
Venous invasion				**0.030***
Positive	32	6	26	
Negative	14	7	7	

Distant metastasis				**0.022***
Positive	23	3	20	
Negative	23	10	13	
Differentiation				**0.036***
Well	7	3	4	
Moderate	22	9	13	
Poor	17	1	16	
TNM stage (AJCC)				**0.022***
I and II	23	10	13	
III and IV	23	3	20	
CRP (mg/l)				0.624
< 10	33	10	23	
≥ 10	13	3	10	
CEA (ng/ml)				0.295
< 5	30	10	20	
≥ 5	16	3	13	
CA199 (U/ml)				
< 37	6	2	4	0.767
≥ 37	40	11	29	
CA125 (U/ml)				0.146
< 35	24	9	15	
≥ 35	22	4	18	
CA242 (U/ml)				0.720
< 20	16	4	12	
≥ 20	30	9	21	
CA211 (ng/ml)				0.299
< 5	34	11	23	
≥ 5	12	2	10	

*** p < 0.05, ** p <0.01**

Table 3. Association between circulating miR-1290 expression and clinicopathologic characteristics in 50 colorectal cancer patients.

Variable	Total (n=50)	miR-1290 expression		p value
		Low (n=23)	High (n=27)	
Age (year)	64.00±1.62	63.26±2.46	64.63±2.19	0.679
Gender				0.057
Male	33	12	21	
Female	17	11	6	
BMI (kg/m^2)	23.83 (21.33-25.54)	24.24 (20.96-27.64)	23.36 (21.45-25.24)	0.483
Smoke				0.741
Yes	23	10	13	
No	27	13	14	
Alcohol				0.990
Yes	13	6	7	
No	37	17	20	
Hypertension				0.309
Yes	19	7	12	
No	31	16	15	
Diabetes				0.777
Yes	5	2	3	
No	45	21	24	
Location				0.555
colon	24	10	14	
rectum	26	13	13	
Tumor size (cm)	3.50 (3.00-3.05)	3.00 (2.30-4.00)	4.00(3.00-5.00)	**0.037***
Lymphatic invasion				**0.019***
Positive	22	6	16	
Negative	28	17	11	
Venous invasion				**0.019***
Positive	22	6	16	
Negative	28	17	11	

Distant metastasis				**0.041***
Positive	16	4	12	
Negative	34	19	15	
Differentiation				**0.025***
Well	1	1	0	
Moderate	39	21	18	
Poor	10	1	9	
TNM stage (AJCC)				**0.047***
I and II	25	15	10	
III and IV	25	8	17	
CRP (mg/l)				0.918
< 10	41	19	22	
≥ 10	9	4	5	
CEA (ng/ml)				**0.022***
< 5	33	19	14	
≥ 5	17	4	13	
CA199 (U/ml)				0.400
< 37	41	20	21	
≥ 37	9	3	6	
CA125 (U/ml)				0.351
< 35	49	23	26	
≥ 35	1	0	1	
CA242 (U/ml)				0.183
< 20	48	23	25	
≥ 20	2	0	2	
CA211 (ng/ml)				0.650
< 5	47	22	25	
≥ 5	3	1	2	

*** p < 0.05**

Table 4. Association between circulating miR-1290 expression and clinicopathologic characteristics in 50 gastric cancer patients.

Variable	Total (n=50)	miR-1290 expression		p value
		Low (n=33)	High (n=17)	
Age (year)	63.74±1.62	65.58±1.78	60.18±3.19	0.115
Gender				0.616
Male	26	18	8	
Female	24	15	9	
BMI (kg/m^2)	22.33 (20.50-25.11)	22.27 (20.38-25.06)	22.48 (20.49-25.50)	0.690
Smoke				0.334
Yes	13	10	3	
No	37	23	14	
Alcohol				0.520
Yes	12	7	5	
No	38	26	12	
Hypertension				**0.031***
Yes	12	11	1	
No	38	22	16	
Diabetes				0.134
Yes	4	4	0	
No	46	29	17	
Tumor size (cm)	4.00 (1.93-5.50)	4.50 (2.00-5.45)	4.00 (0.70-7.00)	0.498
Lymphatic invasion				0.261
Positive	29	21	8	
Negative	21	12	9	
Venous invasion				0.370
Positive	25	18	7	
Negative	25	15	10	
Distant metastasis				0.613
Positive	14	10	4	
Negative	36	23	13	

Differentiation				0.526
Well	9	5	4	
Moderate	12	7	5	
Poor	29	21	8	
TNM stage (AJCC)				0.754
I and II	28	19	9	
III and IV	22	14	8	
CRP (mg/l)				0.218
< 10	47	32	15	
≥ 10	3	1	2	
CEA (ng/ml)				0.235
< 5	43	27	16	
≥ 5	7	6	1	
CA199 (U/ml)				0.410
< 37	41	26	15	
≥ 37	9	7	2	
CA125 (U/ml)				0.963
< 35	41	27	14	
≥ 35	9	6	3	
CA242 (U/ml)				0.692
< 20	46	30	16	
≥ 20	4	3	1	
CA211 (ng/ml)				0.486
< 5	45	29	16	
≥ 5	5	4	1	

*** p < 0.05**

Figures

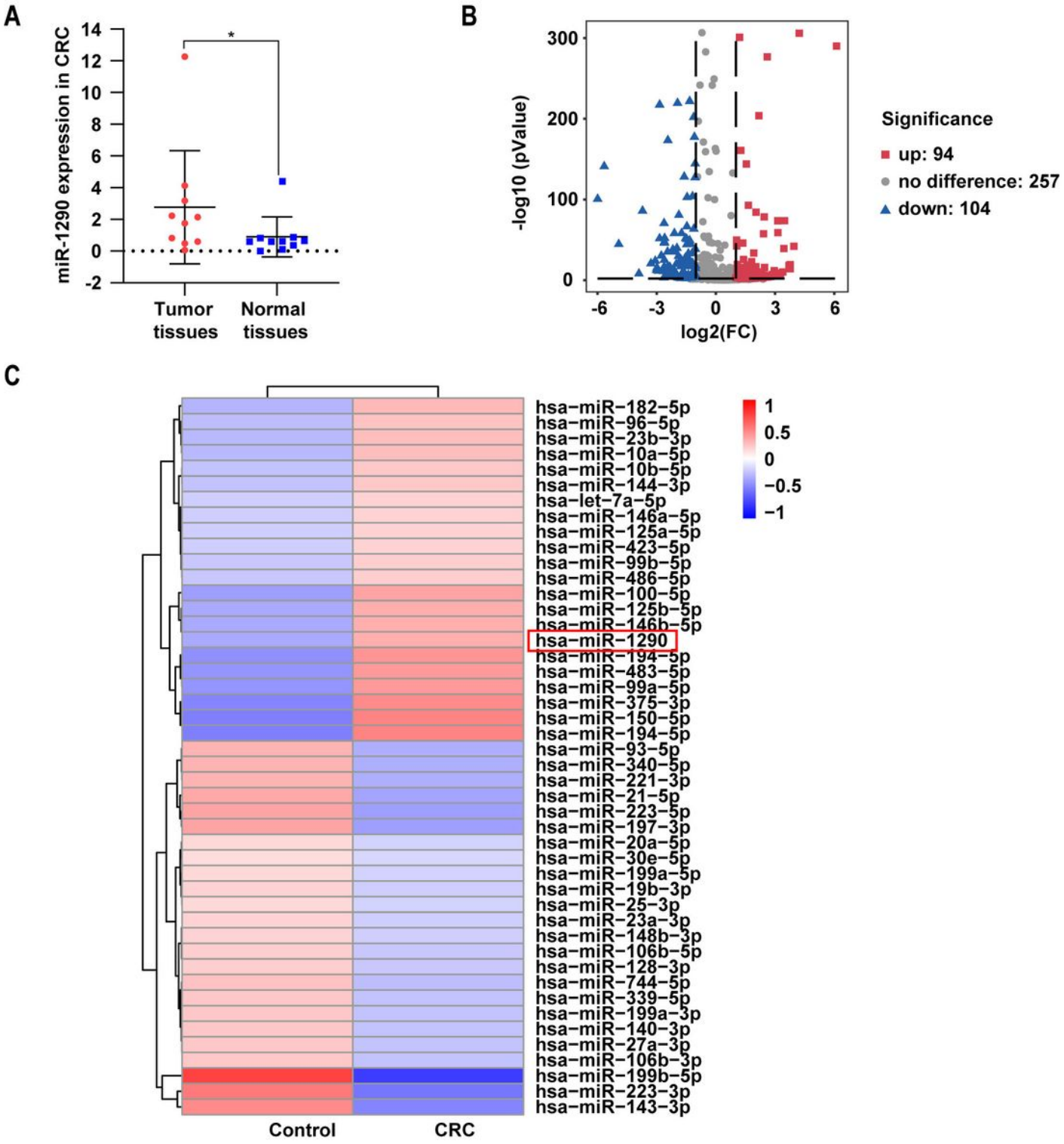

Figure 1

miR-1290 was upregulated in tumor tissues and serum of CRC patients. (a) Expression of miR-1290 in tumor tissues and paired normal tissues (n=10) ($p < 0.05$). (b) Volcano plot of differentially expressed miRNAs ($p<0.01$, $|\log2FC| > 1$). (c) Heatmaps of cluster analysis for differentially expressed miRNAs with high expression levels ($p<0.01$, $|\log2FC| > 1$). Blue color represents a lower expression level and red color represent a higher expression level. * $p < 0.05$.

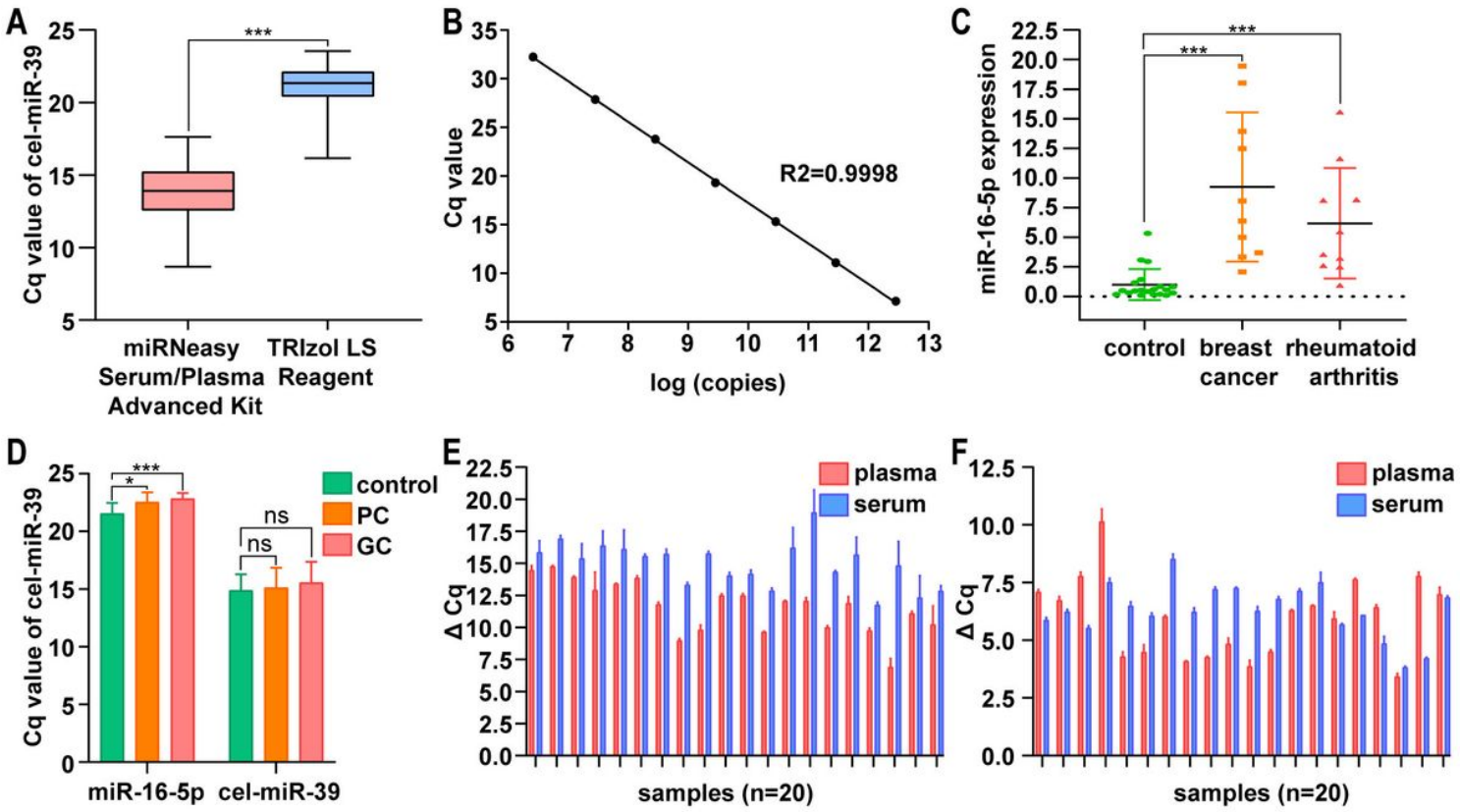

Figure 2

The establishment and evaluation of circulating miRNA detection methodology. (a) Extraction efficiency was compared between the miRNeasy Serum/Plasma Advanced Kit and TRIzol LS by RT-qPCR. (b) The standard curve of miR-1290 standards with different concentrations (100nM, 10nM, 1nM, 10pM, 1pM, 0.1pM), y=-4.173x+58.96, R2=0.9998. (c) Expression levels of circulating miR-16-5p were analyzed in breast cancer (n=10) and rheumatoid arthritis (n=10) by RT-qPCR. (d) Analysis of expression levels of endogenous and exogenous controls in PC, GC and control group by RT-qPCR. (d) Expression levels of miR-1290 were assessed in the serum and plasma of the same individuals by RT-qPCR, with miR-16-5p as an endogenous control. (e) Expression levels of miR-1290 were assessed in the serum and plasma of the same individuals by RT-qPCR, with cel-miR-39 as an exogenous control. * $p < 0.05$, *** $p < 0.001$, ns: no significance.

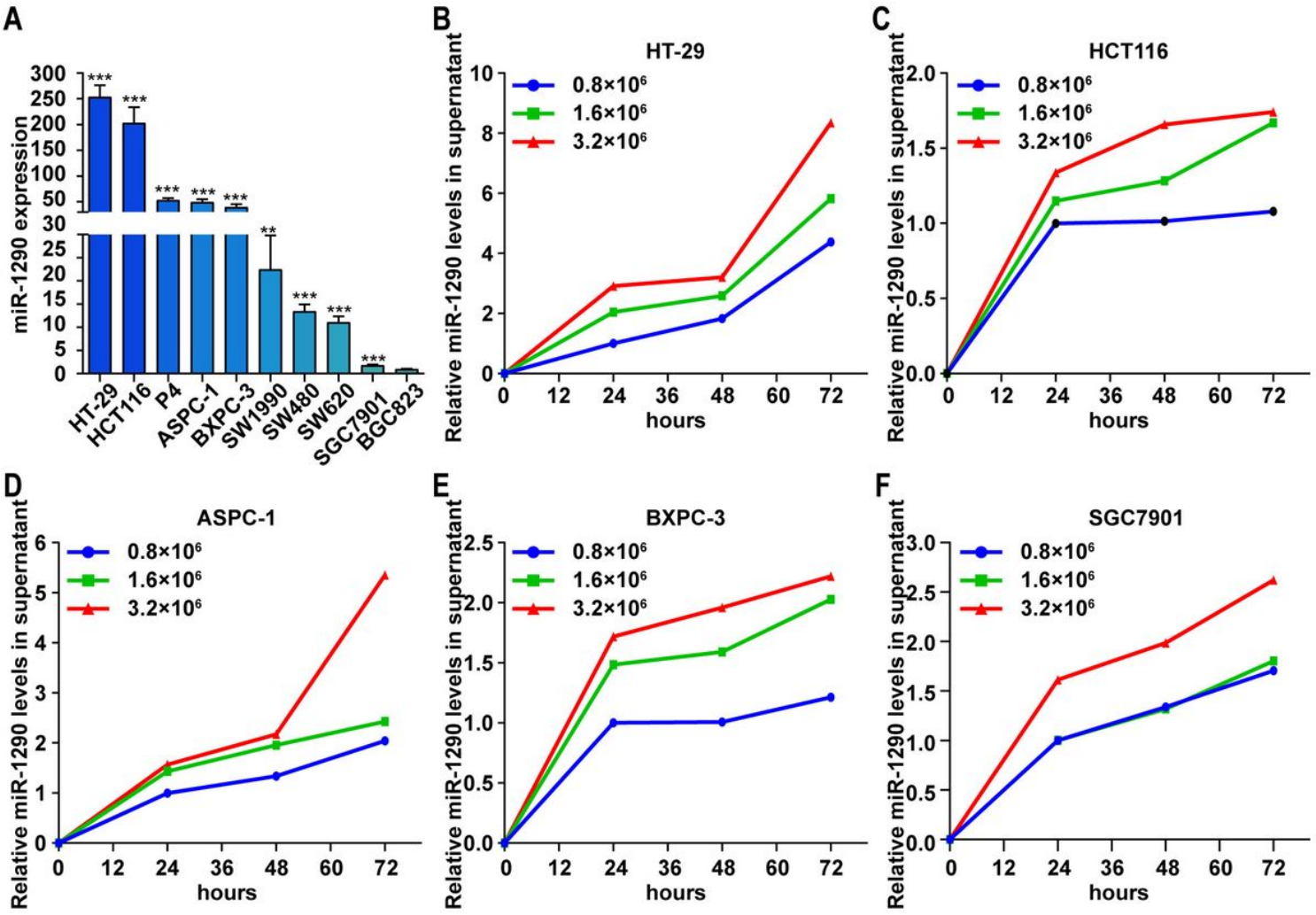

Figure 3

Human gastrointestinal tumor cells express and secret miR-1290. (a) Expression levels of miR-1290 were analyzed in different gastrointestinal cancer cells by RT-qPCR, including HT-29, HCT116, P4, ASPC-1, BXPC-3, SW1990, SW480, SW620, SGC7901, and BGC823. miR-1290 expression in HCT116, HT29, P4, ASPC-1 and BXPC-3 were relatively high. (b) miR-1290 expression levels in cellular supernatant of HT-29 when cultured with different cell densities for 24h, 48h, and 72h. (c) miR-1290 expression levels in cellular supernatant of HCT116 when cultured with different cell densities for 24h, 48h, and 72h. (d) miR-1290 expression levels in cellular supernatant of ASPC-1 when cultured with different cell densities for 24h, 48h, and 72h. (e) miR-1290 expression levels in cellular supernatant of BXPC-3 when cultured with different cell densities for 24h, 48h, and 72h. (f) miR-1290 expression levels in cellular supernatant of SGC7901 when cultured with different cell densities for 24h, 48h, and 72h. ** $p < 0.01$, *** $p < 0.001$.

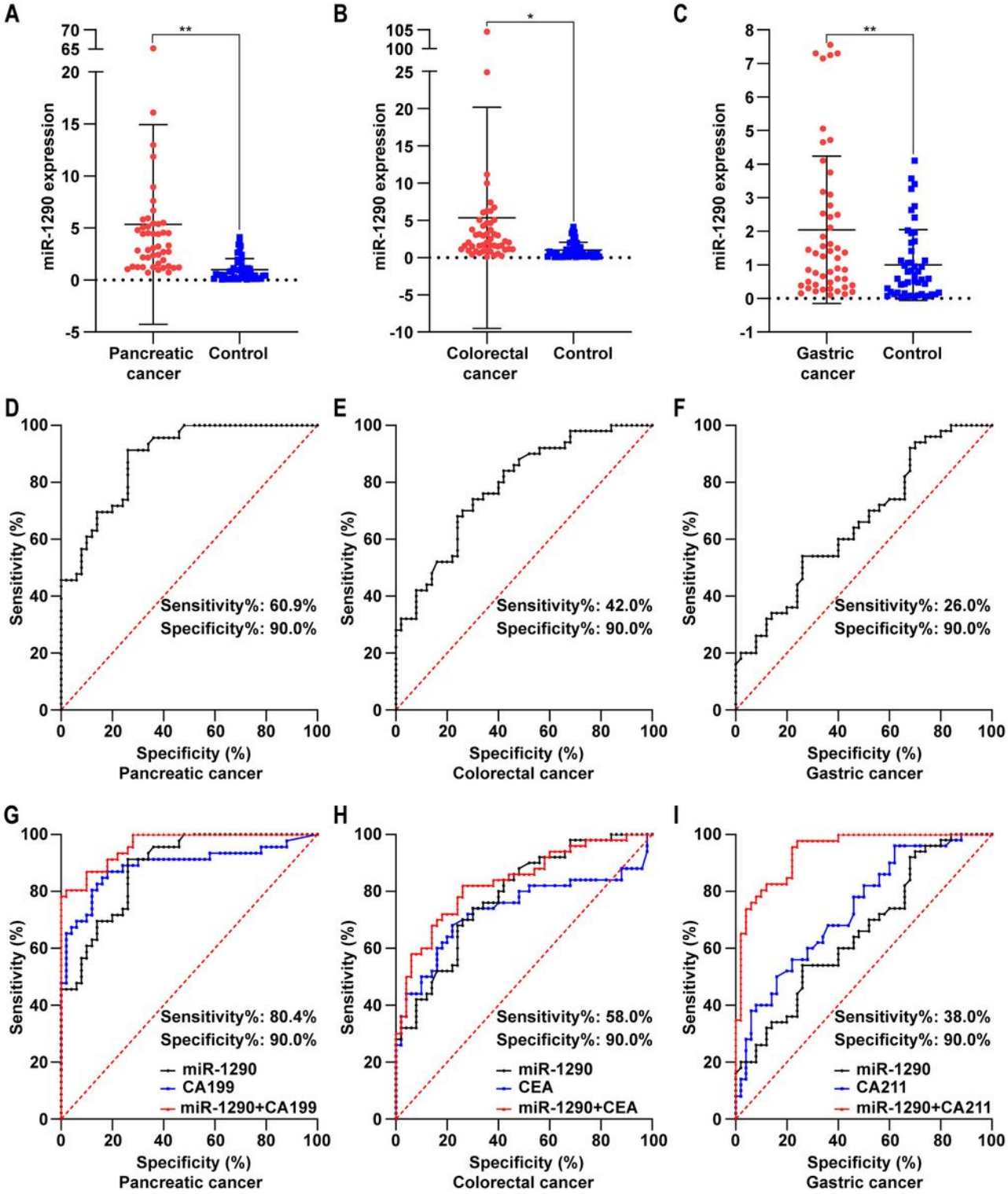

Figure 4

Circulating miR-1290 is a potential diagnostic biomarker in gastrointestinal tumors. (a) Serum miR-1290 expression levels in PC patients (n=46) and healthy controls (n=50) were analyzed by RT-qPCR. (b) Serum miR-1290 expression levels in CRC patients (n=50) and healthy controls (n=50) were analyzed by RT-qPCR. (c) serum miR-1290 expression levels in GC patients (n=50) and healthy controls (n=50) were analyzed by RT-qPCR. (d) Circulating miR-1290 yielded AUC value of 0.8857 with 67.4% sensitivity and 90.0% specificity

in distinguishing PC patients from healthy controls ($p < 0.001$). (e) Circulating miR-1290 yielded AUC value of 0.7852 with 48.0% sensitivity and 90.0% specificity in distinguishing CRC patients from healthy controls ($p < 0.001$). (f) Circulating miR-1290 yielded AUC value of 0.6576 with 34.0% sensitivity and 90.0% specificity in distinguishing GC patients from healthy controls ($p < 0.001$). (g) Sensitivity and specificity of circulating miR-1290 combined with CA199 were 80.4% and 90.0% with the AUC value of 0.9626 in PC ($p < 0.0001$). (h) Sensitivity and specificity of circulating miR-1290 combined with CEA were 58.0% and 90.0% with the AUC value of 0.8348 in CRC ($p < 0.0001$). (i) Sensitivity and specificity of circulating miR-1290 combined with CA211 were 38.0% and 90.0% with the AUC value of 0.7788 in PC ($p < 0.0001$). * $p < 0.05$, ** $p < 0.01$.

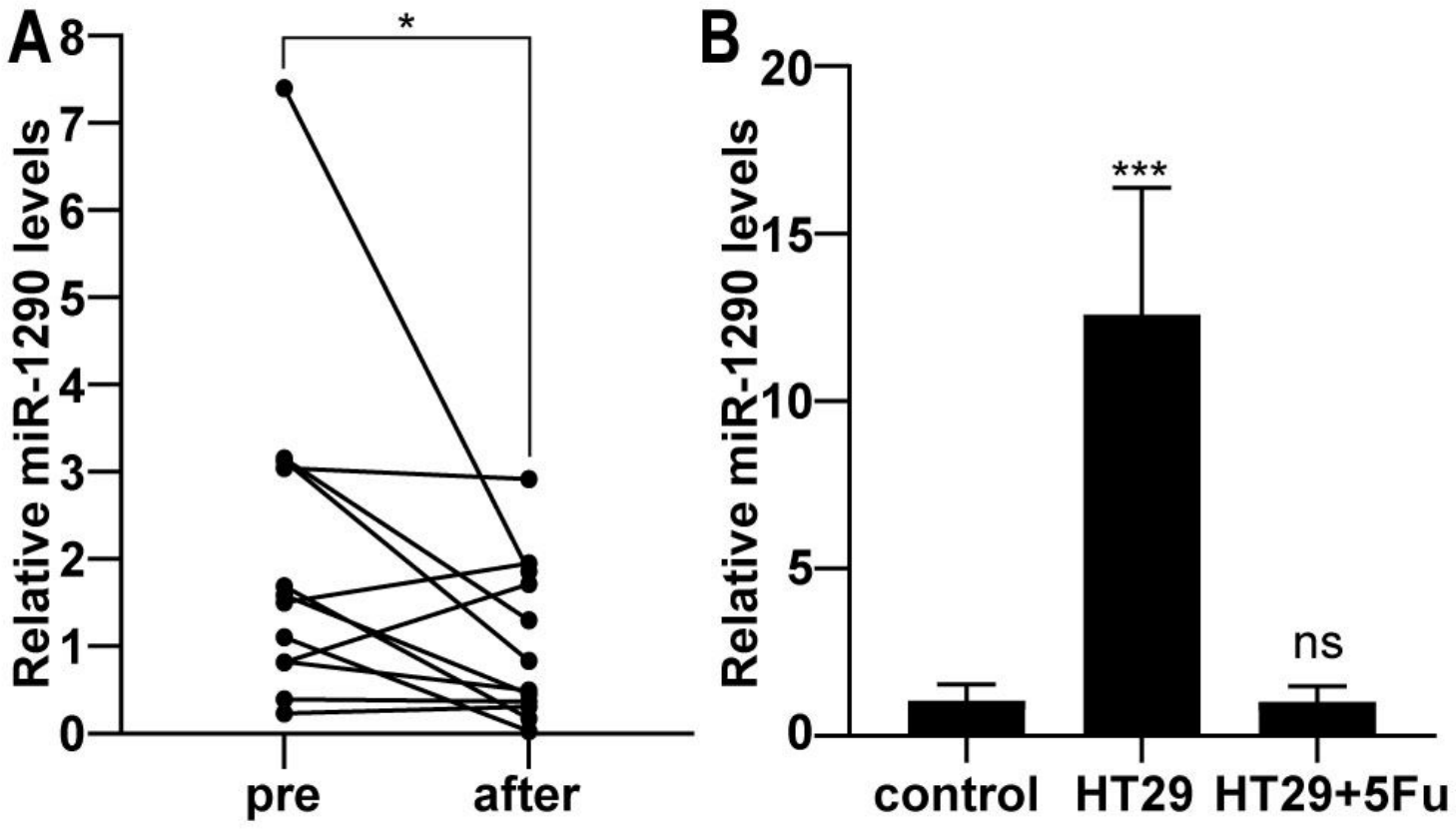

Figure 5

Circulating miR-1290 is a promising biomarker for disease surveillance. (a) Changes of serum miR-1290 levels in CRC patients after surgery were determined by RT-qPCR (n=10). (b) The fluctuation of circulating miR-1290 during disease progression and drug treatment in the xenograft mouse model. * $p < 0.05$, *** $p < 0.001$.

Supplementary Files

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