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ORIGINAL ARTICLE

Plasma miR-1, but not Extracellular Vesicle miR-1, Functions as a Potential Biomarker for Colorectal Cancer Diagnosis

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SUMMARY

Background: **miRNAs have been proved to function as diagnostic biomarkers. Extracellular vesicles (EVs) are carriers of miRNAs. This study aimed to investigate the diagnostic potential of miR-1 in plasma and extracellular vesicles (EVs) for patients with colorectal cancer (CRC).**
Methods: **Bioinformatics analysis was used to find a target miRNA and its potential functions. miR-1 was then detected in plasma and EV from 49 control samples and 40 CRC samples. Next, the diagnostic potential of plasma and EV miR-1 were compared based on common biomarkers including CEA and CA211.**
Results: **miR-1 was differentially expressed in CRC. Target gene and function analyses showed that it might participate in cell migration and the regulation of mRNA splicing via the spliceosome. Plasma miR-1 levels in CRC samples were significantly higher than those in control samples, whereas EV miR-1 levels were not statistically different. Based on receiver operating characteristic (ROC) curve analysis, comparing their predictive power compared to that of CEA and CA211, plasma miR-1 performed better and EV miR-1 performed worse.**
Conclusions: **Our data indicate that plasma miR-1, but not EV miR-1, could function as a potential biomarker for CRC diagnosis.**
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KEY WORDS

miR-1, biomarker, colorectal cancer, extracellular vesicle

LIST OF ABBREVIATIONS

EV - extracellular vesicle
CRC - colorectal cancer
ROC - receiver operating characteristic
AUC - the area under the ROC
KEGG - Kyoto Encyclopedia of Genes and Genomes
GO - Gene Ontology annotation
PPI - protein-protein interaction

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INTRODUCTION

Colorectal cancer (CRC) is a common digestive system malignancy that causes approximately 700,000 deaths each year worldwide [1]. With development and economic progression, Chinese people have changed their lifestyles, and this is related to an increasing incidence of CRC [2]. In addition, genetic factors play an important role in CRC. For example, patients with Lynch syndrome, an autosomal dominant inherited disease, account for almost 6% of all Chinese CRC cases [3].

In addition to the high incidence of CRC, approximately 50% of patients were found to have distant metastases upon initial diagnosis [4]. For the diagnosis of CRC, colonoscopy is the most commonly performed screening test. Further, with polypectomy, colonoscopy can also become a treatment especially for those patients with bleeding symptoms [5]. However, similar to other endoscopy methods, colonoscopy is limited by the requirement for trained colonoscopists, uncomfortable or painful experiences, relatively high risk, and low cost-effectiveness [6]. Imaging techniques including PET, CT, and X-ray can also be used for CRC screening. X-rays are easy to perform in China, but it can miss some small lesions. Moreover, PET/CT has a high sensitivity for CRC detection, but its high cost and the unavailability of machines limit its clinical application for routine inspection [7]. Molecular biomarkers are also options for CRC detection. For example, CEA and CA 19-9 were reported to be associated with the presence and severity of colorectal neoplasia [8]. However, the low detection rate has limited the use of these biomarkers. A fast, convenient, accurate method for CRC screening is necessary.

miRNAs are small RNAs of approximately 20 bp that bind the 3′-UTR to regulate target gene expression at either the translation or post-transcription level [9]. There have been many reports of miRNAs that could be used as biomarkers. EVs (extracellular vesicles) are small vesicles that are shed from all types of live cells. They contain and deliver protein, mRNA, miRNA, and other molecules to distant target cells to participate in pathway regulation. For miRNAs, they can be separate from or encapsulated in EVs and discharged outside the cell and even into the blood. By searching public databases, we found miR-1 might be a potential biomarker for CRC. In this study, we detected the levels of miR-1 in both plasma and EVs from control and CRC samples. We also compared the diagnostic power of miR-1 to that of other molecular biomarkers.

MATERIALS AND METHODS

Subjects

From December 2018 to June 2019, 89 subjects including 40 CRC and 49 control were recruited from the Department of Gastrointestinal Surgery, Affiliated Hospital of Jiangnan University. The Ethics Review Board of Affiliated Hospital of Jiangnan University approved the study. Informed consent was obtained from all participants and the subjects were treated according to the Declaration of Helsinki. The clinical parameters including gender, age, stage, grade, tumor size, and biomarkers were collected by reviewing the medical records.

Sample collection and plasma processing

Blood was drawn from both control and CRC subjects into EDTA blood tubes. For all CRC subjects, the blood samples were collected at the time of diagnosis and before any treatment. The tubes were transferred to the lab and centrifuged at 1,600 × g for 10 minutes within 12 hours. Plasma was stored at -80°C and processed within 15 days.

Extracellular vesicle isolation and identification

Before isolation, plasma was centrifuged at 10,000 × g for 10 minutes at 4°C. For RNA isolation and detection, 200 µL from the supernatant was used. For EV isolation and detection, 800 µL from the supernatant was used. A total of 400 µL 30% PEG4000 (1 M NaCl) was added to 800 µL plasma and mixed upside down. After incubation at 4°C for 3 hours, the mixture was centrifuged at 3,000 g for 15 minutes at 4°C. The total number of pellets were used for RNA detection.

EVs from one control subject and one CRC subject were lysed by RIPA buffer (Biochem, China), and the concentration was assayed by BCA protein assay kit (Thermo, USA). The signature proteins including TSG-101 (Abcam, Cambridge, Mass) and CD9 (Abcam, Cambridge, Mass) were detected by western blot.

The concentration and size distribution of EVs were analyzed by the Nano platform (iZON Science). First, standard particles were used to calibrate the NP100 nanopores of the system. The PBS, which had been repeatedly filtered by 0.22 µm filter, was used to dilute the EV pellets to the proper concentration. The concentration and size distribution were detected by the particle analyzer and the supporting software.

RNAiso Plus (Takara, China) was used to isolate total RNA from 200 µL plasma or EVs according to the instructions. The Hairpin-it miRNAs qPCR Quantitation Kits (Genepharma, China) were used to detect the miRNA levels. As there was no recognized exosomal reference miRNA, we diluted synthetic miRNAs to 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} nmol and established standard curves for absolute quantification.

Bioinformatics analysis

The target genes of miR-1 were predicted by TargetScan, Pictar, miRmap, miRanda, and microT in miRWalk [10]. KEGG (Kyoto Encyclopedia of Genes and Genomes), Gene Ontology annotation (GO), and protein-protein interaction (PPI) analyses were performed for these intersecting genes based on the four databases.

Table 1. Summary of subject characteristics.

Total subjects, n	
Male	44
Female	45
Age (years)	
Median	61
Range	21 - 85
Diagnosis, n	
Colon cancer	20
Rectal cancer	20
Benign lesion	49
Pathology, n	40
Adenocarcinoma	35
NA	5
Stage, n	
Tis	3
T1	5
T2	6
T4	2
T4a	18
Grade, n	
0	3
I	11
II	13
III	5
IV	2
Tumor size (cm)	
Median	4.4
Range	1.2 - 8

Statistical analysis

Data were analyzed using GraphPad Prism and R project for Statistical Computing. The k-scores of proteins were calculated by Cytoscape and MCODE was used to find the key sub-network of these proteins. The nonparametric Mann-Whitney U test was used to compare miRNA expression between the groups, and $p < 0.05$ was considered significant. Pearson's correlation analysis was used to analyze the receiver operating characteristic (ROC) curve, and the area under the ROC (AUC) was calculated to establish the potential of miR-1 to distinguish CRC patients from the control group.

RESULTS

Description of subjects

Table 1 shows the characteristics of the 89 subjects enrolled in this study. The subjects included 44 males and 45 females from 21 to 85 years of age. Forty subjects were diagnosed with colon or rectal cancer and 35 of these had adenocarcinoma with an average tumor size of 4.5 cm.

EV isolation and identification

EVs were isolated from plasma according to the procedure shown in Figure 1A. The size distribution indicated that the pellets contained vesicles ranging from 80 to 280 nm (Figure 1B). We also used western blotting to detect signature proteins including TSG101 and CD9, using GAPDH as a reference (Figure 1C).

Target miRNA searching

We next analyzed the miRNA data from CRC and control samples using the GEO database. Considering differences among different races and regions, we limited the search to samples from China to improve study accuracy. Finally, we obtained four studies including GSE120300, GSE35982, GSE45349, and GSE72281 from GEO database. We summarized these data in Figure 2A. A large number of upregulated and downregulated miRNAs were identified in these data sets. To narrow down the results, we attempted to find intersecting data among these four studies (Figure 2B). When, using a p-value cutoff of less than 0.05 for all four studies, no results were obtained. Moreover, miR-1 was the only differentially-regulated molecule with a p-value less than 0.05 in three of four studies. The detailed expression of miR-1 from the four studies is shown in Figure 2C.

Bioinformatics analysis

TargetScan, Pictar, miRmap, miRanda, and microT were used for miR-1-target gene prediction. The interaction networks from these five analyses resulted in 117 genes (Figure 3A). Then, we performed GO and KEGG analysis based on these genes. GO analysis includes biological process, cellular component, and molecular function profiles. This showed that these genes participate in cell migration, junction, and molecular binding (Figure 3B, C, and D). Further, KEGG pathway analysis showed that these genes participate in the PI3K-Akt signaling pathway, spliceosome, and proteoglycans in cancers (Figure 3E).

We further investigated the function of these genes at the protein level using STRING [11] (Figure 4A). The k-score was used to evaluate the importance of the genes in the network (Figure 4B). Of all genes, eight had a k-score of 7 and formed a sub-network (Figure 4C). Except for MATR3, the other seven genes were involved in regulating mRNA splicing via the spliceosome.

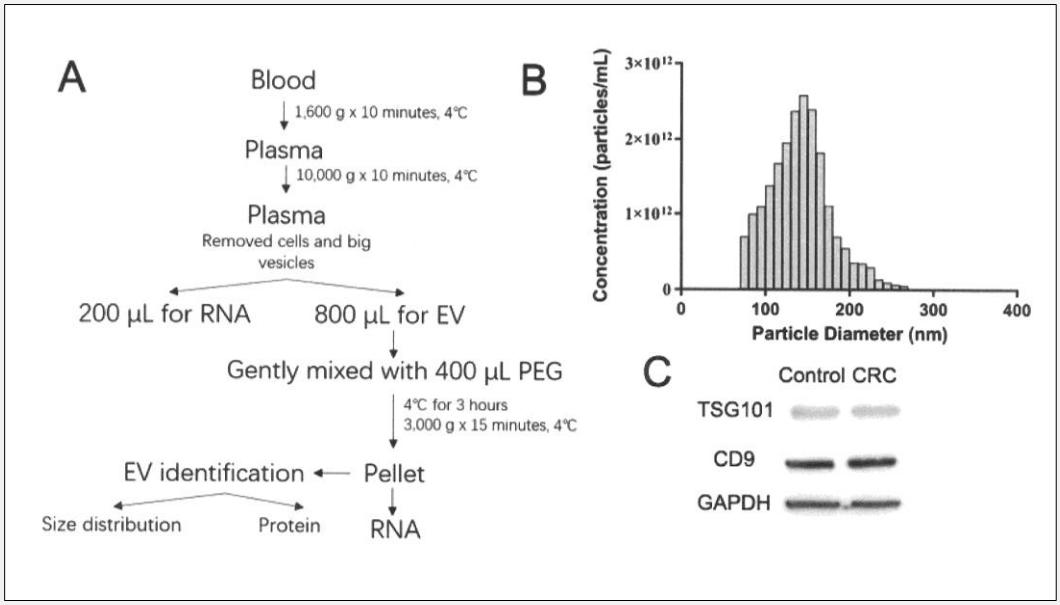

Figure 1. Extracellular vesicle (EV) isolation and identification.

A. The procedure of EV isolation from colorectal cancer (CRC) patients. B. The size distribution of EVs. C. Western blot results of EVs.

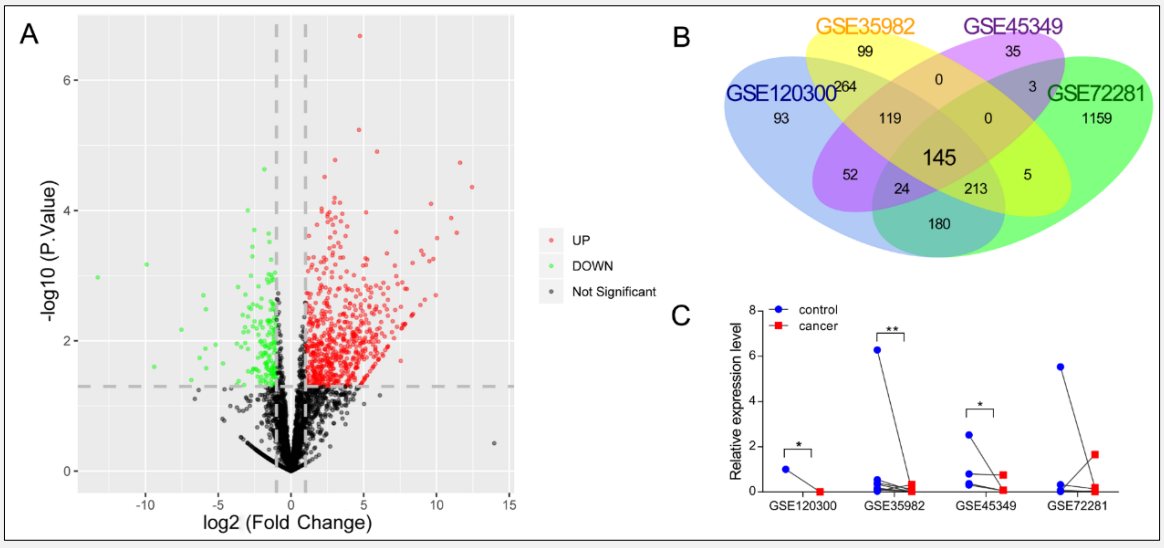

Figure 2. Screening of differentially regulated miRNAs between colorectal cancer and control subjects from the GEO database.

A. miRNA expression in the four tested data sets. p-value < 0.05 and |logFC| ≥ 1 were set as the threshold. Red, upregulated; green, downregulated; black, not significant. B. The interactions among the four studies. C. The relative expression of miR-1 based on the four studies. * - p < 0.05; ** - p < 0.01.

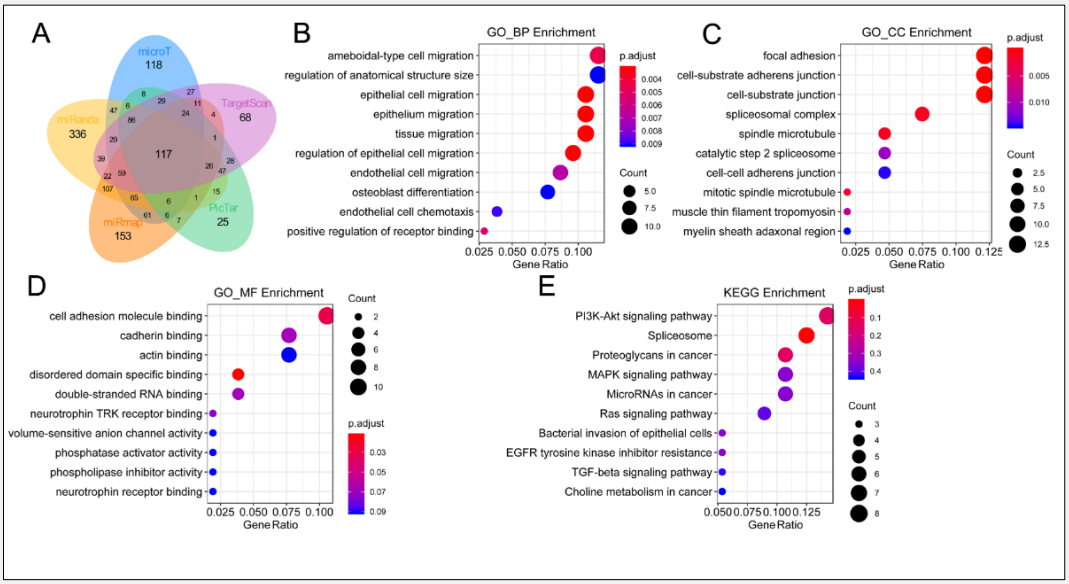

Figure 3. Target gene prediction and functional analysis of differentially expressed genes between colorectal cancer and control subjects.

A. Target gene prediction using TargetScan, Pictar, miRmap, miRanda, and microT. B. GO analysis of biological processes. C. GO analysis of cellular components. D. GO analysis of molecular functions. E. KEGG pathway analysis.

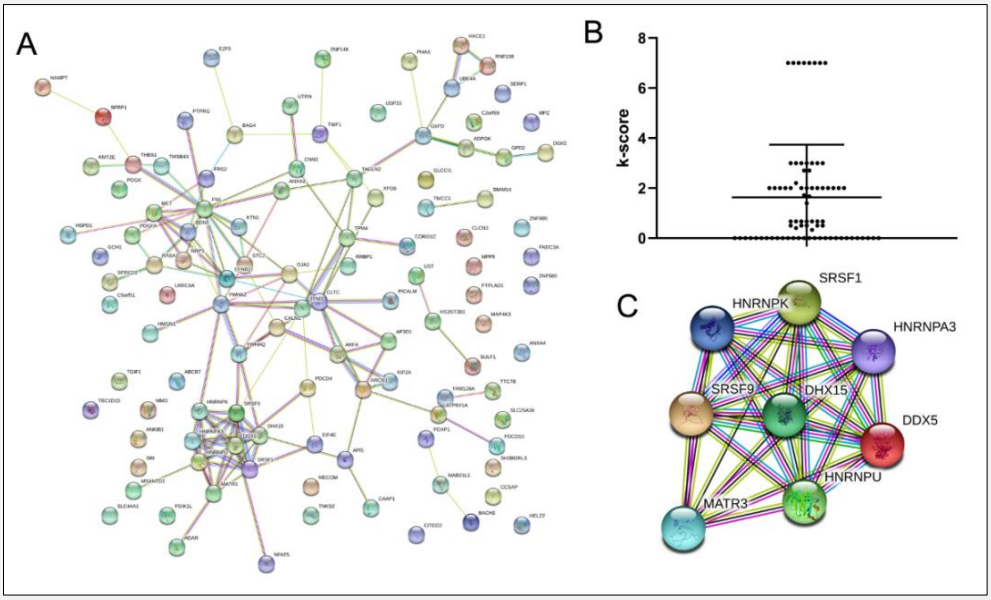

Figure 4. Protein-protein interaction (PPI) analysis of target genes that were differentially expressed genes between colorectal cancer and control subjects.

A. The PPI network of target genes. B. k-score of the target genes based on MCODE. C. Gene cluster based on MCODE results with a k-score > 7.

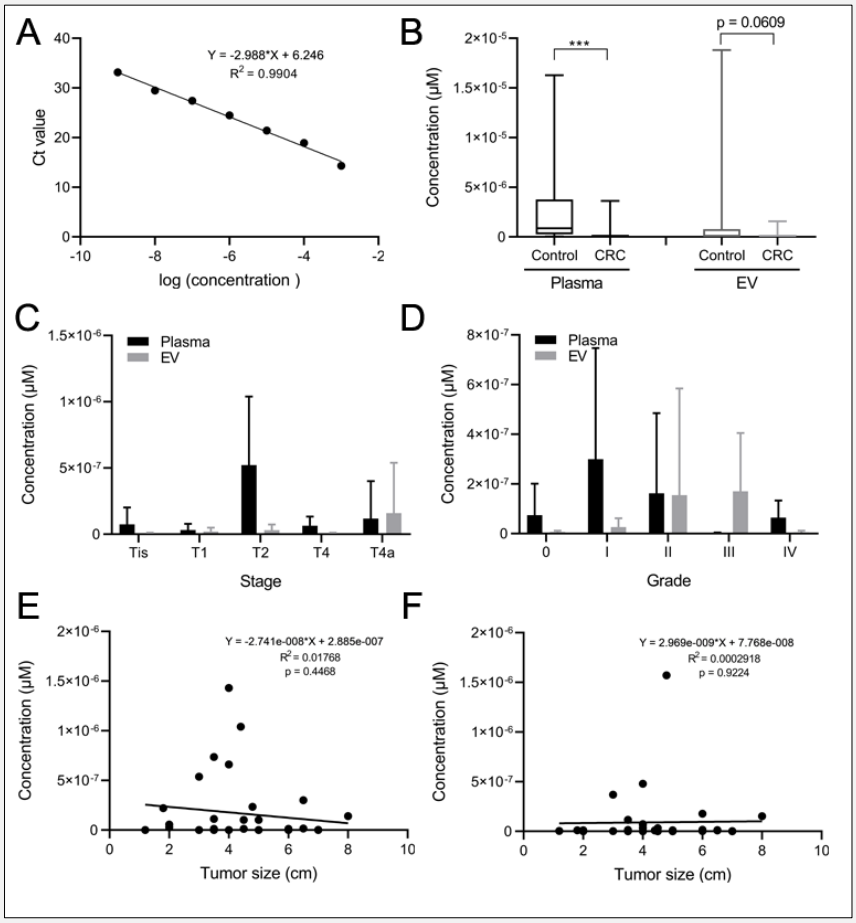

Figure 5. miR-1 levels in plasma and extracellular vesicles (EV) of patients with colorectal cancer (CRC).

A. The standard curve of miR-1. B. Concentrations of miR-1 in plasma and EVs from control and CRC samples. C, D. Plasma and EV miR-1 levels based on different stages (C) and grades (D). E, F. Correlation regression analyses of tumor size vs. miR-1 plasma (E) and EV (F) levels. * - $p < 0.001$.**

Detection of miR-1 in plasma and EV

First, we used synthetic miR-1 to establish a standard curve for its expression (Figure 5A). Then, we detected miR-1 levels in the plasma and EVs from control and CRC samples (Figure 5B). The results showed the miR-1 levels in the plasma of control samples were significantly higher than those in CRC samples ($p < 0.001$), whereas miR-1 levels in EVs were not significantly different between control and CRC samples ($p = 0.0609$). We further compared the miR-1 levels from plasma and EVs based on different stages and grades of CRC samples. The results showed an increase in stage T2 of plasma miR-1 and stage T4a of EV miR-1 (Figure 5C). Both plasma and EV miR-1 showed an increase from grade 0 to grade I disease (Figure 5D). We also determined whether there was relationship between miR-1 levels and tumor size. Pearson's correlation analysis showed that neither plasma nor EV miR-1 levels were related to tumor size ($p = 0.4468$ and $p = 0.9224$, respectively, Figure 5E and F).

Comparison of miR-1 and common biomarkers

Some common biomarkers including CEA, AFP, TPSA, and others were also detected in these samples (Figure 6A). CEA and CA211 had the highest detection rates of 14/40 and 12/40, respectively. ROC analysis was then used to compare the diagnostic efficiencies of miR-1, CEA, and CA211. miR-1 plasma and EV levels exhibited AUCs of 0.8954 and 0.6327, respectively ($p < 0.001$ and $p = 0.032$; Figure 6B and C). In contrast CEA and CA211 had AUCs of 0.7388 and 0.6454, respectively ($p = 0.0001$ and $p = 0.0187$; Figure 6D and E).

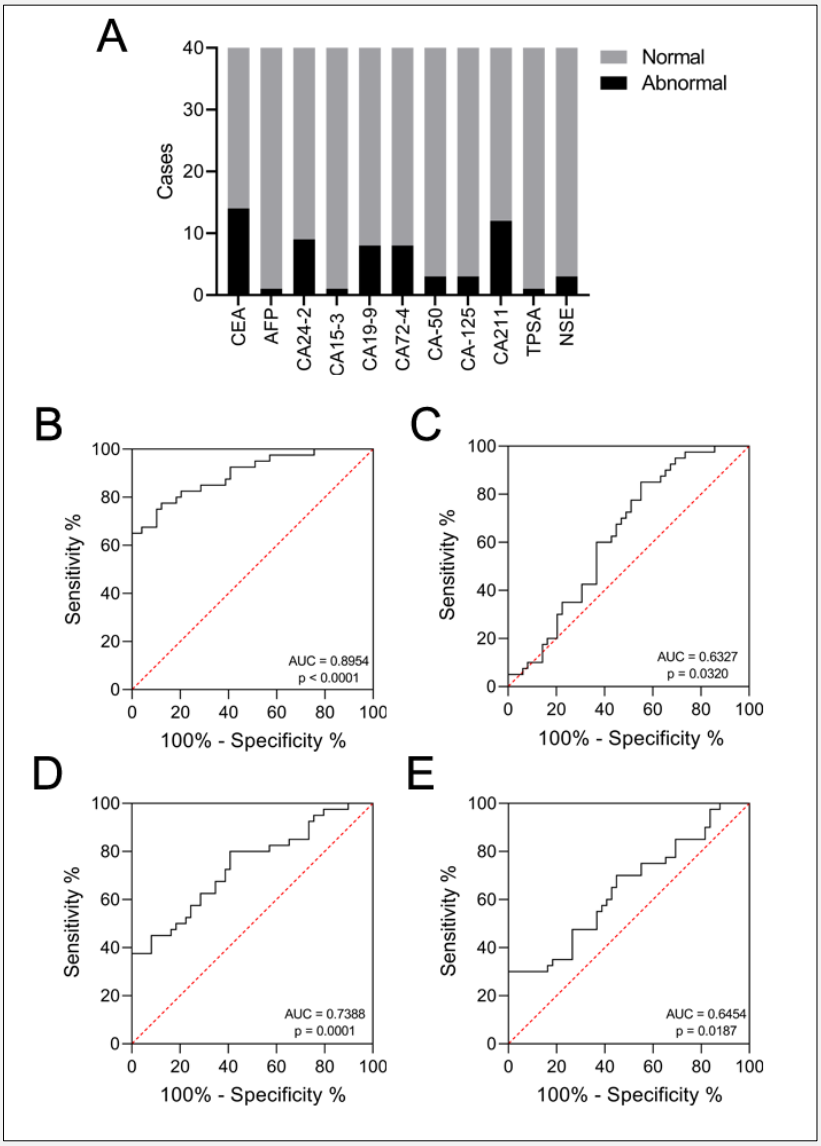

Figure 6. Receiver operating characteristic (ROC) curve analysis of miR-1, CEA and CA211 in colorectal cancer patients.

A. The detection rate of common biomarkers. B - E. ROC curves of plasma miR-1 (B), EV miR-21 (C), CEA (D), and CA211 (E).

DISCUSSION

miRNAs are dysregulated in many types of diseases, especially cancers. This molecule functions as a tumor suppressor in lung cancer, CRC, gastric cancer, and other cancers [12,13]. Accordingly, we analyzed the potential functions of this marker. Target gene prediction for miR-1 gave 117 results, and these genes were mainly related to cell adhesion, junctions, and migration. The PI3K-Akt signaling pathway was the most enriched pathway based on KEGG analysis, and this axis is related to autophagy, epithelial-mesenchymal transition, and cell cycle transition in CRC [14,15]. PPI analysis resulted in a key sub-network of regulators of mRNA splicing via the spliceosome, including SRSF1, HNRNPK, HNRNPA3, SRSF9, DHX15, DDX5, and HNRNPU. Interestingly, DDX5 was previously associated with poor prognosis for CRC [16]. Moreover, HNRNPK is also a prognostic and predictive biomarker for CRC [17], in addition to being the target of DAB2IP, lnc-

RNA91H, and MYC-regulated long noncoding RNAs [18-20]. These regulatory networks suggest new directions for further research.

miR-1 was found to be decreased in CRC tissues in several studies [21-23]. Some studies [24,25] reported that its expression in tissues is associated with the grade and stage of CRC. In serum or plasma, miR-1 was found to function as a biomarker of myocardial injury [26], hepatocellular carcinoma [27], and acquired aplastic anemia [28]. miR-1 was also detectable in exosomes or EVs from serum and plasma samples. However, there has been no report of plasma miR-1 functioning as a biomarker for CRC diagnosis, and no comparison of miR-1 levels between plasma and EVs has been performed. This is thus the first study to indicate that plasma miR-1 levels could be used as a biomarker for CRC diagnosis, in addition to showing that it is superior to EV miR-1 levels for this application.

CEA, AFP, NSE, CA211, and others are recognized tumor markers and are widely used clinically for different diseases including CRC [29,30]. In our study, there were more than 10 abnormal results for CEA and CA211. Meanwhile, AFP, CA15-3, and TPSA gave only one abnormal result. Further, ROC curve analysis showed that plasma miR-1 had better diagnostic power than CEA, CA211, and EV miR-1. Specifically, the Youden index reached a maximum when miR-1 was 1.442×10^{-7} μM with a sensitivity of 77.5% (95% CI, 62.50 - 87.68%) and a specificity of 87.76% (95% CI, 75.76 - 94.27%).

However, there were some limitations to this study. First, the samples included were not sufficient. The lack of stage T3 specimens and the low numbers of samples representing other stages and grades made further statistical analysis impossible. Thus, we need to expand our sample group in a future study. Second, control samples from healthy individuals are necessary. Third, some studies have indicated that some other miRNAs could also be used for CRC diagnosis. A comparison of these miRNAs with miR-1 would improve the quality of this study. Lastly, in this study, we isolated RNA from 200 μL of plasma and EVs from 800 μL of plasma. With the same isolation and detection method, miR-1 levels from plasma ($1.719 \times 10^{-6} \pm 3.477 \times 10^{-7}$ μM) were higher than those from EVs ($5.511 \times 10^{-7} \pm 2.263 \times 10^{-7}$ μM) ($p = 0.0015$). In addition, plasma miR-1 performed better than EV miR-1 for CRC diagnosis. These results indicated that miR-1 might predominantly reside in the plasma outside of EVs. To verify this, ultracentrifugation might be a good choice for further study, as it could provide EVs and EV-free plasma.

CONCLUSION

Plasma miR-1, but not EV miR-1, could be a potential diagnostic biomarker for CRC. Moreover, plasma miR-1 was found to have better diagnostic power than other common biomarkers including CEA and CA211. This information could be used clinically for CRC diagnosis.

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Author Contributions:
XBH and BCQ designed this research. SXM and YZH collected the samples and the information of patients. ZTT and LB performed most of the experiments. GS and SYF performed the EV related experiments. BCQ and XBH organized the results and wrote the article.

Declaration of Interest:
There are no conflicts to disclose for all authors.

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