Clinical Trial/Experimental Study

Medicine

OPEN

MicroRNA-375 in extracellular vesicles – novel marker for esophageal cancer diagnosis

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Abstract

Background: MicroRNAs have been confirmed to function as diagnostic biomarkers for esophageal cancer (EC). This study aimed to investigate the diagnostic potential of miR-375 in the plasma or extracellular vesicles (EVs) of esophageal cancers (ECs).

Methods: miRNAs with diagnostic potential were identified through public database searches and validated through clinical sample testing. The diagnostic value of miR-375 in plasma and EVs was evaluated via receiver operating characteristic analysis and area under the curve. In addition, expression and survival analyses of the top ten target genes of miR-375 were conducted using the cancer genome atlas database.

Results: MiR-375 was identified as a potential biomarker for ECs by searching the gene expression omnibus database. Results of clinical sample measurements showed that miR-375 in plasma or EVs was significantly different between ECs and controls ($P < .01$), but did not differ by gender or age. receiver operating characteristic analysis demonstrated that miR-375 in EVs could function as a diagnostic marker for ECs, with a higher area under the curve (0.852) than that in plasma. The expression and survival analysis of the top ten target genes for miR-375 showed that only EIF4G3 was significantly associated with survival ($P < .05$).

Conclusion: This research shows that miR-375, particularly in EVs, could serve as a biomarker for the diagnosis of ECs.

Abbreviations: AFP = α-fetoproteinAUC = area under the curve, CA = carbohydrate antigen, CEA = carcinoembryonic antigen, ECs = esophageal cancers, EVs = extracellular vesicles, GEO = gene expression omnibus, PPI = protein-protein interaction, RT-PCR = real-time quantitative polymerase chain reaction, ROC = receiver operating characteristic curve, TCGA = the cancer genome atlas, TMs = tumor markers.

Keywords: diagnostic biomarker, esophageal cancer, extracellular vesicle, MicroRNA-375

1. Introduction

Esophageal cancer (EC) is a malignant tumor of the gastrointestinal tract that readily arises in the esophageal mucosa. The overall 5-year survival rate of patients is only 15% to 25%.[1,2] Globally, approximately 300,000 people die each year from EC.[3] Typical EC diagnostic methods include X-ray barium meal angiography, esophagoscopy, and exfoliative cytology.[4] These methods are invasive, costly, and have missed diagnoses, which limits their clinical application.[5,6] Traditional serum tumor markers (TMs) can be used to diagnose and detect dynamic changes in tumors, but they have insufficient sensitivity and predictive value.[7,8] As a result, more sensitive and specific tumor markers are predicted to contribute to the early diagnosis of esophageal cancer (EC).

Studies have shown that abnormal expression of miRNAs is related to the occurrence, invasion and metastasis of cancer (as promoters or repressors) and is a potential tumor markers.[9–11] Their importance in various cancers has been recognized,[12,13] including lung cancer,[14] gastric cancer,[15] colorectal cancer,[16] esophageal cancer, and precancerous lesions. MiRNA-mediated gene regulation plays an important role in the occurrence and development of ECs and the abnormal expression of specific miRNAs is closely related to diagnosis, prognosis, and response to chemotherapy.[17] Further understanding of miRNA expression profiles will help us screen for better biomarkers for the diagnosis, classification and diagnosis of EC.

MiRNAs have been discovered not only in tissues, but also in body fluids.[18] Circulating miRNAs are encapsulated in RNA-binding multiprotein complexes and/or extracellular vesicles

JC, ZC, and JH contributed equally to this work.

The authors have no funding and conflicts of interest to disclose.

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Blood sample and clinical data collection were approved by the Ethics Review Board of the Naval Medical Center of PLA, Second Military Medical University (Shanghai, China), which number was "AF-HEC-020." As approved by the Ethics Review Board, oral informed consent for publication was obtained from all participants.

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How to cite this article: Chen J, Cai Z, Hu J, Zhou L, Zhang P, Xu X. MicroRNA-375 in extracellular vesicles – novel marker for esophageal cancer diagnosis. Medicine 2023;102:5(e32826).

Received: 3 November 2022 / Received in final form: 10 January 2023 / Accepted: 11 January 2023

http://dx.doi.org/10.1097/MD.0000000000032826

(EVs), making them highly stable.[19] EVs are vesicles approximately 50 to 200nm in size and can be secreted by a variety of mammalian cells.[20] The molecular cargo part of an EV reflects not only the contents of its original cell but also changes in conditions (stress or disease).[21] Studies have shown that miRNAs isolated from EVs have higher diagnostic sensitivity and specificity than those isolated from plasma.[22,23] Accordingly, EVs have attracted interest as noninvasive biomarkers for a variety of pathological processes.[24]

MiRNA sequencing data of a large number of diseases, including EC, are publicly available in the gene expression omnibus (GEO) database. In this study, we screened differentially expressed miRNAs (miR-375) between ECs and normal controls from the GEO database and used it as a potential molecular target. Previous studies have confirmed that miR-375 is a tumor suppressor and its expression is significantly down-regulated in EC.[25,26] Therefore, in this study, blood samples were collected from patients with EC, and in combination with the results of bioinformatics analysis, miRNA-375 expression was detected in plasma and EVs to validate its diagnostic value for ECs.

2. Material and Methods

2.1. Subjects and sample collection

From January 2018 to December 2020, 37 subjects diagnosed with esophageal cancer and 26 controls were recruited from the Naval Medical Center of PLA, Second Military Medical University (Shanghai, China). EC patient exclusion criteria included: the admitting diagnosis was not EC; presence of 2 or more types of diseases including EC; deficient clinical and pathological information; and patients treated with radiation and chemotherapy before specimen collection. Healthy individuals and patients with benign lesions formed the control samples. Clinical data for this study population are provided in Table 1, including tumor stage, gender, age, and so on. Informed consent was obtained from all participants for this study and the study was conducted with approval from the ethics committee of the naval medical center of PLA (Reference number: AF-HEC-020). The patients received therapy in accordance with the declaration of Helsinki.

Ethylene diamine tetraacetate acid EDTA blood tubes (BD Company, USA) were used for blood sampling, and all samples were transferred to the laboratory in an ice pack within 24 hours. Subsequently, blood samples were centrifuged at 1600 g for 10 minutes to obtain plasma, which was stored at -80°C for later use.

2.2. Selection of GEO dataset

Microarray-based gene expression data of ECs were obtained from the GEO database. The following keywords were used to search the GEO database: (Esophageal cancer OR esophageal squamous cell carcinoma) AND (Homo sapiens) AND (microRNA OR miRNA). This study incorporated the microarray dataset of miR-375 expression between EC and adjacent non-tumor tissues (the non-tumor tissue 3cm away from the lesion).

2.3. EV isolation

Studies have demonstrated that the enrichment of EVs by polyethylene glycol precipitation is straightforward, inexpensive and suitable for subsequent miRNA analysis.[27] Isolation of EVs was performed according to Yuan et al, and the methods description partly reproduces their wording.[28] Thawed plasma samples were first centrifuged at 2000g for 10 minutes at 4°C and then 10000g for 30 minutes to eliminate the cells and apoptotic bodies. Subsequently, the supernatant was lightly mixed 2:1 with 30% PEG4000 (Sigma-Aldrich, USA) which formulated from 1M NaCl and incubated for 3 hours at 4°C. Ultimately, the pellet was collected for 15 minutes by centrifugation at 3000g for subsequent RNA detection.

2.4. RNA extraction

RNAiso Plus (Takara, Japan) was used for the isolation of RNA from EVs and plasma. Specifically, 1mL RNAiso Plus is required for RNA isolation from 200μL plasma or EV pellets. After blending the sample and RNAiso Plus with a microelectric tissue homogenizer (Kimble Corporation, USA), add 200μL chloroform and mix gently. After incubating at room temperature for 5 minutes, the mixture was centrifuged at 12,000 g for 15 minutes at 4°C. Transfer all the supernatant to a new 1.5mL

Table 1

Summary of subject characteristics.

	Total		Esophageal cancer		Control	
	n	%	n	%	n	%
Gender	63		37		26	
Male	41	65.1%	24	64.9%	17	65.4%
Female	22	34.9%	13	35.1%	9	34.6%
Age(yr)						
Median age	59.6		62.0		57.8	
Age range	19–86		27–86		19–75	
Histology subtype						
Squamous cell carcinoma	-		23	62.2%	-	
Adenocarcinoma	-		14	37.8%	-	
Tumor stage						
Stage I/ II	-		17	45.9	-	
Stage III/ IV	-		20	54.1	-	
TMs (mean, [range])						
AFP (ng/ mL)	-		3.068 (1.14–6.61)		-	
CEA(ng/ mL)	-		2.018 (0.52–3.95)		-	
CA125(U/ mL)	-		9.862 (3.90–24.7)		-	
CA19-9(U/ mL)	-		8.342 (0.00–45.7)		-	

Control, healthy individuals and patients with benign lesions. Benign lesions including pulmonary infection and bronchiectasis.

AFP = α-fetoprotein, CA = carbohydrate antigen, CEA = carcinoembryonic antigen, TMs = tumor markers.

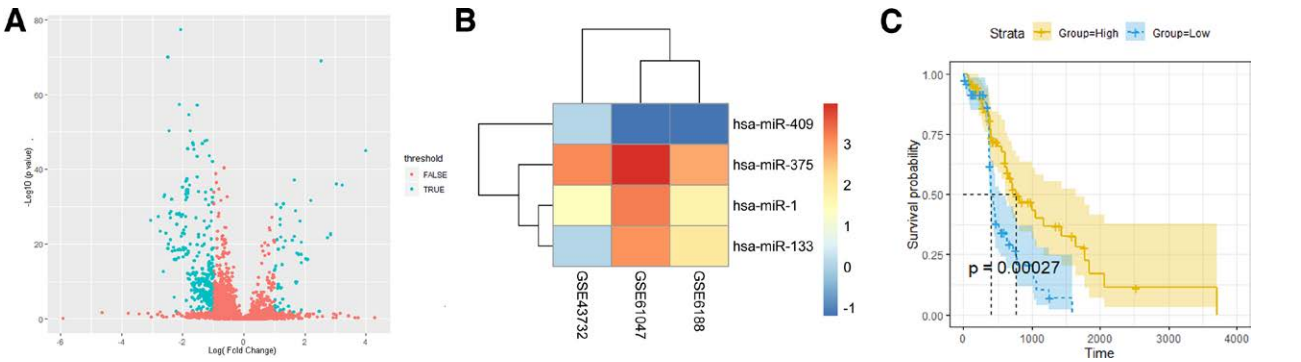

Figure 1. Screening of differentially-regulated miRNAs between control and esophageal cancer subjects from the GEO database. (A) The expression of miRNA in 3 data. (B) The expression of 4 miRNAs in 3 data. (C) The survival analysis of miR-375. The yellow area indicates high expression, while the blue represents low expression.

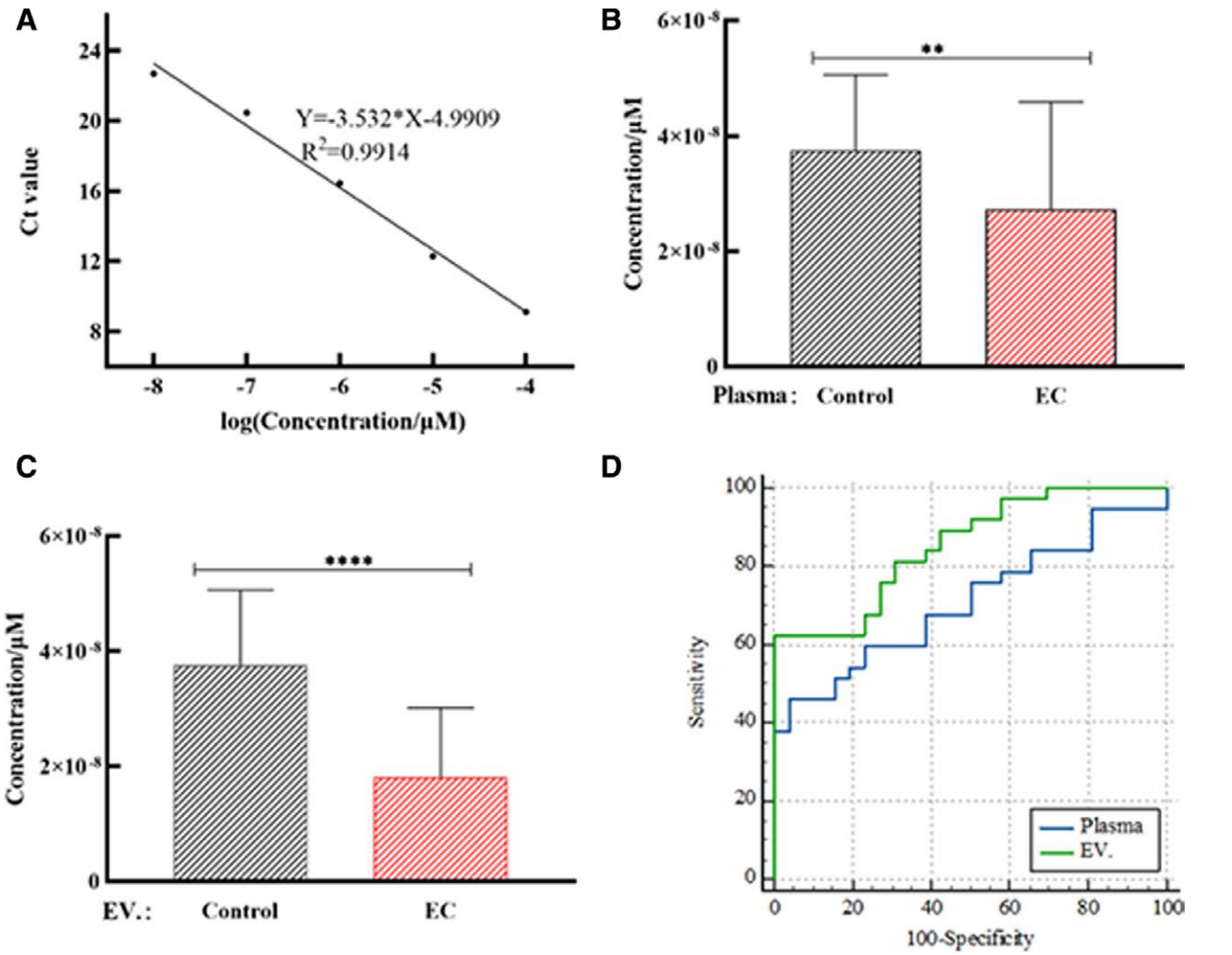

Figure 2. Expressions of miR-375 in plasma and EVs. Figures 2A, 2B and 2C are created using Prism 8.0 (GraphPad Software, USA). Figure 2D is created using SPSS v.22.0 (IBM, Armonk, NY). (A) The standard curve of miR-375. (B) The comparison of miR-375 in plasma. (C) The comparison of miR-375 in EVs. (D) The ROC curve of miR-375 in plasma and EVs. ROC = receiver operating characteristic.

centrifuge tube, add an equal volume of isopropanol, mix well, and incubate at –20°C overnight. The next day, the solution was centrifuged at 12,000g for 15 minutes at 4°C. After discarding the supernatant, the RNA was washed in DEPC water with 75% ethanol. To remove the residual liquid, centrifuge at 12,000g for 5 minutes at 4°C again, air dry the pellet and dissolve in 10μl DEPC water. RNA concentration was measured using the Qubit RNA XR Assay Kit (Invitrogen, USA).

2.5. Reverse transcription (RT) and quantitative PCR analyses

Expression levels of miRNAs in samples were determined using the Hairpin-it miRNAs qPCR Quantitation Kits (Genepharma Corporation, Shanghai, China) according to the manufacturer's instructions. Synthetic miR-375, purchased from Genepharma Corporation, was diluted to different concentrations (10^{-3} to

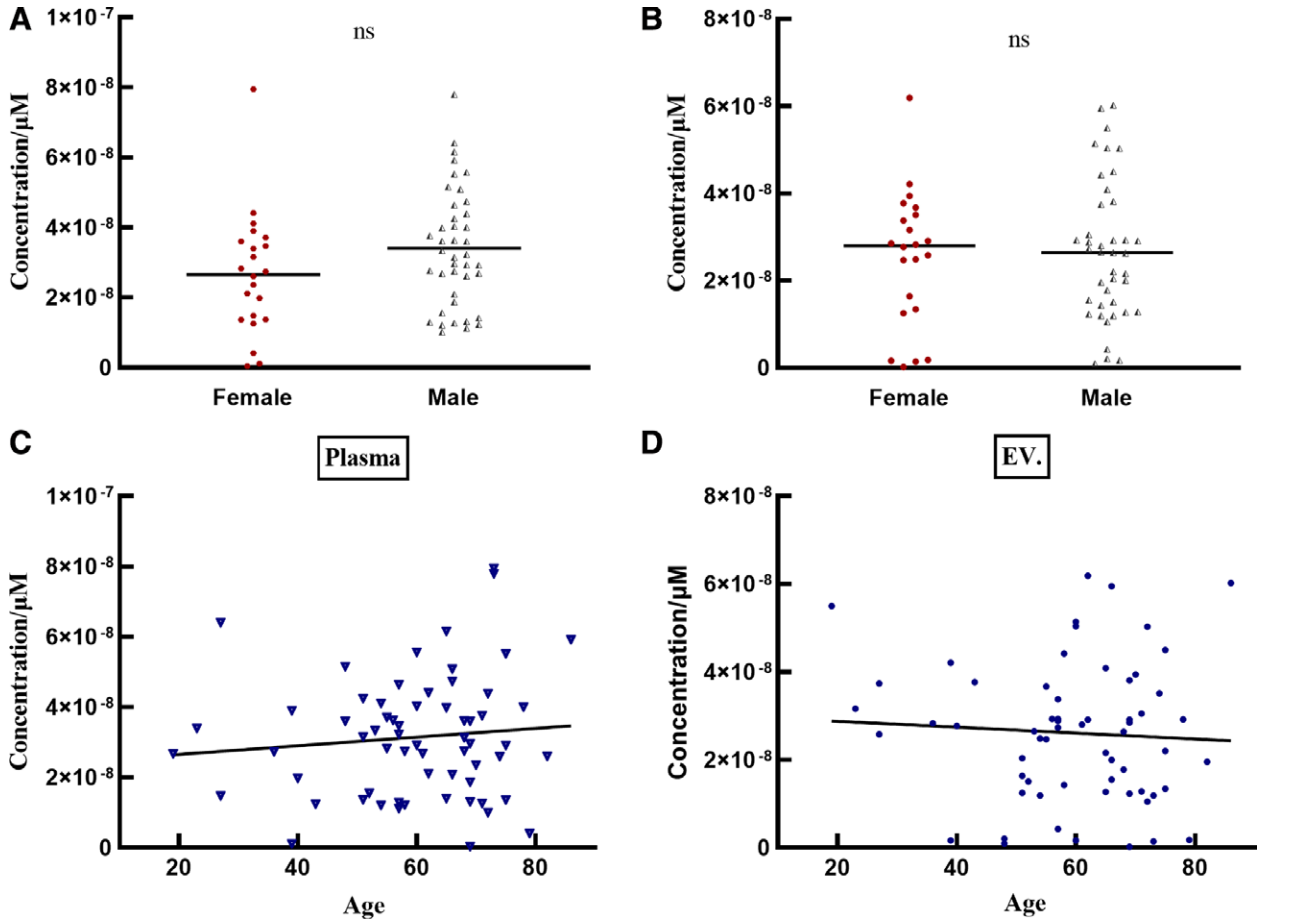

Figure 3. The correlation of miR-375 in plasma and EVs with gender and age. Graphics are created using Prism 8.0 (GraphPad Software, USA). (a) The expression of miR-375 in EVs for different genders. (b) The expression of miR-375 in plasma for different genders. (c) The correlation of age and miR-375 expression in EVs. (d) The correlation of age and miR-375 expression in plasma. (ns, not significant.).

10^{-8}μM) to construct the standard curve, which was used to calculate the concentration of miR-375 in plasma and EVs. The real-time quantitative polymerase chain reaction (RT-PCR) was carried out in 10μL volumes, which contained 2μL 5x MMLV RT Buffer, 0.375μL dNTP(10 mm), 0.6μL miRNA RT primers(1μM), 0.25μL RNasin (40U/μL), 0.1μL MMLV Reverse Transcriptase, 4.675μL RNase-free H_2O and 2μL RNA (1–2μg) of samples or synthetic miR-375. The reaction program of RT-PCR was as follows: 24°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes and then storage at 4°C.

The Ct values were then obtained by qPCR, and the final reaction volume used for the qPCR system was 20μL (7500 Sequence Detection System; applied Biosystems), including 10μL 2x real-time PCR master mix buffer, 0.4μL miRNA specific primer set (10μm), 0.2μL miRNA specific probe (10μm), 0.4μL ROX reference dye (50x), 0.2μL Taq DNA polymerase (5U/μL), 3μL cDNA (products of the above RT-PCR) and 5.8μL sterilized H_2O. The programs were as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 12 sec and 62°C for 40 sec. The sequences were used as following: 5'-GTATCGTTTGTTCGTTCGGCTC-3' (forward) and 5'-TATGCTTGTTCACGACACCTTCAC-3' (reverse) for specific primer set; CCC + TA + TCCAAGCA + TACAGAC + TCACG for specific probe.

2.6. Detection of TMs

The levels of TMs (including α-fetoprotein [AFP], carcinoembryonic antigen [CEA], carbohydrate antigen [CA] 125 and CA19-9) were detected by electrochemiluminescence, which was performed by the laboratory of naval medical center of the second military medical university. Blood samples were collected after diagnosis and before any treatment. The samples were left at room temperature for 20 minutes and centrifuged at 1600g for 10 minutes. All samples must be tested within 1 day.

2.7. Bioinformatics analysis

The expression data of the target miRNA with diagnostic potential for EC were obtained from the GEO database (https://www.ncbi. nlm.nih.gov/geo/) using R v3.5.3 (https://cran.r-project.org/bin/windows/base/old/3.5.3/), and the target genes of this miRNA were predicted by TarBase (https://dianalab.e-ce.uth.gr/tools/tarbasev8),[29] Targetscan (https://www.targetscan.org/)[30] and microT-CDS(https://dianalab.e-ce.uth.gr/tools/microT-CDS).[31] The Kyoto encyclopedia of genes and genomes (KEGG; https://www.kegg.jp/kegg/kegg1.html), (gene annotation; https://david.ncifcrf.gov/), and (protein-protein interaction; https://cn.string-db.org/) were used for the analysis of target gene functions. The expression of genes was analyzed by UALCAN (http://ualcan.path.uab.edu/).[32]

2.8. Statistical analysis

Data were reported as the mean ± SEM. The nonparametric Mann–Whitney *U* test was employed to compare the miRNA expression between pairwise and the statistical analyses were

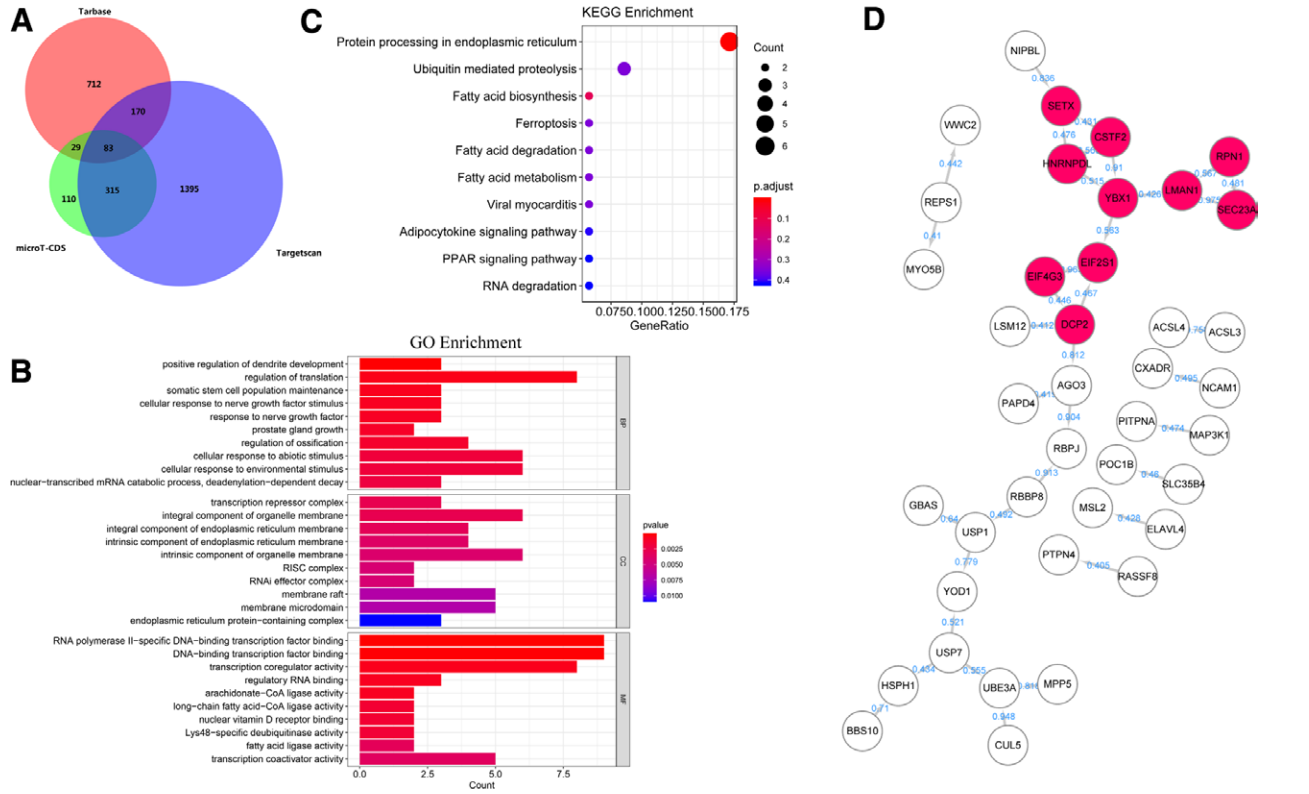

Figure 4. Bioinformatics analysis of miR-375 and its target genes. (A) Prediction of miR-375 target genes and their connections. (B) GO analysis of the molecular functions, cellular components and biological processes of target genes. (C) KEGG pathway analysis of target genes. (D) PPI analysis of the target genes. Blue numbers, the combination score. MCODE results were tagged with red based on a k score > 2. GO = gene annotation, KEGG = Kyoto encyclopedia of genes and genomes, PPI = protein-protein interaction.

performed using Prism 8.0 (GraphPad Software, USA). A significance level of 0.05 was considered to be significant for all calculations. The correlation between miRNA expression and gender or age were processed with Pearson correlation analysis. The receiver operating characteristic (ROC) was performed using the SPSS v.22.0 software package (IBM, Armonk, NY). Assessing the potential of miR-375 for EC diagnosis relies on the area under the curve (AUC). Overall survival was calculated from the ECs and control subjects from the cancer genome atlas (TCGA) database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), and its distribution was analyzed using the Kaplan–Meier method and compared with the 2-sided log-rank test. *p represents $P < .05$. **p represents $P < .01$. ***p represents $P < .001$. ****p represents $P < .0001$.

3. Results

3.1. Description of subjects

Subjects aged 19 to 86 years old participated in this study, including 26 controls and 37 EC patients (Table 1). For EC subjects, 17 subjects were in stage I/II, 20 subjects were in stage III and IV. TMs including AFP, CEA, CA125, and CA19-9 were also detected.

3.2. Search for target miRNA

From the GEO database, GSE61047, GSE43732 and GSE6188 were selected for the target miRNA searching. The 3 data involved 154 pairs of tissues from para-cancer and esophageal cancer samples. The summarized data was presented in Figure 1A. Setting $P < .05$ and | fold of change |≥ 2 as the threshold, we identified 5 miRNAs, namely hsa-miR-1, hsa-miR-133b, hsa-miR-375, hsa-miR-137 and hsa-miR-409-5p. Hsa-miR-137 was detected only in GSE6188 and GSE43732, but not in GSE71047. Consequently we omitted hsa-miR-137 from further screening. According to the expression level of the 4 miRNAs, we eventually selected hsa-miR-375, which was upregulated most in the 3 data (10.59 ± 4.674-fold of change), and this "upregulation" refers to the level of miR-375 in the adjacent tissues relative to the cancer tissues (Fig. 1B). Survival analysis of data from the TCGA database for EC revealed that patients with high miR-375 level had longer survival ($P < .001$, Fig. 1C).

3.3. Detection of miR-375 in plasma and EVs

Synthetic miR-375 was diluted to different concentrations, and the standard curve was established based on the results of qPCR (Fig. 2A). The expression of miR-375 in EVs and plasma was both significantly decreased in EC samples than in controls (Fig. 2B, C), and the ROC curves of miR-375 in plasma and EVs are shown in Figure 2D. The AUC of miR-375 in plasma and EVs was 0.707 and 0.852. However, there was no statistical difference in AUC between miR-375 in plasma and EVs ($P = .067$).

The relationship of miR-375 in EVs and plasma was also evaluated with gender or age. The results confirmed that there was no difference in the expression of miR-375 in plasma or EVs in females and males (Fig. 3a, b), and age was also not linearly correlated with the miR-375 expression; the correlation coefficients were 0.102 and 0.061, respectively (Fig. 3c, d).

3.4. Bioinformatics analysis

TarBase, Targetscan and micro T-CDS were used to search the target genes of miR-375, and a total of 83 interacting target

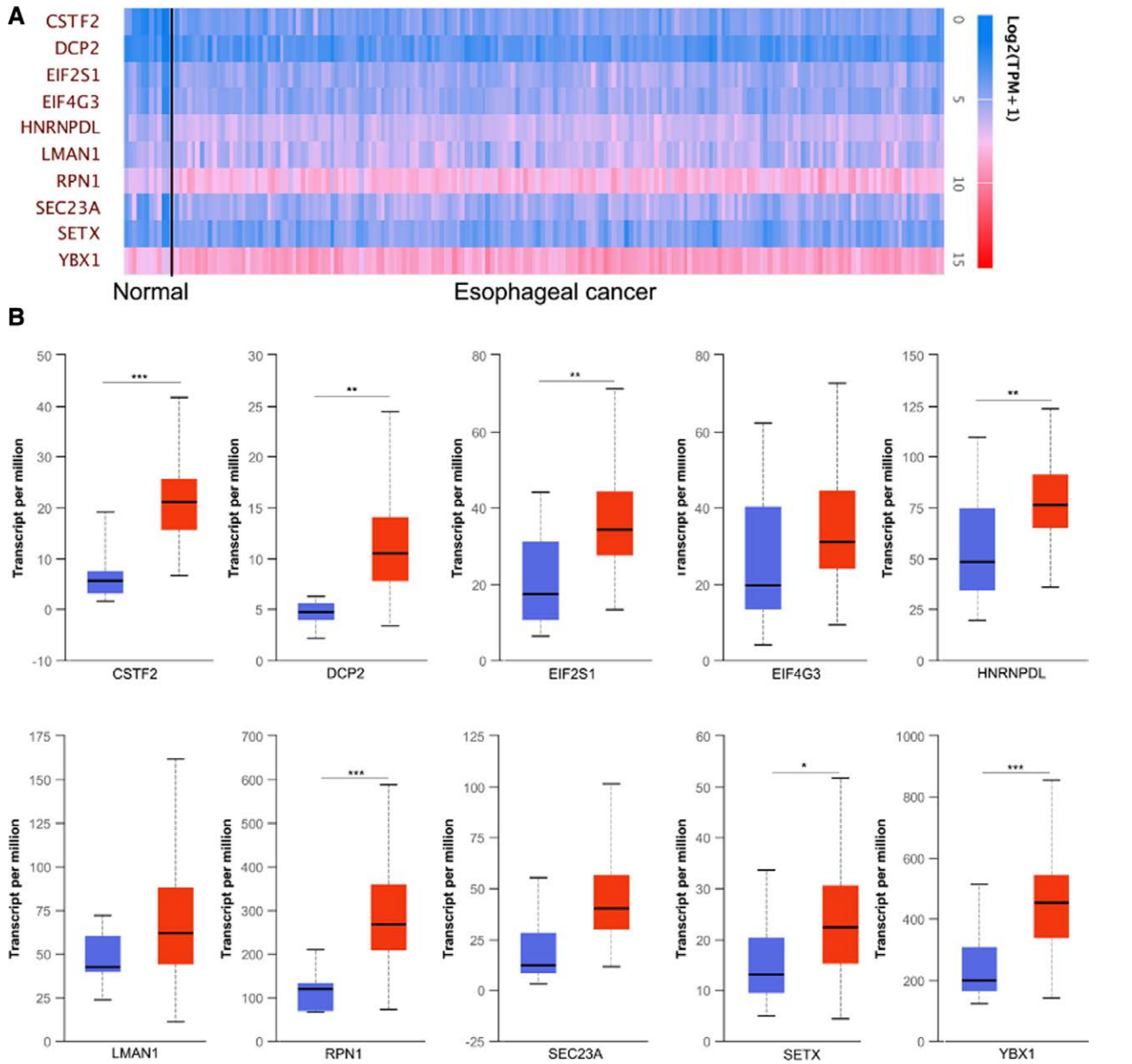

Figure 5. The expression of ten genes from the TCGA database. (A) The expression pattern of ten genes in esophageal cancer. (B) The individual expression of the ten genes. Blue, normal samples. Red, EC samples. TCGA = the cancer genome atlas.

genes were obtained (Fig. 4A). Gene annotation enrichment analysis was used for gene annotation (Fig. 4B). Nine of these genes are related to the regulation of translation for biological processes. In cellular components, the integral and intrinsic components of the organelle were the most enriched annotation. In molecular function, more than 8 genes were associated with polymerase II transcription. Kyoto encyclopedia of genes and genomes pathway enrichment analysis found that "protein processing in the endoplasmic reticulum" was the most enriched pathway (Fig. 4C). And protein-protein interaction analysis obtained the network of proteins (Fig. 4D). Further analysis of the ten genes indicated that they may participate in protein processing in the endoplasmic reticulum and RNA transport. Then the expression of the ten genes in esophageal cancer from the TCGA database was shown in Figure 5A. CSTF2, DCP2, EIF2S1, HNRNPDL, RPN1, SETX, and YBX1 significantly increased expression in EC samples than controls (Fig. 5B). We further performed the survival analysis of ten genes (Fig. 6). Only EIF4G3 had a P value < .05, which indicated it might be related to high expression of EIF4G3 might indicate a poor prognosis.

4. Discussion

Aberrant miRNA expression is associated with tumorigenesis, tumor progression, cancer prognosis, and response to therapy.[33] In this study, the diagnostic value of miR-375 was evaluated in 37 EC patients and 26 healthy controls. Kaplan–Meier analysis showed that low miR-375 expression was positively correlated with short survival time in patients with esophageal cancer. This may be related to the hypermethylation of miR-375 promoter region in esophageal cancer tissues, which inhibits cell colony formation, cell motility and proliferation, and tumor formation and metastasis.[34] Subsequently, validation in clinical blood samples revealed that miR-375 in plasma or EVs was significantly different between ECs and controls (P < .01), but did not differ by gender or age. The

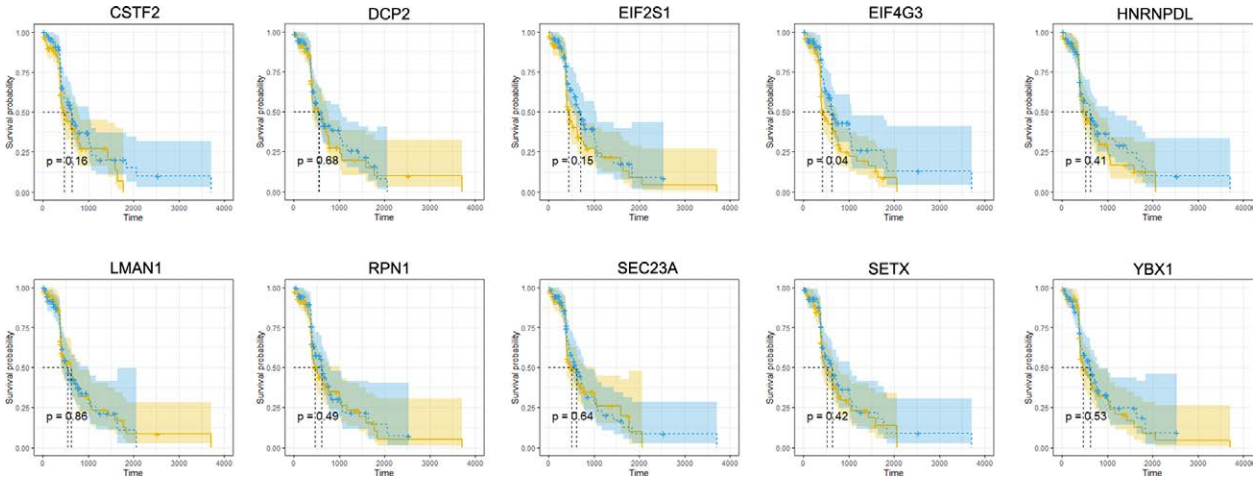

Figure 6. Survival analysis of ten genes. The yellow area indicates high expression, while the blue represents low expression.

specific reason for the low expression of Mir-375 in EC is unclear and requires further study.

TMs are commonly used in routine tumor examinations.[35] In this study, we collected TMs results from 37 patients with EC subjects (Table 1). Only one of these subjects had abnormal CA19-9 results, and all had AFP, CEA or CA125 values within normal ranges. The low detection rate of the TMs signals indicates the need for higher specificity approach. EVs are small vesicles that transport miRNAs from the source to the target cells, and encapsulated miRNAs have been demonstrated to serve as tumor biomarkers.[36] For example, Madhavan et al[37] confirmed that a set of EV-miRNA markers could help diagnose pancreatic cancer. MiR-375 in plasma and EVs showed significant differences between the control and EC groups. The membrane structure of EVs provides protection to cellular contents and ensures their stability.[38] The main evaluative criteria of diagnostic value of miRNAs is AUC in the ROC analysis. The AUC of miR-375 in plasma and EVs was 0.707 and 0.852. Therefore, we concluded that miR-375 in exosomes has better diagnostic value.

Bioinformatics analysis of miR-375 was performed to predict its target genes and determine its potential functions. Target gene and functional analyses indicated that miR-375 may be involved in protein processing of organelle membranes and is related to the regulation of translation. All target genes were abnormally expressed in EC samples, and the differences were statistically significant except for EIF4G3, LMAN1, and SEC23A. Survival analysis of these ten genes revealed that only EIF4G3 was significantly associated with survival ($P = .04$). EIF4G3 is a eukaryotic translation initiation factor, and Huang et al found that inhibiting the expression of EIF4G3 could induce apoptosis and slow down tumor growth.[39,40]

MiR-375 is a vital cancer-associated RNA,[41] which has its own promoter and can be transcribed independently,[42] suggesting that the downregulation of miR-375 may be due to abnormalities in the promoter region in ECs. Hence, reduced miR-375 levels may be a potential diagnostic marker for early detection of EC.[17] Bioinformatics analysis showed low expression of miR-375 and overexpression of EIF4G3, which may indicate poor prognosis of patients with EC. Based on TCGA database, there was no significant difference in EIF4G3 expression between EC and control samples, which may be due to the regulation of EIF4G3 expression by miR-375 at the post-transcriptional level. Consequently, in future studies, the targeting relationship between miR-375 and EIF4G3 should be verified in vivo and in vitro.

The main limitation of this study is the sample size. Recruitment of these 63 eligible subjects, including 37 patients diagnosed with EC and 26 controls, took more than 2 years. In future research, the sample size will be increased to improve the accuracy of the results.

5. Conclusions

In conclusion, our results show that miR-375 in plasma and EVs is differentially expressed in ECs and controls. MiRNA-375 in EVs may be a novel marker for EC diagnosis and its diagnostic value is superior to miR-375 in plasma. Moreover, bioinformatics analysis indicated a possible pathway for miR-375, which will facilitate further research.

Author contributions

Conceptualization: Jie Chen, Zhigang Cai.
Data curation: Jie Chen, Zhigang Cai, Jing Hu, Lixia Zhou, Peili Zhang, Xiaoping Xu.
Investigation: Zhigang Cai, Jing Hu, Lixia Zhou, Peili Zhang, Xiaoping Xu.
Methodology: Jie Chen, Zhigang Cai, Jing Hu, Xiaoping Xu.
Project administration: Zhigang Cai, Jing Hu.
Validation: Jie Chen, Zhigang Cai, Jing Hu, Lixia Zhou.
Writing – original draft: Jie Chen, Xiaoping Xu.
Writing – review & editing: Xiaoping Xu.

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