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Original Paper

Potential Targets and Clinical Value of MiR-224-5p in Cancers of the Digestive Tract

Lu Zhang[a] Lan-shan Huang[a] Gang Chen[a] Zhen-bo Feng[a]

[a]Department of Pathology, First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region, China

Key Words
Microrna • MiR-224-5p • Digestive system cancers • Bioinformatics

Abstract
Background/Aims: MicroRNAs participate in various biological processes in malignant tumors. However, the mechanisms of miR-224-5p in digestive system cancers are not fully understood. A comprehensive investigation of the clinical value and potential targets of miR-224-5p in cancers of the digestive tract is necessary. ***Methods:*** Expression profiling data and related-prognostic data of miR-224-5p were acquired from Gene Expression Omnibus, The Cancer Genome Atlas, ArrayExpress, and published literature. The potential target mRNAs of miR-224-5p were predicted using bioinformatics methods and finally annotated using Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. ***Results:*** MiR-224-5p is up-regulated in digestive system cancers (SMD=0.69, 95% CI: 0.43-0.96, P<0.0001) and exhibits a moderate diagnostic ability (AUC=0.84, 95% CI: 0.80-0.87). Our data also demonstrated that miR-224-5p is statistically significantly correlated with overall survival univariate analysis (HR=1.69, 95% CI: 1.15-2.49, P=0.007) and multivariate analysis (HR=2.39, 95% CI: 1.74-3.30, P<0.0001). In total, 388 potential miR-224-5p target mRNAs were predicted by bioinformatics methods. GO annotation analysis revealed that the top terms of miR-224-5p in biological process, cellular component and molecular function were *system development*, *neuron part*, and *transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding*, respectively. Moreover, eight pathways were identified in KEGG pathway enrichment analysis. ***Conclusions:*** MiR-224-5p is up-regulated and has the potential to become a diagnostic and prognostic biomarker in digestive system cancers. MiR-224-5p might play vital roles in cancers of the digestive tract but the exact molecular mechanisms need further study and verification.

Introduction

Digestive system cancers have the common histological origin of ducts and glands. According to the latest Cancer Statistics, a total of 310, 440 estimated new cases and 157, 700 estimated deaths occurred as a result of digestive system cancers in the United States [1]. Similarly, the morbidity and mortality of digestive system cancers are also increasing in

Zhen-bo Feng
Department of Pathology, the First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning, Guangxi Zhuang Autonomous Region 530021, (P.R. China)
Fax +86 771 5352194, E-Mail Fengzhenbo_GXMU@163.com

China. From top to bottom, esophageal cancer (ESCA), gastric cancer (GC), hepatocellular carcinoma (HCC), biliary tract cancer (BTC), pancreatic cancer (PC), and colorectal cancer (CRC) are the most commonly diagnosed cancers in the digestive tract. However, their prognoses remain fairly poor due to increasing prevalence, late diagnosis, continuous drug resistance and recurrence. Thus, it is necessary to discover potential novel diagnostic and prognostic biomarkers for digestive system cancers.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that are typically 19–24 nucleotides in length. MicroRNAs regulate possible target mRNAs. Numerous studies have concentrated on the roles of miRNAs, indicating that miRNAs participate in a variety of biological processes and signal pathways [2-4]. The roles of miRNAs in the diagnosis, prognosis, and therapeutic prediction of human malignancies have also been discovered [5-7].

The mature miRNA microRNA-224-5p (miR-224-5p, previously named miR-224) participates in a series of biological processes, including cell proliferation, migration and invasion, in various malignancies [8-12]. To date, numerous independent small sample studies have assessed the role of miR-224-5p in digestive system cancers, such as GC [13], CRC [14], HCC [15] and ESCA [16]. However, no comprehensive investigation of the clinical value and potential targets of miR-224-5p in digestive system cancers has been reported to date.

In this study, we first gathered data sources from the Gene Expression Omnibus (GEO), The Cancer Genome Atlas (TCGA), ArrayExpress, and related-prognostic published literature on miR-224-5p to estimate the clinical value of miR-224-5p in digestive system cancers (Fig. 1). Twelve miRNA-target prediction programs were applied to predict the potential target mRNAs of miR-224-5p, and the results were merged to make the conclusion more credible. Subsequently, bioinformatics analyses, including Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, of potential miR-224-5p target mRNAs were performed to investigate the relevant signaling pathways of miR-224-5p target genes and the potential molecular mechanisms of miR-224-5p in digestive system malignancies.

Materials and Methods

Data collection

MiR-224-5p microarray and RNA-seq datasets of digestive system cancers were collected from GEO (http://www.ncbi.nlm.nih.gov/geo/), TCGA (http://cancergenome.nih.gov/) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). Related prognostic studies of miR-224-5p in digestive system cancers were also identified using PubMed, Web of Science, Wiley Online Library, Cochrane Library, Science Direct, Chinese CNKI and Wan Fang database searches of publications up to July 25, 2017. The search strategy was as follows: (malignant* OR neoplasm* OR carcinoma OR cancer OR tumor) AND (miR-224 OR miRNA-224 OR microRNA-224 OR miR-224-5p OR miRNA-224-5p OR microRNA-224-5p).

Selection criteria

The inclusion criteria for microarray and RNA-seq datasets of miR-224-5p in digestive carcinomas were as follows: (1) cancer group samples were identified as a digestive system carcinoma; (2) the species were humans; (3) each dataset included a cancer group and a noncancerous group; (4) expression profiling data of miR-224-5p could be acquired.

The following inclusion criteria for related-prognostic data from published studies of miR-224-5p in digestive system cancers were employed: (1) study objects were histologically proven as a digestive system carcinoma; (2) study objects were humans; (3) the association between miR-224-5p expression and survival outcome was revealed; (4) sufficient survival data to evaluate the Hazard Ratio (HR) and 95% Confidence Interval (CI) were available.

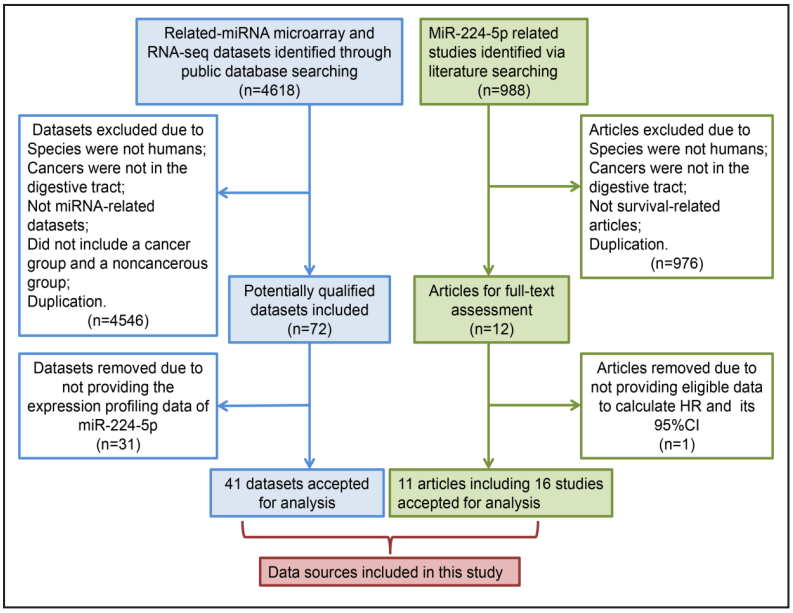

Fig. 1. Flow-chart of data sources included in this study.

Data extraction

Two reviewers collected the data individually from all included studies. The main extracted data in the miR-224-5p expression microarray or RNA-seq datasets consisted of data source, country, cancer type, platform, sample sizes and the expression data of miR-224-5p. For related-prognostic published literatures and datasets, data, such as first author, country, cancer type, test method, sample size, analysis method, HR and the 95% CI, were evaluated. Considering discrepancies, disagreements were determined via discussion.

Target mRNAs prediction

Potential target mRNAs of miR-224-5p were predicted by miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), which incorporates 12 existing miRNA-target prediction programs: miRWalk, miRDB, miRMap, miRNAMap, miRanda, mirbridge, Microt4, Pictar2, PITA, RNA22, RNAhybrid, and Targetscan. Only genes that were simultaneously predicted by at least eight online prediction tools were selected for further functional and pathway enrichment analysis.

GO annotation and KEGG pathway enrichment analysis

To explore functional annotation and pathway enrichment of the extracted target mRNAs of miR-224-5p, we performed GO annotation and KEGG pathway enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/), a functional enrichment analysis web tool. In DAVID, GO annotation analysis was divided into three GO categories, including Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). KEGG pathway enrichment analyses were listed according to P-value. Only entries with a P-value <0.05 were chosen for analysis.

Statistical analysis

The expression profiling data of miR-224-5p were log2-transformed. SPSS 22.0 (IBM, New York, USA) was applied to calculate the mean and standard deviation of miR-224-5p expression values. Stata Version 12.0 (StataCorp, College Station, TX, USA) was used to assess the pooled Standard Mean Deviation (SMD) with its 95% CI. When SMD was greater than zero and its 95% CI did not contain a zero, we could draw the conclusion that miR-224-5p expression level in the cancer group was increased compared with the normal control group, and the result was statistically significant.

To investigate the diagnostic value of miR-224-5p in digestive system neoplasms, Summary Receiver Operating Characteristic (SROC) curves and the Areas Under the Curves (AUCs) with their 95% CIs were analyzed using Stata Version 12.0. An AUC value of 0.5~0.7 suggested poor evidence, 0.7~0.9 represented moderate evidence, and greater than 0.9 indicated high evidence for diagnosis.

HRs with 95% CIs were pooled to evaluate the prognostic value of miR-224-5p in digestive system neoplasms and were acquired via three methods: (1) directly reported in the literature; (2) extracted from

Table 1. Characteristics of 41 microarray and RNA-seq datasets included in this study. BTC: biliary tract cancer; CRC: colorectal cancer; ESCA: esophageal cancer; GC: gastric cancer; PC: pancreatic cancer; HCC: hepatocellular carcinoma; SD: standard deviation; Mean1±SD1: expression level of miR-224-5p in the cancer group; Mean2±SD2: expression level of miR-224-5p in the normal control group

Number	Data source	Country	Cancer type	Platform	Cancer group	Normal control	Mean1±SD1	Mean2±SD2
1	GEO:GSE47764	China	BTC	GPL11487	3	3	4.87±0.58	5.26±1.23
2	GEO:GSE53870	China	BTC	GPL18118	63	9	9.08±0.33	9.32±0.20
3	GEO:GSE7828	USA	CRC	GPL4700	79	79	9.23±0.70	9.23±0.74
4	GEO:GSE10259	USA	CRC	GPL4411	59	7	8.32±1.10	6.95±0.25
5	GEO:GSE28364	Italy	CRC	GPL13328	40	40	8.33±1.42	11.01±1.15
6	GEO:GSE35834	Italy	CRC	GPL8786	55	23	4.32±1.57	2.39±0.74
7	GEO:GSE35982	China	CRC	GPL4133	8	8	7.72±1.48	5.11±0.43
8	GEO:GSE49246	China	CRC	GPL17496	40	40	9.51±0.32	9.51±0.32
9	GEO:GSE54088	Germany	CRC	GPL8178	9	10	12.68±0.80	11.70±0.83
10	GEO:GSE54632	China	CRC	GPL8786	5	5	4.08±0.13	3.97±0.13
11	GEO:GSE68377	China	CRC	GPL8786	7	7	3.99±0.16	4.00±0.16
12	GEO:GSE83924	Hungary	CRC	GPL16384	20	20	7.60±0.87	6.26±0.55
13	GEO:GSE98406	USA	CRC	GPL16384	14	14	5.66±0.31	5.22±0.10
14	GEO:GSE13937	USA	ESCA	GPL8835	27	25	8.31±1.13	7.94±1.17
15	GEO:GSE26595	South Korea	GC	GPL8179	60	8	9.89±1.36	7.99±0.81
16	GEO:GSE26645	China	GC	GPL11487	4	4	2.37±0.65	2.47±1.33
17	GEO:GSE28700	Taiwan	GC	GPL9081	20	20	2.37±1.52	1.66±1.11
18	GEO:GSE33743	Portugal	GC	GPL14895	37	4	7.77±0.52	7.26±0.34
19	GEO:GSE54397	South Korea	GC	GPL15159	16	16	6.07±2.39	5.36±0.92
20	GEO:GSE63121	China	GC	GPL8786	15	15	2.61±0.42	2.62±0.34
21	GEO:GSE67354	South Korea	GC	GPL19952	5	5	8.08±0.56	8.38±1.04
22	GEO:GSE32678	USA	PC	GPL7723	25	7	5.51±1.42	3.95±1.50
23	GEO:GSE41369	Italy	PC	GPL16142	9	9	6.50±0.75	5.99±1.34
24	GEO:GSE43796	South Korea	PC	GPL15159	6	5	8.42±1.23	6.65±0.52
25	GEO:GSE60978	Norway	PC	GPL15159	51	6	7.40±0.74	6.27±1.04
26	GEO:GSE62452	USA	PC	GPL6244	69	61	2.70±0.67	2.67±0.58
27	GEO:GSE6857	USA	HCC	GPL4700	238	241	9.83±0.86	10.01±0.76
28	GEO:GSE10694	China	HCC	GPL6542	78	88	11.59±0.97	10.68±0.14
29	GEO:GSE12717	USA	HCC	GPL7274	10	6	9.55±2.18	4.78±1.40
30	GEO:GSE21362	Japan	HCC	GPL10312	73	73	7.10±1.85	5.87±1.39
31	ArrayExpress:E-MTAB-298	None	CRC	A-MEXP-1738	12	4	8.60±1.15	8.11±0.50
32	ArrayExpress:E-MEXP-3515	None	CRC	A-MEXP-1797	8	8	6.36±1.20	7.28±0.74
33	ArrayExpress:E-GEOD-35602	Japan	CRC	GPL8227	17	8	4.99±1.39	2.18±1.42
34	ArrayExpress:E-GEOD-30454	Spain	CRC	GPL8179	42	20	12.55±0.68	12.27±0.21
35	ArrayExpress:E-TABM-341	USA	GC	A-MEXP-620	184	169	8.54±1.16	7.90±1.09
36	TCGA (2016)	USA	BTC	None	36	9	8.21±1.27	6.98±1.23
37	TCGA (2016)	USA	CRC	None	619	11	7.88±1.47	3.66±1.34
38	TCGA (2016)	USA	ESCA	None	187	13	7.81±2.25	5.14±1.46
39	TCGA (2016)	USA	GC	None	446	45	5.75±1.85	5.20±1.46
40	TCGA (2016)	USA	PC	None	179	4	7.05±1.33	6.84±1.67
41	TCGA (2016)	USA	HCC	None	364	50	8.88±2.04	6.49±1.40

survival curves using Engauge Digitizer Version 4.1 (http://digitizer.sourceforge.net/) [17]; (3) calculated according to the expression level of miR-224-5p via Kaplan-Meier analysis. We estimated the pooled HR in digestive system cancers using Stata Version 12.0. When the pooled HR was greater than 1 and its 95% CI did not contain a 1, the data suggested that increasing miR-224-5p levels predict poor survival.

Heterogeneity in the studies was assessed using the chi-square (x^2) test of Cochran's Q [18] and inconsistency index (I^2) [19], and a P-value less than 0.05 or I^2 greater than 50% indicated significant heterogeneity in the study, and a random-effects model was applied. If the P-value was greater than 0.05 or I^2 was less than 50%, the fixed-effects model was selected. Subgroup analyses were applied to assess the origin of heterogeneity when significant heterogeneity existed.

Sensitivity analysis was used to evaluate the robustness of the pooled overall result by excluding studies one by one and comparing the pooled results of different models. Publication bias was estimated using Egger's and Begg's bias tests and visually represented by a funnel plot. A P-value less than 0.05 indicated statistically significant publication bias, and the trim and fill method was used to evaluate the number of missing studies and recalculate the pooled variables. All the results were performed using Stata version 12.0.

Results

MiR-224-5p expression levels in cancers of the digestive tract

A total of 41 miRNA expression microarray datasets and RNA-seq data were identified in this study, and the characteristics are presented in Table 1. As shown in Fig. 2, up-regulation of miR-224-5p was observed in the digestive cancer group compared with the

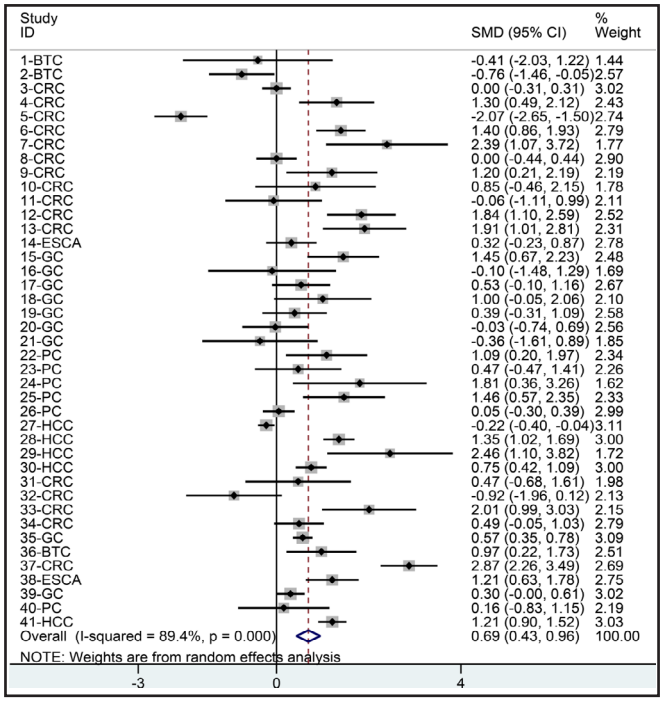

Fig. 2. Forest plot of datasets evaluating miR-224-5p expression between digestive system cancer groups and normal control groups (random-effects model).

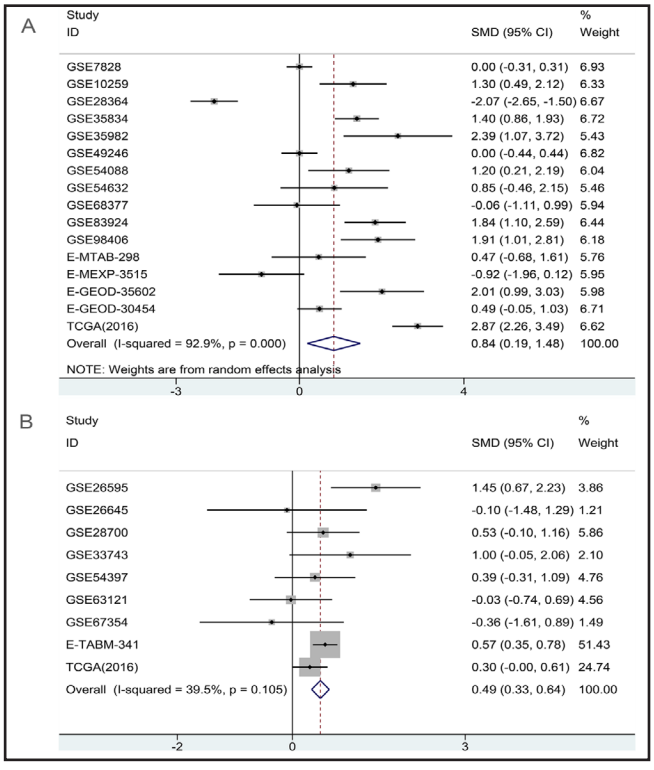

Fig. 3. The expression level of miR-224-5p in CRC and GC. A. Forest plot of datasets evaluating miR-224-5p expression between CRC and normal control groups. B. Forest plot of datasets evaluating miR-224-5p expression between GC and normal control groups.

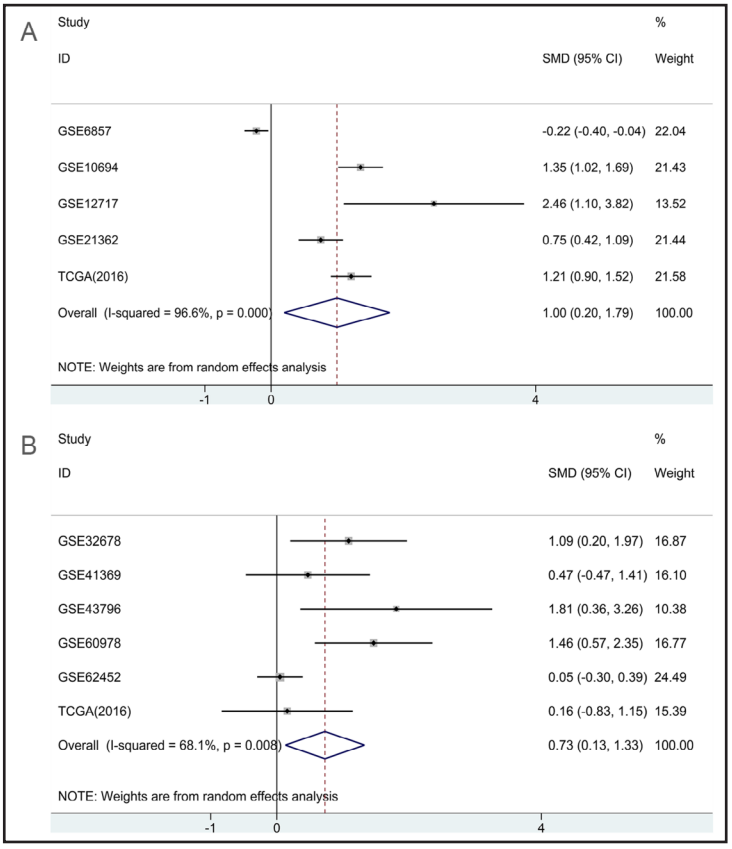

Fig. 4. The expression level of miR-224-5p in HCC and PC. A. Forest plot of datasets evaluating miR-224-5p expression between HCC and normal control groups. B. Forest plot of datasets evaluating miR-224-5p expression between PC and normal control groups.

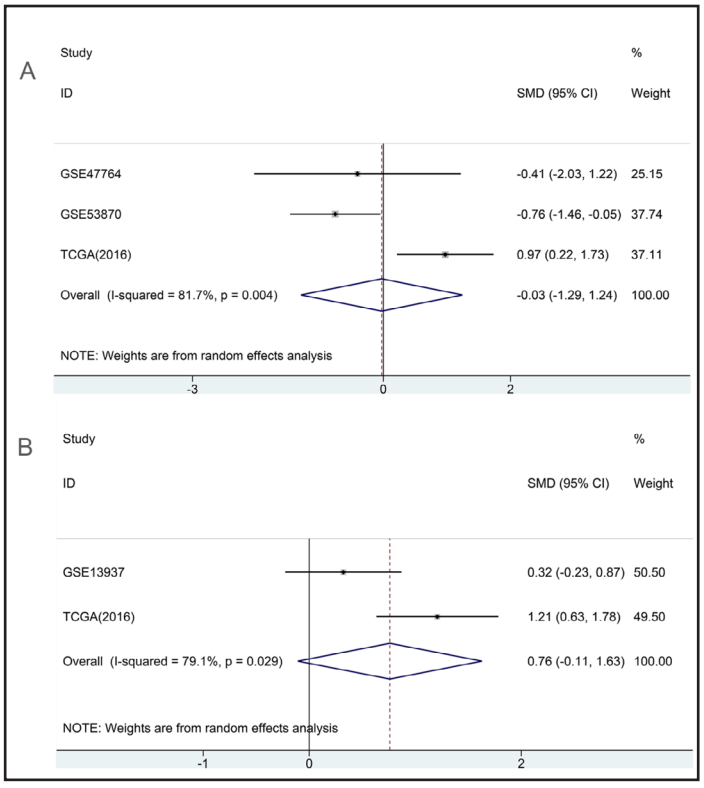

Fig. 5. The expression level of miR-224-5p in BTC and ESCA. A. Forest plot of datasets evaluating miR-224-5p expression between BTC and normal control groups. B. Forest plot of datasets evaluating miR-224-5p expression between ESCA and normal control groups.

normal control group, and the overall result was statistically significant (SMD=0.69, 95% CI: 0.43-0.96, P<0.0001). Significant heterogeneity occurred in individual datasets, so a random-effects model was applied ($P_{heterogeneity}$<0.0001, I^2=89.4%). Furthermore, subgroup analyses based on cancer type were constructed. Statistical significance was observed among four cancer types and their corresponding noncancerous groups, including CRC (P=0.011; Fig. 3A), GC (P<0.0001; Fig. 3B), HCC

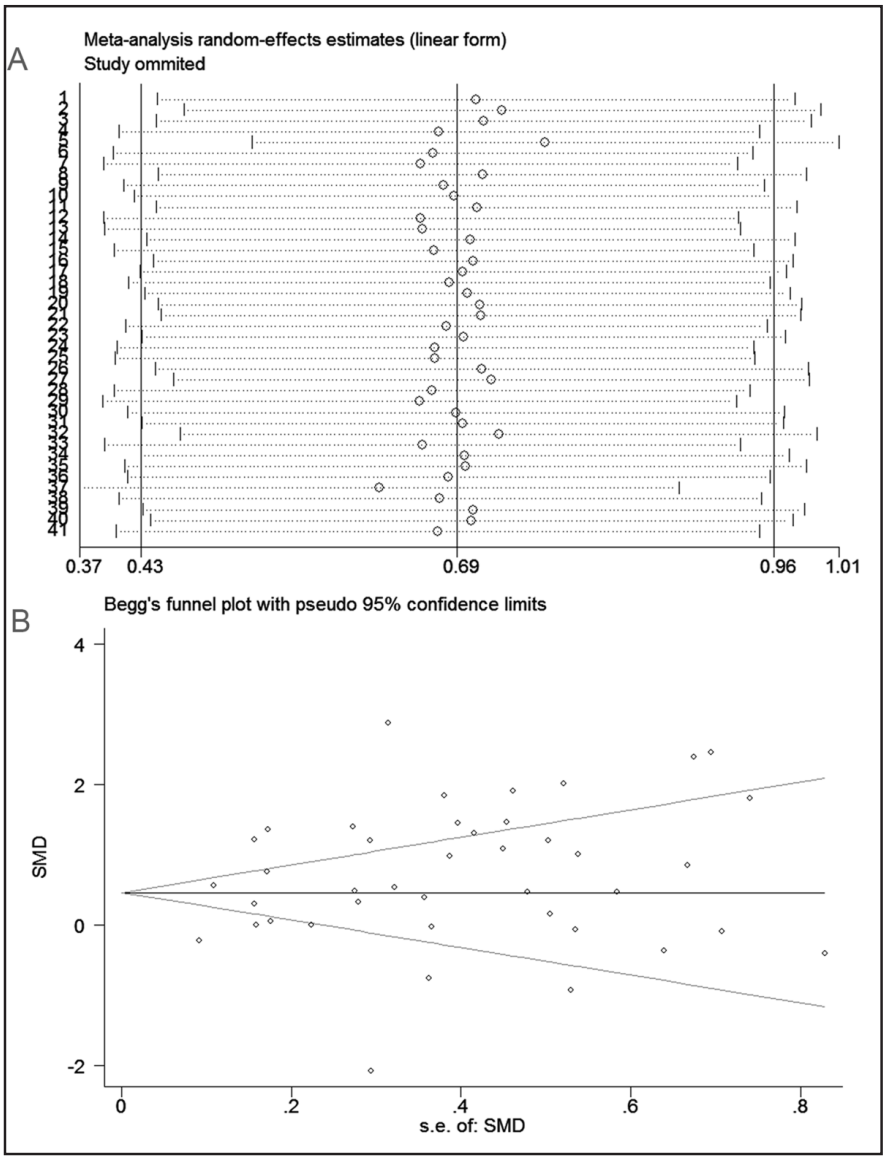

Fig. 6. Sensitivity analysis and publication bias test for the 41 included datasets. A. The results of the sensitivity analysis using a random-effects model. B. Funnel plot of the 41 included datasets.

(P=0.014; Fig. 4A), and PC (P=0.017; Fig. 4B). SMDs with 95% CIs were 0.84 (0.19-1.48), 0.49 (0.33-0.64), 1.00 (0.20-1.79), and 0.73 (0.13-1.33), respectively. Regarding BTC (P=0.969; Fig. 5A) and ESCA (P=0.086; Fig. 5B), no statistical significance was observed between cancer and noncancerous groups, and SMDs with 95% CIs were -0.03 (-1.29-1.24) and 0.76 (-0.11-1.63), respectively.

Next, sensitivity analysis was applied to estimate the robustness of the pooled SMD of miR-224-5p expression in all datasets. The results indicated that the pooled overall SMD was stable (Fig. 6A). P-values of Egger's and Begg's bias tests were 0.077 and 0.762, respectively, and the funnel plot was approximately symmetric (Fig. 6B), indicating that no publication bias existed regarding miR-224-5p expression.

MiR-224-5p diagnostic value in cancers of the digestive tract

To investigate the diagnostic value of miR-224-5p in cancers of the digestive tract, SROC curves and the AUCs with their 95% CIs were analyzed in our study. The overall AUC of the including 41 datasets was 0.84 (95% CI: 0.80-0.87) (Fig. 7). Next, we generated different SROC curves based on different cancer types. In SROC curves analyses of CRC, GC, HCC and PC, the AUCs with the 95% CIs were 0.86 (0.82-0.88), 0.74 (0.70-0.78), 0.82 (0.78-0.85) and

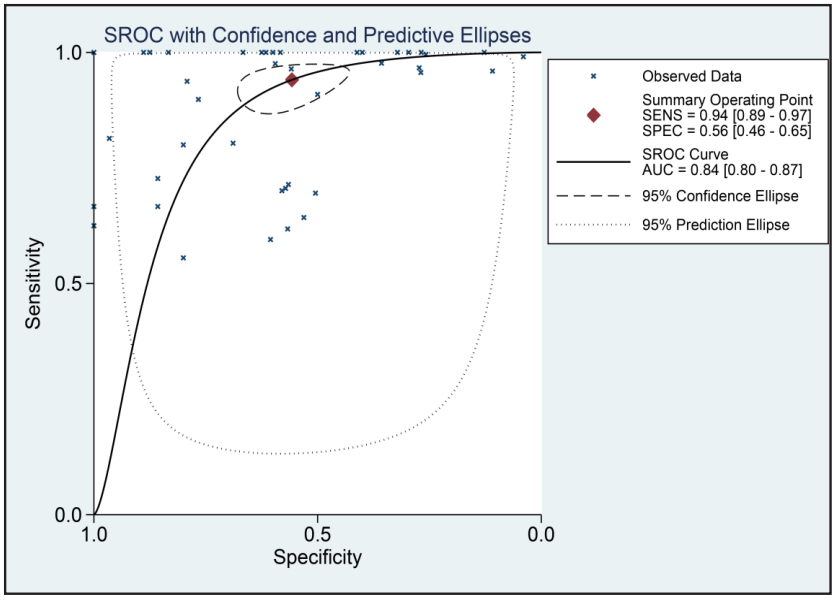

Fig. 7. SROC curve for digestive system cancer patients from normal controls based on miR-224-5p expression.

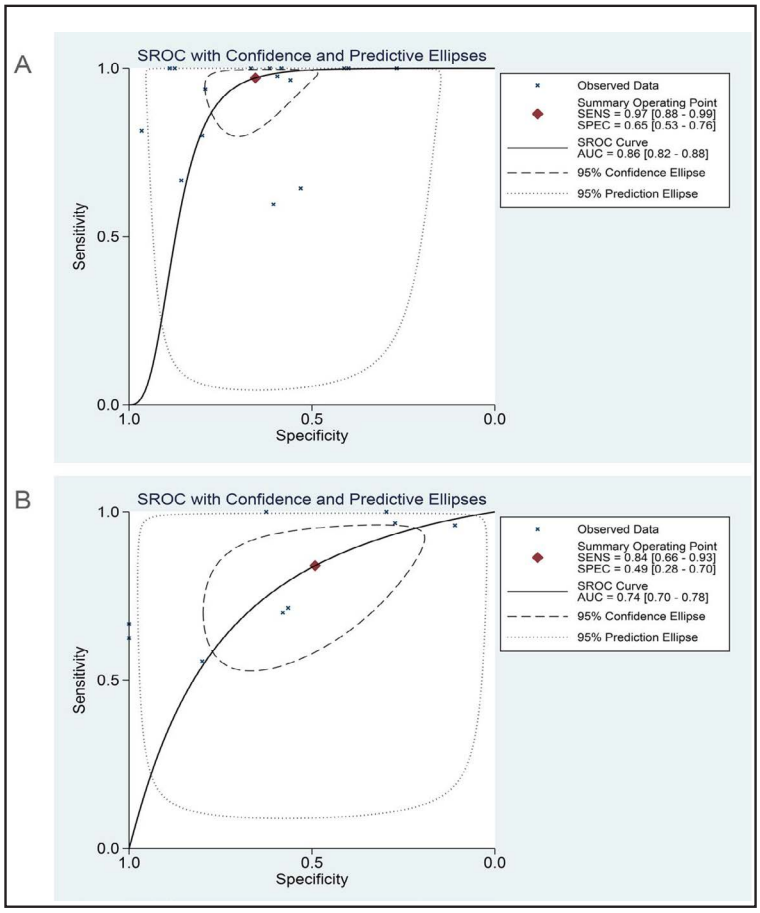

Fig. 8. SROC curves for CRC and GC patients from normal controls based on miR-224-5p expression. A. The diagnostic ability of miR-224-5p in CRC. B. The diagnostic ability of miR-224-5p in GC.

0.82 (0.79-0.85), respectively (Fig. 8A-8B, Fig. 9A-9B). Regarding BTC and ESCA, we did not perform SROC curve analysis given that the datasets only included three and two samples, respectively.

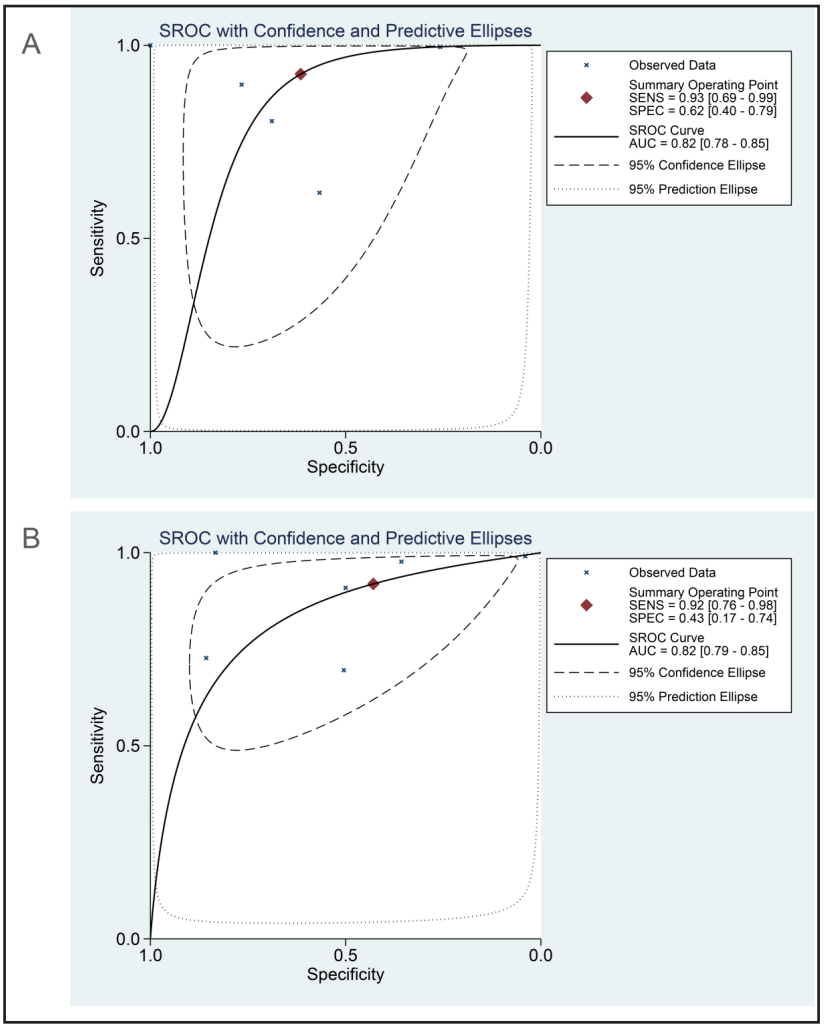

Fig. 9. SROC curves for HCC and PC patients from normal controls based on miR-224-5p expression. A. The diagnostic ability of miR-224-5p in HCC. B. The diagnostic ability of miR-224-5p in PC.

MiR-224-5p prognostic value in cancers of the digestive tract

To estimate miR-224-5p prognostic value in cancers of the digestive tract, the available data that exhibited miR-224-5p prognostic merit based on published studies, microarrays and RNA-seq data were collected. In total, 16 studies from 11 published manuscripts and two studies from two microarrays were included in this prognostic analysis. The characteristics of the included studies are presented in Table 2.

In the overall survival (OS) univariate analysis of 17 studies included from all 18 studies, the pooled HR and 95% CI was 1.69 (1.15-2.49) (P=0.007) (Fig. 10A), suggesting that increasing miR-224-5p levels predict unfavorable overall survival in patients with digestive system cancers. Simultaneously, in the OS multivariate analysis of eight studies selected from 18 studies, fixed-effects model results confirmed that increased miR-224-5p expression is associated with unfavorable overall survival in digestive system cancer patients (HR: 2.39, 95% CI: 1.74-3.30, P<0.0001; Fig. 10B). However, progression-free survival (PFS) and disease-free survival (DFS) analysis of six records assessed by both univariate and multivariate analyses revealed no statistically significant differences between miR-224-5p expression and PFS and DFS (HR: 1.07, 95% CI: 0.38-3.02, P=0.893; Fig. 10C). Furthermore, we performed subgroup analyses based on cancer type of the pooled OS analysis, including 25 records, using both univariate and multivariate analyses. As shown in Fig. 11, elevated miR-224-5p expression predicts poor overall survival in CRC, and the result was statistically significant (HR: 2.52, 95% CI: 1.80-3.53, P<0.0001). Nevertheless, no statistically significant differences were observed between miR-224-5p expression levels and the prognosis of patients with

Table 2. Characteristics of included studies in the prognostic analysis of miR-224-5p. GC: gastric cancer; CRC: colorectal cancer; HCC: hepatocellular carcinoma; BTC: biliary tract cancer; ESCA: esophageal cancer; OS: overall survival; DFS: disease-free survival; PFS: progression-free survival; SC: survival curve; HR: hazard ratio; 95% CI: 95% confidence interval; U: univariate analysis; M: multivariate analysis

First author (publication year)	Country	Cancer type	Test method	Sample size	Analysis method	HR (95% CI)
Zhang Y et al(2016)	China	GC	qRT-PCR	160	SC	OS (U) 0.83 (0.28 to 2.44)
Smid D et al(2016)	Czech	GC	qRT-PCR	41	SC	OS (U) 2.03 (0.80 to 5.14)
Shao CM et al(2012)	China	GC	ISH	112	SC	OS (U) 2.54 (1.38 to 4.68)
Ling H et al(2015)	Italy set 1	CRC	qRT-PCR	54	Report	OS (U/M) 3.32 (1.15 to 9.59) / 2.77 (0.95 to 8.105)
	Italy set 2		qRT-PCR	68	Report	OS (U/M) 4.41 (1.03 to 18.84) / 4.14 (0.96 to 17.76)
	UK		qRT-PCR	41	Report	OS (U) 4.92 (1.31 to 18.46)
	Romania		qRT-PCR	38	Report	OS (U/M) 4.25 (1.01 to 18.89) / 1.76 (0.36 to 8.64)
	Austria		qRT-PCR	74	Report	OS (U/M) 2.14 (1.21 to 3.77) / 2.36 (1.32 to 4.21)
	USA		RNA-seq	143	Report	OS (U/M) 2.99 (1.08 to 8.23) / 2.88 (0.97 to 8.56)
Adamopoulos PG et al(2015)	Greece	CRC	qRT-PCR	115	Report	OS (U/M) 4.08 (1.68 to 9.88) / 4.41 (1.72 to 11.34) DFS (U/M) 3.52 (1.20 to 10.33) / 4.61 (1.41 to 15.09)
Zhang GJ et al(2013)	China	CRC	qRT-PCR	108	SC	DFS (U) 2.04 (0.63 to 6.58)
Liao WT et al(2013)	China	CRC	qRT-PCR	110	SC	OS (U) 1.90 (0.92 to 3.89)
Yuan K et al(2013)	USA	CRC	qRT-PCR	108	SC	OS (U) 0.61 (0.33 to 1.12) DFS (U) 0.64 (0.23 to 1.78)
Gyongyosi B et al(2014)	Italy	HCC	qRT-PCR	20	Report	OS (U/M) 0.24 (0.07 to 0.79) / 0.11 (0.013 to 0.913) PFS (U/M) 0.28 (0.09 to 0.92) / 0.125 (0.015 to 1.034)
Liu M et al(2014)	China	HCC	qRT-PCR	136	Report	OS (U) 0.700 (0.553 to 0.886)
Zhuang LP et al(2015)	China	HCC	qRT-PCR	182	Report	OS (U/M) 2.188 (1.264 to 3.786) / 2.085 (1.142 to 3.807)
Zhang M et al(2015)	China	BTC	Microarray	63	Calculation	OS (U) 1.01 (0.53 to 1.91)
Mathe EA et al(2009)	USA	ESCA	Microarray	27	Calculation	OS (U) 1.43 (0.54 to 3.82)

GC (HR: 1.83, 95% CI: 0.99-3.37, P=0.052) and HCC (HR: 0.84, 95% CI: 0.38-1.86, P=0.666). Regarding BTC and ESCA, only one available study was included in the present investigation to discover the prognostic value of miR-224-5p. The HRs with 95% CIs were 1.01 (0.53-1.91) (P=0.976) and 1.43 (0.54-3.82) (P=0.474), respectively.

Moreover, sensitivity analysis was performed with all 25 records of OS univariate and multivariate analyses, and the pooled HRs were not significantly altered, indicating the stability of the result (Fig. 12A). In the publication bias analysis, no significant indication of publication bias was observed with Begg's test (P=0.528), whereas Egger's test revealed a potential publication bias (P=0.007). After performing the trim and fill method, four missing studies were identified, but the newly pooled result (HR: 1.62, 95% CI: 1.20-2.19) only minimally changed from the original result (HR: 1.84, 95% CI: 1.33-2.54), indicating that the original result was minimally affected by publication bias. The funnel plot after performing the trim and fill method is presented in Fig. 12B.

Potential target mRNAs of miR-224-5p

Potential target mRNAs of miR-224-5p were predicted using 12 existing miRNA-target prediction programs as described. Only genes that were simultaneously predicted by at least eight prediction solutions were selected. Accordingly, 388 mRNAs were recognized as potential target mRNAs of miR-224-5p for further analysis.

GO annotation and KEGG pathway enrichment analysis

GO annotation and KEGG pathway enrichment analysis of the 388 mRNAs were constructed using DAVID. The results of GO annotation enrichment analysis are summarized in Table 3-5 and Fig. 13. Regarding BP term annotation, the potential target mRNAs of miR-224-5p focused on a series of biological processes involved in development and transcription (P<0.001). Regarding CC term annotation, the target genes were significantly enriched in the neuron, cell, organelle, intracellular and somatodendritic compartments (P<0.001). Regarding MF term annotation, the target genes significantly altered the activity of transcriptional activators, protein kinases, phosphotransferases and protein serine/threonine kinases, and the binding of protein, DNA, heterocyclic compounds and organic

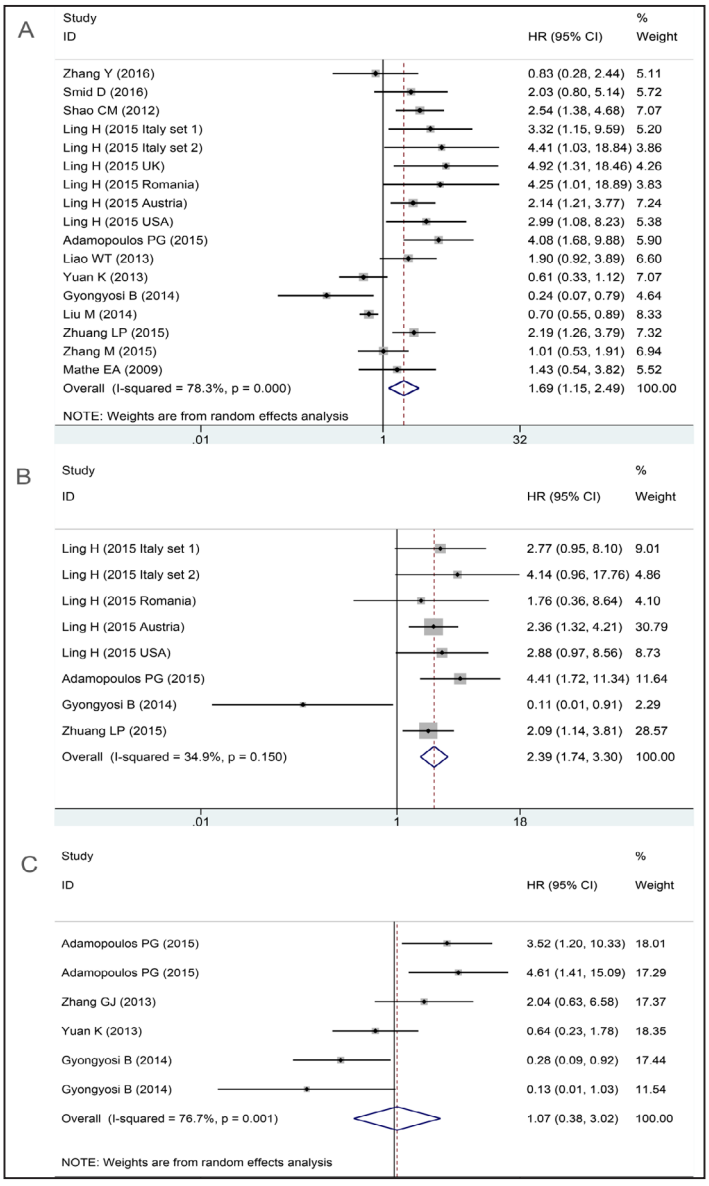

Fig. 10. Forest Plots estimated the prognostic value of miR-224-5p in digestive system cancers. A. Association between miR-224-5p overexpression and overall survival based on univariate analysis. B. Association between miR-224-5p overexpression and overall survival based on multivariate analysis. C. Association between miR-224-5p overexpression and disease-free survival and progression-free survival based on both univariate and multivariate analyses.

cyclic compounds (P<0.001). In KEGG pathway enrichment analysis, the results were chiefly concentrated in proximal tubule bicarbonate reclamation, ubiquitin-mediated proteolysis, PI3K-Akt signaling pathway, glioma, axon guidance, aldosterone-regulated sodium reabsorption, bile secretion and measles (P<0.05, Table 6 and Fig. 14).

Discussion

MiRNAs play vital roles in various human cancers and participate in several biological processes, such as cell differentiation, proliferation, mobility, and survival [20]. In addition, miRNAs are involved in tumorigenesis and progression of malignancies via different mechanisms, including control of cell cycle [21, 22], apoptosis [23, 24], autophagy [25], epithelial-mesenchymal transition [26], drug resistance [27] and metabolic reprogramming [28]. All of the above data indicate that miRNAs have the potential to be novel diagnostic and prognostic biomarkers for malignant tumors.

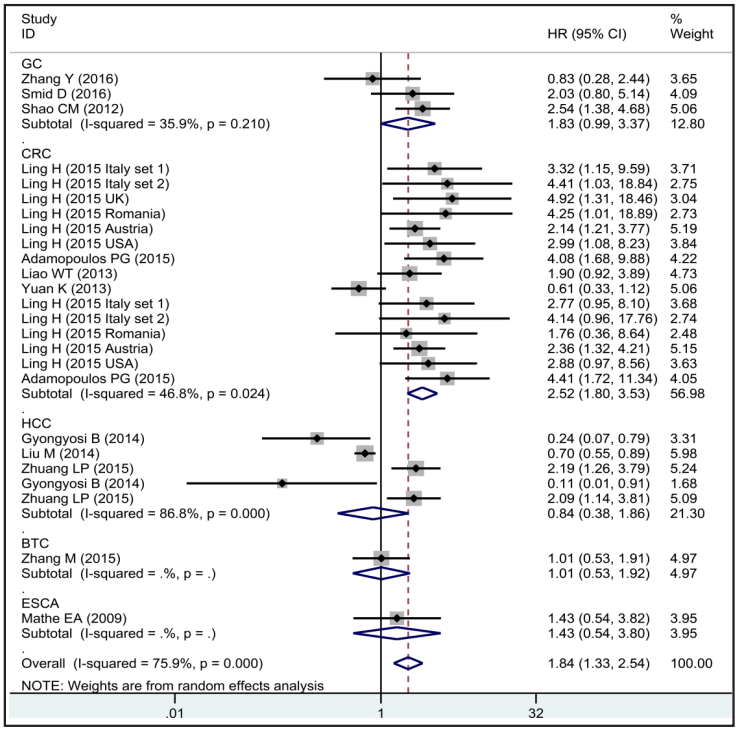

Fig. 11. Subgroup analysis by cancer type evaluated the association between miR-224-5p overexpression and overall survival based on both univariate and multivariate analyses.

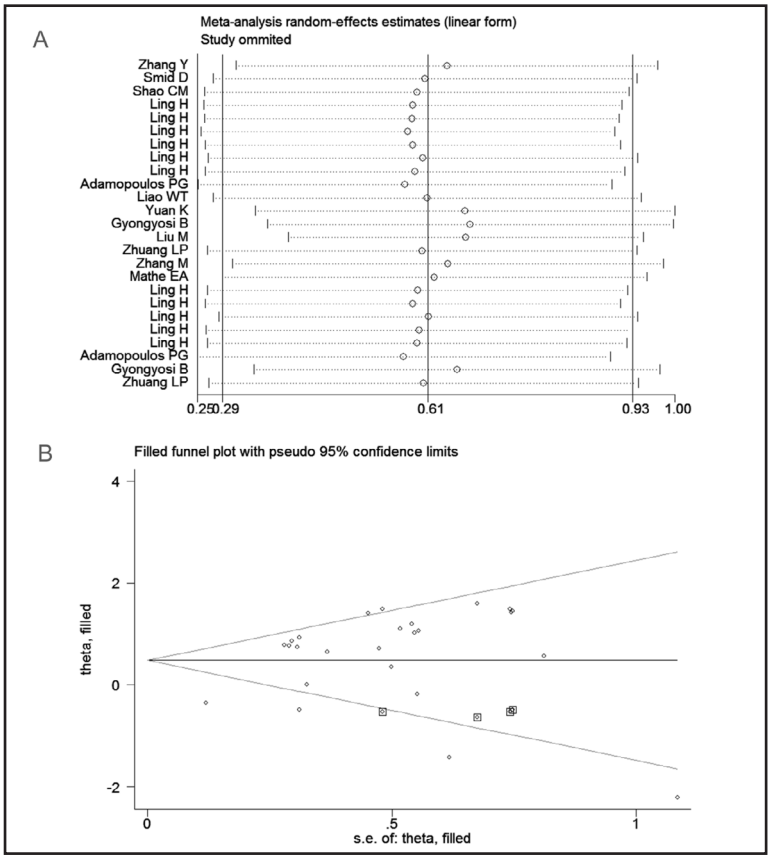

Fig. 12. Sensitivity analysis and publication bias test for all 25 records of overall survival based on both univariate and multivariate analyses. A. The results of the sensitivity analysis based on a random-effects model. B. Funnel plot after performing the trim and fill method for the included studies.

Increasing evidence demonstrates that miR-224 may be involved in the tumorigenesis and progression of various malignant tumors and participate in several biological processes.

Table 3. GO enrichment analysis (Biological Process) of the potential miR-224-5p target mRNAs constructed by DAVID

Biological Process	term	Count	P-value	Genes
GOTERM_BP_ALL	GO:0048731~system development	134	3.77E-08	GLDN, HMGCR, F2RL1, GDF5, MEGF11, HOXD10, SERPINE1, RNF38, RALA, LRRC55, etc.
GOTERM_BP_ALL	GO:0007275~multicellular organism development	144	2.99E-07	GLDN, HMGCR, F2RL1, GDF5, PRTG, MEGF11, HOXD10, SERPINE1, RNF38, RALA, etc.
GOTERM_BP_ALL	GO:0048468~cell development	73	5.24E-07	GLDN, F2RL1, WASF2, GDF5, NCS1, KCNJ10, SDC4, HOXD10, TMF1, SPRY3, etc.
GOTERM_BP_ALL	GO:0048856~anatomical structure development	156	6.11E-07	GLDN, HMGCR, RP2, F2RL1, PRTG, GDF5, MEGF11, HOXD10, SERPINE1, RNF38, etc.
GOTERM_BP_ALL	GO:0044767~single-organism developmental process	156	6.27E-07	GLDN, HMGCR, RP2, F2RL1, PRTG, GDF5, MEGF11, HOXD10, SYP, SERPINE1, etc.
GOTERM_BP_ALL	GO:0007399~nervous system development	78	6.35E-07	SLC5A3, GLDN, GDF5, NCS1, CXCR2, KCNJ10, SDC4, HOXD10, SPRY3, SLC1A2, etc.
GOTERM_BP_ALL	GO:0032502~developmental process	159	8.00E-07	GLDN, HMGCR, RP2, F2RL1, PRTG, GDF5, MEGF11, HOXD10, SYP, SERPINE1, etc.
GOTERM_BP_ALL	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	46	1.53E-06	AKNA, ELF4, F2RL1, BMPR2, HOXD10, ATF2, NFATC2IP, TCF21, PAX9, ASH2L, etc.
GOTERM_BP_ALL	GO:0006366~transcription from RNA polymerase II promoter	67	2.37E-06	CREBRF, AKNA, ELF4, F2RL1, GDF5, HSBP1, HOXD10, TMF1, NFATC2IP, ASH2L, etc.
GOTERM_BP_ALL	GO:0045893~positive regulation of transcription, DNA-templated	54	2.91E-06	AKNA, ELF4, F2RL1, HOXD10, NFATC2IP, ASH2L, PAX9, SERPINE1, DHX33, PHOX2B, etc.

A number of studies have identified the aberrant expression of miR-224 in different types of neoplasm. For example, up-regulated miR-224 expression levels were noted in cervical cancer [9], ovarian cancer [8], breast cancer [29], lung cancer [30] and glioma [31]. In contrast, down-regulated miR-224 expression levels were discovered in prostate cancer [32], diffuse large B cell lymphoma [33], and meningioma [34]. Regarding the clinical value of miR-224 in digestive system cancers, Yu et al. [35] performed in situ hybridization to identify the expression level of miR-224 in samples from 130 HCC patients and found that miR-224 was significantly increased in HCC tissues compared with adjacent noncancerous liver tissues. Wang et al. [36] discovered a trend of miR-224 expression (HCC tissues> paired adjacent nontumorous liver > normal liver) based on 100 HCC and paired adjacent nontumorous liver samples and 40 healthy liver tissues using qRT-PCR. Meanwhile, Adamopoulos et al. [37] identified the expression level of miR-224 in 115 CRC samples with 66 adjacent nontumor mucosae samples and observed significantly up-regulated miR-224 expression in CRC tissues compared with adjacent non-cancer mucosae. Liao et al. [38] found that miR-224 expression levels were significantly increased in 43 CRC samples compared with corresponding normal tissues via qRT-PCR. Simultaneously, He et al. [39] demonstrated that miR-224 was highly expressed in 29 GC samples compared with adjacent non-tumor tissues by qRT-PCR. Moreover, Mees et al. [40] verified significant overexpression of miR-224 in pancreatic ductal adenocarcinoma (PDAC) cell lines using microarray and qRT-PCR.

In the present study, we gathered a significant amount of data regarding miR-224-5p expression in digestive system cancers from GEO, ArrayExpress and TCGA public databases, including 41 microarray and RNA-seq datasets. Then, we performed a comprehensive meta-analysis of miR-224-5p expression data from all 41 microarray and RNA-seq datasets. The results indicated that miR-224-5p expression was significantly up-regulated in digestive system cancers compared with the noncancerous group. According to subgroup analyses based on cancer type, the up-regulated expression of miR-224-5p was verified in CRC, GC, HCC, and PC. The results were consistent with the previous studies mentioned above and were credible based on the large sample size in the study. However, no statistically significant differences in BTC and ESCA were observed. Considering that only three and two datasets were included in the analysis of BTC and ESCA, the stability of the pooled results is questionable. Therefore, further studies investigating miR-224-5p expression levels in BTC and ESCA are necessary to validate our conclusions.

Previous studies have suggested that miR-224 may be a prospective biomarker for the diagnosis of HCC [41-43]. Okajima et al. [44] performed a systematic review of microRNAs in plasma and identified miR-224 as a sensitive biomarker for detecting and monitoring HCC. However, the diagnostic value of miR-224-5p in the other digestive system cancers remains

Table 4. GO enrichment analysis (Cellular Component) of the potential miR-224-5p target mRNAs constructed by DAVID

Cellular Component	term	Count	P-value	Genes
GOTERM_CC_ALL	GO:0097458~neuron part	47	1.35E-04	TNFRSF21, PHLPP2, SRSF10, AGFG1, BMPR2, NCS1, IGF2BP1, KCNJ10, GABBR2, ITSN1, etc.
GOTERM_CC_ALL	GO:0044464~cell part	343	1.48E-04	DYNC1LI1, ATP1B3, GLDN, ATP1B2, PITPNA, RP2, GDF5, MEGF11, WTAP, ITSN1, etc.
GOTERM_CC_ALL	GO:0043005~neuron projection	37	1.77E-04	TNFRSF21, SRSF10, BMPR2, NCS1, IGF2BP1, GABBR2, ITSN1, HNRNPA3, SYP, SLC1A2, etc.
GOTERM_CC_ALL	GO:0005623~cell	343	2.12E-04	DYNC1LI1, ATP1B3, GLDN, ATP1B2, PITPNA, RP2, GDF5, MEGF11, WTAP, ITSN1, etc.
GOTERM_CC_ALL	GO:0043226~organelle	288	2.80E-04	DYNC1LI1, ATP1B3, PITPNA, RP2, WTAP, ITSN1, HOXD10, SSR1, MAP3K7, SYP, etc.
GOTERM_CC_ALL	GO:0043227~membrane-bounded organelle	270	4.43E-04	DYNC1LI1, ATP1B3, PITPNA, RP2, ITSN1, WTAP, HOXD10, SSR1, MAP3K7, SYP, etc.
GOTERM_CC_ALL	GO:0005622~intracellular	305	5.90E-04	DYNC1LI1, ATP1B3, ATP1B2, PITPNA, RP2, GDF5, WTAP, ITSN1, HOXD10, SSR1, etc.
GOTERM_CC_ALL	GO:0036477~somatodendritic compartment	27	6.67E-04	AGFG1, SRSF10, BMPR2, NCS1, IGF2BP1, ELOVL5, TRIM9, CAMK2D, INPP5F, PAFAH1B1, etc.
GOTERM_CC_ALL	GO:0044424~intracellular part	298	8.70E-04	DYNC1LI1, ATP1B3, ATP1B2, PITPNA, RP2, WTAP, ITSN1, HOXD10, SSR1, MAP3K7, etc.
GOTERM_CC_ALL	GO:0043231~intracellularmembrane-bounded organelle	246	1.09E-03	DYNC1LI1, ATP1B3, RP2, ITSN1, WTAP, HOXD10, SSR1, SYP, MAP3K7, EPC2, etc.

Table 5. GO enrichment analysis (Molecular Function) of the potential miR-224-5p target mRNAs constructed by DAVID

Molecular Function	term	Count	P-value	Genes
GOTERM_MF_ALL	GO:0001077~transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	19	1.60E-06	AKNA, PLAG1, PHOX2B, EGR2, KLF13, ELF4, SOX11, NR4A3, NPAS4, FOSB, etc.
GOTERM_MF_ALL	GO:0001228~transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	22	3.35E-06	MAF, AKNA, MAFG, PLAG1, PHOX2B, EGR2, KLF13, ELF4, SOX11, FOSB, etc.
GOTERM_MF_ALL	GO:0000982~transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	21	2.45E-05	AKNA, PLAG1, PHOX2B, EGR2, KLF13, ELF4, SOX11, NR4A3, NPAS4, FOSB, etc.
GOTERM_MF_ALL	GO:0004672~protein kinase activity	29	1.59E-04	CDK19, PRPF4B, NUAK1, BMPR2, STK17A, TRIB1, MAP3K7, IRAK3, PAK2, CAMK2D, etc.
GOTERM_MF_ALL	GO:0005515~protein binding	251	1.92E-04	DYNC1LI1, ATP1B3, GLDN, ATP1B2, RP2, GDF5, UHRF1BP1, ITSN1, WTAP, SSR1, etc.
GOTERM_MF_ALL	GO:0016773~phosphotransferase activity, alcohol group as acceptor	32	3.05E-04	CDK19, PRPF4B, NUAK1, BMPR2, STK17A, TRIB1, MAP3K7, IRAK3, PAK2, CAMK2D, etc.
GOTERM_MF_ALL	GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding	19	3.66E-04	AKNA, PLAG1, PHOX2B, EGR2, KLF13, ELF4, FOSB, NR4A3, NPAS4, MBD2, etc.
GOTERM_MF_ALL	GO:0004674~protein serine/threonine kinase activity	22	3.89E-04	CDK19, PIK3CG, PRPF4B, NUAK1, TAOK1, BMPR2, CDK9, CDK6, STK17A, MAP3K7, etc.
GOTERM_MF_ALL	GO:1901363~heterocyclic compound binding	152	4.36E-04	CAST, AKNA, DYNC1LI1, GNA14, PRPF4B, HMGCR, RP2, HBS1L, HOXD10, MAP3K7, etc.
GOTERM_MF_ALL	GO:0097159~organic cyclic compound binding	153	5.78E-04	CAST, AKNA, DYNC1LI1, GNA14, PRPF4B, HMGCR, RP2, HBS1L, HOXD10, SYP, etc.

Table 6. KEGG pathway enrichment analysis of the potential miR-224-5p target mRNAs constructed by DAVID

Category	term	Count	P-value	Genes
KEGG pathway				
KEGG_PATHWAY	hsa04964:Proximal tubule bicarbonate reclamation	4	1.01E-02	ATP1B3, ATP1B2, GLS, SLC4A4
KEGG_PATHWAY	hsa04120:Ubiquitin mediated proteolysis	8	1.88E-02	ANAPC1, TRIM37, UBE2D3, UBE2G1, UBA2, UBE2J1, NEDD4L, BRCA1
KEGG_PATHWAY	hsa04151:PI3K-Akt signaling pathway	14	1.89E-02	PPP2R1B, PIK3CG, PHLPP1, PHLPP2, CDK6, BRCA1, ATF2, NRAS, LPAR5, PRLR, etc.
KEGG_PATHWAY	hsa05214:Glioma	5	3.95E-02	PIK3CG, NRAS, PDGFRA, CAMK2D, CDK6
KEGG_PATHWAY	hsa04360:Axon guidance	7	4.00E-02	NRAS, PAK2, EFNB3, GSK3B, DPYSL5, DPYSL2, ARHGEF12
KEGG_PATHWAY	hsa04960:Aldosterone-regulated sodium reabsorption	4	4.15E-02	PIK3CG, ATP1B3, ATP1B2, NEDD4L
KEGG_PATHWAY	hsa04976:Bile secretion	5	4.75E-02	ATP1B3, ATP1B2, HMGCR, SLC4A4, SLC10A2
KEGG_PATHWAY	hsa05162:Measles	7	4.83E-02	MAP3K7, PIK3CG, TACR1, GSK3B, CDK6, ADAR, CD28

unclear to date. In the present study, we performed systemic and integrated research to reveal the prospective diagnostic merit of miR-224-5p. The results indicated that miR-224-5p exhibited moderate diagnostic ability (AUC=0.84, 95% CI: 0.80-0.87) of digestive system cancers. In subgroup analyses based on cancer type, the AUCs with the 95% CIs

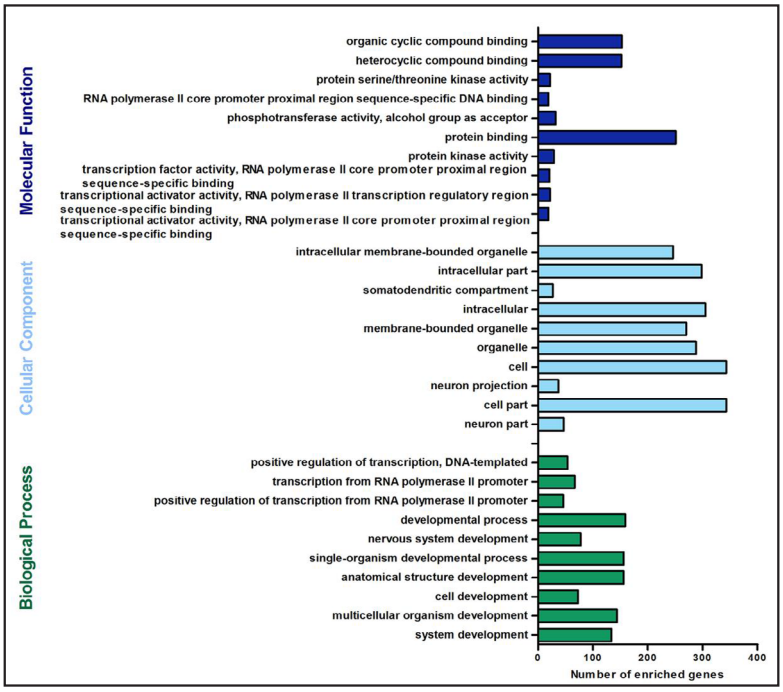

Fig. 13. Top ten GO enrichment analysis terms of potential miR-224-5p target genes identified by DAVID.

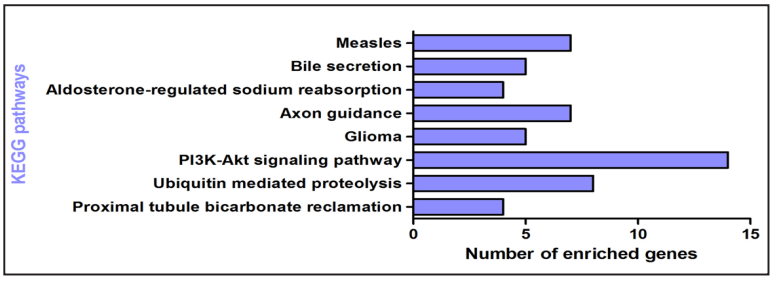

Fig. 14. The statistically significant KEGG pathways of potential miR-224-5p target genes identified by DAVID.

were 0.86 (0.82-0.88), 0.74 (0.70-0.78), 0.82 (0.78-0.85) and 0.82 (0.79-0.85) in CRC, GC, HCC and PC, respectively. We took advantage of the large sample size in this study when drawing conclusions about the diagnostic merit of miR-224-5p in digestive system cancers. Nevertheless, the detection precision of high-throughput technology, including microarray analysis and RNA sequencing, may be less precise than qRT-PCR methods. Therefore, further researches based on qRT-PCR are necessary for exploring the diagnostic capability of miR-224-5p.

A previous review reported by Chen et al. [45] demonstrated that miR-224 may be a potential therapeutic target for malignant tumors via targeting associated genes and pathways. In our study, we conducted a meta-analysis including 16 studies from the literature and two studies from microarrays to quantitatively estimate the prognostic capability of miR-224-5p. In the OS univariate and multivariate analyses, the pooled HRs and 95% CIs were 1.69 (1.15-2.49) (P=0.007) and 2.39 (1.74-3.30) (P<0.0001), suggesting that increased miR-224-5p expression predicts poor overall survival in patients with digestive system cancers. Subgroup analyses suggested that miR-224-5p might be a prospectively prognostic biomarker for patients with CRC (HR: 2.52, 95% CI: 1.80-3.53, P<0.0001). Three and five eligible studies were included in the prognostic analyses of GC and HCC patients, respectively. However, only one study was available that reported the prognostic merit of miR-224-5p in patients with both BTC and ESCA. No eligible study is available on the prognostic performance of miR-224-5p in PC patients. Thus, the reliability of some of our results remains inadequate due to the number of included studies. More studies are required

to explore the prognostic merit of miR-224-5p in patients with different types of digestive system cancers.

MiR-224 targets several important genes and pathways in the tumorigenesis and progression of malignant tumors, such as mTOR, Wnt/β-catenin signaling, KRAS, and SMAD4 [13, 14, 46, 47]. To reveal the potential target mRNAs and pathways of miR-224-5p, we used bioinformatics methods that can supplement the limitations of experimental time and cost. The results revealed that 388 potential target mRNAs of miR-224-5p were predicted by 12 existing miRNA-target prediction programs and were simultaneously predicted by at least eight prediction solutions. Then, we performed GO annotation and KEGG pathway enrichment analysis of the 388 mRNAs using DAVID. Among GO annotation enrichment analysis of the miR-224-5p target mRNAs, biological process development and transcription were the most enriched terms in BP term annotation. In addition, neuron, cell, organelle, intracellular and somatodendritic compartments were significantly identified in CC term annotation. In MF term annotation, the target genes were significantly focused on the activity of transcriptional activators, protein kinases, phosphotransferases and protein serine/threonine kinases, as well as the binding of protein, DNA, heterocyclic compounds and organic cyclic compounds. In the KEGG pathway enrichment analysis of miR-224-5p target genes, eight pathways were identified, including proximal tubule bicarbonate reclamation, ubiquitin-mediated proteolysis, PI3K-Akt signaling pathway, glioma, axon guidance, aldosterone-regulated sodium reabsorption, bile secretion and measles. All the results demonstrated that miR-224-5p may affect the tumorigenesis and progression of malignant tumors by targeting multiple genes and signaling pathways. The inter-connections of "miRNA-mRNA-function" are known as miRNAs involved in the post-transcriptional regulation of genes by inducing degradation or repressing translation and regulating numerous genes associated with different biological processes. In our study, 388 mRNAs were identified using bioinformatics methods. The inter-connections between these newly identified targets and functions were discovered by performing GO annotation and KEGG pathway enrichment analysis. Some of the results are consistent with previously published studies, whereas others must be experimentally verified. Hence, further studies are essential to validate our findings.

Some limitations in several aspects of the study should be noted. First, in the analyses of the clinical value of miR-224-5p, heterogeneity of the pooled SMDs and pooled HRs was inevitable for a number of reasons, such as diverse databases from which the expression profiles were acquired, and different methods used for HR extraction. When a P-value was less than 0.05 or I^2 was greater than 50% for a heterogeneity test, a random-effects model was chosen to reduce the effects of heterogeneity. Second, in the subgroup analyses, the sample size of some cancer types was really small, which may affect the stability of our pooled results. Therefore, more high-quality reports are needed to validate the clinical value of miR-224-5p in various types of digestive system malignancies. Third, the credibility of miR-224-5p target gene prediction results was increased by combining 12 miRNA-target prediction programs. However, no *in vitro* and *in vivo* experimental verification was a weakness in the present study. Thus, further studies are required to confirm the target genes and pathways of miR-224-5p and the molecular mechanism within the network.

In summary**,** the current study gathered 41 microarray and RNA-seq datasets and 11 published studies to validate the up-regulation of miR-224-5p and discover its diagnostic and prognostic value in cancers of the digestive tract. Twelve miRNA-target prediction programs were used to predict the potential target mRNAs of miR-224-5p. In total, 388 target mRNAs, which were simultaneously predicted by at least eight prediction programs, were extracted for GO annotation and KEGG pathway enrichment analysis. The results highlighted the multiple genes and signaling pathways targeted by miR-224-5p. However, the exact molecular mechanisms of miR-224-5p in digestive system cancers remain confusing and demand further study. We hope our study may provide a basis for further clinical application of miR-224-5p in digestive system cancers.

Disclosure Statement

The authors declare no Disclosure Statement.

Acknowledgements

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