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MiR-421 expression independently predicts unfavorable overall survival in patients with esophageal adenocarcinoma

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Abstract. – OBJECTIVE: This study aimed to examine the prognostic value of miR-421 in terms of overall survival (OS) and recurrence free survival (RFS) in ESAD and its potential regulatory network.

PATIENTS AND METHODS: An in-silico analysis was conducted using data from large databases, including The Cancer Genome Atlas-Esophageal Carcinoma (TCGA-ESCA), Starbase 3.0 and GeneMANIA.

RESULTS: Both esophageal adenocarcinoma (ESAD) and esophageal squamous cell carcinoma (ESCC) tissues had significantly upregulate miR-421 expression, compared with adjacent normal tissues. Upregulated miR-421 expression was associated with shorter OS, but not RFS in ESAD. In patients with ESCC, no difference in miR-421 expression was observed regards to OS or RFS status. Univariate and multivariate analysis showed that high miR-421 expression was independently associated with shorter OS (HR: 2.77, 95%CI: 1.41-5.46, $p<0.01$), after adjustment of histological grade and pathological stages. The predicted regulatory network of miR-421 in ESAD includes both tumor suppressors and oncogenes, which makes the role of miR-421 quite mysterious in this cancer.

CONCLUSIONS: MiR-421 expression might serve as a valuable prognostic biomarker in patients with ESAD. But future molecular studies are required to explore the exact regulatory effect of it.

Key Words
MiR-421; overall survival; esophageal adenocarcinoma.

Introduction

Esophageal adenocarcinoma (ESAD) is a highly lethal carcinoma[1]. Among the patients with advanced ESAD, the 5-year overall survival (OS) rate is lower than 20%[2]. The survival rate significantly drops when the patients have malignant tumor behaviors such as regional lymph-node invasion and distant metastases[3]. During the past decades, the incidence of ESAD has been increasing globally as a result of the growing prevalence of the risk factors, such as gastroesophageal reflux, Barrett's esophagus, smoking and obesity[4]. Among the risk factors, Barrett's esophagus increases the risk of ESAD by about 10- to 40-fold compared with that of the general population. Currently, the treatment of ESAD still highly relies on surgical resection and radiation. The Tumor lymph Nodes Metastases (TNM) staging system is still fundamental to make treatment strategy and to estimate prognosis[5]. However, there are significant prognostic discrepancies in patients with the same TNM stage[6-8]. To improve risk prediction, it is quite necessary to explore other prognostic biomarkers.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, typically comprised of 18-22 nucleotides. They usually bind to the complementary sequences in the 3′ untranslated region (3′ UTR) of the targeting genes and negatively regulate their expression by suppressing translation or inducing mRNA destabilization[9]. They can act as either oncogenes or tumor suppressors, depending on the downstream targets and the specific tumor environment[10,11]. In addition, they also might be valuable prognostic markers in several cancers[12-16].

One recent study examined the miRNA expression profiles between Barrett's esophagus and ESAD tissues and found that miR-421 is the most differentially expressed miRNA[17]. Its expression was detected in 98% of cancer tissues but was only detected in 16% of Barrett's esophagus

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tissues[17]. However, its prognostic value in ESAD has not been explored. In this study, using data in The Cancer Genome Atlas-Esophageal Carcinoma (TCGA-ESCA), we examined the prognostic value of miR-421 in terms of OS and recurrence free survival (RFS) in ESAD and also analyzed its potential regulatory network using an in-silico analysis.

Patients and methods

This study was approved by the Ethics Committee of West China Hospital, Sichuan University, China.

Secondary analysis using data from TCGA-ESCA

The level-3 data in TCGA-ESCA were acquired with the use of the UCSC Xena browser (https://xenabrowser.net/). Only the cases with RNA-seq data of miRNA expression and without neoadjuvant treatment were included in this study. Based on these criteria, 94 esophageal ESCC cases (with 2 adjacent normal tissues) and 88 ESAD cases (with 10 adjacent normal tissues) were included. Their clinicopathological data, including age at initial diagnosis, gender, histological grade, the history of esophageal cancer, pathological stages, the presence of residual tumor, Barrett's esophagus, reflux history, smoking history, postoperative drug therapy, radiation therapy, primary therapy outcome success, RFS and OS survival data were downloaded for re-analysis. Primary therapy outcome was defined as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD).

Bioinformatic analysis of the possible targets of miR-421

The potential targets of miR-421 in cervical cancer were identified using Starbase 3.0 (http://starbase.sysu.edu.cn/)[18]. Briefly, the miRNA-targets were predicted using multiple target-predicting programs, including PITA, RNA22, miRmap, DIANA-microT, miRanda, PicTar and TargetScan. Only the targets with binding sites of Ago protein (analyzed using the crosslinking-immunoprecipitation and high-throughput sequencing data) were considered as the potential targets[18]. Then, the expression of these genes in TCGA-ESAD were extracted. Linear regression models were constructed to analyze the correlation between the expression of miR-421 and those genes. The genes that were moderately and negatively correlated with miR-421 expression (Pearson's r value $\leq$ -0.35 and $p<0.01$) were considered as high potential targets.

Bioinformatic analysis using GeneMANIA

The genes that were co-expressed, co-localized or genetically interacted with the high potential targets of miR-421 were identified using GeneMania (http://genemania.org/), which is based on data collected from hundreds of data sets, including GEO, BioGRID, Pathway Commons and I2D, as well as organism-specific functional genomics data sets[19].

Statistical analysis

Statistical analysis was performed by using SPSS 25.0 software package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.04 (GraphPad Inc., La Jolla, CA, USA). The group difference was analyzed using Welch's unequal variances *t*-test. Kaplan-Meier (K-M) curves of OS were generated using GraphPad Prism, by setting median miR-421 expression as the cutoff. Log-rank test was performed to calculate the significance of the difference between the curves. The clinicopathological parameters between patients with high or low miR-421 expression were compared using Chi-squared test by two-sided Fisher's exact test. Univariate and multivariate Cox regression models were applied to assess the prognostic value of miR-421 expression. $p<0.05$ was considered statistically significant.

Results

MiR-421 was significantly upregulated in both ESAD and ESCC tissues compared with adjacent normal tissues

Using RNA-seq data of miRNA expression in TCGA-ESCA, we compared the expression of miR-421 in ESAD (N=88) and ESCC (N=94) tissues and their adjacent normal tissues respectively. Results showed that both ESAD and ESCC tissues had significantly upregulated miR-421 expression ($p<0.01$, Figure 1A-C).

The ESAD cases with poor OS had significantly upregulated miR-421 expression

We further examined the difference of miR-421 expression in patients with different survival outcomes. In patients with ESAD, the deceased cases had substantially higher miR-421 expression compared with the living cases (Figure 2A).

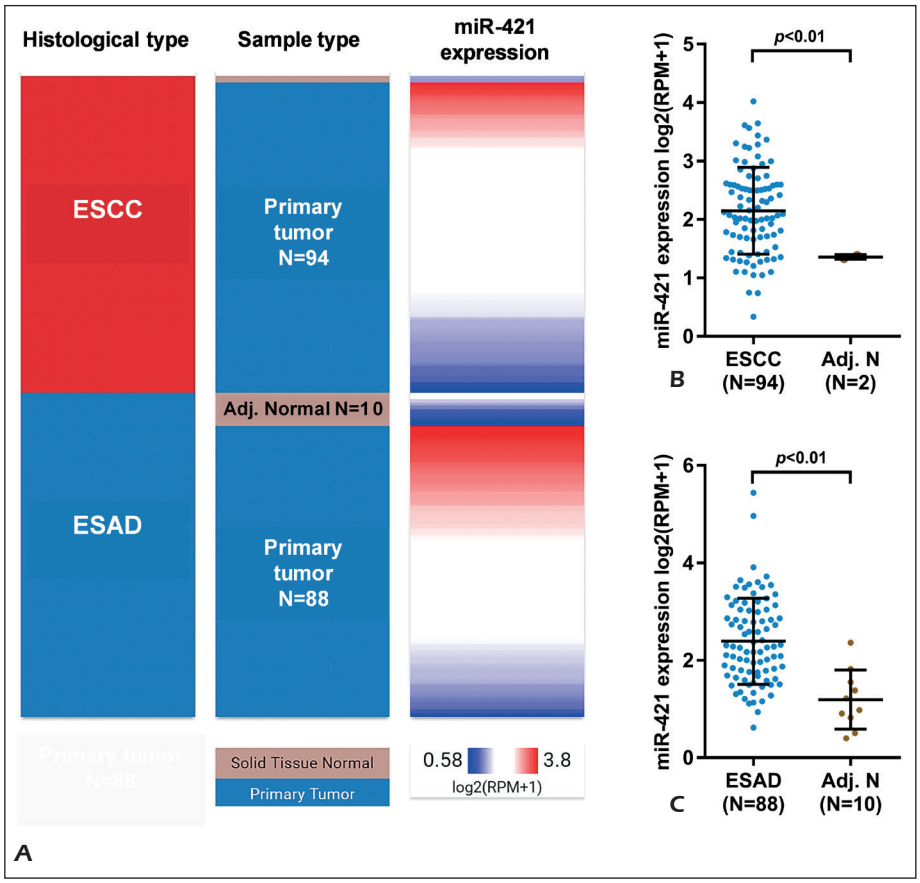

Figure 1. The expression profile of miR-421 in ESAD and ESCC tissues compared with their respective adjacent normal tissues (**A**) Heatmap showing the expression of miR-421 in ESAD (N=88) and ESCC (N=94) and their adjacent normal tissues. B-C. Pots chart comparing the expression of miR-421 in ESCC (**B**) and ESAD (**C**) and their adjacent normal tissues (N=2 and 10 respectively).

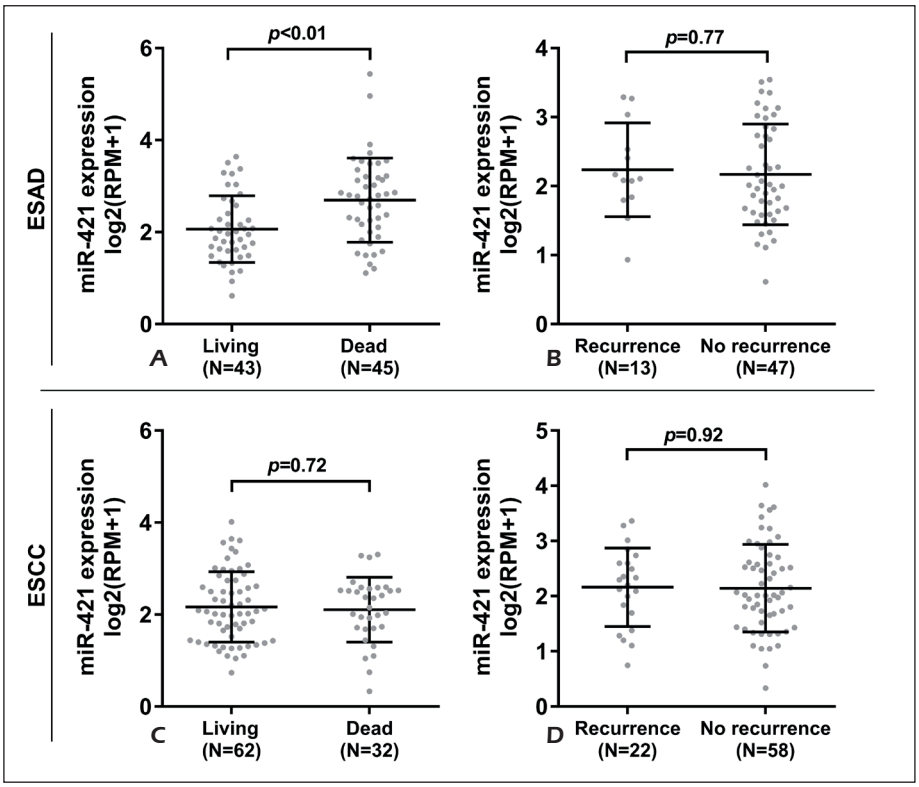

Figure 2. The ESAD cases with poor OS had significantly upregulated miR-421 expression. **A-D**, Comparison of miR-421 expression in ESAD (A-B) and ESCC (C-D) patients according to their OS status (**A** and **C**) or RFS status (**B** and **D**).

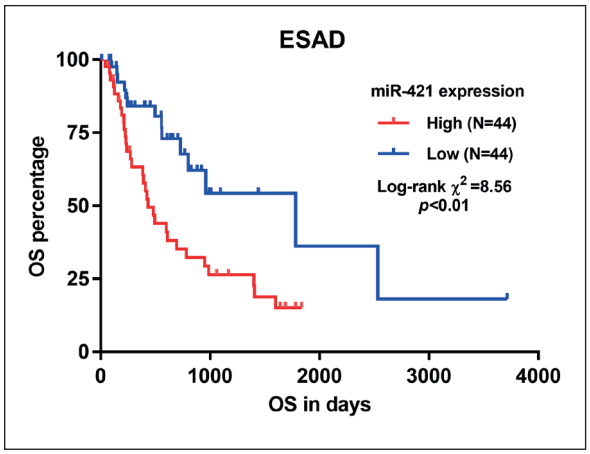

Figure 3. K-M curves of OS in ESAD patients. Patients were separated into two groups according to median miR-421 expression.

However, the difference was not found in patients with different RFS status (Figure 2B). In patients with ESCC, no difference in miR-421 expression was observed regards to OS or RFS status (Figure 2C-D).

MiR-421 expression was independently associated with shorter OS in ESAD patients

Since the deceased ESAD patients had significantly increased miR-421 expression, we then assessed its prognostic value by generating the K-M survival curves. By setting median miR-421 expression as the cutoff, we found that the high miR-421 expression group had a significantly shorter OS ($p<0.01$, Figure 3). The clinicopathological parameters between the high and low miR-421 expression groups were compared in table I. Chi-square analysis found that the high miR-421 expression group had a higher ratio of death (31/44 *vs.* 14/44, $p<0.01$) but no significant differences in the parameters between the two groups (Table I), suggesting that these two groups were comparable.

By performing univariate analysis, we found that high histological grade (G3), advanced pathological stages (III/IV) and high miR-421 expression were associated with unfavorable OS in ESAD patients (Table II). Multivariate analysis showed that high miR-421 expression was independently associated with shorter OS (HR: 2.77, 95%CI: 1.41-5.46, $p<0.01$), after adjustment of histological grade and pathological stages.

In-silico analysis of the potential targets of miR-421 in ESAD and the possible regulatory network

We further explored the potential regulatory network of miR-421 in ESAD. Using Starbase 3.0, we searched the predicted targets of miR-421 using PITA, RNA22, miRmap, DIANA-microT, miRanda, PicTar and TargetScan. Then, only the predicted target sites with binding sites of Ago protein were identified as the potential targets (N=3117) (Figure 4A). Among these genes, 2906 had RNA-seq data in TCGA-ESAD (Figure 4B). Then, we generated linear regression models to analyze the correlation between the expression of miR-421 and these genes. By setting Pearson's $r \leq -0.35$ and $p<0.01$ as the criteria, we identified 26 high-potential targeting genes, including SLMAP, SH3RF1, CHD9, FA2H, ZBTB7A, PARP4, ADAM9, RBL2, KLF3, ARNTL, BCL11B, FAM120A, GALNT7, FGD4, HECA, BCAR3, FOXO1, SECISBP2, CD59, FEM1C, PCMTD1, TRAK1, ALDH1A3, MEF2A, CAPN8 and NR1D2 (Figure 4C). Then, we constructed a two-layer model to show the potential regulatory network of miR-421 in ESAD (Figure 4D). The inner layer only included the high potential targets, while the outer layer included the genes that were co-expressed, co-localized or genetically interacted with inner layer members, including NPTN, BLZF1, DNAJB4, CBX7, RNF13, ARL6IP5, GDE1, CLN5, NR3C1, NEK7, RABGAP1L, RNF6, TRIM38, PHKB, NR3C2, ACER2, SUSD6, TMEM178B, SSFA2, CCNYL1 (Figure 4D).

Discussion

In this study, we indicated that miR-421 was significantly upregulated in ESAD tissues compared with that in adjacent normal tissues. In addition, we showed that its expression was independently associated with unfavorable OS, after adjustment of histological grade and pathological stage. These findings suggest that the miR-421 might be a specific prognostic biomarker in ESAD. Its prognostic value has also been observed in some cancers. For instance, miR-421 expression was upregulated in circulating tumor cells (CTCs) from gastric cancer patients[20] and its upregulation in non-small cell lung cancer was independently associated with poor OS (HR=1.991, 95% CI=1.046-3.791, p=0.036)[21].

Table I. Comparison of clinicopathological parameters between patients with high or low miR-421 expression.

Parameters Age (Mean ± SEM)		miR-421 expression High (N=44) 66.00±1.70	 Low (N=44) 67.27±1.90	 *p*-value 0.62
Gender	Female	4	8	0.35
	Male	40	36	
Histologic grade	G1/G2	16	15	0.60
	G3	17	11	
	No data	11	18	
Smoking history	2/3/4	18	31	0.32
	1	12	12	
	no data	14	1	
Reflux history	No	17	15	0.35
	Yes	17	26	
	No data	10	3	
Pathologic stage	III/IV	18	16	0.63
	I/II	15	19	
	Discrepancy/no data	11	9	
History of esophageal cancer	No	20	28	1.00
	Yes	3	5	
	No data	21	11	
Radiation therapy	No	37	30	0.29
	Yes	3	6	
	No data	4	8	
Postoperative drug therapy	No	35	30	0.75
	Yes	5	6	
	No data	4	8	
Residual tumor	R0	28	30	0.71
	R1	5	3	
	RX/no data	11	11	
Primary therapy outcome	PD+SD	2	7	1.00
	CR+PR	7	18	
	no data	35	19	
Recurrence status	No	18	29	1.00
	Yes	5	8	
	no data	21	7	
Living Status	Living	13	30	<0.01
	Dead	31	14	

Smoking history: 1: lifelong non-smoker; 2: current smoker; 3. Current reformed smoker (for>15 yrs); 4. Current reformed smoker (for≤15 yrs); RX: the presence of residual tumor cannot be assessed; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Previous studies reported that miR-421 acts as either an onco-miR or tumor suppressor in different cancers. MiR-421 is significantly upregulated in pancreatic cancer and inhibits the expression of DPC4/Smad4 protein, which is a critical tumor suppressor involved in the progression of pancreatic cancer[22]. miR-421 is involved in epithelial-mesenchymal transition in breast cancer[23] and also promotes breast cancer progression by inhibiting caspase-10 expression[24]. Its ectopic expression in biliary tract cancer directly downregulates the expression of FXR protein and promotes cell proliferation, colony formation and migration of biliary tract cancer cells[25]. However, over-expression of miR-421 significantly suppresses cell viability, delays cell cycle, reduces glycolysis and inhibits migration of prostate cancer cells, via targeting NRAS, PRAME, CUL4B and PFKFB2[26] and SPINK1[27]. It also suppresses the proliferation and metastasis of colorectal cancer cells by targeting MTA1[28]. Its downregulation is associated with N-Myc induced therapeutic resistance in neuroendocrine prostate cancer[29]. By these findings, we infer that the functional role of miR-421 might be cancer specific.

Table II. Univariate and multivariate analysis of OS in patients with ESAD.

Parameters	Univariate analysis				Multivariate analysis			
	p	HR	95%CI (lower/upper)		*p*	HR	95%CI (lower/upper)	
Age	0.27	0.99	0.96	1.01				
Gender Female vs. Male	0.96	0.98	0.35	2.76				
Histologic grade G3 vs. G1/G2	**0.02**	2.36	1.16	4.80	**0.02**	2.53	1.20	5.35
History of esophageal cancer No vs. Yes	0.91	1.06	0.39	2.88				
Pathologic stages III/IV vs. I/II	**<0.01**	3.35	1.58	7.12	**<0.01**	3.50	1.61	7.61
Residual tumor No vs. Yes	0.07	0.45	0.19	1.06				
Reflux History No vs. Yes	0.76	1.11	0.56	2.21				
Smoking History Yes vs. No	0.41	1.38	0.64	2.96				
Postoperative drug therapy No vs. Yes	0.89	0.94	0.39	2.26				
Radiation therapy No vs. Yes	0.58	1.34	0.47	3.78				
Primary therapy outcome success SD+PD vs. CR+PR	0.65	0.74	0.20	2.70				
miR-421 expression High vs. Low	**<0.01**	2.55	1.33	4.89	<0.01	2.77	1.41	5.46

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Since miRNAs influence the translation or stability of transcripts by partial sequence complementarity, one miRNA can potentially regulate hundreds of targeting genes[30-32]. Although we found that miR-421 might have potential prognostic value in ESAD, its exact targeting genes have not been explored. Therefore, we further constructed a potential regulatory network of miR-421 in ESAD using an in-silico analysis. There are both tumor suppressors and oncogenes among the members of the inner layer. For example, ZBTB7A has been demonstrated as a context-dependent cancer gene that shows tumor suppressive activity in PTEN-null tumors[33] and its mutations stimulate cancer cell proliferation by promoting glycolysis[34]. ARNTL is an important component of the circadian regulatory network and acts as a critical tumor suppressor via activating the p53 signaling pathway[35,36]. pRb2/p130 (encoded by RBL2 gene) has been characterized as a tumor suppressor, by inducing cell cycle arrest and cell apoptosis[37,38]. ESCC patients with pRb2/p130 (encoded by RBL2 gene) expression have significantly better OS than the counterparts without pRb2/p130 expression[39]. FOXO1 is a tumor suppressor in cervical cancer[40], ovarian cancer[41] and colon cancer[42]. Therefore, we speculated that miR-421 might exert oncogenic effects in ESAD via regulating the expression of multiple tumor suppressors. However, some members of the inner layer act as oncogenes. For example, ZBTB7A plays as an oncogene in non-small cell lung cancer and breast cancer[43,44]. CHD9 and ADAM9 are two known cancer driver genes[45,46]. FAM120A is required for IL13-induced cell migration, invasion, and survival of colon cancer cells [47]. CD59 expression is associated with radioresistance of ESCC[48]. Besides, one recent study found that upregulated FOXO1 is associated with malignant behaviors of ESAD, such as advanced tumor stages and shorter OS[49]. These clues make the role of miR-421 quite mysterious. Therefore, future molecular studies are required to figure out the exact regulatory effect of miR-421 in ESAD.

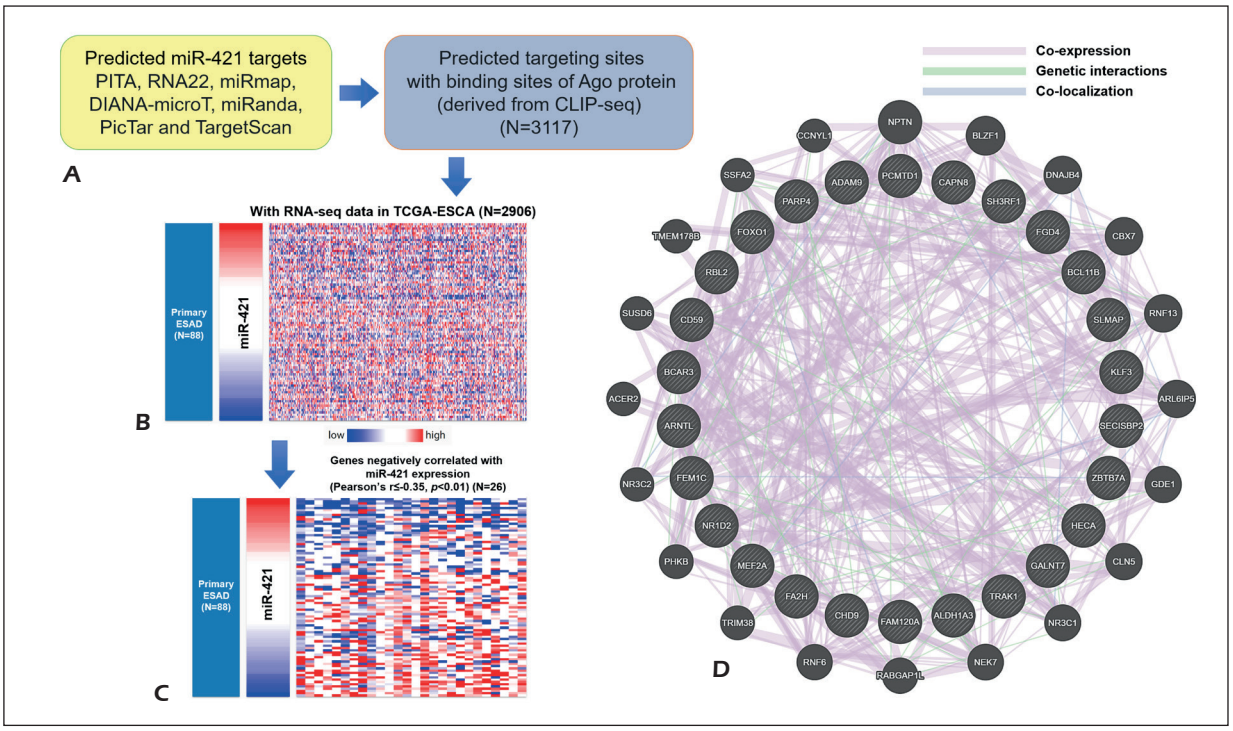

Figure 4. In-silico analysis of the potential targets of miR-421 in ESAD and the possible regulatory network. **A-C**, The screening process of the high potential targets of miR-421 in ESAD. Identification of the possible predicted targets of miR-421 intersecting the predicted target sites with binding sites of Ago protein (N=3117) (**A**). Heatmap showing the expression of predicted genes with RNA-seq data (N=2906) in TCGA-ESAD (**B**). Heatmap showing the expression of high potential target genes (N=26), by setting the following criteria: Pearson's r≤-0.35, $p<0.01$ (**C**). **D**, Identification of the genes that were co-expressed, co-localized or genetically interacted with the high potential targets of miR-421.

Conclusions

MiR-421 expression might serve as a valuable prognostic biomarker in patients with ESAD. But its regulatory network in ESAD needs to be explored in the future.

Conflict of Interests

None declared.

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