LIMD2 Is a Prognostic and Predictive Marker in Patients With Esophageal Cancer Based on a Cerna Network Analysis

Yuanmei Chen

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Xinyi Huang

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Kunshou Zhu

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Changkun Li

Fujian Normal University - Qishan Campus

Haiyan Peng

Fujian Normal University - Qishan Campus

Lin Chen

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Zhengrong Huang

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Yangfan Zhang

Fujian Normal University - Qishan Campus

Guibin Weng

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Tianya Xiao

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Junqiang Chen

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital

Yuanji Xu (✉ xuyuanji@fjmu.edu.cn)

Fujian Medical University cancer Hospital: Fujian Provincial Cancer Hospital <https://orcid.org/0000-0002-7194-0433>

Primary research

Keywords: Esophageal cancer (ECA), competing endogenous RNA (ceRNA), LIM domain containing 2 (LIMD2), prognostic, predictive

Posted Date: June 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-568786/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Frontiers in Genetics on November 18th, 2021. See the published version at <https://doi.org/10.3389/fgene.2021.774432>.

Abstract

Background. Globally, esophageal cancer (ECA) is the seventh most common cancer and sixth most common cause of cancer-associated mortality. However, there are no reliable prognostic and predictive molecular markers for ECA; in addition, the pathogenesis of ECA are not fully elucidated. This study was to explore the potential prognostic and predictive markers of ECA, as well as investigate the mechanisms of ECA.

Methods. The expressions of circular RNAs (circRNAs), microRNAs (miRNAs), and messenger RNAs (mRNAs) of ECA and control groups were obtained from the RNA-sequencing (RNA-seq) data of our hospital, the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) datasets. Analyses of differentially-expressed genes, the circRNA-miRNA-mRNA competing endogenous RNA (ceRNA) network, and functional/pathway enrichment were conducted. The key targets in the ceRNA network that showed significant results in survival Cox regression analyses were selected. Furthermore, analyses of immune infiltration and autophagy genes related to the key targets were performed.

Results. Seven circRNAs, 22 miRNAs, and 34 mRNAs were identified as vital genes in ECA; the nuclear factor- κ -gene binding (NF- κ B) and phosphatidylinositol-3 kinase/protein kinase B (PI3K-Akt) signaling were identified as the most enriched pathways. In addition, LIM domain containing 2 (*LIMD2*) was an independent predictor of prognosis in ECA patients, and closely associated with immunity and autophagy. Moreover, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) revealed significant upregulation of *LIMD2* expression in ECA tissues.

Conclusions. ECA may be closely correlated with NF- κ B and PI3K/Akt signaling. In addition, *LIMD2* could be a potential prognostic and predictive marker of ECA. Our study may provide novel insights into the prognosis and treatment of ECA by its pathogenesis.

Background

Esophageal cancer (ECA), including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), is the seventh most common malignant tumor in the world and the sixth most common primary cause of tumor-associated death, especially in Asia [1]. In 2018, approximately 572,034 new ECA cases were diagnosed (accounting for 3.2% of all malignancies), and 508,585 deaths were caused by ECA (accounting for 3.3% of all malignancies) [1]. Surgical removal of tumor is an effective and conventional treatment for most types of ECA. Although advances in surgical procedures, chemoradiotherapy and immunotherapy have helped improve the overall survival (OS) of patients, the current 5-year survival rates of ECA patients still range from 15–25% [2]. Therefore, in-depth exploration of potential prognostic and predictive markers, therapeutic targets, and mechanisms of ECA is a key imperative to improve the treatment outcomes and prognosis of ECA patients.

Due to the rapid advances in microarray and RNA-sequencing (RNA-seq) technology, multiple circular RNAs (circRNAs), microRNAs (miRNAs) and messenger RNAs (mRNAs) have been identified as important

genes in ECA [3]. CircRNAs are endogenous non-coding RNAs with closed-loop structures without 5' caps and 3' tails [4]. Recent studies have found that the mRNA targeted by miRNAs in the circRNA-miRNA-mRNA competing endogenous RNA (ceRNA) regulatory network could serve as a key therapeutic target in cancer. In addition, circRNAs were shown to play a vital role in the occurrence and development of cancers by positively or negatively regulating the circRNA-miRNA-mRNA ceRNA axis or by acting as protein "sponges" in cancer cells. CircRNAs in the ceRNA network may indirectly regulate those target mRNAs by serving as miRNA "sponges" [5, 6]. In a study, low expression of large tumor suppressor kinase 1 (LATS1) was found to be related to tumor stage and poor prognosis of gastric cancer patients; in addition, circLARP4 was found to inhibit the malignant biological behavior of gastric cancer as a tumor suppressor via modulation of the circLARP4/miR-424-5p/LATS1 axis [7].

Genome-wide analyses of ECA have indicated complex mutation situations and discovered significant mutations in genes (including *TP53*, *MLL2*, *NOTCH1*, *PTEN*), repeated copy number amplifications in *SOX2*, *TERT*, *FGFR1*, as well as frequent deletion of *RB1* [8, 9]. However, previous ECA genome studies had some limitations, including relatively small sample size, low-coverage whole-genome sequencing, and one-sided analysis based on only chip data or sequencing data. Therefore, more comprehensive studies are required to overcome the shortcomings of previous studies.

The purpose of our study was to explore the potential prognostic and predictive markers and to unravel the mechanisms of ECA from our RNA-seq data, the Gene Expression Omnibus (GEO), and The Cancer Genome Atlas (TCGA) database. Differentially expressed circRNAs (DEcircRNAs), differentially expressed miRNAs (DEmiRNAs), and differentially expressed mRNAs (DEmRNAs) were identified using Limma R package, and the circRNA-miRNA-mRNA ceRNA regulatory network was constructed based on these differentially expressed genes. In addition, the enriched pathways were investigated by performing multiple functional and pathway enrichment analyses. Furthermore, the key target mRNAs in the ceRNA regulatory network were determined using survival Cox regression analyses. Finally, analyses of immune infiltration autophagy genes related to the key targets were performed. Our study may provide novel insights into the prognosis and treatment of ECA by its pathogenesis.

Materials & Methods

Sample collection

Between July and December 2020, a total of 26 pairs of tumor and adjacent tissues were collected from patients with pathologically confirmed ECA at the Department of thoracic surgery, Fujian Cancer Hospital. The ethics committee of the Fujian Cancer Hospital approved the use of human tissues (project ethics number: SQ2020-063-01), and informed consent was acquired. The study methodologies conformed to the standards set by the Declaration of Helsinki.

Datasets

The chip data of circRNAs expression profiles, GSE131969 (GPL 19978), were downloaded from the GEO [10] (<http://www.ncbi.nlm.nih.gov/geo>). The miRNA and mRNA expression and clinical information in the TCGA (<https://tcga-data.nci.nih.gov/tcga/>) were obtained from University of California Santa Cruz (USCS) Xena (<https://xenabrowser.net>). The circRNA microarray data included three ECA tissues and three adjacent normal esophageal tissues. The miRNA sequencing data included 185 ECA samples and 13 adjacent samples, and the mRNA sequencing data included 162 ECA samples and 11 adjacent samples.

Differential expression analyses

The Limma R package (version 4.0.2) [3] and the edgeR R package (version 3.14.0) [11] was used for differential analyses. The expressions of all differentially expressed genes (DEGs) were visualized by volcano maps and two-way clustering heat maps. Principal component analysis (PCA) was conducted with the “pca3d” R package to explore gene expression patterns of ECA and normal groups.

Construction of the ceRNA and circRNA-RBP network

The miRanda (<http://www.miranda.org/>) were used to predict the target miRNAs of the DEcircRNAs. Target mRNAs of DEmiRNAs were predicted using miRWalk (version 2.0) [12]. The databases used for targeted mRNA prediction were miRWalk, MicroT4, miRanda, mirbridge, miRDB, miRMap, and Targetscan databases. Overlapping mRNAs in 6 or more databases were considered as the target mRNAs. Moreover, the ceRNA network was constructed and visualized by Cytoscape (version 3.6.1) [13]. The potential RNA binding proteins (RBPs) were analyzed by the CatRAPID (http://service.tartagliolab.com/page/catrapid_group) database. Star Rating Score > 2 was considered indicative of strong binding effect. The Cytoscape software was used to construct the circRNA-RBP binding network.

Functional and pathway enrichment analyses

The mRNAs in the ceRNA network were analyzed using Search Tool for the Retrieval of Interacting Genes (STRING) [14], Database for Annotation, Visualization, and Integrated Discovery (DAVID) [15], and Metascape [16]. Metascape, DAVID, and STRING databases were used to conduct analyses based on the DisGeNET (version 7.0) [17], Gene Ontology (GO) [18], biological processes (BPs), Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST) database (version 2) [19], and Kyoto Encyclopedia of Genes and Genomes (KEGG) [20] analyses.

Gene Set Variation Analysis (GSVA)

The enrichment scores of pathways were calculated using the GSVA [21], and a scoring matrix was obtained. Based on the matrix, the difference analysis was performed using Limma and visualized with a two-way clustering heat map.

Survival analysis

Based on the optimal cutoff calculated by the survminer R package [22], the patients were divided into low and high gene expression groups. The difference between groups was evaluated using the log-rank test with the survival (version 3.2-7) R package [23], and target genes that showed significant association with survival were chosen ($p < 0.05$). Further, single-factor and multi-factor Cox regression analyses were performed on mRNAs with significant results. Finally, LIM domain containing 2 (*LIMD2*) with significant results was selected as a key target.

Immune infiltration related analysis

Based on the TCGA database, the CIBERSORT [24] were used to perform immune infiltration related analysis, and immune cells were visualized between groups. Based on the ratio of various immune cells, the spearman correlation analysis was performed between immune cells and between targets and immune cells.

Autophagy correlation analysis

The autophagy-related genes were obtained from Human Autophagy Database (HADb) (<http://www.autophagy.lu/>) database. The expression of key target *LIMD2* and autophagy-related genes were analyzed by Spearman correlation analysis, and the p-values were adjusted by the BH method.

Gene Set Enrichment Analysis (GSEA)

GSEA [25] (<http://www.broadinstitute.org/gsea/index.jsp>), performed by R package cluster Profiler (version 3.8.0) [26], was used to analyze the significant function and pathway difference between high- and low- *LIMD2* groups. P value < 0.05 and FDR q-value < 0.25 were set as the cut-off criteria.

Single-factor and multi-factor Cox regression analyses

Among the clinical variables, age, M stage, N stage, T stage, sex, tumor stage, and *LIMD2* expression were selected for single factor regression analysis. Multivariate regression analyses were conducted using variables that showed significant results in single-factor regression analysis.

Statistical analysis

Statistical analyses were performed using the R software (version 3.6.1), GraphPad Prism (version 8.0.1.244), SPSS (version 24.0), and the bioinformatics tools mentioned above. Differential expressions of genes were obtained by two-tailed Student's t test. Benjamini and Hochberg FDR method were conducted to adjust the P-values. Enrichment analyses were analyzed using the hypergeometric test and Bonferroni correction. The outcomes were showed as the mean $\pm$ SD. For analyses, the paired Student's t-test and Wilcoxon rank sum test were used between groups, and P less than 0.05 was regarded as statistically significant. * $P < 0.01$, ** $P < 0.001$.

Other procedures

Protocols for other procedures were provided in the Supplementary Material.

Results

Data collection and preprocessing

The differences between the clustering of the circRNAs (Fig. 1A, 1D), mRNAs (Fig. 2A, 2D), and miRNAs (Fig. 3A) of the ECA and adjacent samples were analyzed. The results showed that most principal components could separate or tended to separate ECA samples from normal esophageal tissues.

Identification of DEcircRNAs, DEmiRNAs, and DEmRNAs

CircRNAs in GSE131969 and RNA-seq data (P value < 0.05 and $|\log_2 \text{FC}| > 1.5$), mRNAs in RNA-seq data (P value < 0.05 and $|\log_2 \text{FC}| > 1$) and the TCGA dataset (P value < 0.05 and $|\log_2 \text{FC}| > 1.5$), as well as miRNAs in the TCGA (P value < 0.05 and $|\log_2 \text{FC}| > 1.5$) were analyzed. A total of 368 DEcircRNAs were identified in the GSE131969 (Fig. 1B); 493 DEcircRNAs were identified in RNA-seq data (Fig. 1E); 1576 DEmRNAs were identified in RNA-seq data (Fig. 2B); 2450 DEmRNAs were identified in the TCGA (Fig. 2E); 86 DEmRNAs were identified in the TCGA (Fig. 3B). The DEcircRNAs in the GEO (Fig. 1C) and RNA-seq data (Fig. 1F), DEmRNAs in RNA-seq data (Fig. 2C) and TCGA (Fig. 2F), as well as DEmiRNAs in TCGA (Fig. 3C) were visualized using the hierarchical cluster maps. Seven up-regulated circRNAs (Fig. 1G), zero down-regulated circRNAs (Fig. 1H) as well as 61 up-regulated mRNAs (Fig. 2G) and six down-regulated mRNAs (Fig. 2H) were significantly differentially expressed in both datasets. Finally, seven DEcircRNAs in the GSE131969 (Fig. S1A) and RNA-seq data (Fig. S1B) were visualized.

Construction of the ceRNA network and circRNA-RBP network

The miRNAs targeted by the seven DEcircRNAs were predicted and 22 circRNA-miRNA pairs were identified. The mRNAs targeting the miRNAs were identified, and 34 mRNAs were obtained through the intersection with DEmRNAs (Fig. 4D). Subsequently, the differential expression of the 34 mRNAs in the TCGA database (Fig. S3A) and RNA-seq data were visualized (Fig. S3B). A ceRNA network were established based on seven circRNAs, 22 miRNAs, and 34 mRNAs (Fig. 3D). The circRNA-RBP network was constructed, including seven circRNA and 37 RBPs (Fig. 3E).

Functional and pathway enrichment analyses

The enriched terms analyzed based on the DisGeNET database are shown in Fig. 4A, including Adult T-Cell Lymphoma and Childhood Langerhans Cell Histiocytosis. The enriched GO terms are shown in Fig. 4B, including positive regulation of cell adhesion and positive regulation of protein kinase B signaling. The enriched terms analyzed based on the TRRUST database were SP1, RELA, and NFKB1 (Fig. 4C). Moreover, GSEA showed enrichment of ether lipid metabolism and acute myeloid leukemia in the cancer group (Fig. 4E). Based on the DAVID database, the outcomes showed that mRNAs were enriched in positive regulation of T cell activation and positive regulation of cell migration (BP), extracellular space and plasma membrane (Cellular Components, CC), DNA binding (Molecular Functions, MF), and NF-kappa B signaling pathway (KEGG, Fig. S2A). Based on the STRING database, the results

showed that targets were enriched in positive regulation of lymphocyte activation and positive regulation of immune system process (BPs), extracellular space (CCs), protein binding and DNA binding (MFs), NF-kappa B pathway and ether lipid metabolism (KEGG), regulation of PI3K/AKT signaling (Record control the memory, RCTM, Fig. S2B).

Survival analysis

Targets that showed a significant association with survival ($p < 0.05$) included *LIMD2*, ARL11, and SERPINB7 (Fig. 5A-L). *LIMD2* with significant regression result was selected as a key target.

Immune infiltration analysis

The composition of immune tumor microenvironment (TME) cells in the ECA and normal groups was analyzed. We observed significant differences in TME cells infiltration and composition in the two groups, including T cells CD4 memory activated and macrophages M1 (Fig. 6A). The R2 of TME cells was also calculated, and the results showed that T cells CD4 memory activated had statistical significance (Fig. 6B). A positive relationship was observed between *LIMD2* and T cells CD4 memory activated (Fig. 6C). These analyses indicated a positive correlation of ECA group with immune-relevant signatures.

Analysis of LIMD2 and autophagy-related genes

Several studies have indicated the importance of autophagy in the development of ECA [27, 28]. We investigated the relationship between *LIMD2* and autophagy-related genes (Fig. 7A-I). The top nine autophagy-related genes that showed a positive relationship with *LIMD2* were identified, including BCL2, CCL2, and RGS19.

GSEA

The results demonstrated that the ECA patients with high expression of *LIMD2* showed enrichment of the following pathways: systemic lupus erythematosus, DNA replication, and T cell receptor signaling pathway (Fig. S4A-I).

Cox regression analysis

Univariate and multivariate Cox regression analyses were performed to explore independent predictors of poor survival in ECA patients. A total of 162 ECA patients was included in the regression analysis. On univariate regression analysis, *LIMD2*, M stage, N stage, and tumor stage showed a strong association with OS. Based on the results of univariate Cox analysis, multivariate regression analysis was performed to analyze the effect of *LIMD2* and other related clinical phenotypes on the prognosis of ECA. On multivariate regression analysis, *LIMD2* showed a significant correlation with OS (Table 1, Fig. S5). The results showed that *LIMD2* is an independent predictor of poor OS in ECA patients.

Table 1

Univariate analysis and multivariate analysis for prognostic factors in overall survival patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95 % CI	P value	HR	95 % CI	P value
Age						
>=60	0.9911	0.574–1.711	0.974			
Tumor_cental_location						
Mid	8.6e-01	0.391–1.893	0.709			
Proximal	3.75e-08	0-Inf	0.997			
M stage						
M1	5.32	2.449–11.576	2.45e-05	2.149	0.9242–4.997	0.0756
N stage						
N1-N3	3.504	1.82–6.744	0.000175	2.088	0.946–4.611	0.0685
T stage						
T3-T4	1.2498	0.719–2.173	0.43			
Gender						
Male	2.366	0.852–6.572	0.0984			
Tumor stage						
Stage iii-stage iv	3.398	1.9-6.076	3.68e-05	2.078	0.98–4.406	0.0564
Alcohol history						
Yes	0.674	0.386–1.177	0.166			
bmi						
>30	0.3895	0.122–1.244	0.1116			
25–30	0.462	0.1757–1.216	0.1177			
18.5–25	0.456	0.181–1.152	0.0968			
LIMD2						

HR, Hazard Ratio; CI, Confidence Interval.

Variables	Univariate analysis			Multivariate analysis		
	HR	95 % CI	P value	HR	95 % CI	P value
Low	2.9065	1.413–5.981	0.00375	2.629	1.2486–5.537	0.0109
HR, Hazard Ratio; CI, Confidence Interval.						

Validation of LIMD2 by qRT-PCR

To verify the RNA-seq and bioinformatics results, the target *LIMD2* was chosen for validation by quantitative reverse-transcription polymerase chain reaction

(qRT-PCR) with primers (Table 2). The results showed significantly higher expression of *LIMD2* in ECA tissues compared with normal tissues (Fig. 8).

Table 2
The primers of *LIMD2* and *GAPDH* mRNA.

Primers	Forward primers(5'to 3')	Reverse primers(5'to 3')
QPCR- <i>LIMD2</i>	TTTTCACAACCTCTTGCTTCTGC	AACCCCTCGTCGTAGTTGCCT
QPCR- <i>GAPDH</i>	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Discussion

In this study, 7 circRNAs, 22 miRNAs, and 34 target mRNAs in the circRNA-miRNA-mRNA ceRNA regulatory network were identified as crucial genes in ECA; in addition, the nuclear factor- κ B (NF- κ B) and phosphatidylinositol-3 kinase/protein kinase B (PI3K-Akt) signaling were identified as the most enriched pathways. Survival Cox regression analyses indicated that target *LIMD2* in the ceRNA network may act as an independent predictor of poor OS in ECA patients. On further analyses of immune infiltration and autophagy genes related to target *LIMD2*, *LIMD2* showed a close linkage with immunity and autophagy in ECA. Furthermore, we verified the high expression level of *LIMD2* by qRT-PCR based on 20 pairs of ECA and normal samples. Hence, *LIMD2* is a potential promising molecular marker of prognostic significance in ECA.

The ceRNA networks may play a vital role in the development of cancer [29]. Seven circRNAs, 22 miRNAs and 34 mRNAs were identified in the ceRNA network. The increase of has_circ_0000154 (circDCAF6) identified in our study was related to tumor invasion, positive lymph node metastasis, and higher TNM stages in GC patients. It could serve as an independent prognostic indicator [30]. A total of 13 identified mRNAs (*HMGA2*, *CCL22*, *CDKN2D*, *CEACAM6*, *DKK2*, *KIT*, *KRT14*, *LEF1*, *LPCAT1*, *NCCRP1*, *NRXN1*, *PPP1R18*) were found to be related to ECA. In a previous study, High-mobility group AT-hook 2 (*HMGA2*) was shown to regulate transcription by inducing structural alterations in the chromatin [31]. Several

studies have shown that *HMGA2* is re-expressed in most tumors and plays a vital role in tumorigenesis [32]. The stability of *HMGA2* may be regulated by hepatitis B X-interacting protein (*HBXIP*) via the Akt-PCAF pathway, thereby promoting the growth of ECA cells [33]. All 22 DE miRNAs in the ceRNA network have been reported to be related to cancer. MiR-141-3p plays an important role in various carcinomas [34], and it was found to be highly upregulated in ECA cells [35]. A recent study reported that miR-141-3p may inhibit the expression of pleckstrin homology domain leucine-rich repeat protein phosphatase-2 (*PHLPP2*), a negative regulator of the PI3K/AKT signaling, and could serve as a biomarker in ECA [36].

CircRNAs with RBP binding sites can act as sponges for RBPs and may indirectly modulate their functions [5]. In this study, the six identified RBPs (SRSF1, SRSF2, PCBP2, TIA1, FUS, FMR1) were closely related to ECA. PCBP2 performs multiple functions, such as stabilization of mRNAs and silencing or promotion of translation [37]. Several studies have shown that PCBP2 may promote tumor growth. Ye et al. found that PCBP2 regulates the proliferation and apoptosis of ESCC cells and may serve as a novel therapeutic target in ESCC [37]. Our findings are consistent with those of the previous study.

In our study, multiple diseases were enriched in ECA, including Adult T-cell lymphoma (ATL), Childhood Langerhans cell histiocytosis, and classical Hodgkin's lymphoma that are strongly associated with immune cells. ATL is a T-cell lymphoproliferative tumor of mature CD4 + CD25 + T cells [38], and Langerhans cell histiocytosis is characterized by the accumulation of Langerhans cells, antigen-presenting cells [39]. Our results also revealed that many enriched GO and KEGG terms were closely related to immune response, including positive regulation of T cell activation and positive regulation of immune system process. GSEA showed that immunity was strongly associated with ECA, including systemic lupus erythematosus and T cell receptor signaling pathway. The NF- κ B signaling pathway and PI3K/AKT signaling were the most enriched pathways. Zheng et al. depicted the entire immune landscape, including the innate and acquired immune cell map, in ESCC and adjacent tissues; their work revealed that ESCC is enriched in immune-suppressive cell mass [40]. Tong et al. revealed that 14-3-3 ζ may enhance the invasion and growth of ESCC cells by inhibiting the S1PR2 protein expression via the NF- κ B pathway [41]. GSVA indicated enrichment of ether lipid metabolism in ECA. Disorders of ether lipid metabolism are vital signs of tumors, which serves as the basis of tumor pathogenicity [42]. Cao et al. found that lncRNAs may interact with their adjacent coding RNAs to modulate ether lipid metabolism [43].

Furthermore, we found that overexpression of 12 genes was strongly related to poor OS. We included these 12 mRNAs in Cox regression analyses, and identified *LIMD2* as a vital target. *LIMD2* an oncogene in a variety of tumors [44], in previous studies, expression of *LIMD2* in lung [45] and thyroid cancer tissues [46] showed a strong association with cell movement, metastatic potential, and tumor grade. Zhang et al. revealed that high expression of *LIMD2* may enhance the progression of non-small cell lung carcinoma (NSCLC); in addition, overexpression of *LIMD2* was closely related to lymph node metastasis, distant metastasis, and advanced stage. The survival time of NSCLC patients with overexpression of *LIMD2* was shorter than that of patients with lower expression, indicating that *LIMD2* may serve as a therapeutic target in NSCLC [45]. Araldi et al. found that *LIMD2* may strengthen phosphorylation of kinases related to

epithelial-mesenchymal transition (EMT) and invasion, and modulate the crucial steps in the metastatic cascade of thyroid cancer cells through the mitogen-activated protein kinase (MAPK) crosstalk [47]. Previous studies have demonstrated a close relationship between *LIMD2* and T cells CD4 memory activated, and positive relationship of *LIMD2* with nine autophagy-related genes. Yu et al. found that the immunity high group of cutaneous melanoma specimens had the highest level of CD4 memory activated T cells, and the OS rate was poor [48]. A recent research unraveled the connection between clinical information and immune signatures in GC. They noticed that the high-risk group showed greater proportion of CD4 memory activated T cells and M1 macrophages [49]. Bcl2-associated athanogene 3 (BAG3) was shown to be involved in multiple biological processes, including cell proliferation, cell vitality, and apoptosis [50]. Compared with Barrett's metaplasia (BE) and normal samples, the amount of T lymphocytes with downregulation of bcl2 was notably increased in EAC [51].

Nevertheless, some limitations of our study should be considered while interpreting the results. Firstly, owing to the use of several datasets in our analyses, the effect of inter-batch differences on our results cannot be ruled out. In addition, further study with a larger sample size is required to verify the high expression of *LIMD2* in ECA. Finally, further in vitro and in vivo biological experiments are required for in-depth characterization of the functions of the identified targets and potential mechanisms in ECA.

Conclusions

we identified 7 circRNAs, 22 miRNAs, and 34 mRNAs in the ceRNA network in ECA. The NF- κ B and PI3K/Akt signaling were the most enriched pathways in ECA. Furthermore, *LIMD2* was notably upregulated in ECA tissue samples, and may serve as a potential prognostic and predictive markers that is closely associated with immunity and autophagy. Our study provides insights into the pathogenesis, prognosis, and therapeutic strategy for ECA.

Abbreviation

ATL Adult T-cell lymphoma

BAG3 Bcl2-associated athanogene 3

BE Barrett's metaplasia

BPs Biological processes

CC Cellular components

ceRNA Competing endogenous RNA

CI Confidence interval

circRNAs circular RNAs

DAVID Database for Annotation, Visualization, and Integrated Discovery

DEcircRNAs Differentially expressed circRNAs

DEGs Differentially expressed genes

DEmiRNAs Differentially expressed miRNAs

DEmRNAs Differentially expressed mRNAs

EAC Esophageal adenocarcinoma

ECA Esophageal cancer

EMT Epithelial-mesenchymal transition

ESCC Esophageal squamous cell carcinoma

GEO Gene Expression Omnibus

GO Gene ontology

GSEA Gene Set Enrichment Analysis

GSVA Gene Set Variation Analysis

HADb Human Autophagy Database

HBXIP Hepatitis B X-interacting protein

HMGA2 High-mobility group AT-hook 2

HR Hazard ratio

KEGG Kyoto Encyclopedia of Genes and Genomes

KM Kaplan-Meier

LATS1 Large tumor suppressor kinase 1

LIMD2 LIM domain containing 2

MAPK Mitogen-activated protein kinase

MF Molecular Functions

miRNAs microRNAs

mRNAs messenger RNAs

NES Normalized enrichment score

NF- κ B Nuclear factor- κ -gene binding

NSCLC Non-small cell lung carcinoma

OS Overall survival

PCA Principal component analysis

PHLPP2 Pleckstrin homology domain leucine-rich repeat protein phosphatase-2

PI3K-Akt Phosphatidylinositol-3 kinase/protein kinase B

qRT-PCR quantitative reverse-transcription polymerase chain reaction

RBPs RNA binding proteins

RCTM Record control the memory

RNA-seq RNA-sequencing

STRING Search Tool for the Retrieval of Interacting Genes

TCGA The Cancer Genome Atlas

TME Tumor microenvironment

TRRUST Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining USCS
University of California Santa Cruz

Declarations

Ethics approval and consent to participate

The ethics committee of Fujian Cancer Hospital approved the human tissue samples related to this work (project ethics number: SQ2020-063-01).

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the [GEO] repository, [<http://www.ncbi.nlm.nih.gov/geo>] [10]. Reference Other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported in part by grants from the Natural Science Foundation of Fujian Province, China (#2020J011123) and Fujian Provincial Health and Family Planning Commission Research Talent Training Program, China (#2020GGA017).

Authors' contributions

YC conceived and designed the experiments, performed the analyses, and was a major contributor in writing the manuscript. XH designed the experiments, performed the analyses, analyzed the data, prepared figures and tables, and was a major contributor in writing the manuscript. KZ conceived and designed the experiments, analyzed the data, and was a contributor in writing the manuscript. CL analyzed the data. HP prepared figures and tables. LC was responsible for data collection. ZH prepared figures. YZ was responsible for data collection. GW analyzed the data. TX prepared tables. JC conceived the experiments and substantively revised the manuscript. YX conceived and designed the experiments, substantively revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Medjaden Bioscience Limited (MJD) for the paper revision support.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA & Jemal A (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68, 394-424.
2. Pennathur A, Gibson MK, Jobe BA & Luketich JD (2013) Oesophageal carcinoma. *Lancet* 381, 400-12.
3. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W & Smyth GK (2015) Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43, e47.
4. Kristensen LS, Andersen MS, Stagsted LVW, Ebbesen KK, Hansen TB & Kjems J (2019) The biogenesis, biology and characterization of circular RNAs. *Nat Rev Genet* 20, 675-691.
5. Zhang J, Liu H, Hou L, Wang G, Zhang R, Huang Y, Chen X, Zhu J (2017) Circular RNA_LARP4 inhibits cell proliferation and invasion of gastric cancer by sponging miR-424-5p and regulating LATS1

- expression. *Mol Cancer* 16, 151.
6. Wu Y, Xie Z, Chen J, Chen J, Ni W, Ma Y, Huang K, Wang G, Wang J, Ma J et al. (2019) Circular RNA circTADA2A promotes osteosarcoma progression and metastasis by sponging miR-203a-3p and regulating CREB3 expression. *Mol Cancer* 18, 73.
 7. Yang C, Yuan W, Yang X, Li P, Wang J, Han J, Tao J, Li P, Yang H, Lv Q et al. (2018) Circular RNA circ-ITCH inhibits bladder cancer progression by sponging miR-17/miR-224 and regulating p21, PTEN expression. *Mol Cancer* 17, 19.
 8. Chang J, Tan W, Ling Z, Xi R, Shao M, Chen M, Luo Y, Zhao Y, Liu Y, Huang X et al. (2017) Genomic analysis of oesophageal squamous-cell carcinoma identifies alcohol drinking-related mutation signature and genomic alterations. *Nat Commun* 8, 15290.
 9. Cancer Genome Atlas Research Network, Analysis Working Group: Asan University, BC Cancer Agency, Brigham and Women's Hospital, Broad Institute, Brown University, Case Western Reserve University, Dana-Farber Cancer Institute, Duke University, Greater Poland Cancer Centre et al. (2017) Integrated genomic characterization of oesophageal carcinoma. *Nature* 541, 169-175.
 10. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M et al. (2013) NCBI GEO: archive for functional genomics data sets-update. *Nucleic Acids Res* 41, D991-5.
 11. Robinson MD, McCarthy DJ & Smyth GK (2010) EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139-40.
 12. Dweep H & Gretz N (2015) MiRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods* 12, 697.
 13. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B & Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 13, 2498-504.
 14. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P et al. (2019) STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res* 47, D607-D613.
 15. Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC & Lempicki RA (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4, P3.
 16. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C & Chanda SK (2019) Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun* 10, 1523.
 17. Piñero J, Ramírez-Anguita JM, Saüch-Pitarch J, Ronzano F, Centeno E, Sanz F & Furlong LI (2020) The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Res* 48, D845-D855.
 18. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT et al. (2000) Gene ontology: tool for the unification of biology. *The Gene Ontology*

Consortium. Nat Genet 25, 25-9.

19. Han H, Cho JW, Lee S, Yun A, Kim H, Bae D, Yang S, Kim CY, Lee M, Kim E et al. (2018) TRRUST v2: an expanded reference database of human and mouse transcriptional regulatory interactions. Nucleic Acids Res 46, D380-D386.
20. Kanehisa M, Furumichi M, Tanabe M, Sato Y & Morishima K (2017) KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res 45, D353-D361.
21. Hänzelmann S, Castelo R & Guinney J (2013) GSEA: gene set variation analysis for microarray and RNA-seq data. BMC Bioinformatics 14, 7.
22. [Internet] Alboukadel K, Marcin K & Przemyslaw B (2020) survminer: Drawing Survival Curves using 'ggplot2'. R package version 0.4.8. <https://CRAN.R-project.org/package=survminer/>
23. [Internet] Therneau T (2020) A Package for Survival Analysis in R. R package version 3.2-7. <https://CRAN.R-project.org/package=survival/>
24. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, Hoang CD, Diehn M & Alizadeh AA (2015) Robust enumeration of cell subsets from tissue expression profiles. Nat Methods 12, 453-7.
25. Reimand J, Isserlin R, Voisin V, Kucera M, Tannus-Lopes C, Rostamianfar A, Wadi L, Meyer M, Wong J, Xu C et al. (2019) Pathway enrichment analysis and visualization of omics data using g:Profiler, GSEA, Cytoscape and EnrichmentMap. Nat Protoc 14, 482-517.
26. Yu G, Wang L, Han Y & He Q (2012) ClusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16, 284-7.
27. Chen C, Chen S, Cao H, Wang J, Wen T, Hu X & Li H (2020) Prognostic significance of autophagy-related genes within esophageal carcinoma. BMC Cancer 20, 797.
28. Li Z, Dong K, Guo P, Tan Z, Zhang F, Tian Y & Lv H (2020) Identification of Autophagy-Related Genes and Small-Molecule Drugs in Esophageal Carcinoma. Med Sci Monit 26, e921855.
29. Liu G, Sun J, Yang ZF, Zhou C, Zhou PY, Guan RY, Sun BY, Wang ZT, Zhou J, Fan J et al. (2021) Cancer-associated fibroblast-derived CXCL11 modulates hepatocellular carcinoma cell migration and tumor metastasis through the circUBAP2/miR-4756/IFIT1/3 axis. Cell Death Dis 12, 260.
30. Wu L, Liu D & Yang Y (2019) Enhanced expression of circular RNA circ-DCAF6 predicts adverse prognosis and promotes cell progression via sponging miR-1231 and miR-1256 in gastric cancer. Exp Mol Pathol 110, 104273.
31. Mansoori B, Mohammadi A, Ditzel HJ, Duijf PHG, Khaze V, Gjerstorff MF & Baradaran Behzad (2021) HMGA2 as a Critical Regulator in Cancer Development. Genes (Basel) 12, undefined.
32. Lu W, Huang Z, Wang J & Liu H (2021) Long non-coding RNA DANCR accelerates colorectal cancer progression via regulating the miR-185-5p/HMGA2 axis. J Biochem undefined, undefined.
33. Wu Y, Wang X, Xu F, Zhang L, Wang T, Fu X, Jin T, Zhang W & Ye L (2020) The regulation of acetylation and stability of HMGA2 via the HBXIP-activated Akt-PCAF pathway in promotion of esophageal squamous cell carcinoma growth. Nucleic Acids Res 48, 4858-4876.

34. Huang S, Wa Q, Pan J, Peng X, Ren D, Huang Y, Chen X & Tang Y (2017) Downregulation of miR-141-3p promotes bone metastasis via activating NF- κ B signaling in prostate cancer. *J Exp Clin Cancer Res* 36, 173.
35. Phatak P, Noe M, Asrani K, Chesnick IE, Greenwald BD & Donahue JM (2021) MicroRNA-141-3p regulates cellular proliferation, migration, and invasion in esophageal cancer by targeting tuberous sclerosis complex 1. *Mol Carcinog* 60, 125-137.
36. Ishibashi O, Akagi I, Ogawa Y & Inui T (2018) MiR-141-3p is upregulated in esophageal squamous cell carcinoma and targets pleckstrin homology domain leucine-rich repeat protein phosphatase-2, a negative regulator of the PI3K/AKT pathway. *Biochem Biophys Res Commun* 501, 507-513.
37. Ye J, Zhou G, Zhang Z, Sun L, He X & Zhou J (2016) Poly (C)-binding protein 2 (PCBP2) promotes the progression of esophageal squamous cell carcinoma (ESCC) through regulating cellular proliferation and apoptosis. *Pathol Res Pract* 212, 717-25.
38. Mehta-Shah N, Ratner L & Horwitz SM (2017) Adult T-Cell Leukemia/Lymphoma. *J Oncol Pract* 13, 487-492.
39. Rayamajhi SJ, Shahi RR, Maharjan S, Sharma S & Sudhir SKC (2020) Muscle Infiltrative Adult Multisystem Langerhans Cell Histiocytosis Detected on Fluorodeoxyglucose Positron Emission Tomography/ Computed Tomography - A Rare Case. *Indian J Nucl Med* 35, 342-344.
40. Zheng Y, Chen Z, Han Y, Han L, Zou X, Zhou B, Hu R, Hao J, Bai S, Xiao H et al. (2020) Immune suppressive landscape in the human esophageal squamous cell carcinoma microenvironment. *Nat Commun* 11, 6268.
41. Tong S, Chen S, Xu K, Fang B, Wang S & Wang J (2018) 14-3-3 ζ promotes esophageal squamous cell carcinoma invasion by repressing S1PR2 protein expression through NF- κ B signaling. *Arch Biochem Biophys* 643, 7-13.
42. Piano V, Benjamin DI, Valente S, Nenci S, Marrocco B, Mai A, Aliverti A, Nomura DK & Mattevi A (2015) Discovery of Inhibitors for the Ether Lipid-Generating Enzyme AGPS as Anti-Cancer Agents. *ACS Chem Biol* 10, 2589-97.
43. Cao W, Wu W, Shi F, Chen X, Wu L, Yang K, Tian F, Zhu M, Chen G, Wang W et al. (2013) Integrated analysis of long noncoding RNA and coding RNA expression in esophageal squamous cell carcinoma. *Int J Genomics* undefined, 480534.
44. Wang F, Li Z, Xu L, Li Y, Li Y, Zhang X, Wang Y & Liu D (2018) LIMD2 targeted by miR-34a promotes the proliferation and invasion of non-small cell lung cancer cells. *Mol Med Rep* 18, 4760-4766.
45. Zhang F, Qin S, Xiao X, Tan Y, Hao P & Xu Y (2019) Overexpression of LIMD2 promotes the progression of non-small cell lung cancer. *Oncol Lett* 18, 2073-2081.
46. Pinheiro Dos Santos MJC, Bastos AU, da Costa VR, Delcelo R, Lindsey SC, Colozza-Gama GA, Peng H, Rauscher FJ, Oler G & Cerutti JM (2018) LIMD2 is overexpressed in BRAF V600E-Positive papillary thyroid carcinomas and matched lymph node metastases. *Endocr Pathol* 29, 222-230.
47. Araldi RP, de Melo TC, Levy D, de Souza DM, Maurício B, Colozza-Gama GA, Bydlowski SP, Peng H, Rauscher FJ & Cerutti JM (2020) LIMD2 Regulates Key Steps of Metastasis Cascade in Papillary

Thyroid Cancer Cells via MAPK Crosstalk. Cells 9, undefined.

48. Yu J, Xie M, Ge S, Chai P, Zhou Y & Ruan J (2020) Hierarchical Clustering of Cutaneous Melanoma Based on Immunogenomic Profiling. *Front Oncol* 10, 580029.
49. Liu C, Chen B, Huang Z, Hu C, Jiang L & Zhao C (2020) Comprehensive analysis of a 14 immune-related gene pair signature to predict the prognosis and immune features of gastric cancer. *Int Immunopharmacol* 89, 107074.
50. Rosati A, Graziano V, De LV, Pascale M & Turco MC (2011) BAG3: a multifaceted protein that regulates major cell pathways. *Cell Death Dis* 2, e141.
51. Berndt U, Philipsen L, Bartsch S, Hu Y, Röcken C, Bertram W, Hämmerle M, Rösch T & Sturm A (2010) Comparative Multi-Epitope-Ligand-Cartography reveals essential immunological alterations in Barrett's metaplasia and esophageal adenocarcinoma. *Mol Cancer* 9, 177.

Figures

Figure 1

The differences between the clustering of the circRNAs

Figure 2

The differences between the clustering of the circRNAs

Figure 3

DEmRNAs in RNA-seq data (Fig. 2C) and TCGA (Fig. 2F), as well as DEmiRNAs in TCGA (Fig. 3C)

Figure 4

The enriched terms analyzed based on the DisGeNET database are shown in Fig. 4A, including Adult T-Cell Lymphoma and Childhood Langerhans Cell Histiocytosis. The enriched GO terms are shown in Fig. 4B, including positive regulation of cell adhesion and positive regulation of protein kinase B signaling. The enriched terms analyzed based on the TRRUST database were SP1, RELA, and NFKB1 (Fig. 4C). Moreover, GSVA showed enrichment of ether lipid metabolism and acute myeloid leukemia in the cancer group (Fig. 4E).

Figure 5

Targets that showed a significant association with survival ($p < 0.05$) included LIMD2, ARL11, and SERPINB7 (Fig. 5A-L).

Figure 6

We observed significant differences in TME cells infiltration and composition in the two groups, including T cells CD4 memory activated and macrophages M1 (Fig. 6A). The R2 of TME cells was also calculated, and the results showed that T cells CD4 memory activated had statistical significance (Fig. 6B). A positive relationship was observed between LIMD2 and T cells CD4 memory activated (Fig. 6C).

Figure 7

Several studies have indicated the importance of autophagy in the development of ECA [27, 28]. We investigated the relationship between LIMD2 and autophagy-related genes (Fig. 7A-I).

Figure 8

The results showed significantly higher expression of LIMD2 in ECA tissues compared with normal tissues (Fig. 8).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials.docx](#)