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Analysis of Lncrna-Mirna Interactions in Esophageal Squamous Cell Carcinoma

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Abstract: objective: The study identify hub genes and vital signal pathways by integrated bioinformatics analysis of GEO and TCGA, in order to obtain new prognostic markers for esophageal squamous cell carcinoma. Method: We use R package Limma (chip data) or edgeR[12] (sequencing data) to calculate the differentially expressed mRNA and lncRNA between GEO esophageal squamous cell carcinoma dataset (GSE23400, GSE38129) and TCGA esophageal squamous cell carcinoma dataset. The genes with significant mean difference (ANOVA) (P value =<0.05) in all samples were analyzed by PCA. According to the correlation between lncRNA and mRNA expression, Cytoscape v3.7 was used to construct lncRNA-mRNA network. We construct a PPI network for the most tightly regulated mRNA and lncRNA by the string online tool . Use Cytoscapev3.7 to screen out the hub genes (here, select the top 10 genes with greater connectivity). GO and KEGG enrichment analysis for hub gene. Based on TCGA clinical data, clinical prognostic analysis of the hub genes of the selected COL protein family was performed. Results: We found that lncRNA-RP11-863P13.3,RP11-576I22.2,CTD-2171N6.1 could co-regulate the up-regulation of 16 genes such as MMP11, THY1, and down-regulated lncRNA is the closest to gene regulation is MAMDC2- AS1 interacts with 19 genes including FHL1 and TGFBR3. We determined that COL5A2, COL3A1, COL1A1 and other hub genes are most affected in the lncRNA-mRNA regulatory network of esophageal squamous cell carcinoma. The GO and kegg enrichment analysis of COL family genes showed that extracellular matrix organization, fibrillar collagen trimer, and extracellular matrix structural enrichment scores were the highest. Survival analysis showed that high expression of the four genes COL1A1, COL1A2, COL3A1, COL5A2 can significantly shorten the disease-free survival period of esophageal squamous cell carcinoma, and the prognosis of patients becomes worse. Conclusion: lncRNA-RP11-863P13.3, RP11-576I22.2, CTD-2171N6.1 may promote changes in ECM traits by up-regulating COL1A1,COL1A2,COL3A1,COL5A2, leading to poor prognosis in patients with esophageal squamous cell carcinoma, and could be potential biomarkers for ESCC patients's prognosis.

1. Introduction

Esophageal carcinoma (EC) is a prevalent and multiple malignancy in the world [1]. In Chinese mainland, it ranks the third and fourth respectively in mobility and mortality [2], and most cases suffer from esophageal squamous cell carcinoma (ESCC) which accounts for around 90% of all global EC cases [3]. Generally, patients suffering from ESCC can have a 5-year survival up to 90%. While in China, the 5-year survival rate is only less than 20%, as 50% of ESCC cases are developing tumor metastasis when diagnosed, making surgery and chemoradiotherapy of limited efficacy [4, 5]. Notably, early diagnosis and distant metastasis emerge as determinant factors for cancer prognosis, and relevant screening indicators as well as therapeutic targets are in turn in an urgent need.

Non-coding RNA (ncRNA) as known is essential in initiation and progression of tumors [6]. A previous study by our research group, who devote to ncRNA in tumor for a long-term, reported that, microRNA-192 (miR-192) shows significantly decreased expression in acute myeloid leukemia, and it is involved in tumor cell proliferation and cell cycle progress by interaction with cyclin CCNT2 [7]. Long non-coding RNA (lncRNA) NKILA also downregulates in ESCC, which results in blocked cell proliferation and migration dependent on suppressed NF-κB signaling pathway, ultimately leading to advanced tumor malignant progression [8]. Circular RNA CIRS-7 as reported is contrarily a promoter for ESCC growth and metabolism via the miR-7/HOXB13 axis [9]. At present, ncRNA has been emerging as a research hotspot in the field of tumor, but identification of lncRNA in ESCC as a tumor biomarker has barely been reported.

In the present study, two popular bioinformatics databases, Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA), were consulted to dig out the differential expression profiles of lncRNA and messenger RNA (mRNA) in ESCC. With the performance of a series of bioinformatics analyses hub genes associated with ESCC survival were identified, and further certified in their feasibility as Prognostic biomarkers.

2. Methods

2.1 Data Collection and Preprocessing

Unstandardized Series Matrix File of interest microarrays and Counts data of ESCC were respectively accessed from GEO (http://www.ncbi.nlm.nih.gov/geo/) and TCGA databases (https://portal.gdc.cancer.gov/). Quartile standardization [11] for acquired microarray data was initially conducted using the normalize Between Arrays in Limma package [10], and then gene expression matrix was obtained by log2 transfer.

2.2 Correlation Analysis

Principal component analysis (PCA) is a data dimension reduction method characterized by unsupervised feature learning, and it can provide data classification according to expression data from target samples. Here, we applied PCA to intuitively observe sample distribution in the experimental and control groups. The outliers were removed, while the sample sets of high similarity were identified for further analysis. The sample genes we applied to perform PCA were of significance in analysis of variance (ANOVA) (P value =< 0.05), with all genes implicated if no duplicates. It is recognized that correlation in between-sample gene expression level can be an important indicator for experiment reliability and rational sample selection. The correlation coefficient can describe the similarity between samples, and when it approaches 1, the between-sample similarity comes with a high degree. Here, we used gene expression data to

calculate the correlation coefficient between samples and made them visualized in a heat map.

2.3 Differential Gene Screening and Lncrna-Mrna Network Construction

Sample grouping was firstly performed, followed by differential gene expression analysis under R package Limma (microarray data) or edgeR (sequencing data) [12]. LncRNA and mRNA of interactions were analyzed and then intuitively displayed in a lncRNA-mRNA network by Cytoscape v3.7 [13].

2.4 Protein-Protein Interaction (Ppi) Network Analysis

Candidate genes were projected on STRING [14] online website, and the proteins meeting Required Confidence (combined score) > 0.7 were taken to construct a PPI network visualized by Cytoscape for typology structure analysis. It is known that most biological networks are scale-free networks, allowing the Connectivity Degree in network statistics an important parameter to identify the vital nodes, what we call hub proteins, that involved in PPI networks [15]. We here performed node analysis from the constructed PPI network based on the scale-free property, to finally find the hub proteins of interest.

2.5 Enrichment Analysis for Gene Biological Function

Biological function enrichment analysis for candidate genes here were completed with methods of Gene Ontology (GO) [16] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases [17]. Fisher's exact test was performed to find certain function terms of the highest connectivity with a gene set, with P-value calculated to identify the statistical significance.

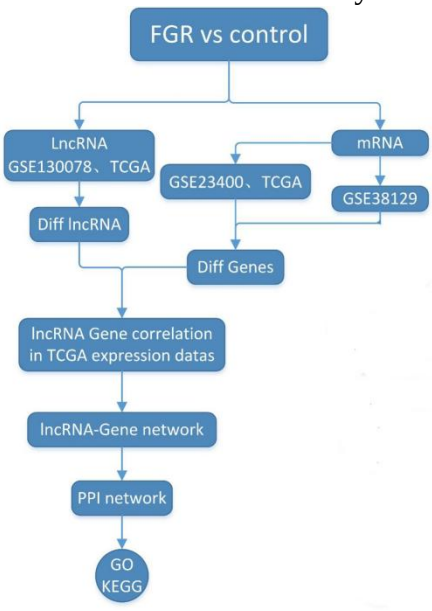

Fig.1 Analysis Process

3. Results

3.1 Differential Mrna Screening

Microarrays GSE23400 and GSE38129 from GEO database and ESCC dataset from TCGA

database were collected, and the protein-coding genes in TCGA data were filtered out by gencode v22 annotation files. From PCA analysis, between-sample correlation in these three datasets was better in cancer cases, relative to normal or adjacent tissues (Figure 2). Suitable samples were collected based on their phenotypes, and the gene expression data implicated were log2 transferred, and then subjected to differential gene expression analysis by Limma (microarray data) or edgeR (sequencing data, at least 1 gene set of CPM > 10 retained). Differential genes from the three datasets were detailed in Table 1. Clustering analysis for the differential genes was presented in Figure 3, with the mRNAs of relatively significant difference marked. Additionally, 517 differential genes, including 326 differentially unregulated and 191 differentially down-regulated, were shared by these three datasets as shown in Figure 4.

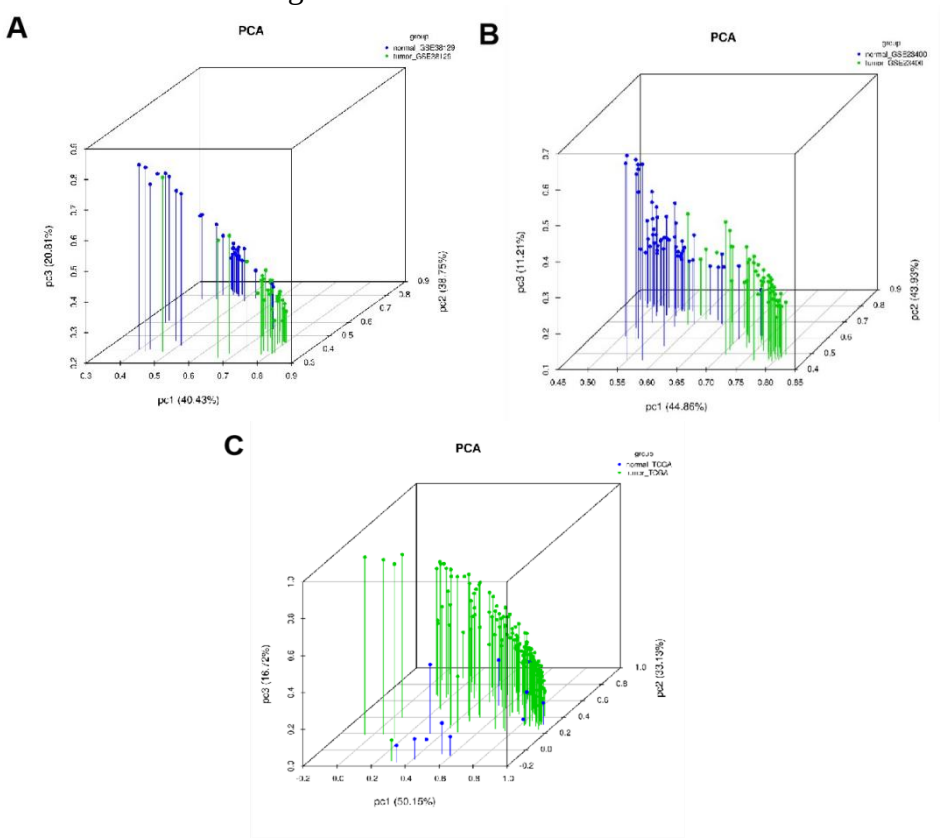

Fig.2 Pca Analysis

Table 1 Differential Genes

Data information		Threshold	Count of differential genes			Sample size	
Data source	Platform	Fold change (FC)	P_value	Up	Down	tumor	normal
GSE23400	GPL96	1.5	0.05	614	570	53	53
GSE38129	GPL571	1.5	0.05	1056	990	30	30
TCGA	HTSeq	1.5	0.05	1417	1630	161	12

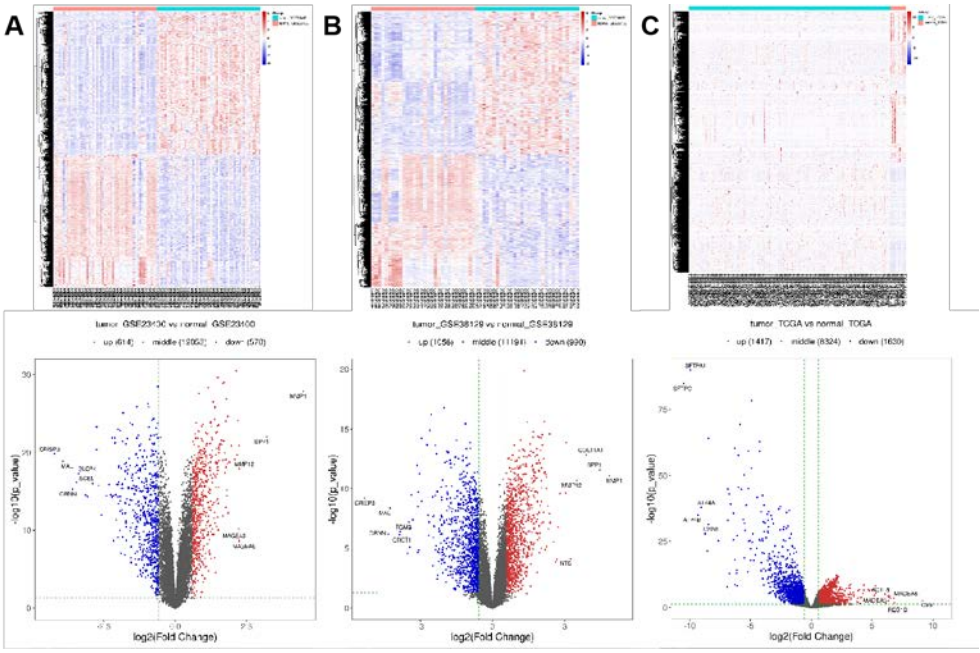

Fig.3 Mrna Difference Analysis Heat Map and Volcano Map

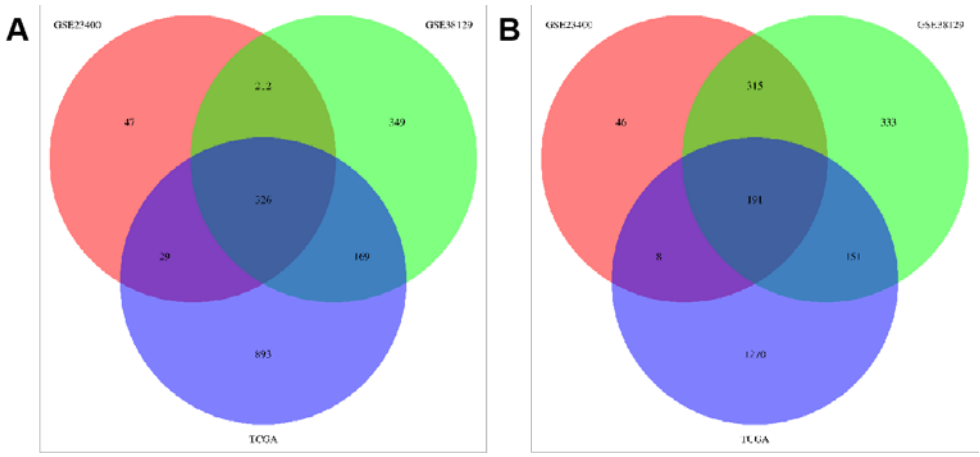

Fig.4 Differential Mrna Intersection Venn Diagram

3.2 Differential Lncrna Screening

Table 2 Differential Lncrnas

Data information		Threshold for	Count of differential lncRNAs			Sample size	
Data source	Platform	(FC)	P_value	Up	Down	tumor	normal
GSE130078	HiSeq 2000	1.5	0.05	512	302	23	23
TCGA	HTSeq	1.5	0.05	1266	1000	161	12

LncRNA microarray GSE130078 from GEO database and lncRNA data from TCGA filtered out by gencode v22 annotation were combined. Similarly, suitable samples were collected, and gene expression following log2 transfer was loaded on edgeR (at least 1 gene set of average CPM > 10 retained) for differential analysis. Differential lncRNAs screened from the two datasets were listed in Table 2. The screened lncRNAs were processed by clustering analysis and the genes of relative significance were marked (Figure 4). Overall, 241 differential lncRNAs were overlapped in the two datasets, including 184 up-regulated and 57 down-regulated (Figure 5), and prepared for follow-up

network analysis.

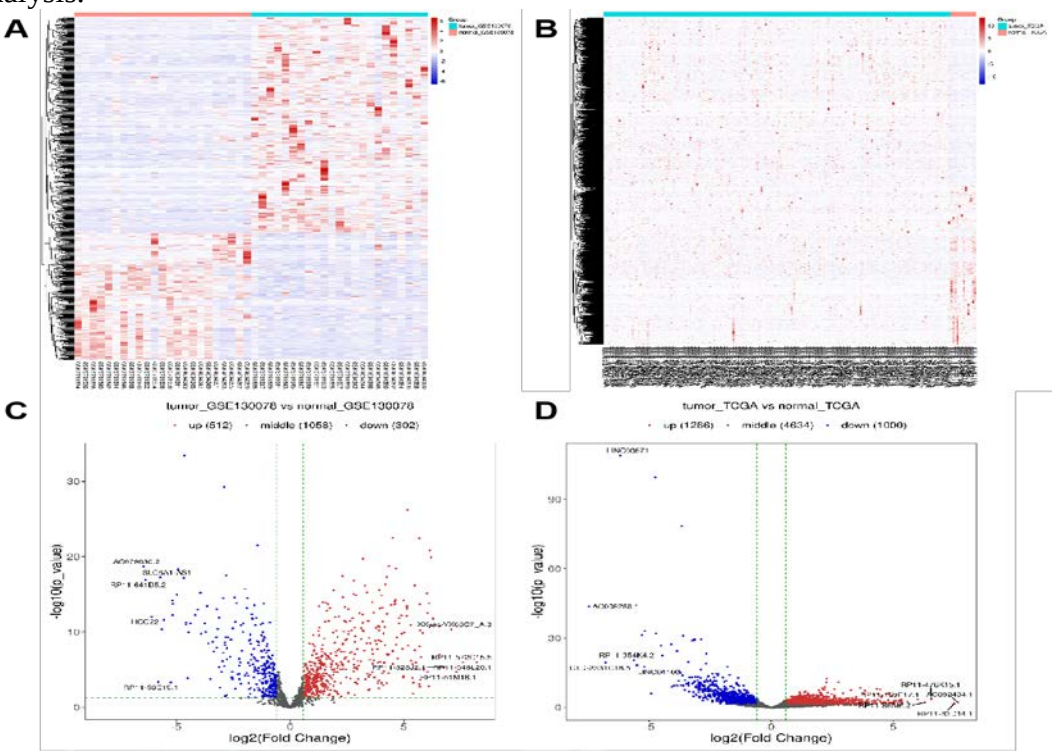

Fig.5 Lncrna Difference Analysis Heat Map and Volcano Map

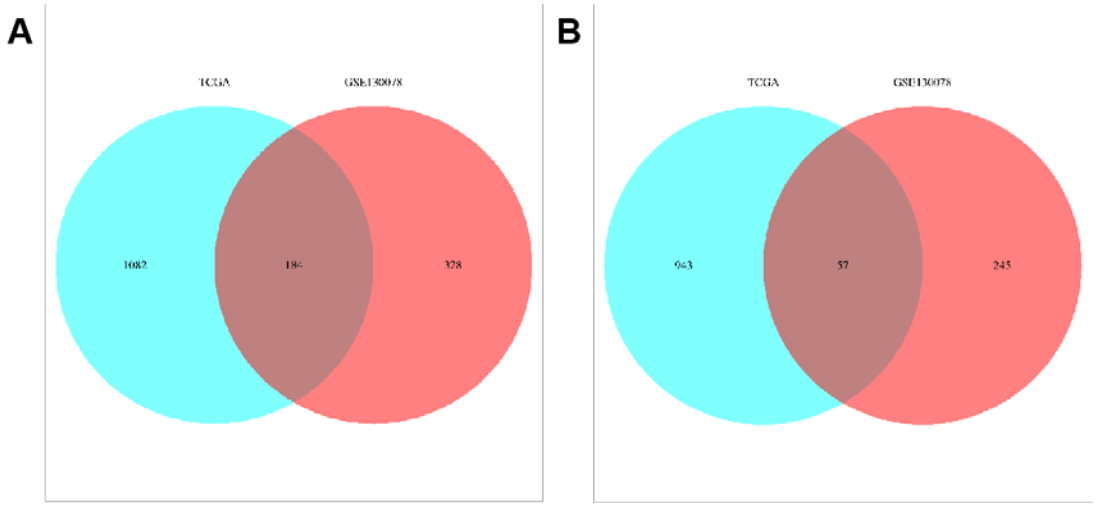

Fig.6 Differential Lncrnas Intersection Venn Diagram

3.3 Lncrna-Mrna Network Analysis

Correlation between the above identified lncRNAs and mRNAs was firstly calculated in TCGA database, and the lncRNA-mRNA pairs meeting correlation coefficient over 0.7 were selected. Then, the selected lncRNA-mRNA pairs were visualized on Cytoscape (Figure 7). From the network we constructed, we noted that lncRNAs RP11-863P13.3, RP11-576I22.2 and CTD-2171N6.1 could cooperate to mediate the up-regulation of 16 genes including MMP11 and THY1. While in differentially down-regulated lncRNAs, MAMDC2-AS1 was the master lncRNA that could interact with 19 genes including FHL1 and TGFBR3. Subsequently, mRNAs which were the most

correlated with lncRNA were projected onto STRING website for PPI analysis. The top 10 hub genes from the PPI network were identified based on their Connectivity Degree, as revealed in Figure 8A. From the 10 hub genes, COL5A2, COL3A1 and COL1A1 were identified to suffer the greatest effect in the lncRNA-mRNA network. GO annotation and KEGG pathway enrichment analysis were carried out for the genes in PPI network. In GO analysis, three aspects including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) were considered. As analyzed, extracellular matrix organization in BP (Figure 8B), fibrillar collagen trimer in CC (Figure 8C), and extracellular matrix structural constituent conferring tensile strength in MF (Figure 8D) were the biological functions highest enriched. KEGG analysis results uncovered that the genes in the PPI network were highly activated in pathways involved in protein digestion and absorption (Figure 8E). Finally, combined with clinical information collected from TCGA, the survival significance of the hub genes from COL protein family was evaluated, and it was noted that their high expression was significantly associated with shortened disease-free survival of ESCC cases (Figure 9). This indicated that the network we constructed is of certain clinical significance.

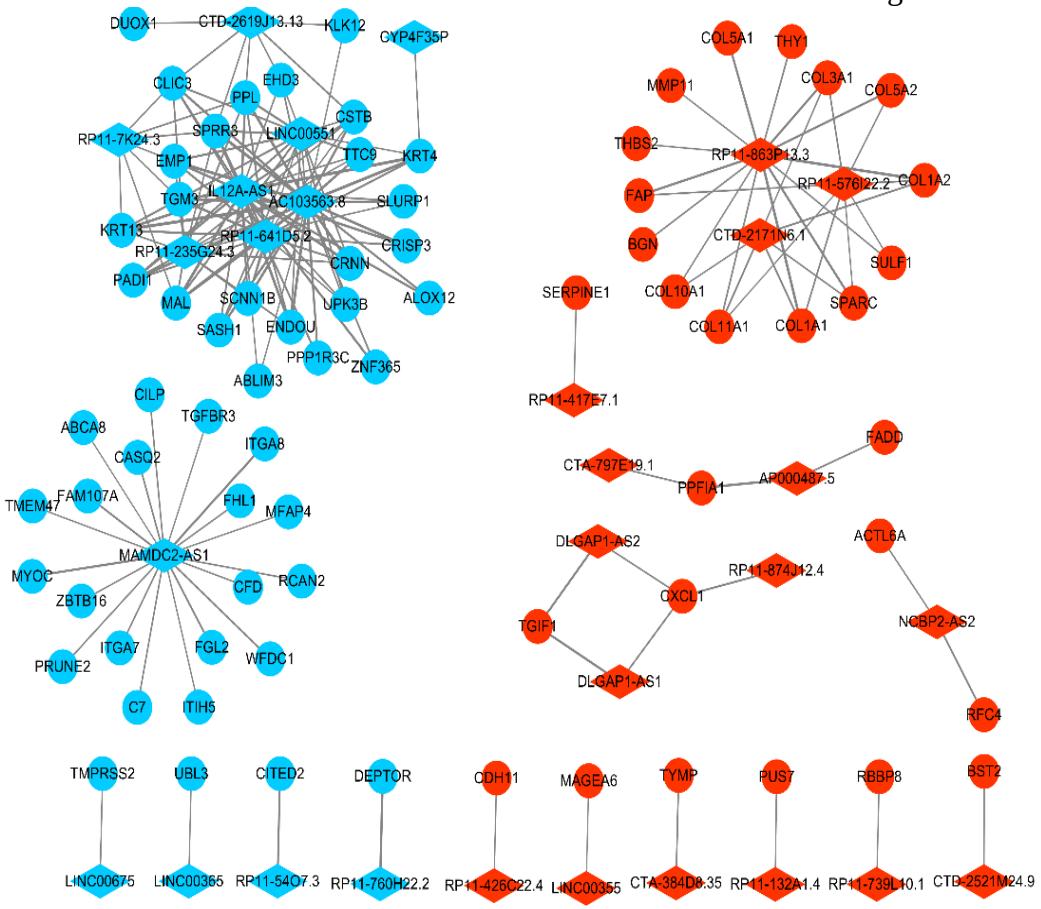

Fig.7 Lncrna-Mrnaregulation Network

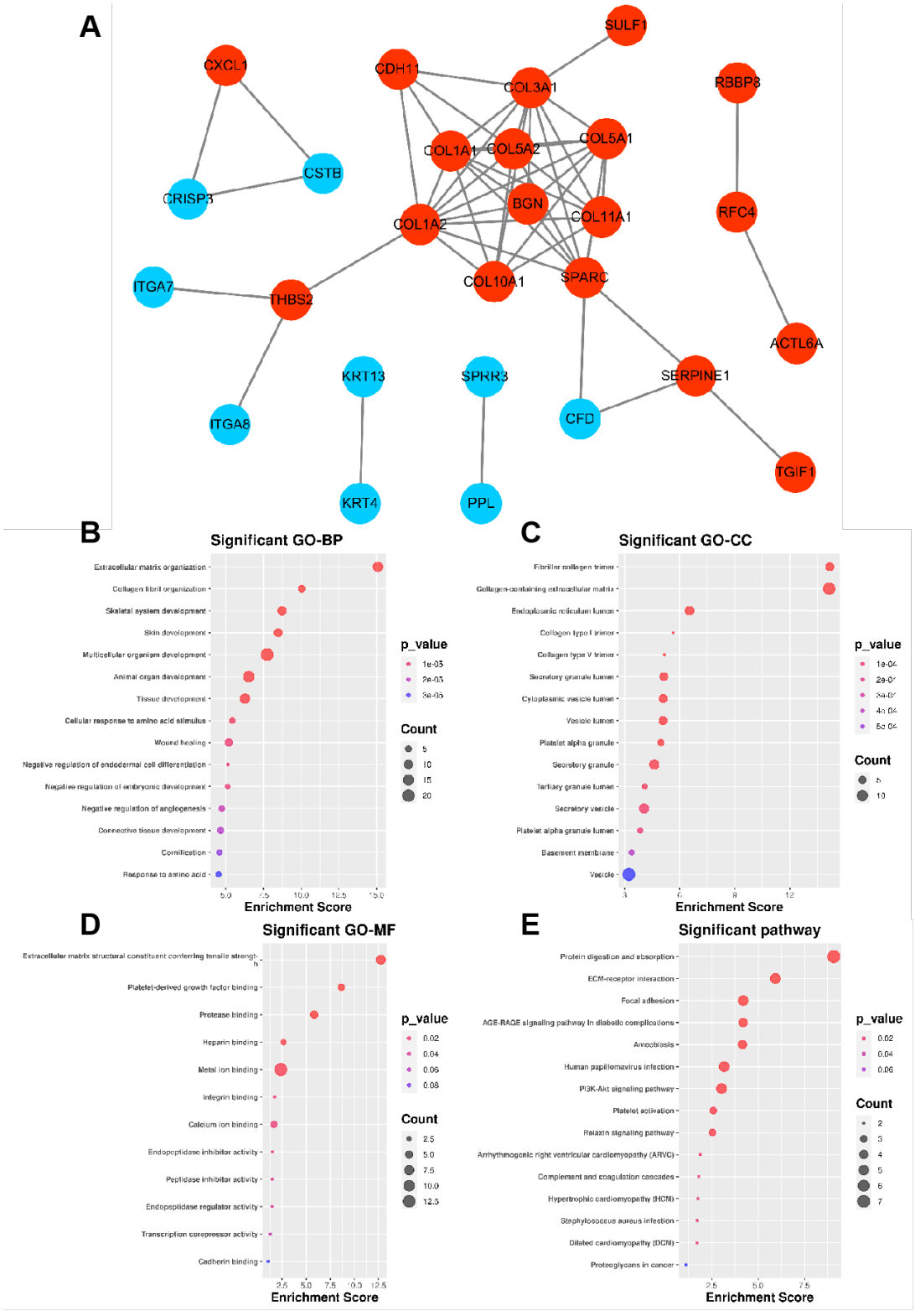

Fig.8 Ppi Network and Hub Gene Pathway Enrichment Analysis

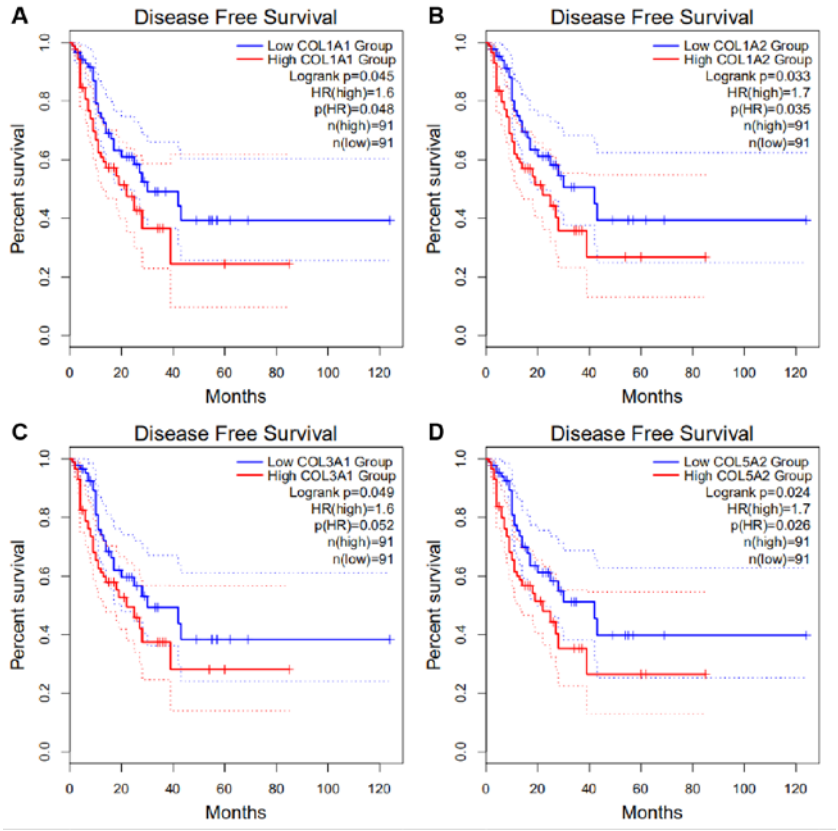

Fig.9 Prognostic Analysis of Hub Gene Dfs

4. Discussion

As we know, lncRNA in hepatocellular carcinoma (HCC) has been rarely characterized. There was a study which constructed a 6-lncRNA signature to predict the recurrence-free survival of HCC, and Wu et al. validated it in a certain population with resectable HCC tumors [19, 20]. Different with previous research, this study constructed a lncRNA-mRNA interaction network in ESCC cases, to screen hub genes for survival prediction. Such approach, from the perspective of cell function correlation, is more precise than single identification of lncRNA signature and is of higher clinical significance. In the present study, we firstly screened lncRNAs and mRNAs which were differentially expressed in ESCC tumor tissue and adjacent tissue in GEO and TCGA databases. Then, the correlation of lncRNA with mRNA in TCGA was analyzed, with the lncRNA-mRNA pairs of correlation coefficient over 0.7 visualized by Cytoscape. PPI analysis was subsequently carried out on STRING website, followed by GO annotation and KEGG pathway enrichment analysis. Finally, we probed into the survival significance of lncRNA-related hub genes in TCGA-derived clinical samples.

As uncovered, COL1A1-represented COL collagen protein family were in the majority of the hub genes we identified. COL gene encodes pre alpha-1 chain of collagen type 1, including two alpha-1 chain and one alpha-2 chain involved in its triple helical structure. Collagen type 1 is present in most connective tissues known as fibril-forming collagen and it is rich in skeleton, cornea, dermis and tendon [21-23]. Mutations in such gene is associated with I-IV osteogenesis imperfecta, VIIA Ehlers-Danlos syndrome, classical Ehlers-Danlos syndrome, Caffey syndrome and idiopathic osteoporosis. Translocation of chromosome 17 and 22, positions for the gene and platelet-derived growth factor, is associated with a specific type of skin tumor named as dermatofibrosarcoma

protuberans, which is triggered by the aberrant expression of growth factors. The two transcripts generated by alternative polyadenylation signal have been identified as this gene [24-27]. We here based on TCGA clinical data found that COL1A1, COL1A2, COL3A1 and COL5A2 all could significantly reduce the disease-free survival of ESCC cases when up-regulated, and finally contributed to poor clinical outcome. Therefore, we reasoned that COL collagen protein family is intimately correlated with poor survival of ESCC sufferers.

Extracellular matrix (ECM) is an insoluble structural component essential for the formation of mesenchymal and epithelial vascular matrix, mainly composed of collagen, elastin, proteoglycan and glycoprotein [28]. Research revealed that ECM can affect multiple biological processes, such as cell differentiation, proliferation, adhesion, morphogenesis and phenotype expression [29]. The occurrence, development, invasion and metastasis of malignant tumors often come with the changes in ECM and cell surface receptor expression [30]. In normal liver cells, no basal membrane is present, and the specific integrin receptor α6β1 of laminin (LN) is not expressed. While in HCC, LN and α6β1 not only present increased expression but also negatively correlate with the survival of HCC sufferers, indicating that HCC cells might receive signal from LV via α6β1 receptors, allowing the essential role of LN and α6β1 in HCC cell invasion [31]. The onset of HCC generally accompanies with portal vein invasion, intrahepatic metastasis, and extra hepatic lung and bone metastasis, while tumor invasion, metastasis and postoperative recurrence are main factors leading to poor outcome. Degradation of ECM by matrix metalloproteinase (MMPs) is one of the key steps in tumor cell invasion and metastasis, and most malignancies accompany with increased MMPs secretion and activity [32]. Our enrichment results revealed that ECM-related pathways, extracellular matrix organization, fibrillar collagen trimer and extracellular matrix structural, were of the highest enrichment score, which demonstrated that COL1A1-representative COL family can be involved in ESCC occurrence and progression via mediating ECM properties, to finally affect cancer prognosis.

Epigenetics is a biological study that describes the inheritable changes in gene expression with no alterations in DNA sequence. It is essential for cell growth and differentiation, as well as for tumor initiation and progression. The main mechanisms of epigenetics include DNA modification, histone modification, and newly discovered ncRNA [33]. NcRNA refers to the functional RNA molecule that cannot be translated into proteins, mainly falls in small interfering RNA, miRNA, Piwi interacting RNA (piRNA) and lncRNA which are common and have regulatory function [34]. A large number of studies indicate that ncRNA is increasingly important in epigenetics. LncRNA is a kind of ncRNA with a length over 200 nucleotides. As reported, lncRNA is a vital player involved in regulating various life activities, including dosage compensation effect, epigenetic regulation, cell cycle and cell differentiation, making it a hotspot in the field of genetics. In Tongji University, integrated genomic data were once used to find out lncRNAs significantly associated with cancers, and two lncRNAs in prostate cancer were discovered [35]. LncRNA GLCC 1 and HSP 90 as reported can bind to form RNA-protein complex which helps stabilize the ubiquitination degradation of c-Myc in cytoplasm, and further induce the occurrence of colorectal cancer and glucose metabolism after promoting the transcription of its target gene LDHA [36]. The binding of HNF1A-AS1 with Egr1 enhances the CD34-mediated p21 ubiquitination and degradation, up-regulating the expression of cyclin-dependent kinase 2 (CDK2), CDK4, and cyclin E1, suppressing p21 expression, and ultimately advancing gastric cancer malignancy [37, 38]. All above mentioned studies indicate that lncRNA can mediate the malignant progression of tumors by regulating gene expression in multiple approaches. The data obtained here revealed that lncRNAs RP11-863P13.3, RP11-576I22.2 and CTD-2171N6.1 all could up-regulate the expression of COL collagen family members including COL1A1, and these three RNAs were also highly expressed in ESCC tissues. Hence, we believed that the three RNAs can contribute to poor outcome of ESCC

cases via up-regulating COL1A1, COL1A2, COL3A1, COL5A2 and changing ECM properties.

In all, this study used bioinformatics analysis to identify three lncRNAs which are closely associated with the poor survival of ESCC cases. However, relevant mechanisms have not been further probed by in vivo and in vitro experiments due to diverse objective causes. In the future, we will carry out more in-depth research regarding the mechanisms in regulating relevant molecules identified in this study.

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