Identification of Novel Transcription Factor-microRNA-mRNA Co-regulatory Networks in Pulmonary Large-cell Neuroendocrine Carcinomas

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Abstract

Background: Large-cell neuroendocrine carcinoma (LCNEC) of the lung is a rare neuroendocrine neoplasm. Previous studies have shown that microRNAs can be widely involved in tumor regulation through targeting critical genes. However, it is unclear about which miRNAs play vital roles in LCNEC's pathogenesis and how they interact with transcription factors (TFs) to regulate the cancer-related genes.

Methods: To clarify the new TFs-miRNA-target gene feed-forward loops (FFLs) model of LCNEC, we integrated multi-omics data from GEO, TRRUST, TRED, and miRTarBase database. First, expression profile datasets for mRNAs (GSE1037) and miRNAs (GSE19945) were downloaded from the GEO database. Overlapping differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) were identified through integrative analysis. The target genes of the FFL being obtained from the miRTarBase database, the GO and KEGG functional enrichment analysis was performed on the target gene. Then, we screened for key miRNAs in the FFL and perform gene regulatory network analysis based on key miRNAs. Finally, the TF-miRNA-target gene FFLs were constructed by the hypergeometric test.

Results: A total of 343 DEGs and 60 DEMs were identified in LCNECs compared to normal tissues, including 210 downregulated, 133 upregulated genes and 29 downregulated, 31 upregulated miRNAs. Additionally, 55 DEMs were predicted through miRanda, mirDB, and TargetScan to be associated with the LCNEC 77 key genes. Finally, ETS1-miR195-CD36 and E2F3-miR195-CD36 from the TF-miRNA-mRNA FFLs were constructed and are significantly enriched in focal adhesion, cytokine-receptor interaction, hematopoietic cell lineage, and transforming growth factor (TGF)- β signaling pathways. Survival analysis showed that the differential expression of the multi-effect factors ETS1, CD36, and hsa-miR-195 in the FFL significantly affected the survival time of LCNEC patients.

Conclusion: In summary, we construct the TF-miRNA-target regulatory network, which is helpful to understand the complex LCNEC regulatory mechanisms. The expression difference of regulated multi-effect target factors ETS1, CD36, and hsa-miR-195 has a significant impact on the survival and development of LCNEC.

Introduction

Pulmonary high-grade neuroendocrine carcinoma includes the following two clinicopathological entities classified based on their morphological and biological features: large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancer (SCLC) [1, 2]. LCNEC and SCLC share several histological features, including rosette formation, molding of nuclei, and lack of apparent glandular formation and keratinization [3, 4]. LCNEC, diagnosed in approximately 3% of all patients with lung cancer, is generally associated with a high metastases rate and poor survival [5, 6]. It is characterized by occult onset, strong invasiveness, and poor survival regardless of operation, chemotherapy, radiotherapy, or other treatments [7]. There is a paucity in the understanding of the precise molecular mechanisms about LCNEC. Therefore, it is urgent to find the biological marker for early diagnosis and novel treatment targets of LCNEC.

Transcription factors (TFs) are DNA-binding proteins that can function as tumor suppressors or oncogenes [8]. TFs play significant roles in the regulation of gene expression and can induce avoidance of apoptosis and uncontrolled growth [9]. A study has demonstrated the presence of thyroid transcription factor-1 (TTF-1) in a significant subset of pulmonary neuroendocrine tumors, including 35% of typical carcinoids, 100% of atypical carcinoids, 75% of large cell neuroendocrine tumors, and 95% of small cell carcinomas [10]. Huang *et al.* found that POU2F3 is an essential master regulator of cell identity in the neuroendocrine variant of SCLC. Epithelial-mesenchymal transition (EMT) plays an important role in tumor invasion and metastasis, and it is one of the important mechanisms for many tumor cells to acquire invasion and metastasis capabilities [11]. Snail, a major transcription factor regulator of EMT, directly inhibits the expression of E-cadherin (E-cad) and upregulates the expression of interstitial cell marker molecules such as N-cadherin (N-cad), thereby promoting the oncogenesis of NSCLC [12]. Jin *et al.* found that metformin-induced repression of miR-381-YAP-Snail axis activity can disrupt NSCLC growth and metastasis. Thus, they believe that the miR-381-YAP-Snail signal axis may be a suitable diagnostic marker and a potential therapeutic target for lung cancer [13]. Previous research reported Foxp3 regulates T cells and natural killer cells to play a role in pulmonary adenocarcinoma [14]. Shih *et al.* validated that MYC, YAP1, or MMP13 overexpression increased the incidence of lung adenocarcinoma brain metastasis by functional assessment in patient-derived xenograft mouse models [15].

microRNAs (miRNAs) are short, noncoding RNAs consisting of 18–25 nucleotides that regulate the translation of mRNAs [16]. Mature miRNAs can recognize and bind to the 3' untranslated region of mRNAs and regulate translation at the posttranscriptional level through repression or degradation of the targeted mRNAs [17]. Recent studies have documented a relationship between the aberrant expression of a class of miRNA and the pathogenesis of many human cancers, including lung cancer [18, 19]. Lee *et al.* found that the miR-21 and miR-155 were differentially expressed according to the histologic subtypes of pulmonary neuroendocrine tumors, and the expression level of miR-21 was significantly higher in carcinoid tumors with lymph node metastasis than in carcinoid tumors without lymph node

metastasis [20]. Other links between lung cancer and miRNA have been reported, including let-7 in lung cancers [21], high expression and oncogenic function of mir-17-92 cluster in human B cell lymphomas as well as in lung cancers [22]. The expression of five miRNAs (hsa-mir-155, hsa-mir-17-3p, hsa-let-7a-2, hsa-mir-145, and hsa-mir-21) is statistically altered in lung cancers and also has a prognostic impact on survival [18].

Regulatory network analysis, such as feedback loop and feed-forward loop (FFL), is a powerful way to investigate the underlying global relationship of molecular networks. miRNA-transcription factor (TF) coregulation is one of the important FFL types. Transcription factors (TFs) and microRNAs (miRNAs) are two kinds of crucial regulators for transcriptional and post-transcriptional regulations. Understanding the cross-talks between the two regulators and their targets is critical to reveal the complex molecular regulatory mechanisms of cancers. Numerous studies have revealed that TFs regulate gene expression by interacting with miRNAs [23]. There are currently no detailed reports of the TFs and TF-miRNA-mRNA network in LCNECs.

Recently, by identifying key cancer-related genes, noncoding RNAs, and TFs, bioinformatics analysis has gradually been utilized to explore the mechanisms of oncogenesis and progression of cancers. In this study, we investigated the comprehensive miRNA-TF co-regulatory network in LCNEC. Firstly, we identified the potential targets of LCNEC-related TFs and miRNAs. We used a public database to find the TFs that regulate the dysregulated genes. miRNAs were further predicted according to the TFs that targeted the DEGs, and interactions between miRNAs and TFs were predicted with online tools miRanda, mirDB, and TargetScan. This integration with experimental data from patients is a complement to the FFL studies which mostly relied on the predicted regulation information by reducing false positives. Then, based on our findings, a TF-miRNA-mRNA network was constructed to reveal a transcriptional regulation model and identify key genes and miRNAs associated with LCNEC. To verify the implication of these components, we further explored the associations between the expression level of identified components and LCNEC survival and significant signal pathways. This study established a valuable LCNEC progress regulation network, which help to reveal the complicated regulatory mechanisms and find out new markers or targets for the diagnoses and treatments for LCNEC.

Methods

Collection of datasets

A flowchart of the study design is shown in Fig. 1. The mRNA expression profile (GSE1037) and miRNA expression profile (GSE19945) were downloaded from the GEO database (<http://www.ncbi.nlm.nih.gov/gds/>). All of the datasets were selected based on the following criteria: (1) there were no fewer than six samples, (2) the samples were from human LCNEC tissue, and (3) the datasets included case-control groups.

Screening LCNEC-related miRNAs and genes

To obtain the dysregulated miRNAs in LCNEC, we searched the miR2Disease (<http://www.mir2disease.org/>), PhenomiR2.0 (<http://mips.helmholtz-muenchen.de/phenomir>), and HMDD2.0 (<http://www.cuilab.cn/hmdd>) by using the keywords “large-cell neuroendocrine carcinoma” or “LCNEC”. Finally, mapping unique miRNAs were retrieved as LCNEC-related miRNAs. The R limma 10.0 package in Bioconductor version 3.10 (<http://www.bioconductor.org/>) was utilized to identify DEGs and aberrantly expressed miRNAs in LCNEC tissues compared with normal lung tissues. The Benjamini and Hochberg false discovery rate (FDR) and adjusted p -values (adj. p) were applied to provide a balance between the probability of obtaining false positives and the probability of discovering statistically significant genes. The cutoff values for aberrantly expressed miRNAs were a $|\log_2FC| \geq 2$ and $FDR < 0.05$, and for mRNAs were a $|\log_2FC| \geq 1$ and an $FDR < 0.05$.

Function enrichment analysis

To investigate the potential biological function and pathways, the Online Database for Annotation, Visualization and Integrated Discovery (DAVID; <https://david-d.ncifcrf.gov/>) was used to analyze and visualize Gene Ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Identification of critical TF-miRNA-mRNA regulation loops

The FunRich (<http://funrich.org>) analysis was used to predict the differential miRNA transcription factors (overlapping with DEGs). The TargetScan (http://www.targetscan.org/vert_72/), miRanda (<http://www.microrna.org/microrna/home.do>), and mirDB (<http://mirdb.org/>)

databases were used to predict biological targets (overlapping with DEGs) of DEMs. Finally, a TF-miRNA-mRNA network was constructed to show the potential molecular mechanisms in LCNEC's oncogenesis and progression.

Results

Microarray datasets from the GEO

The mRNA expression profiles were obtained from the GSE1037 dataset, and miRNA profiles were obtained from the GSE19945 dataset. There are 19 normal and 8 LCNEC tissues included in the GSE1037 dataset; while 20 Barrett's esophagus, 8 normal and 11 LCNEC tissues were included in the GSE19945 dataset.

Screening LCNEC-related TFs and miRNAs

To identify DEGs, the GEO mRNA expression profile datasets were analyzed. A total of 343 DEGs were obtained finally by examining genes from GSE1037 dataset (Fig. 2B). Among the DEGs, 133 genes were upregulated in LCNEC, while the remaining 210 genes were downregulated ($|\log_2FC| \geq 1$, adjusted p -value < 0.05). Top down-regulated genes (*HBE1*, *TIMP3*, *SPATA31A3*, *LYVE1*, *FMO2*, *SUSD2*, *SLPI*, *FOLR1*, *PEBP4*, *AQP1*, *CAV2*, *PMP2*, *ADARB1*, *LIMCH1*, *HOPX*, *ABCA8*, *ADAMTSL3*, *A2M*, *ANGPTL1*) and top up-regulated genes (*DSP*, *NBPF14*, *FOXI1*, *S100A7*, *NUF2*, *UBE2C*, *TOP2A*, *TEX101*, *ZWINT*, *STMN1*) were identified in the DEGs dataset (Table 1). Specially, TF *FOXI1* significantly overexpressed in LCNEC. A total of 60 DEMs were identified between the LCNEC and normal tissues. Among them, 29 miRNAs were significantly downregulated in LCNEC, while the other 31 miRNAs were upregulated ($|\log_2FC| \geq 1$, adjusted p -value < 0.05) (Fig. 2A).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses

The online database of DAVID was used to explore the enrichment analysis of GO and KEGG in the key modules. Figure 3 shows the enrichment results of GO and KEGG pathways. The mRNA pathway enrichment analysis found that the top terms were: negative regulation of phosphorylation, negative regulation of protein phosphorylation, cell-cell adherens junction, and cell-cell junction. Several transcription factors-associated terms were also observed, such as transcription factor activity, sequence-specific DNA binding, transcriptional activator activity, and RNA polymerase II transcription factor binding. KEGG pathway analysis was conducted to determine the signaling cascades related to the identified genes; by using $p < 0.05$ as the threshold value, the following significantly enriched pathways were identified: cell adhesion molecules, MAP-activated protein kinase (MAPK) signaling, proteoglycans in cancer, Hippo signaling, and human T-cell leukemia virus 1 infection.

MiRNA pathway enrichment analysis found that the KEGG pathways were mainly manifested in the focal adhesion, cytokine-receptor interaction, hematopoietic cell lineage, and transforming growth factor (TGF)- β signaling pathways, while the biological processes found based on GO analysis mainly included response to wounding, inflammatory response, cell adhesion, biological adhesion, and vasculature development.

Construction of TF regulatory network

The regulatory network of TF-miRNA-target gene was established, as shown in Fig. 4, involving 5 TFs (E2F3, MYB, ETS1, TCF7L2, FOSB), 7 miRNAs (has-miR-195-5p, has-miR-200c-3p, has-miR-18a-5p, has-miR-7-1-3p, has-miR-183-5p, has-miR-96-5p, has-miR-141-3p), and 12 hub genes (TAOK1, STMN1, MAPK7, DUSP6, FZD3, BIRC5, F3, PDE4D, RGL1, CD36, GRIA1, IL6R). The key regulatory network modules included ETS1-miR195-CD36, TAOK1-miR7-1-3P-GRIA1, E2F3-miR195-CD36, and TEAD1-miR30A-CTHRC1.

Discussion

LCNEC of the lung is a rare but highly aggressive tumor. LCNECs presents with a high gene mutation rate, and genetic alterations have a predictive value on chemotherapy outcome [24]. However, the pattern and function of these dysregulated mRNAs and miRNAs have not been fully recognized. In this study, we identified 343 DEGs (including 133 upregulated and 210 downregulated genes) and 60 DEMs (including 31 upregulated and 29 downregulated miRNAs) in the LCNEC tissues compared with normal tissues.

We found several critical genes and pathways in the transcriptome with biological significance in this study. First, Stathmin 1 (STMN1), a member of the Stathmin family, acts as a microtubule destabilizer to regulate the cell cycle. The activation of STMN1 pathway promotes

lung cancer cell invasion, migration and resistance to tyrosine kinase inhibitors [25, 26]. STMN1 overexpression predicts poor survival in multiple solid tumors including lung cancer [27]. High STMN1 expression is also associated with cancer progression and chemoresistance in lung squamous cell carcinoma [28]. Similar to our results, Shimizu *et al.* reported that all 17 LCNEC samples expressed a high level of STMN1 in their study, indicating that STMN1 might be a potential diagnostic marker for high-grade lung neuroendocrine tumors [29]. Second, Wnt signaling pathway. It plays a role in the development of lung cancer [30]. Wnt inhibitory factor 1 (WIF1) directly binds to Wnt protein to inhibit Wnt signaling pathway, functioning as a tumor suppressor. WIF1 is downregulated in both the lung squamous cell carcinomas and adenocarcinomas, and negative WIF1 expression is significantly associated with high malignancy and metastasis of lung cancers [31]. However, WIF1 expression is not associated with survival [31]. Third, tissue inhibitor of metalloproteinases (TIMPs). TIMPs are the most important inhibitors of metalloproteinase function, and TIMP3 is commonly downregulated in most cancer types [32]. TIMP3 expression is also low in lung cancer and has a negative correlation with cancer stage and prognosis [33]. Yun *et al.* found that interleukin-32 gamma suppressed lung tumor development by upregulating TIMP3 [34]. Fourth, caveolins (CAVs), including CAV1, CAV2 and CAV3, are a family of proteins known to regulate cholesterol distribution, signal transduction, cell migration and endocytic vesicular trafficking [35]. For female lung cancer patients who never smoked, low expression of CAV1 was associated with a worse overall survival [36]. And the overexpression of CAV1 inhibited lung adenocarcinoma cell proliferation [37]. Finally, NUF2. NUF2 was found in most of the GO terms. Many studies have clarified the relationship between NUF2 and cancer initiation or development. In an association study, Xu *et al.* found that NUF2 as a prognostic marker in breast cancer [38]. The relationship between NUF2 and cancer was also demonstrated by Wang *et al.* NUF2 is a valuable prognostic biomarker to predict early recurrence of hepatocellular carcinoma after surgical resection [39]. However, the association between NUF2 and LCNEC metastasis remains unclear, and further studies are still needed.

Of the ten top upregulated miRNAs, several have been demonstrated to be onco-miRNAs in lung cancer. MiR-1290 and miR-1246 promote cell stemness and invasiveness of non-small cell lung cancer (NSCLC) [40]. MiR-301b-3p contributes to cell proliferation, invasion, drug resistance of NSCLC through repressing transforming growth factor-beta receptor II (TGFB2) [41]. MiR-183-5p and miR-18a-5p induce NSCLC progression by targeting PTEN and interferon regulatory 2 (IRF2), respectively [42, 43]. The suppression of miR-96-5p inhibited epithelial-mesenchymal transition (EMT) and metastasis of NSCLC [44]. The downregulated miRNAs identified in this study also function as tumor suppressors in lung cancer. MiR-144-5p increased the radiosensitivity of NSCLC cells by repressing activating transcription factor 2 (ATF2) [45] and miR-451a decreased doxorubicin resistance in lung cancer via suppressing c-Myc [46]. MiR-144-3p and miR-30a-3p inhibited the progression of lung cancer via targeting EZH2 and DNA methyltransferase 3a [47, 48]. Long noncoding RNA RMRP sponged miR-1-3p to promote NSCLC cell proliferation and invasion [49]. Moreover, miR-126-5p, miR-486-5p, miR-338-3p, miR-126-3p and miR-145-5p have all been shown to block the progression of lung cancer [50–53].

The pathway enrichment analysis revealed that the altered signaling pathways in LCNEC mainly focused on focal adhesion, cytokine-receptor interaction, hematopoietic cell lineage, and TGF- β signaling pathway, while the biological process mainly included response to wounding, inflammatory response, cell adhesion, biological adhesion, and vasculature development. Focal adhesion is a process of cellular attachment by linking the actin cytoskeleton to components of the extracellular matrix via integrins, and Focal adhesion kinase (FAK) plays a pivotal role in focal adhesion regulation [54]. FAK promotes lung cancer progression and drug resistance [55, 56], and is essential for the formation of an aggressive phenotype of NSCLC with mutant *KRAS* [57]. TGF- β signaling pathway regulates cell apoptosis, motility, invasion, extracellular matrix production, angiogenesis and immune function. In lung adenocarcinoma cells, TGF- β signaling pathway mediates EMT process mostly via the Smad pathways [58]. However, the activation of TGF- β signaling pathway is suppressed in NSCLC cells [59]. Recently, Li *et al.* analyzed eight studies involving 579 patients with lung cancer and concluded that high TGF- β expression as an indicator of poor survival [60].

We identified TF-miRNA-mRNA regulation loops, such as module ETS1-miR195-CD36, TAOK1-miR7-1-3P-GRIA1, E2F3-miR195-CD36, and TEAD1-miR30A-CTHRC1, which might play important roles in LCNEC. And 12 hub mRNAs were selected out, including TAOK1, STMN1, MAPK7, DUSP6, FZD3, BIRC5, F3, PDE4D, RGL1, CD36, GRIA1 and IL6R. ETS1 overexpression in breast cancers is associated with invasive features and predicts poor prognosis [61]. ETS1 also promotes NSCLC cell migration and invasion [62]. MAPK7, also known as ERK5. Jiang *et al.* showed that ERK5 was activated during lung cancer development, and ectopic expression of ERK5 promoted cell proliferation and G2/M cell cycle transition. Besides, they found that ERK5 is a potential regulator of radiosensitivity [63]. Moncho-Amor *et al.* showed that DUSP6 plays a major role in the regulation of cell migration, motility and tumor growth in NSCLC cells [64].

Our study has some limitations. First, the number of samples is relatively small, which may cause some bias. Second, the verification experiments are lacking, and wet laboratory experiments are warranted to confirm these newly found targets.

Conclusions

Overall, bioinformatics analysis of mRNA and miRNA expression profiles identified some TF-miRNA-mRNA regulation loops that may play important roles in LCNEC metastasis. These results might be helpful in understanding the genetic mechanisms of LCNEC and might suggest potential targets in the clinical treatment.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval and consent details

Not applicable

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

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Authors' contribution statement

Cunliang Cai, Mingqiang Zhang and Xiangdong Mu coordinated, designed, drafted and edited the manuscript.

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Figures

Figure 1

A flowchart of the study design

A Diff miRNA((Normal VS Tumor)

B

Diff Genes Heatmap(|FoldChange|>2,FDR<0.0001)

Figure 2

Expression profiles of distinct RNAs. (A) Heat map and volcano map show differential expression of miRNA in normal and cancerous tissues. (B) Heat map and volcano map show differential expression of mRNA in normal and cancerous tissues.

Figure 3

Gene Ontology (GO) enrichment annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of pathological progression of LCNEC. The top terms were synapse (GO:0045202), cytoskeleton (GO:0005856), postsynaptic density (GO:0014069), cell-cell adherens junction (GO:0005913), dendrite (GO:0030425), axon (GO:0030424), and neuron projection (GO:0043005). (A-B) GO terms. (C-D) KEGG terms. Significantly enriched pathways featured $p < 0.05$. The analysis was conducted using DAVID (<https://david.ncifcrf.gov/summary.jsp>) database.

Figure 4

Construction of TF regulatory network (A) TF-miRNA networks were constructed for LCNEC based on TF, TF regulated miRNAs and their inside regulations. The butterfly represents the miRNA, the hexagon represent the transcription factors, and the lines represent the regulatory relationship of the transcription factors to the miRNA. Red nodes indicated TFs/miRNAs that were continuously up-regulated and green nodes indicated TFs/miRNAs that were continuously down-regulated.. (B) miRNA-mRNA mediated networks were constructed for LCNEC based on DEMs, DEMs target genes and their inside regulations. The circles indicate miRNA, the butterflies indicate mRNA, and the lines indicate the regulatory relationship of miRNA to mRNA. Red nodes indicated miRNAs/genes that were continuously up-regulated and green nodes indicated miRNAs/genes that were continuously down-regulated. In miRNA-mRNA networks, the miRNA represses gene expression. (C) TF-miRNA-mRNA regulatory networks were constructed for LCNEC based on TF-miRNA networks, TF-mRNA networks, miRNA-mRNA networks and their inside interaction relationship. The circles indicate the metabolic pathways involved in the regulation of genes, and the arrows indicate the relationship of transcription factors or miRNA-regulated genes. Butterfly transcription factor arrows point to rectangular mRNA, indicating that TF regulates this gene expression. Butterfly transcription factor arrows point to rectangular miRNAs, indicating that transcription factors regulate these miRNAs expression. The hexagonal miRNA arrow points to a rectangular mRNA or butterfly transcription factor, indicating that miRNA regulates the corresponding target genes or TFs. Red nodes indicated miRNAs/genes that were continuously up-regulated and green nodes indicated miRNAs/genes that were continuously down-regulated., the same node color indicates their positive regulation, and the different node color indicates their negative regulation.