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Integrated Analysis of Non-Small Cell Lung Cancer Through Competing Endogenous RNA Network and Its Implications in Immune Evasion Regulation

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Abstract

Lung cancer is a complex disease integrated with diverse histological and molecular types that have clinical relevance. Non-small cell lung cancer (NSCLC), comprised of adenocarcinoma (LUAD), squamous cell carcinoma (LUSC), and large cell carcinoma (LCC), accounts for the majority of lung cancers. This study harnesses the recent surge in biomarker identification, exploring the promising potential of the competing endogenous RNA (ceRNA) network in deciphering the intricate interplay among lncRNA-miRNA-mRNA components, providing insights into the tumorigenesis of NSCLC. Datasets encompassing differentially expressed mRNA, miRNAs, and lncRNAs were curated from the Gene Expression Omnibus (GEO), Differentially Expressed miRNAs in human Cancers (dbDEMC), and The Cancer Genome Atlas (TCGA) databases. miRNAs were selected with respect to their established roles in immune regulation, pathways, and the tumor microenvironment (TME), and an integrative analysis was performed, based on which a comprehensive ceRNA regulatory network was constructed using Cytoscape software with 26 lncRNAs, 13 miRNAs, and 143 target genes. Gene Ontology (GO)-based functional enrichment analysis focusing specifically on immune system processes uncovered genes in pathways associated with immune cell differentiation, cytokine signaling, the immune response to cancer, and so on, positioning them as potential key biomarkers within the tumor microenvironment. Furthermore, a novel lncRNA, ALAL1, supported by a lone study, was known to bind with the SART3 gene in NSCLC. Simultaneously, another study also established a connection between the SART3 gene and miR-34a. Leveraging these individual interactions, this study revealed a previously unexplored link between ALAL1, SART3, and miR-34a. This plausible ceRNA interaction is suggested to potentially exert a regulatory influence on the progression of NSCLC cells. These computationally inferred interactions, though promising, necessitate rigorous clinical validation. However, the identified biomarkers lay the groundwork for subsequent research, offering valuable insights that can guide future investigations in the realm of NSCLC.

Keywords: NSCLC, ceRNA Network, ALAL-1, Biomarkers, Network Biology

Introduction

Non-small cell lung cancer (NSCLC) presents a formidable challenge due to the diverse patient groups with varying smoking histories. There are only a few cases of NSCLC associated with genetic aberrations in non-smokers.Understanding these profiles opens up new avenues for personalized treatment strategies and for improving diagnostics in non-smoker patientswith NSCLC (1-2).

Investigations into the interplay between NSCLC and the immune system have revealed significant impacts on clinical outcomes. Qinet al. detail on mechanisms like MHC-I (major histocompatibility complex Class I) alterations, tumor-infiltrating cytotoxic T cells (CTLs) affected by Treg (regulatory T cells) recruitment, and the TCR (T cell receptor) complex altered by tumor-

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associated myeloid cells, suppressing antigen-specific T cell responses.These mechanisms contributed to hindering immune recognition and creating an immunosuppressive environment in the tumor (3).Recent studies have identified that, active immune escape mechanisms exist even in pre-invasive lesions progressing to NSCLC histotypes, including reduced cytolytic CD8+ T cells and Natural Killer (NK) cells, increased PD-1expressing CD8+ T cells, andHLA loss of heterozygosity (LOH) associated with increased PD-L1 expression. These mechanismspotentially influence the progression to LUAD and LUSC. Furthermore, intra-tumor immunological heterogeneity, which ischaracterized by complex immune landscapes, is found to be increased in aggressive stages (4).

The intricacies of the RNA world surpass initial molecular biology assumptions, challenge conventional beliefs, and reveal its role in specific regulatory processes with functional significance (5). Key RNA molecules, including long non-coding RNA (lncRNA), microRNA (miRNA), and messenger RNA (mRNA), emerge as crucial players in gene expression regulation, and aberrations in these RNA molecules are implicated in various cancer-related pathways (6). An intriguing facet lies in the ability of lncRNAsto act as miRNA sponges. By competitively binding to miRNAs, lncRNAs can regulate intracellular miRNA activity, which in turn influences the respective mRNA expression (7). This dynamic interaction extends to complex competing endogenous RNA (ceRNA) mechanisms, facilitating cross-talk among diverse RNA types, including lncRNA, miRNA, mRNA, and others. By analyzingceRNA networks, researchers are exploring the potential to identify novel biomarkers for early cancer detection and intervention, including NSCLC.

Dysregulation of these networks contributes to disease onset and impacts various biological mechanisms associated with tumorigenesis, with the expression profiles of ceRNA elements varying across diverse cancer subtypes (8-9). For example, in breast cancer (BC), MALAT1 acts as an oncogene through the miR-561-3p/TOP2A axis, leading to increased proliferation and malignancy (10). Conversely, in glioma, MALAT1 functions as a tumor suppressorby downregulating mir-155, which in turn inhibits FBXW7, reducing tumorigenicity (11). In NSCLC, MALAT1 plays a dual role as a predictive marker for metastasis and a potential therapeutic target(12).

Dysregulated ceRNA networks in NSCLC have been linked to promoting cell proliferation, survival, invasion, and metastasis. For instance, upregulated lncRNAs like ADAMTS9-AS2 and H19 disrupt the miRNA-mediated pathways, driving cell proliferation and survival (13). They are also implicated in driving metastasis via the axes like lncRNA LEF1-AS1/miR-489/SOX4 pathway (14) and inducing therapy resistance through anti-apoptotic gene expressionand signaling pathway alterations such as the NF-kB pathways, exemplified by lncRNA ATP2B1/miR-222-5p/TAB2 and lncRNA HUWE1/miR-222-5p/TAB1 (15). The seceRNA components, involving lncRNAs, miRNAs and mRNAs, couldbe a promising avenue for discovering biomarkers, facilitating the early diagnosis of lung cancer. Moreover, studies suggest the need for effective strategies aimed at overcoming the challenges related to drug resistance in malignant diseases.The lncRNA and miRNA interactions, acting as ceRNA elements, could emerge as one of these strategiesby understanding the mechanisms that drives therapy resistance in these diseases, thereby, improving treatment, including for NSCLC (16-17)

This study investigates the intricate interplay of lncRNA-miRNA-mRNA components in NSCLC tumorigenesis. By identifying potential biomarkers and analyzing the ceRNA network, we aim togain valuable clinical insights. Specifically, the miRNAswere selected for their significant impact on immune regulation and, generally,

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the tumor microenvironment (TME). An integrative analysis was then undertaken, serving as the foundation for the construction of a comprehensive competing endogenous RNA (ceRNA) regulatory network. Furthermore, gene ontology (GO) analysis specifically focused on immune system processes revealed a prominent enrichment of genes associated with the complex TMEand its impact on various immune responses in NSCLC.

Materials and Methods

Data Screening for mRNA, miRNA, and lncRNA Expression Profile

In this study, the data for mRNA expression profiles were acquired from the Gene Expression Omnibus (GEO) database, a public repository of gene expression data (http://www.ncbi.nlm.nih.gov/geo/). The chosen dataset was GSE19804, undertaken on the GPL570 platform; Affymetrix Human Genome U133 Plus 2.0 Array. It focused on female, non-smoker NSCLC patients from Taiwan. The miRNA expression profiles necessary for understanding potential mRNA interactions were obtained from thedatabase of Differentially Expressed MiRNAs in human Cancers (dbDEMC); (https://www.biosino.org/dbDEMC/index). The search involved the retrieval of experiment ID EXP00710, comprising a sample case of NSCLC patients, analyzed using a microarray platform. For lncRNA expression profile data, The Cancer Genome Atlas (TCGA) database, a comprehensive resource providing genomic and clinical data from cancer patients; (https://portal.gdc.cancer.gov/), was used. The retrieval process involved parameters related to sample characteristics and experimental methods. A flowchart of this study was drawn to explain the analytical process (Figure 1).

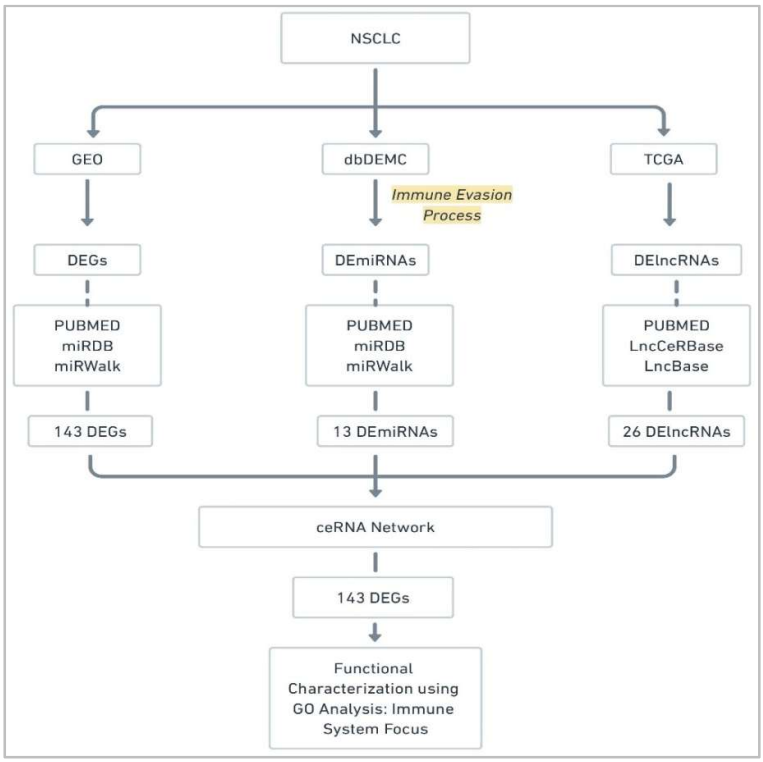

Figure 1: Overall Workflow of the study

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Differential Expression Analysis

To identify the differentially expressed genes (DEGs) between tumor samples and normal samples (normal: 60, tumor: 60), we employed the GEO2R tool, a specialized analysis tool integrated within the GEO database, designed for the comparison of gene expression profiles in different experimental conditions (https://www.ncbi.nlm.nih.gov/geo/geo2r/). The samples were appropriately grouped and subjected to GEO2R, utilizing the Benjamini Hochberg method, also known as the False Discovery Rate (FDR) method, for p-value correction. It is a statistical technique used to control the rate of false positives in multiple hypothesis testing, with a significance cut-off of 0.05.

Literature Mining for DEmiRNAs and DElncRNAs

We conducted a thorough literature study to identify differentially expressed miRNAs (DEmiRNAs) and differentially expressed lncRNAs (DElncRNAs), that aligned with our research objectives. The original DEmiRNAs dataset was refined, and those with established roles in immune regulation and the tumor microenvironment were prioritized. For DElncRNAs, the analysis targeted candidates with potential binding affinity to the previously identified miRNAs, specifically those crucial to NSCLC and, more broadly, lung cancer.

Construction of ceRNA-Interaction Network

To identify key components for the ceRNA network, we employed a combined approach involving database mining and literature studies. For miRNA-mRNA interactions, we conducted a predictive analysis using the databases miRWalk v2.0 (http://mirwalk.umm.uni-heidelberg.de/) and miRDB v6.0 (https://mirdb.org/). For lncRNA-miRNA interactions, screening was conducted using LncCeRBase v1.0 (http://www.insect-genome.com/LncCeRBase/front/) and the DIANA-LncBase v3.0 database (https://diana.e-ce.uth.gr/lncbasev3). Analogous interactions in other cancer types were also extrapolated in this study, focusing on their specific interactions.

An in-depth analysis of interactions among the selected lncRNA-miRNA-mRNA components was conducted, and the final ceRNA network was then constructed and visualized using the Cytoscape v3.10.0 software (https://cytoscape.org). Cytoscape is an open-source software platform designed for visualizing and analyzing complex networks, including biological networks. While Cytoscape offers a range of network layout algorithms, we opted for a simpler approach that aligned with our study objectives. This involved directly importing the curated interactions between lncRNAs, miRNAs, and mRNAs from the selected databases mentioned above. This decision reflects our focus on maintaining an integrity of the curated interactions while ensuring clear visualization of the network.

Gene Ontology-Based Functional Enrichment Analysis

Gene Ontology (GO) is a knowledge base that offers a standardized framework for characterizing gene functions across various species. Our study aimed to explore genes associated with immune-related processes in NSCLC. To accomplish this, we utilized the ClueGO v2.5.10 plug-in,a functional enrichment analysis tool within the Cytoscape software (https://apps.cytoscape.org/apps/cluego), for a targeted GO analysis focused on immune system processes. In particular, the analysis incorporated the Bonferroni step-down approach; a statistical method that adjusts significance thresholds for multiple hypothesis testing to reduce the risk of false positives, for p-value correction, and a two-sided hypergeometric test to enhance the statistical robustness of GO term enrichment.

Results and Discussion

DifferentialExpression Analysis of Genes in NSCLC

The gene expression data retrieved from the GEO database were analyzed using the GEO2R tool, and the resulting DEGs across 120 samples, divided into normal and tumor groups (60 samples each), were obtained (Figure 2). Subsequently, this initial

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list of DEGs was refined based on our research focus, forming the basis for further investigation.

Literature Mining

From the intensive literature analysis, we identified significant DEmiRNAs with a potential impact on immune regulation within the tumor microenvironment, specifically targeting those associated with the immune evasion process. This comprehensive study led to the selection of 13 miRNAs, recognized for their involvement in immunosuppression (18), immune cell development (19), interplay in cancer-immune talk (20), and other immunomodulatory roles in NSCLC. Simultaneously, among the lncRNAs collected from the TCGA database, we selected the DElncRNAs that showed significant binding affinity with the 13 chosen DEmiRNAs, as identified through literature mining.

Establishment of the ceRNA-Interaction Network

The screening process uncovered key components essential for constructing the ceRNA network. Based on the 13 miRNAs selected, predictive analysis employing databases and literature studies was conducted. The implicated interactions were primarily associated with NSCLC. Previous investigations have delved into these molecular relationships in NSCLC and also in diverse cancer types. In this study, therefore, interactions from other cancer types, including hepatocellular carcinoma, were incorporated, considering the shared targets.

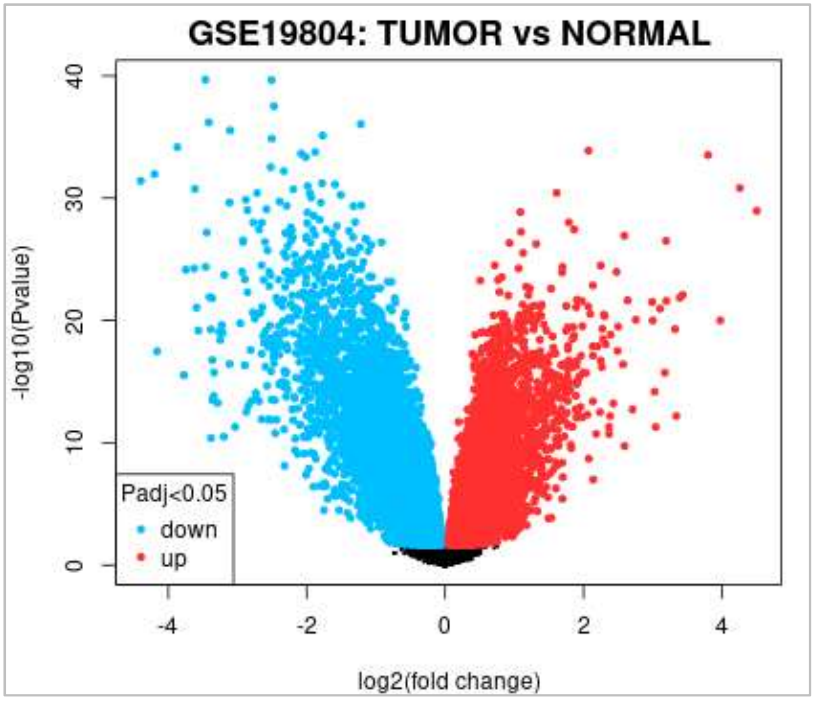

Figure 2: Volcano Plot of Differentially Expressed mRNAs; blue dots represent genes that are significantly downregulated, while red dots indicate genes that are significantly upregulated. The x-axis displays the log2 fold change, and the y-axis displays the negative logarithm of the p-value. Gene above the horizontal line are statistically significant ($p<0.05$).

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Through the identification of potential relationships between miRNA/lncRNA and miRNA/mRNA, we constructed a ceRNA network encompassing 26 lncRNAs, 13 miRNAs, and 143 mRNAs. This network was then visualized using Cytoscape v3.10.0 software. This analytical framework integrated the interacting components, providing an enriched perspective on the regulatory landscape within the established ceRNA network (Figure 3).

Within the network of constructed ceRNA interactions, miR-34a has prominently assumed the role of a central hub miRNA; this centrality is attributed to the extensive relationship it establishes within the network. miR-34a is primarily recognized as a tumor suppressor in NSCLC (21). SIRT1 (Sirtuin 1) has been identified as a proposed target for miR-34a in NSCLC. The study conducted by Yamakuchi et al. explores the intricate interaction between miR-34a and SIRT1. The study has identified that miR-34a is known to inhibit SIRT1, thereby augmenting p53 activity and counteracting SIRT1's conventional role in deacetylating p53 to impede apoptosis (22). Lin et al., in alignment with this perspective, suggested that, in NSCLC, SIRT1 serves as a viable target for miR-34a to induce apoptosis by reducing SIRT1 expression (23). Further exploring the targets of miR-34a revealed key genes, namely FOXP3 (Forkhead Box P3), PRF1 (Perforin 1), and WNT1 (WNT Family Member 1), as expounded by Forough et al. (24). In accordance with findings by Peng et

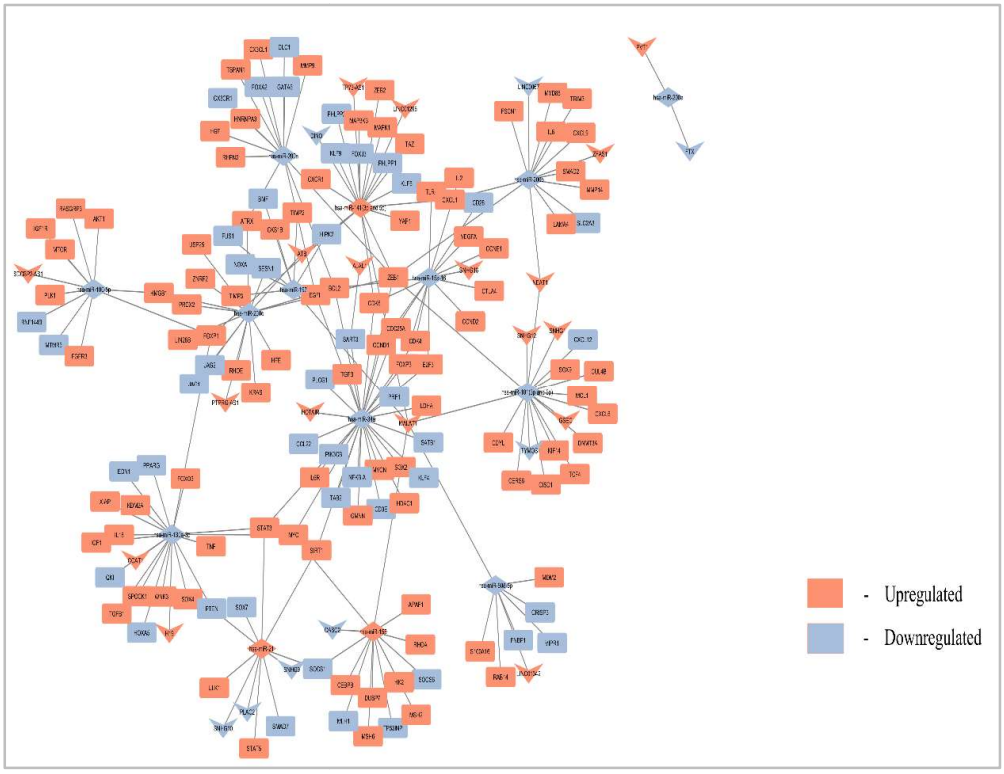

Figure 3: The ceRNA-Interaction Network in Cytoscape; shapes represent the RNA types (V-shape for lncRNAs, diamond for miRNAs, and round rectangle for mRNAs), and colors indicate the regulation type (red color represents upregulated, and blue color represents downregulated).

Non-Small Cell Lung Cancer

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al., it was found that the aberrant expression of FOXP3, a regulator of T-cells (Tregs), was identified in lung cancer cells. The interaction between miR-34a and FOXP3 elucidates a downregulatory effect, mitigating the overexpression of FOXP3. This interaction was implicated with relevance to the pathogenesis of rheumatoid arthritis (25). PRF1, involved in T-cell cytotoxicity, may be subject to downregulation by miR-34a. Altered expression of PRF1 may indeed impact T-cell cytotoxic activity, potentially deviating from its presumed anti-tumor immune function. This dynamic suggests a nuanced role for miR-34a in influencing cancer cell viability. Additionally, WNT1, a constituent of the WNT signaling pathway, underscores an oncogenic potential in NSCLC. WNT1 overexpression culminates in the hyperactivation of the WNT1 pathway, contributing to a poor prognosis in NSCLC patients (26). Given that WNT1 is a validated target of miR-34a, the latter's regulatory influence may reduce the expression of WNT1, presenting a prospect for a more favorable outlook in NSCLC patients.

In the context of the ceRNA network, the lncRNA is implicated as having an inhibitory influence on miRNA, directing a reversal in function that leads to the restoration of the initial activity of the target genes. HOTAIR was implicated in lung cancer cells, known to regulate processes such as initiation, proliferation, and invasion(27). miR-34a emerges as a feasible target for the lncRNA HOTAIR identified through the database search. Consequently, it can be inferred that when HOTAIR targets miR-34a, it causes reactivation of the target genes to fulfill their primary functions. This phenomenon, however, assumes a deleterious role in anti-tumor activity, as lncRNA HOTAIR promotes the tumorigenic property by indirectly activating the oncogenic genes through the modulation of miR-34a. This insight is supported by a study conducted by Fang et al. (28).

Most importantly, our study has revealed a novel mechanistic interplay involving the lncRNA ALAL1 (Amplified lncRNA associated with lung cancer 1), the SART3 (Squamous cell carcinoma antigen recognized by T-cells 3) gene, and miR-34a. ALAL1, ascertained in lung cancer cells through a singular study (29), emerges as a frequently amplified oncogenic lncRNA, transcriptionally activated by nuclear factor-kappa B (NF-kB). Primarily functioning within the cytoplasm, ALAL1 exerts its influence on SART3, which further regulates the subcellular localization of the USP4(Ubiquitin-Specific Protease 4) gene. Specifically, within NSCLC cells, overexpressed ALAL1 demonstrates an inverse correlation with immune infiltration, implying a significant role in immune evasion. Concurrently, an additional study highlights the roles of SART3 and miR-34a in NSCLC. SART3 is particularly associated with squamous cell carcinoma, and its overexpression is associated with elevated miR-34a levels, resulting in the downregulation of its target genes, CDK4 and CDK6, triggering a regulatory response to prevent uncontrolled proliferation and imparting a tumor suppressive effect in NSCLC cells(30). Our study proposes a novel hypothesis, stating that ALAL1 as a ceRNA component may indirectly modulate the function of miR-34aby targeting the SART3 gene. This mechanism implies a potential impact on inhibiting cell cycle arrest, exacerbating uncontrolled proliferation, and facilitating tumor growth in NSCLC cells. Beyond this specific interaction, ALAL1's broader role in NSCLC may be critical. Its ability to function as a ceRNA component suggests that it could be a key player in regulating other NSCLC-associated pathways. These compelling insights, through assimilation of existing literature, have been highlighted with plausible interactions between the molecules following the perceptive identification of common targets and mechanisms; however, the absence of experimental validation in this study emphasizes the need to advance this theoretical paradigm.

miR-155 emerges as another key miRNA in NSCLC cells, known for its oncogenic role. It exerts its influence by downregulating tumor suppressor genes like SOCS1 (Suppressor of Cytokine Signaling

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1), activating the NF-kB pathway, and cytokine production (31). Its inverse correlation with TGF-β expression influences responses to immune checkpoint inhibitors (32). SOCS1 in NSCLC is known for inhibiting the FAK-dependent signaling pathway (33). Additionally, CASC2 is also associated with tumor growth and poor survival in NSCLC (34). Yuan et al. have elucidated the CASC2/miR-155/SOCS1 axis in hepatocellular carcinoma (HCC) development, emphasizing CASC2's role as a tumor suppressor, impeding cell proliferation and migration by binding to miR-155 (35). This leads to the upregulation of SOCS1 expression, the target gene of miR-155, highlighting the ceRNA function of CASC2. This shared mechanism in HCC suggests that while CASC2 may function as a tumor suppressor in HCC, its oncogenic role in NSCLC could reflect different regulatory dynamics. However, this interaction in NSCLC requires clinical validation to confirm its role.

The ceRNA networks in NSCLC are, thus, a key to identifying treatment targets and advancing innovative therapies. In-depth research on these non-coding elements within ceRNA networks provides a critical understanding of the molecular mechanisms, especially those related to immune escape, in NSCLC.

These ceRNA networks are implicated in substantially influencing the immune escape mechanisms in NSCLC through various strategies. For instance, immune checkpoint molecules such as PD-L1 and CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) affect the ability of tumor cells to evade immune surveillance (36). They are also known to modulate immune cell infiltration, mainly affecting the function of cytotoxic T cells, regulatory T cells, and myeloid-derived suppressor cells within the tumor microenvironment (37).

Moreover, these ceRNA networks could regulate cytokine and chemokine production, influencing immune cell activation and migration (38). They could also activate immune suppressive pathways such as TGF-β, which inhibits immune cell function, leading to tumor growth and invasiveness (39). Furthermore, ceRNA networks contribute to immune cell exhaustion by activating immune suppressive pathways and contributing to functional impairment, leading to a reduced immune response against tumors (40).

The findings from this study also holdsignificant potential for advancing personalized treatment strategies in NSCLC. Significant biomarkers within the ceRNA network can be identifiedby gaining insights into the molecular mechanisms driving NSCLC progression. These biomarkers could serve as indicators for disease prognosis and treatment response, which could lead to better patient outcomes. Understanding the dysregulated ceRNA networks in NSCLC is another factor that is essential for developing personalized therapies, addressing individual patient profiles, and thereby improving treatment efficiency while minimizing adverse effects. Additionally, drug resistance remains a significant challenge in NSCLC treatment. By delving into the mechanisms underlying resistance pathways, novel therapeutic strategies implicated in overcoming drug resistance could be developed. Our study also provided insights into the impact of interactions between the ceRNA components on immune-related pathways within the TME. This could shed light on immunomodulatory therapies aimed at enhancing anti-tumor immune responses in NSCLC patients.By targeting some of these specific components involved in immune evasion mechanisms, such as the miRNAs highlighted in our study, known to regulate T cell function and tumor infiltrating lymphocytes, and so on, personalized treatment approaches could be implemented to promote the activity of the patient's immune system against tumor cells. The ceRNA regulatory network within our study could also provide insights into the heterogeneity of NSCLC at the molecular level. By characterizing the molecular profiles of individual tumors based on their ceRNA

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signatures, the distinct molecular subtypes of patients could be classified, allowing for a more precise treatment approach.

Therefore, our study highlights the potential of ceRNA networks in NSCLC for personalized treatment strategies overall, suggesting the importance of integrating molecular insights into clinical settings to improve NSCLC management (41-45).

GO-BasedFunctional Enrichment Analysis

To gain insights into the immune system's role in NSCLC, we performed a GO analysis restricted to immune system processes within the network of DEGs. The results revealed a significant enrichment of terms related to immune cell differentiation and activation, cytokine signaling, and the immune response to cancer (Figure 4). This suggests the ceRNA network may influence various aspects of the immune response in NSCLC, potentially impacting anti-tumor immunity and immune evasion mechanisms.

The focused exploration into the genes associated with these immune-related processes was utilized for further investigation (Table 1), incorporating insights from another study on Lung Squamous Cell Carcinoma (LUSC). Through an intensive literature study, we established the interconnection of identified pathways with immune cell populations enriched in LUSC, providing a comprehensive understanding of the disease landscape.

Integration of the Immune-Infiltrating Cells and GO Enriched Terms to reveal the interconnecting pathways in LUSC and NSCLC

Lung squamous cell carcinoma (LUSC) stands as a significant subtype within NSCLC, presenting a formidable challenge in cancer research that demands a profound comprehension of its underlying mechanisms. Recent advancements in immunogenic research

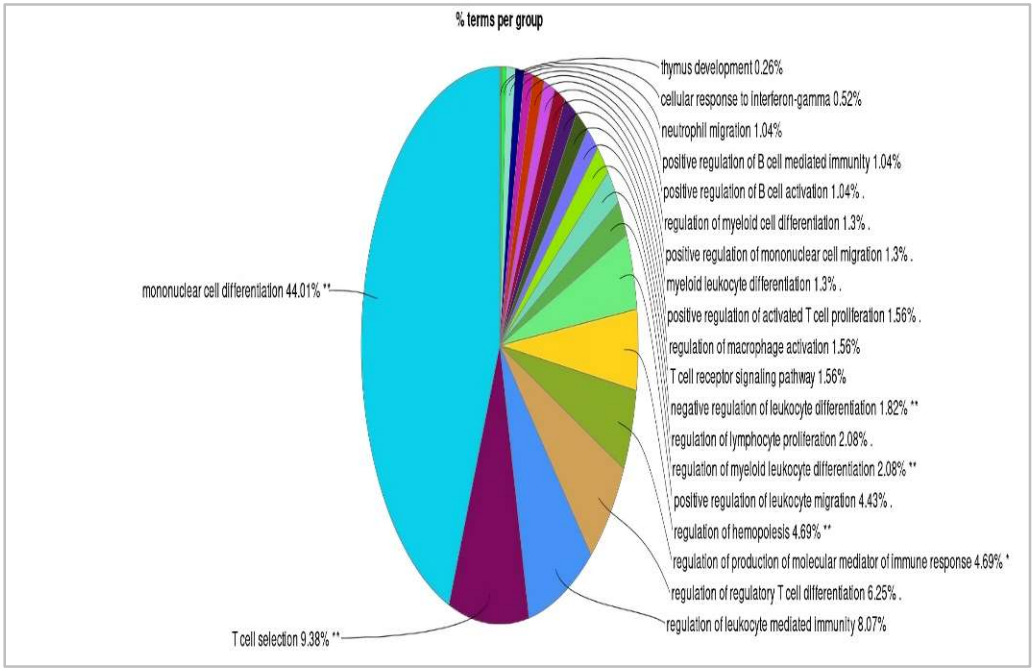

Figure 4: GO Functional Enrichment Analysis of 143 DEGs specifically targeting the immune system processes conducted using ClueGO; Visualization with pie chart. The chart shows the percentage of enriched GO terms within specific cluster identified through the analysis.

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Table 1: Enriched GO Terms and their Associated Genes

GO ID: Description	Associated Genes Found
GO:1903131 Mononuclear cell differentiation	BCL2, CD28, CD3E, CDK6, CEBPB, CTLA4, FOXO3, FOXP1, FOXP3, HMGB1, IL18, IL2, IL6, IL6R, JAG2, KLF6, MMP14, MSH2, MTOR, MYC, PPARG, PRDX2, RHOA, SMAD7, SOCS1, SOX4, STAT3, TGFB1, VEGFA, ZEB1
GO:0045058 T cell selection	BCL2, CD28, CD3E, FOXP3, IL6, IL6R, JAG2, MTOR, RHOA, STAT3
GO:0002703 Regulation of leukocyte mediated immunity	CD28, CX3CR1, CXCL6, FOXP3, HFE, HMGB1, IL18, IL2, IL6, MLH1, MSH2, SMAD7, TGFB1, TLR4, TNF
GO:0045589 Regulation of regulatory T cell differentiation	CD28, CTLA4, FOXO3, FOXP3, IL2, SOCS1, TGFB1
GO:0002700 Regulation of production of molecular mediator of immune response	CD28, FOXP3, HFE, IL18, IL2, IL6, MLH1, MSH2, MYD88, SIRT1, SMAD7, TGFB1, TLR4, TNF
GO:1903706 Regulation of hemopoiesis	CD28, CDK6, CEBPB, CTLA4, FOXO3, FOXP1, FOXP3, HMGB1, HOXA5, IL18, IL2, JAG1, MMP14, MTOR, MYC, NFKBIA, PRDX2, RHOA, SMAD7, SOCS1, SOX4, STAT3, TGFB1, TLR4, TNF, ZEB1
GO:0002687 Positive regulation of leukocyte migration	CX3CL1, CX3CR1, CXCL12, EDN1, HMGB1, IL6, IL6R, MAPK1, MMP14, RHOA, TGFB1, TNF, VEGFA
GO:0045637 Regulation of myeloid cell differentiation	CDK6, CEBPB, FOXO3, FOXP1, HOXA5, JAG1, MTOR, MYC, NFKBIA, STAT3, TGFB1, TLR4, TNF
GO:0050670 Regulation of lymphocyte proliferation	BCL2, CD28, CD3E, CEBPB, CTLA4, FOXP3, HMGB1, IGF1, IL18, IL2, IL6, MYD88, PTEN, TGFB1, TLR4
GO:1902106 Negative regulation of leukocyte differentiation	CDK6, CTLA4, FOXP3, HMGB1, IL2, MYC, PRDX2, SMAD7, SOCS1, TLR4

(*Contd.*)

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Table 1: Enriched GO Terms and their Associated Genes (*Contd.*)

GO ID: Description	Associated Genes Found
GO:0050852 T cell receptor signaling pathway	CD28, CD3E, CTLA4, FOXP3, MAPK1, PLCG1
GO:0043030 Regulation of macrophage activation	CX3CL1, IL6, TLR4
GO:0002573 Myeloid leukocyte differentiation	CDK6, CEBPB, FOXP1, MMP9, MTOR, MYC, PPARG, SIRT1, SOCS1, TGFB1, TLR4, TNF, VEGFA
GO:0071677 Positive regulation of mononuclear cell migration	CX3CR1, CXCL12, HMGB1, RHOA, TGFB1, TNF
GO:0045637 Regulation of myeloid cell differentiation	CDK6, CEBPB, FOXO3, FOXP1, HOXA5, JAG1, MTOR, MYC, NFKBIA, STAT3, TGFB1, TLR4, TNF
GO:0050871 Positive regulation of B cell activation	BCL2, CD28, IL2, IL6, MLH1, MMP14, MSH2, TGFB1, TLR4
GO:0002714 Positive regulation of B cell mediated immunity	CD28, IL2, MLH1, MSH2, TGFB1, TNF
GO:1990266 Neutrophil migration	CCL22, CX3CL1, CXCL1, CXCL6, CXCL9, CXCR1, EDN1, MYD88
GO:0071346 Cellular response to interferon-gamma	CCL22, CX3CL1, EDN1, PPARG, SOCS1, TLR4, TNF
GO:0048538 Thymus development	BCL2, MAPK1, PRDX2

have provided a deeper, more clarified perspective on the tumor microenvironment. Zhao et al. have identified the diverse immune cell populations within LUSC (46). Concurrently, our research delved into the molecular intricacies of NSCLC through gene ontology (GO) analysis. The analysis revealed an intricate network of interconnected pathways that establish links between the immune infiltrating cells and the identified GO pathways.

One such immune-infiltrating cell is the resting CD4 (cluster of differentiation 4) T cells associated with the TP53 mutation in LUSC. The explored key pathways were linked to cytokine secretion and their anti-tumor immune responses. This regulatory influence exerted by resting CD4 memory T

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cells is crucial for maintaining effective humoral immune responses. Another study by Blair et al. explored the nuanced engagement of CTLA-4 with specific monoclonal antibodies that had regulatory effects on resting CD4 T cells, causing inhibition of cell proliferation, cytokine production, and cell cycle progression while maintaining cell viability. The specific suppression of IL-2 (interleukin-2) production can modulate the early signaling activities in these cells (47).

Another cell type found was monocytes, known to intricately govern T cell dynamics. Charron et al. have identified the impact of CD28 stimulation on modulating the expression and function of CD46, potentially affecting the interaction between T cells and monocytes (48). Additionally, blocking monocyte migration via CCR2 (C-C motif chemokine receptor 2) inhibition has been found to have diverse impacts on T-cell responses. Specifically, this leads to a reduction in macrophage-based production of cytokines such as IL-6, TNF-α (tumor necrosis factor-alpha), and so on, highlighting the intricate interplay between monocytes and T cell activity (49). They maintain myeloid cell homeostasis and differentiation, regulating immune cell development within the tumor. STAT3 (signal transducer and activator of transcription 3) and TNFs were among the genes identified to significantly contribute to myeloid cell differentiation. A study revealed that TNF played a crucial role in modulating monocyte differentiation and promoting dendritic cell generation in an inflamed microenvironment (50). Moreover, STAT3 is known as a critical regulator that links oncogenic and myeloid-specific activities to dynamic changes in cellular metabolism. The altered metabolic shift caused by increased tumor growth resulting in increased glycolysis was found to impact monocyte differentiation into macrophages (51). Monocytes were also associated with chemotaxis, emphasizing the common chemokine receptors expressed by them, such as CXCR3 (C-X-C motif chemokine receptor 3) and others. These are associated with the modulation of inflammatory response (52).

Neutrophils constitute another distinct category of immune cells present in LUSC tissues, as mentioned in the study explaining the immune cell populations within LUSC. The presence of neutrophils in the advancedTcell stage indicates their crucial role in the immune microenvironment during the later stages of LUSC. Identified as the main sources of reactive oxygen species (ROS) and cytokines, neutrophils contribute to immunosuppression, impacting cytotoxic T-cells. Their significant association with the tumor microenvironment in smokers with NSCLC has also been investigated, revealing both pro- and anti-tumor roles. In NSCLC, tumor-associated neutrophils (TANs) are identified as the main source of MMP-9 (matrix metalloproteinase-9). Research has shown a positive correlation between increased expression levels of MMP-9 and the invasive characteristics of tumor cells. The enrichment analysis performed in our study showed MMP-9s involvement in muscle cell proliferation, which can reflect its role in tissue remodeling and repair. In the context of NSCLC, the dysregulation of MMP-9 can lead to the degradation of extracellular matrix (ECM) components that correlate with tumor invasion and metastasis. The study also revealed that elevated levels of TGF-β in NSCLC cells further drive tumor progression by stimulating epithelial-mesenchymal transition (EMT), angiogenesis, and metastasis.TGF-β also induces immune suppression, influencing dendritic cells (DCs) and promoting regulatory T cell (T-reg) expansion (53).

Resting mast cells, acting as sentinels, are found to be abundant in LUSC tissues. They are critical regulators of the immune response associated with poor overall survival (OS) and progression-free survival (PFS) in LUSC. It is known as a significant biomarker for predicting survival outcomes in LUSC patients (54). Resting mast cell infiltration is markedly suppressed in NSCLC, and its inhibition is correlated with a subgroup at higher risk. The role of mast

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cells in NSCLC in general is ambiguous, with studies presenting diverse findings and emphasizing the need to define their prognostic value as therapeutic targets (55). CX3CL-1 (C-X3-C motif chemokine ligand 1) is implicated in orchestrating immune responses, particularly influencing the role of mast cells. It plays a crucial role in coordinating the movement of mast cells to the sites of inflammation (56). Moreover,some vascular endothelial growth factor-positive (VEGF-positive) lungadenocarcinomas were observed to trigger the migration of mast cells within the tumor microenvironment (57).

M2 Macrophages were also found in higher proportions in LUSC and NSCLC tissues, associated with poor survival and contributing to tumor progression, angiogenesis, and immunosuppression in NSCLC patients (58). A study by Fei et al. abstracted the role of distinct functional phenotypes of these macrophages, namely M2a, M2b, M2c, and M2d, within lung cancer in general. Specifically, M2a macrophages secrete high levels of TGF-β and IGFs (insulin-like growth factors), contributing to tissue repair and healing. M2b macrophages produce anti-inflammatory molecules such as IL-6, TNF-α, and so on. M2c was identified as being involved in immunosuppression and tissue repair through the initiation of TGF-β and other molecules. Lastly, M2d macrophages that exhibit low expression of CD206 (mannose receptor C type 1) are activated by leukocyte inhibitory factors and TLR (toll-like receptor) ligands. They contributed to immunosuppression and angiogenesis by producing high amounts of TGF-β and VEGF (59). In the context of NSCLC, a finer classification of M2 macrophage subtypes is crucial for a more precise understanding of their distinct functions leading to NSCLC tumorigenesis.

This analysis elucidates the specific molecular mechanisms in NSCLC, which can further be correlated in the context of LUSC mechanisms. This is particularly pertinent, as the role of these immune-infiltrating cells in LUSC has been underexplored in existing studies. The integration of these findings therefore, contributes to discerning LUSC development and progression.

Conclusion

This work highlights the key regulatory elements and intricate mechanisms governing NSCLC through the ceRNA network, fostering potential for future advancements in navigating the complexities of this disease. The computationally inferred interactions, particularly those involving a newly discovered lncRNA, supported by a lone study, present avenues for experimental validation of their roles in tumor microenvironment. The literature search facilitated the prediction of potential interactions; however, the absence of direct algorithmic applications and the lack of experimental validation introduces a gap in the functional relevance of these associations and may limit the depth of our understanding of the underlying network dynamics. The interpretation of the identified pathways and interactions, therefore, remains contingent upon the available literature. To facilitate the experimental validation of the in-silico findings presented in this study, it is crucial to optimize the protocols for extracting the lncRNAs and miRNAs from various tissue and blood samples. Techniques such as quantitative PCR (qPCR) can be employed to measure the abundance of these specific components.However, these identified components could serve as potential biomarkers, laying the groundwork for subsequent research offering valuable insights that can guide future investigations in the NSCLC domain.

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