

INTEGRATED ANALYSIS OF NON-SMALL CELL LUNG CANCER THROUGH COMPETING ENDOGENOUS RNA NETWORK AND ITS IMPLICATIONS IN IMMUNE EVASION REGULATION

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science in Bioinformatics and Data Science

By

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
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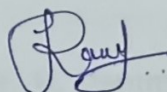
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DECLARATION

I, **RANJINI**, hereby declare that the Project Report entitled **Integrated Analysis of Non-Small Cell Lung Cancer through Competing Endogenous RNA Network and its Implications in Immune Evasion Regulation** done by me under the guidance of **Dr. H. JEMMY CHRISTY**, at **Sathyabama Institute of Science and Technology**, Jeppiaar Nagar, Rajiv Gandhi Road, Chennai-600119, Tamil Nadu, India, is submitted in partial fulfillment of the requirements for the award of **Bachelor of Science degree in Bioinformatics and Data Science**.

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Ranjini



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This is to certify that the dissertation entitled **"Integrated Analysis of Non-Small Cell Lung Cancer through Competing Endogenous RNA Network and its Implications in Immune Evasion Regulation"** was carried out by **Ms. RANJINI (Reg. No. 41738011)**, Department of Bioinformatics and Data Science, Sathyabama Institute of Science and Technology, Chennai in partial fulfillment of the requirements of the award of the **Degree of Bachelor of Science in Bioinformatics and Data Science 2021-2024**, is a bonafide record of the project work done under my guidance and supervision. This thesis or part, therefore, has not been submitted elsewhere for any other degree or diploma.

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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 (dbDEMOC), 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) and KEGG enrichment analysis revealed several crucial pathways, where our study focused specifically on the pathways associated with immune system processes, positioning the genes related to the pathways 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

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LIST OF ABBREVIATIONS

NSCLC	Non-Small Cell Lung Cancer
LUSC	Lung Squamous Cell Carcinoma
LCC	Large Cell Carcinoma
ceRNA Network	Competing Endogenous RNA Network
mRNA	Messenger RNA
miRNA	MicroRNA
lncRNA	Long non-coding RNA
ALAL1	Amplified lncRNA associated with lung cancer 1
SART3	Squamous cell carcinoma antigen recognized by T-cells 3
GEO	Gene Expression Omnibus
dbDEMC	database of Differentially Expressed MiRNAs in human Cancers
TCGA	The Cancer Genome Atlas
WNT1	WNT Family Member 1
NF-κB	Nuclear Factor-kappa B
SOCS1	Suppressor of Cytokine Signaling 1
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
TGF-β	Tumor Growth Factor-beta
PD-L1	Programmed death-ligand 1
CD4	Cluster of Differentiation 4
CCR2	C-C motif chemokine receptor 2
TNF-α	Tumor Necrosis factor-alpha
TANs	Tumor-Associated Neutrophils

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CHAPTER 1

INTRODUCTION

Lung cancer is one of the most widespread and lethal cancers worldwide. It is primarily situated in the lung parenchyma or within the bronchi (*Neeraja et al., 2023*). Lung cancer presents a significant challenge in the field of oncology, characterized by a complex interaction of genetic predispositions, environmental factors, and lifestyle habits (*Charles et al., 2011*). One of the primary reasons for the prominence of lung cancer as a leading health concern is its tendency to evade early detection, often remaining undiagnosed until reaching an advanced stage. This emphasizes the critical importance of timely diagnosis, particularly through targeted screening efforts aimed at high-risk populations, including individuals with a history of smoking, exposure to hazardous chemicals, and other toxic environments (*Reem et al., 2021*).

1.1 BACKGROUND

1.1.1 Epidemiology

- *Rate of Incidence in Lung Cancer*

- Global Incidence Rate of Lung Cancer

Lung cancer is a significant global health challenge, with approximately two million new cases diagnosed worldwide in 2020. It stands as the primary cancer diagnosis globally, impacting individuals of both genders. It was identified that there was a notable decline of incidence among men in specific continents, but in females, although, there has been a noticeable surge in diagnosis (*Chao et al., 2023*)

- Incidence Rate in India

In India, lung cancer was identified to be 5.9% of all cancer cases, ranking as the fourth most common cancer. The incidence of lung cancer is estimated at approximately 70,000 new cases reported each year, with a notable impact in both men and women (*Navneet et al., 2017*)

- *Mortality Rate in Lung Cancer*

- Global Mortality Due to Lung Cancer

Globally, lung cancer is the leading cause of cancer-related deaths for both men and women. In 2018, it was responsible for a significant portion of cancer mortalities, with men being more affected than women. The mortality rate was identified to be 1.8 worldwide. According to the lung cancer statistics retrieved from databases, it was identified that Hungary exhibited the highest death rate (*Hyuna et al., 2020*).

- Mortality Rate in India

The recent data highlighted India's substantial impact on lung cancer mortality, with an estimated number of approximately 18 lakhs deaths, adding significantly to the worldwide toll. A study revealed that in 2022, there was a notable rise in age-standardized mortality rates within the Indian population, specifically observing an increase in males compared to females (*Nath et al., 2022*).

- *Survival Rates and Trends*

According to studies, the 5-year survival rates for lung cancer showed significant improvement, with the increase identified in females whereas in males it was comparatively lesser. It was also identified that the survival rate for adenocarcinoma a subtype of non-small cell lung cancer was more than other subtypes, including small cell carcinoma (*Yan et al., 2024*).

1.1.2 Risk Factors

Lung cancer arises from a complex interplay of environmental and lifestyle factors. At the core of this complex disease is the exposure to various carcinogens, which initiate and drive the carcinogenic processes (**Fig 1.1**).

- Smoking: Smoking tobacco exposes the lungs to a myriad of carcinogens, including tar, nicotine, and various chemical compounds. These substances lead to the genetic mutations and abnormalities that can trigger the development of

lung cancer. Chronic exposure to cigarette smoke, especially over several years, significantly elevates the risk of developing this malignancy.

- **Second-hand Smoking:** Second-hand smoke contains many of the same harmful chemicals found in direct tobacco smoke. When non-smokers inhale these toxins, they can accumulate in the lungs and contribute to cellular damage and inflammation. Prolonged exposure to second-hand smoke, particularly in enclosed or poorly ventilated spaces, increases the risk of lung cancer in non-smokers.
- **Carcinogenic Substances:** Exposure to environmental carcinogens such as radon gas, heavy metals like arsenic, and industrial pollutants can directly damage lung tissue.
- **Genetic and Familial Factors:** Genetic factors can also influence an individual's susceptibility to lung cancer. Certain genetic mutations, such as those affecting tumor suppressor genes or DNA repair mechanisms, can impair the body's ability to prevent or repair DNA damage caused by carcinogens. Family history of lung cancer cases may also suggest a genetic predisposition to the disease, although environmental factors often interact with genetic susceptibility to determine overall risk.
- **Health Conditions:** Chronic lung infections, such as chronic obstructive pulmonary disease (COPD) or tuberculosis, can cause persistent inflammation and damage to lung tissue. This chronic inflammation creates an environment conducive to cancer development, as inflamed cells are more susceptible to genetic mutations and aberrant growth. Furthermore, the conditions affecting the immune system, such as the Human Immunodeficiency Virus (HIV) infection, could compromise its ability to detect and eliminate cancerous cells.

- **Lifestyle Choices:** Lifestyle factors like excessive alcohol consumption can also weaken the immune system, leading to impaired lung function. Chronic alcohol abuse could lead to oxidative stress and inflammation in the lungs, contributing to cellular damage and promoting cancerous changes. Additionally, dietary habits, characterized by a lack of antioxidant-rich foods and essential nutrients, may also contribute to an increased risk of lung cancer. Furthermore, sedentary lifestyles and obesity have been linked to systemic inflammation and metabolic dysfunction, which can indirectly impact lung health and cancer risk (Yaser et al., 2023).

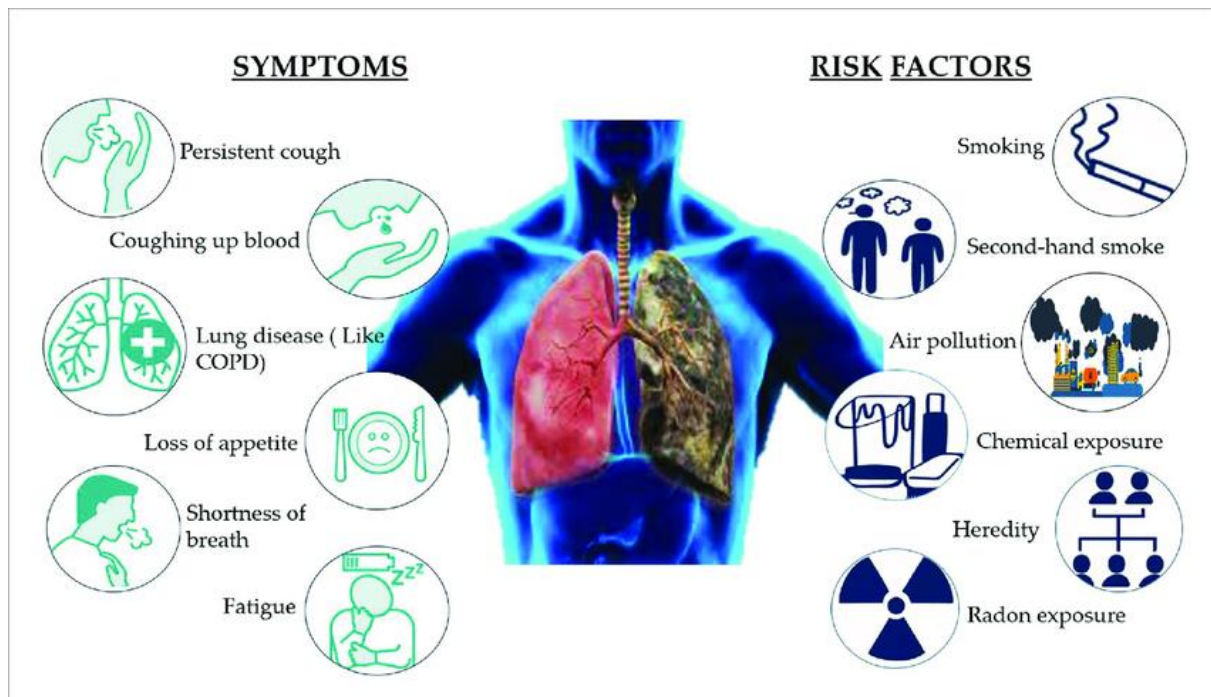


Fig 1.1 The common symptoms and risk factors associated with lung cancer (Anisha et al., 2021)

1.1.3 Pathophysiology

The pathophysiology of lung cancer involves intricate molecular alterations that contribute to the development and progression of the disease. These alterations are often associated with specific histological subtypes and genetic profiles, providing insights into targeted treatment approaches.

In lung adenocarcinoma, driver genetic alterations such as EGFR (Epidermal Growth Factor Receptor) mutations are common and associated with certain characteristics related to the population and medical history of patients. Other oncogenic drivers in adenocarcinoma, including ALK (Anaplastic Lymphoma Kinase), ROS1 (ROS Proto-Oncogene 1, Receptor Tyrosine Kinase), and so on, also plays a critical role and show histological patterns that can guide treatment approaches. It was identified that even microRNAs, small non-coding RNAs, contribute to the pathophysiology of lung adenocarcinoma by regulating certain gene expression. Different histological subtypes of adenocarcinoma were known to have unique miRNA expression profiles, reflecting their diverse molecular characteristics.

Advanced sequencing technologies, mostly employed by TCGA (The Cancer Genome Atlas) research network, provided molecular insights into lung cancer. Squamous cell carcinoma, known for its intricate genetic changes linked to heavy smoking, was identified to uncover significant mutations such as TP53 (Tumor Protein 53), PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), PTEN (Phosphatase and Tensin Homolog), and so on, with a greater number of mutations notably prevalent in TP53 gene.

1.2 CLASSIFICATION

Lung cancer is a combination of diverse malignancies originating from the epithelial cells lining the airways of the lungs. The identification of distinct lung cancer subtypes has led to the understanding of the unique biological characteristics of the disease, therapeutic responses personalized to individual patients.

- *Histological Classification*

The main classification of lung cancer includes Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC).

NSCLC comprises of adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. These subtypes exhibit distinct histological and molecular profiles influencing the treatment strategies. NSCLC is further categorized into Adenocarcinoma, known to originate in the glandular cell, significantly associated with EGFR (epidermal growth factor) and ALK (anaplastic lymphoma kinase) mutations, Squamous cell carcinoma, arising from the cells that compose the lining of the airways, and are mostly linked to smoking and mutations such as TP53 (tumor protein p53), and Large Cell Carcinoma, arising from the large, pluripotent cells within the lung tissue. Compared to other NSCLC subtypes, large cell carcinoma is observed less frequently.

Small Cell Lung Cancer (SCLC) is a rapidly growing, aggressive subtype, characterized by small, round tumor cells that proliferate rapidly. This lung cancer exhibits a strong correlation with smoking behavior.

- *Molecular Classification*

The molecular profile of NSCLC subtypes plays a crucial role in guiding treatment strategies. Adenocarcinoma, in particular, is significantly associated with specific genetic alterations, such as EGFR mutations and ALK rearrangements. EGFR mutations in adenocarcinoma patients have been shown to confer sensitivity to EGFR tyrosine kinase inhibitor (TKI) therapies. Similarly, the presence of ALK rearrangements in adenocarcinoma is advantageous for the use of ALK inhibitor-based treatments. Beyond these well-established biomarkers, including ROS1 (Reactive Oxygen Species 1), PD-L1 (Programmed Death-Ligand 1), are also being explored for their potential implications on treatment approaches in NSCLC (*Shane et al., 2023*).

- *Clinical Staging*

The staging of lung cancer is determined using the TNM classification system, which takes into account various factors, such as the size of the primary tumor (T), the extent of lymph node involvement (N), and the presence of metastatic disease (M). The stage of lung cancer, ranging from Stage 1 (localized disease) to Stage IV (advanced, metastatic disease), is a critical factor that influences the appropriate treatment approaches and the overall prognosis for the patient. (Mahul et al., 2017)

1.3 DIAGNOSTIC METHODS

Lung cancer diagnosis employs a range of techniques, including imaging tests like X-rays CT scans, and PET scans to visualize the lungs and identify suspicious areas. Additionally, sample collection methods, such as bronchoscopy, biopsy, and so on, are used to confirm the presence of cancerous cells. Molecular testing, including EGFR mutation analysis and PD-L1 expression assessment, provides valuable insights into genetic alterations and biomarker profiles guiding the treatment strategies.

1.4 TREATMENT APPROACHES

Lung cancer management follows a multidisciplinary strategy adapted to the individual patient's condition. Early-stage tumors may be treated with surgery to remove the cancerous tissue. Radiation therapy and Chemotherapy are some of the treatment modalities for lung cancer. Other approaches to treating the disease such as immunotherapies are also utilized, either individually or in combination, based on the stage, type of lung cancer, patient's overall health, and treatment objectives.

While this introduction has covered various aspects of lung cancer, the focus of this study is on non-small cell lung cancer (NSCLC). NSCLC comprises a significant portion of lung cancer cases and is distinguished by unique histological and molecular

characteristics that impacts diagnosis, prognosis, and treatment strategies. The subsequent sections delve into NSCLC in detail, exploring its different subtypes, molecular classifications, risk factors, and personalized treatment approaches to NSCLC.

1.5 OVERVIEW OF NON-SMALL CELL LUNG CANCER

NSCLC accounts for approximately 85 percent of all lung cancer, with substantial variations in incidence and mortality rates across different regions.

- In India, NSCLC stand as the fourth most prevalent cancer, with an estimated 70,000 new cases reported annually, highlighting its significant burden on country's public health.
- Concerning trends have emerged, with age-standardized mortality rates for NSCLC showing a concerning rise, particularly among the male population.

As discussed above, NSCLC encompasses several distinct histological subtypes, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. These subtypes exhibit unique histological characteristics and molecular profiles, which holds significance in the treatment strategy approaches. Adenocarcinoma, the most common subtype, is often associated with specific genetic alterations, such as EGFR mutations and ALK rearrangements. Squamous cell carcinoma, on the other hand, is strongly linked to heavy smoking and harbors distinct genetic abnormalities, including TP53 and PI3KCA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) mutations.

1.5.1 Molecular Landscape

Advancements in molecular profiling have revolutionized our understanding of NSCLC, revealing an intricate network of genetic alterations and biomarkers. Just as discussed earlier, the development of driver mutations, such as EGFR, ALK, and emerging biomarkers, including ROS1 and PD-L1, has laid the groundwork for the development of personalized, targeted therapies for specific NSCLC patient subgroups.

1.5.2 Risk Factors, Pathogenesis and Evolving Treatment Strategies

- Tobacco smoking continues to be the primary risk factor for NSCLC, driving a complex, multistep process of the lung cancer development.
- However, other risk factors, such as environmental exposures, genetic predisposition, and underlying health conditions, also play significant roles in the development of NSCLC.
- Understanding these pathogenic mechanisms underlying NSCLC is crucial for the effective prevention strategies and improving treatment outcomes.
- The management of NSCLC, therefore, follows a multidisciplinary approach, with various treatment modalities, including surgery, radiation therapy, chemotherapy, and personalized treatment therapies. The selection of treatment is dependent on the individual patient's disease characteristics, stage, and overall health.

1.5.3 Emerging Biomarkers

The identification and characterization of molecular biomarkers in NSCLC are crucial for driving advancements in personalized cancer care. These biomarkers provide critical insights into underlying genetic and molecular mechanisms influencing the development of disease, facilitating the development of targeted and more effective treatment approaches. Several biomarkers have been identified such as EGFR, ALK, KRAS, and so on, which significantly advanced our understanding of NSCLC (*Maja et al., 2021*).

1.5.4 Genetic Predisposition and Personalized Treatment Approaches

Numerous studies have investigated the genetic differences between smoker and non-smokers with NSCLC. These investigations have revealed distinct molecular profiles and patterns of genetic alterations that characterize these two subgroups of NSCLC patients. Smokers with NSCLC are more likely to harbor mutations in the TP53 and

KRAS (Kirsten rat sarcoma viral oncogene homolog) genes, which are associated with increased genomic instability and more aggressive tumor behavior.

In contrast, non-smokers with NSCLC often exhibit a higher frequency of mutations in the EGFR gene, which is a key driver of tumor growth and proliferation.

The identification of these genetic differences has significant implications for the management and treatment of NSCLC. Patients with EGFR-mutant NSCLC are more likely to respond favorably to targeted therapies, such as EGFR-tyrosine kinase inhibitors, compared to patients with other genetic alterations. Conversely, smokers with TP53 and KRAS mutations may require more intensive treatment regimens and have a poorer prognosis. Understanding the genetic predisposition in smokers and non-smokers with NSCLC is crucial for developing personalized treatment strategies and improving patient outcomes (*Elisabeth et al., 2019*).

1.5.5 Challenges and Limitations in NSCLC Management

The identification of molecular biomarkers is one of the crucial factors for the advancements in personalized treatment in cancer. These biomarkers are associated with the genetic and molecular mechanisms influencing the progression of the disease. The development of the biomarker-derived therapy has led to the strategies for effective treatment.

Despite the significant progress in the field of personalized treatment for NSCLC, there are still several challenges that continue to impede the expanded use of these agents to achieve promising cure for the disease, therefore addressing these challenges is critical. One of the key challenges is resistance, both primary and acquired, which limits the potential for a curative approach in advanced NSCLC. The strategies using combination therapy has shown promise in overcoming this challenge. By combining the drugs targeting distinct pathways, enhanced treatment responses preventing the emergence of resistance, can be achieved.

Additionally, the treatment involving chronic oral administration raises concerns about their associated toxicities. These toxicity issues often make it impractical to use these

agents in combination, limiting the potential cure. Future research prioritizing the understanding of these mechanisms associated with the toxicities of the respective treatment and devising new drug administration schedules facilitating the combination of complementary tyrosine kinase inhibitors (TKIs). Investigating studies involving the patient-specific factors, such as host characteristics and special populations, which contributes to the variations in the toxicity profiles, is crucial.

Another significant challenge in the widespread adoption of personalized treatment methods is the issue of affordability. The high cost of these essential drugs often restricts access, particularly in resource-limited settings, preventing many NSCLC patients from benefiting from these promising curative treatment options. Overall, addressing all these challenges is critical for progressing towards an effective treatment strategy, including targeted therapies for NSCLC (Joel *et al.*, 2022).

1.5.6 Directions and Research Priorities

Just as discussed above, addressing the challenges remain to be one of the significant priorities in NSCLC research. Overcoming resistance mechanisms, optimizing the management of treatment-related toxicities, improving the accessibility and affordability of these novel agents, advancing biomarker-driven approaches to guide personalized treatment selection, and exploring efficient combination strategies (Alex *et al.*, 2020).

1.6 INTERACTIONS BETWEEN NSCLC AND THE IMMUNE SYSTEM

Investigations into the interplay between NSCLC and the immune system have revealed significant impacts on clinical outcomes, particularly in modulating anti-tumor responses. However, NSCLC tumors can also establish an immunosuppressive microenvironment that supports tumor growth and progression.

One key aspect of the NSCLC tumor microenvironment is the presence of high levels of immunosuppressive regulatory T cells (Tregs). These Tregs can directly inhibit the proliferation and function of effector T cells. It also shows increased expression of

immune checkpoint proteins such as the PD-1 on tumor-infiltrating T cells and PD-L1 on the tumor cells. This upregulation of inhibitory checkpoint pathways suppresses the anti-tumor immune response. Additionally, NSCLC tumors cells may downregulate their expression of antigen presentation machinery, helping them evade detection by the immune system.

Despite this immunosuppressive environment, the immune system's involvement in NSCLC is still evident. NSCLC patients exhibit the presence of circulating anti-tumor T cells and antibodies, indicating the immune system's continuous attempt in initiating anti-tumor responses. Furthermore, higher levels of tumor-infiltrating lymphocytes, particularly cytotoxic CD8⁺ (cluster of differentiation) T cells, are associated with improved patient survival outcomes. Conversely, increased infiltration of immunosuppressive cell types like Tregs and protumor macrophages correlates with reduced survival.

Recognizing the critical role of the immune system in NSCLC, significant research efforts are underway to develop innovative immunotherapeutic approaches. This includes the development of immune checkpoint inhibitors that block suppressive pathways like PD-1/PD-L1 and CTLA-4, as well as other strategies such as cancer vaccines and non-specific immune stimulators. The objective of these immunotherapies is to counteract the immunosuppressive tumor microenvironment and harness the potential of the patient's immune system to enhance outcomes in individuals diagnosed with NSCLC (*David et al., 2015*).

1.7 REGULATORY ROLE OF NON-CODING RNAS (NCRNAS) IN CANCER

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 (*Clark et al., 2013*). 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.

The regulatory mechanisms by which ncRNAs influence cancer include modulating the expression of oncogenes and tumor suppressor genes, regulating the cellular processes including, cell proliferation, apoptosis, invasion, angiogenesis, epigenetic modifications, such as DNA methylation and histone modifications, and interacting with other cellular components, such as proteins and other RNAs. Understanding the roles of these ncRNAs in cancer has led to the development of potential diagnostic and therapeutic strategies involving these ncRNAs (*Cornelia et al., 2019*). However, further research is essential for understanding the complex regulatory networks involving ncRNAs in cancer and its application in clinical settings.

1.7.1 Competing Endogenous RNA Mechanism

An intriguing facet lies in the ability of lncRNAs to act as miRNA sponges. By competitively binding to miRNAs, lncRNAs can regulate intracellular miRNA activity, which in turn influences the respective mRNA expression (*Meilian et al., 2021*). 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 analyzing ceRNA networks, researchers are exploring the potential to identify novel biomarkers for early cancer detection and intervention, including NSCLC.

1.7.2 Dysregulated Competing Endogenous RNA Network

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 (*Lin et al., 2022*). For example, in breast cancer (BC), MALAT1 acts as an oncogene through the miR-561-3p/TOP2A axis, leading to increased proliferation and malignancy (*Sara et al., 2023*). Conversely, in glioma, MALAT1 functions as a tumor suppressor by downregulating mir-155, which in turn inhibits FBXW7, reducing tumorigenicity (*Shuanzhu et al., 2016*).

In NSCLC, MALAT1 plays a dual role as a predictive marker for metastasis and a potential therapeutic target (*Wang et al., 2022*).

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 (*Chao et al., 2018*). They are also implicated in driving metastasis via the axes like lncRNA LEF1-AS1/miR-489/SOX4 pathway (*Jiansheng et al., 2019*) and inducing therapy resistance through anti-apoptotic gene expression and signaling pathway alterations such as the NF- κ B pathways, exemplified by lncRNA ATP2B1/miR-222-5p/TAB2 and lncRNA HUWE1/miR-222-5p/TAB1 (*Xiangzhen et al., 2020*). These ceRNA components, involving lncRNAs, miRNAs and mRNAs, could be 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 strategies by understanding the mechanisms that drives therapy resistance in these diseases, thereby, improving treatment, including for NSCLC (*Haizhou et al., 2019*).

CHAPTER 2

LITERATURE SURVEY

Amidst the persisting cases of lung cancer, NSCLC emerges a critical focus in oncology, with smoking being the major risk factor, but, the existing literature highlights a significant portion of NSCLC cases that occur in never-smokers. Understanding the genetic variation between smokers and non-smokers remains a key focus in NSCLC research.

A study by *Piotr (2023)*, explored the genetic variations between smokers and non-smokers. Smoking as a major risk factor in NSCLC, develops the diseases compared to non-smokers. Additionally, the differences in the tumor characteristics suggests that NSCLC in smokers is often associated with squamous cell carcinoma and large cell carcinoma subtypes, strongly linked to smoking, whereas, non-smokers is more frequently associated with the NSCLC subtype, adenocarcinoma. The genetic alterations are one of the critical factors that differentiate smokers and non-smokers. NSCLC in non-smokers is more likely to contain specific genetic mutations, such as EGFR gene, often targeted for specific treatment. The smokers with NSCLC are less likely to contain driver mutations. Furthermore, compared to smoker patients, non-smoker patients with NSCLC often exhibit a better prognosis.

Elisabeth and Martin, (2019). This study represented the distinct and biologically unique subtype of lung cancer, involving the NSCLC patients associated with non-smoker category, especially never-smokers. It was identified that there was a subset of cases occurring in non-smokers, particularly in Asian countries. These non-smoking patients, often female and of East Asian ethnicity, exhibited distinct molecular characteristics and better treatment responses compared to smokers. Environmental factors such as radon and air pollution also play a role in non-smoking lung cancer.

Siddhartha, et al. (2021). Insights from this study include a higher prevalence of clinically significant driver alterations in never-smoker lung adenocarcinoma (LUAD) samples compared to smokers. While some never-smoker samples showed germline alterations in DNA repair genes, the frequency of cancer predisposing germline variants was similar between smokers and never-smokers. Additionally, a subset of never smokers also displayed mutations signature influenced by the passive exposure to cigarette smoke, indicating potential environmental factors in LUAD development among non-smokers.

Pamela and Ignacio, (2017). This study has discussed the significance of crucial biomarkers of lung cancer, particularly NSCLC. The genomic biomarkers with their significant role in the advancement of precision medicine in NSCLC research were highlighted, including EGFR, ALK, KRAS, ROS1, MET, BRAF, P1K3CA, RET proto-oncogene, and others. The identification of the genetic alterations associated with these genes is crucial to specifically address the genetic drivers of NSCLC. For instance, the discovery of EGFR and ALK mutations has provided effective treatment strategies in NSCLC, further, assessing biomarkers such as the PD-L1 has become critical for the personalized treatment approach, including immunotherapy in NSCLC.

Elisa, et al. (2021), uncovered the factors that could sheds light on the factors that drive the tumor growth and treatment resistance in NSCLC. Particularly, the study incorporates the information involving the DNA-and RNA-based next-generation sequencing (NGS) techniques, covering all the predictive and diagnostic markers. RNA-based biomarkers, such as gene expression profiles and miRNAs, is one of the crucial factors for understanding the activity of specific genes and regulatory pathways involved in NSCLC pathogenesis.

A study by *David, et al. (2015),* provided insights into the role of immune system in NSCLC, where it was specified that previously, NSCLC was considered as a non-immunogenic disease. However, the emerging studies have recognized the role of specific immune-evasive mechanisms that contributes to the lack of immune response in NSCLC. Understanding these mechanisms is crucial for therapeutic strategies to overcome NSCLC disease. Investigations into the nuanced interplay relationship

between NSCLC and the immune system have revealed critical evidence of the immune system's significant impact on clinical outcomes. The positive associations observed for diverse immune cell subsets and enhanced survival indicate the significance of these immunological dynamics within the framework of outcomes in NSCLC.

Mengzhu, et al. (2017). In this paper, the comparative analysis of immune-response related genes in the progression of lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), are provided. NSCLC, including its subtypes LUAD and LUSC, is categorized into four stages based on tumor node metastasis classification. Despite their differences, both subtypes are associated with various genes implicated in lung cancer, with the immune system playing a crucial role in their development and progression.

A study by *Lisha, et al. (2023)*, investigated the importance of early-stage diagnosis and effective treatment for LUSC, specifically the need for understanding innate immune responses in the tumor microenvironment. While immune-checkpoint inhibitors are implicated in treating NSCLC, including some forms of LUSC, resistance and variable responses remain challenges. This study systematically examined the potential immunotherapeutic strategies for LUSC management.

Andrea, et al. (2020). highlights the development of immune evasion mechanisms in NSCLC has traditionally been associated with advanced stages of tumor progression. However, recent evidence suggests that active immune escape mechanisms can emerge even in pre-invasive lesions that could later progress to main NSCLC histotypes. These mechanisms include impaired antigen presentation, loss of heterozygosity at the human leukocyte antigen (HLA) region, activation of immune checkpoints, and so on. These processes continue to evolve in the invasive stages of NSCLC and are associated with inter-and intra-tumor heterogeneity.

A study by *Qin, et al. (2016)*, 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-associated myeloid cells to inhibit antigen-specific T cell responses. These

mechanisms contributed to hindering immune recognition and creating an immunosuppressive environment in the tumor. 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-1 expressing CD8+ T cells, and HLA loss of heterozygosity (LOH) associated with increased PD-L1 expression. These mechanisms potentially influence the progression to LUAD and LUSC. Furthermore, intra-tumor immunological heterogeneity, which is characterized by complex immune landscapes, is found to be increased in aggressive stages.

Another study by *Branislava, et al. (2019)*, highlights the role of immune system in NSCLC, influencing the prognosis and response to therapy. Immunological analysis holds great potential in predicting the patient outcomes and guiding immunotherapy. In NSCLC tumors, T cells dominate the immune landscape, with CD4+ and CD8+ T cells being the most abundant subtypes. B cells, macrophages, and natural killer cells also contribute significantly to the immune infiltrate. Notably, the composition of immune cells may vary between the tumor and non-cancerous lung tissue, highlighting the complex interplay between the tumor microenvironment and immune response.

Kajetan, et al. (2023). This study explored the significant role of non-coding RNAs in NSCLC, representing 85% of all cases. They are known to regulate the signaling pathways associated with tumorigenesis. The dysregulated non coding RNAs such as miRNA, lncRNA, and circRNA, influence the progression of NSCLC. These elements can modulate the gene expression by interacting with mRNA and each other, either promoting the oncogenic activity or inhibiting them.

Soudeh, et al. (2020), studied the emergence of non-coding RNAs and its impact on NSCLC. lncRNAs and miRNAs are implicated in the cancer development and progression. They implied that in NSCLC, molecular heterogeneity is notable, with high throughput sequencing that revealed frequent somatic mutations in genes such as TP53, KRAS, and others. Lung adenocarcinoma was identified to often exhibit mutations that activated the RAS signaling pathway. Moreover, Lung squamous cell carcinoma showed complex genomic alterations, including TP53, PTEN, PIK3CA, and

other mutations, showing overexpression in the PI3K/AKT pathway. The role of non-coding RNA is gaining recognition in the NSCLC pathogenesis, with their possible contribution in these genomic alterations.

Dysregulation of the ncRNAs plays a crucial role in the pathogenesis of NSCLC. A study by *Santhosh, et al. (2022)*, revealed the participation of the dysregulated ncRNAs, serving as both therapeutic targets and prognostic markers in NSCLC. Integrative analysis of lncRNAs, miRNAs, and mRNAs has revealed the existence of ceRNA networks, where lncRNAs act as molecular sponges to inhibit the regulatory function of miRNAs on mRNAs. Importantly, some of these networks exhibit subtype-specific functions within NSCLC.

Many studies have delved into the regulatory mechanism of competing endogenous RNA (ceRNA) network, emerging as a significant research focus, especially in the field of cancer therapy and precision medicine. A study by *Xianxian, et al. (2020)*, provides insights into the components of ceRNA network and its implications in tumor progression. In this network, certain ncRNAs such as lncRNAs, circRNAs act as ceRNAs by competitively binding to specific miRNAs, thereby modulating mRNA expression. This intricate interplay contributes significantly to various aspects of tumorigenesis, including proliferation, apoptosis, and drug resistance. Notably, it was identified that miRNAs could also target lncRNAs, forming a regulatory loop within the nucleus. Interestingly, existing evidence suggests that lncRNAs can counteract miRNA function, indicating their antagonistic roles in tumorigenesis, primarily mediated through ceRNA crosstalk.

Another study by *Dandan, et al. (2022)*, highlights the importance of the ceRNA network contributing to the mechanisms driving EGFR-TKI resistance in NSCLC. Addressing therapy resistance in NSCLC is crucial for improving the efficiency of treatments. Understanding the interplay among specific lncRNAs, miRNAs, and mRNAs, could be one of the strategies to identify and target the biomolecular drivers of drug resistance. They identified the complex regulatory pathways implicated in EGFR-TKI resistance, particularly through the core ceRNA network, therefore providing promising avenue for

the development of innovative therapeutic approaches aimed at overcoming resistance in NSCLC patients.

Wei-Jing, et al. (2021). This study delves into the role of the tumor microenvironment and immune-related ceRNA networks in LUAD. Differential gene expression analysis identified key genes, and survival analysis coupled with predicted RNA interaction pairs constructs a prognosis-related ceRNA network. The study further explores the association between the ceRNA network, immune cell infiltration in the tumor microenvironment, and response to immunotherapy.

Yunzhen, et al. (2017). This study focused on the impact of somatic copy number alterations (SCNA) in lncRNAs in LUAD. By analyzing high-throughput sequencing data, it highlights the importance of lncRNAs in cancer biology, particularly in LUAD progression. This study explored how the SCNA lncRNAs regulate gene expression and contribute to cancer progression through various mechanisms, such as cis-regulation, trans-regulation, and ceRNA activity.

A study by *Ding, et al. (2022)*, investigated the role of CNVs in LUSC by constructing a ceRNA network, involving CNV-driven lncRNAs, miRNAs, and mRNAs. This study sheds light on the potential pathogenesis of LUSC mediated by CNV-related lncRNAs and their ceRNA interactions.

Jing Sui, et al. (2016). In this study, researched focused on the understanding of the lncRNAs and its role in LUAD progression. They constructed a ceRNA network with the components from the network associated with LUAD progression. Functional analysis of the ceRNA network revealed enrichment in pathways related to cancer biology, including the p53 signaling pathway, MAPK signaling pathway, and Wnt signaling pathway, highlighting the potential involvement of these pathways in LUAD development and progression. To validate their findings, the researchers performed qRT-PCR analysis on a subset of LUAD samples, confirming the dysregulation of key lncRNAs identified in the ceRNA network. The validation step supported the potential utility of these lncRNAs as diagnostic and prognostic biomarkers for LUAD.

miR-34a is a tumor suppressor miRNA downregulated in various cancers, including NSCLC. A study by *Yang, et al. (2014)*, provided insights into the role of the miR-34a in NSCLC progression. It was shown to regulate cancer stem cells (CSCs), implicated in tumor initiation, progression and therapy resistance. In NSCLC, it was identified that miR-34a negatively regulates the CSC-associated properties and tumor-initiating capacity in NSCLC cells. This suggests that miR-34a may act as tumor suppressor by targeting CSCs in NSCLC, highlighting its potential as a therapeutic target.

Additionally, a study by *Alexia, et al. (2021)*, highlighted the role of miR-34a in predicting the efficacy of immunotherapy in NSCLC patients. miR-34a was known to be emerging as a potential predictive biomarker in NSCLC patients undergoing therapy. The study further indicated that the lower expression levels of miR-34a were associated with shorter progression-free survival, overall survival compared to patients, and non-responsiveness to treatment, with higher miR-34a expression.

Y-L Li, et al. (2017). This study demonstrated the interaction between miR-34a and EGFR, a key player in cancer progression, and its potential involvement in lung tumorigenesis. Knockdown of EGFR was known to inhibit the cell proliferation, promote apoptosis, and arrest the cell-cycle progression. Moreover, EGFR could reverse the suppressive effects of miR-34a overexpression on proliferation and apoptosis. These findings suggested that miR-34a exerts its tumor-suppressive role in NSCLC by targeting EGFR.

There are key genes and pathways associated with NSCLC, with significant roles in various biological processes, signaling pathways and so on. A study by *Tingting, et al. (2019)*, uncovered the crucial genes and pathways involved in NSCLC using bioinformatics analysis of gene expression data. The GO and KEGG pathway analysis revealed enrichment of DNA replication, immune response pathways and others, among differentially expressed genes, indicating their involvement in tumorigenesis. This study incorporated the pathways associated with the genes relative to their study objectives. However, there are numerous other critical genes and pathways that are

associated with NSCLC, implying that NSCLC is a complex disease with diverse molecular mechanisms underlying its development and progression.

Manzar, et al. (2023). This study provided insights into the pathways that plays a crucial role in NSCLC, regulating the cell growth and proliferation. The signaling pathways such as p53, Wnt, EGFR, KRAS/MAPK. STAT3, NF-kB, and PI3K/Akt pathways are crucial targets for therapy, since the alterations in these pathways could promote tumor progression. By targeting specific molecules within these pathways, apoptosis in NSCLC could be induced. However, maximizing the efficiency of pathway inhibitors remains challenging due to acquired resistance and side effects. Overcoming the resistance and utilizing the drug combinations are essential for increasing the effectiveness of personalized therapy in NSCLC.

CHAPTER 3

AIM AND SCOPE OF THE STUDY

3.1 AIM OF THE STUDY

The primary aim of this study is to investigate the role of competing endogenous RNA (ceRNA) network, focusing on the interactions among long non-coding RNA (lncRNA), microRNA (miRNA), and messenger RNA (mRNA) in the tumorigenesis of NSCLC. Specifically, the study aims to identify key biomarkers and understand the regulatory mechanisms underlying NSCLC progression through the ceRNA network, with a particular emphasis on the connection between the immune system processes and the genes associated within the ceRNA network.

3.2 RESEARCH QUESTIONS

- a) What is the significance of ceRNA network in NSCLC, and how do the ceRNA components interact to influence disease progression and tumor microenvironment?
- b) Does the identification of novel ceRNA interactions, such as the one involving the lncRNA ALAL1, the SART gene, and miR34-a, have implications for understanding the mechanisms driving NSCLC progression?
- c) What are the potential biomarker candidates within the ceRNA network that could be used for the early detection, diagnosis, and therapeutic targeting of NSCLC?

- To address these research questions, the following hypothesis are provided:
 - a) The ceRNA network constructed in this study, could play a significant role in NSCLC by regulating key biological processes and signaling pathways, that could influence the disease progression and tumor microenvironment.
 - b) ALAL1's ability to function as a ceRNA component in general, suggests that it could be a key player in regulating NSCLC-associated pathways therefore, in this study, the identification of novel ceRNA interaction, involving the lncRNA ALAL1, the SART gene, and miR34-a, suggests potential implications for understanding the mechanisms that influence the NSCLC progression. These compelling insights, through assimilation of existing literature, have been highlighted with significant interactions between the molecules following the perceptive identification of common targets and mechanisms.
 - c) The comprehensive ceRNA network analysis constructed in our study will reveal key biomarker candidates, including lncRNAs, miRNAs, and their target genes, which could have clinical relevance for NSCLC, particularly those involved in immune system processes.

3.3 SCOPE OF THE STUDY

- 1) The study will utilize publicly available datasets from the Gene Expression Omnibus (GEO), Differentially Expressed miRNAs in human Cancers (dbDEMOC), and The Cancer Genome Atlas (TCGA) to curate differentially expressed mRNAs, miRNAs, and lncRNAs in NSCLC.
- 2) The analysis will focus on miRNAs with established roles in immune regulation, pathways, and the TME, and an integrative analysis will be performed to construct a comprehensive ceRNA regulatory network.

- 3) Gene Ontology (GO)-based functional enrichment analysis will be conducted, with a specific focus on uncovering genes involved in immune system processes and their potential as key biomarkers within the NSCLC Tumor microenvironment.
- 4) The study will also explore a previously unexplored ceRNA interaction involving the novel lncRNA ALAL1, the SART3 gene, and miR-34a, and its potential regulatory influence on NSCLC progression.
- 5) The computationally inferred ceRNA interactions will be discussed in the context of their potential clinical relevance, while highlighting the need for clinical validation.

Overall, this study aims to provide insights into the intricate ceRNA network in NSCLC, to identify promising biomarkers, and lay the groundwork for future investigations in the field of NSCLC research.

CHAPTER 4

MATERIALS AND METHODS

4.1 DATA DOWNLOADING

Collection of mRNA Expression Profile

In this study, mRNA expression profiles for our investigation were sourced from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>), a globally accessible public repository, houses high-throughput gene expression and functional genomics datasets. The chosen series ID (GSE ID) was GSE19804, with the corresponding platform ID (GPL ID) as GPL570; The platform undertaken was Affymetrix Human Genome U133 Plus 2.0 Array, representing a dataset specifically focusing on the expression profiles of non-small cell lung cancer (NSCLC) in female non-smoker patients. All the samples were collected from Taiwan.

Collection of miRNA Expression Profile

The miRNA expression profiles necessary for understanding potential mRNA interactions were obtained from the database of Differentially Expressed miRNAs in human Cancers (dbDEMC; <https://www.biosino.org/dbDEMC/index>). This specialized database is designed to store and display differentially expressed miRNAs in cancers, detected through both high-throughput and low-throughput methods. The search involved retrieval of experiment ID: EXP00710, which comprises a sample case of NSCLC patients comparing tumor vs. normal samples, analyzed using a microarray platform. The resulting dataset involved 89 upregulated and 120 downregulated miRNAs, totaling 209 miRNAs.

Collection of lncRNA Expression Profile

For lncRNA expression profiles, data was obtained from The Cancer Genome Atlas database (TCGA; <https://portal.gdc.cancer.gov/>). A specific search was conducted for general lung cancer data, as these samples would later be filtered based on their molecular interactions. Detailed procedures were followed to navigate the TCGA database and retrieve the necessary lncRNA expression profile, which involved the transcriptome profile, with additional details such as sample characteristics and experimental methods provided in the dataset. The search parameters were optimized to ensure relevance to the study's objectives. A flowchart of this study was drawn to explain the analytical process (**Fig 4.1**).

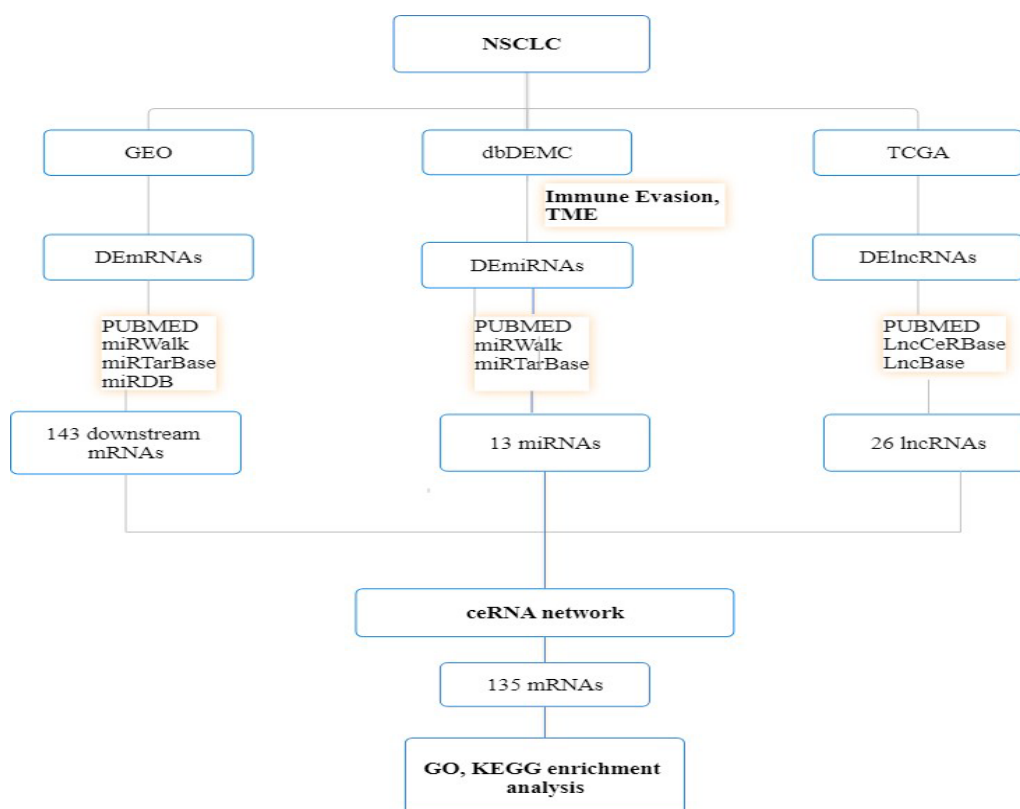


Fig 4.1. Overall Workflow of the study

4.2 DIFFERENTIAL EXPRESSION ANALYSIS

Identification of Differentially Expressed Genes (DEGs)

To identify the differentially expressed mRNAs (DEmRNAs or DEGs) between tumor samples and normal samples (normal: 60, tumor: 60), we employed the GEO2R tool within the GEO database (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>). GEO2R is a specialized analysis tool integrated into the GEO platform, designed for the comparison of gene expression profiles in different experimental conditions. The samples were appropriately grouped and subjected to GEO2R, utilizing the Benjamini Hochberg (FDR) method for p-value, with a significance cut-off of 0.05, therefore facilitating the differential expression analysis. Subsequently, it generated a comprehensive list comprising 54,676 DEGs including both upregulated and downregulated genes. The respective expression fold changes and statistical significance were given along with the result, contributing to the reliability of the data. The resulting list of 54,676 DEGs forms the basis for further analysis in our study.

4.3 LITERATURE MINING: Identification of Differentially Expressed miRNAs (DEmiRNAs) and Differentially Expressed lncRNAs (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. To identify the DEmiRNAs integral to our study's focus, a rigorous literature analysis was conducted. The objective was to specifically target miRNAs with a well-known role in immune regulatory mechanisms and the tumor microenvironment, particularly those contributing to the immune evasion process. By prioritizing miRNAs

intricately linked with immune processes, we aim to elucidate their regulatory connections within the ceRNA network. It ensures that the selected miRNAs play a significant role in the complex interplay between immune system and the tumor microenvironment. Therefore, only those DE miRNAs with established role in immune modulation were retained. This filtering process refines the original miRNA dataset, providing a focused subset aligned to our research objectives.

In pursuit of identifying the DE lncRNAs with potential functional relevance, an extensive literature analysis, similar to the approach for DE miRNAs dataset, was conducted. The selection criteria involved prioritizing lncRNAs that demonstrated binding affinity to the previously identified miRNAs. Further filtration was achieved by focusing on lncRNAs known for their critical involvement in NSCLC, and, more broadly, in lung cancer. The approach aimed to select lncRNAs that exerted a significant impact on NSCLC. The final set of DE lncRNAs, with exploration of their intricate roles within the regulatory network, was obtained.

4.4 SCREENING COMPONENTS FOR CERNA NETWORK CONSTRUCTION

The construction of the regulatory network in our study relied upon the intricate interplay among lncRNA-miRNA-mRNA components, for which, a comprehensive understanding of their binding potential was imperative. This involved a combination of database mining and literature study. For the 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/>). miRWalk integrates predicted and experimentally validated miRNA-target interactions. miRDB is another database for miRNA target prediction and functional annotations. Analogous interactions in other cancer types were also extrapolated in this study focusing on its specific interactions, providing a basis for further investigations particular to NSCLC.

For lncRNA-miRNA interactions, screening was conducted using LncCeRBase v1.0 and DIANA- LncBase v3.0 database. LncCerBase (<http://www.insect-genome.com/LncCeRBase/front/>) provides a curated collection of experimentally

validated lncRNA-miRNA-mRNA interactions, while DIANA-LncBase (<https://diana.e-ce.uth.gr/lncbasev3>), serves as a repository containing experimentally validated miRNA targets within non-coding transcripts. These databases, coupled with additional literature studies, ensured selection of lncRNAs interacting with the specified miRNAs, related to NSCLC.

4.5 CONSTRUCTION OF COMPETING ENDOGENOUS RNA (ceRNA) NETWORK

The comprehensive ceRNA network representing the intricate interactions among lncRNA-miRNA-mRNA components, was developed, following an in-depth analysis of these interacting components. The final ceRNA network was visualized using the Cytoscape v3.10.0 software. It is an open-source bioinformatics software platform that allows visualization and analysis of complex networks to interpret the interconnected relationships within biological systems. This analytical framework integrated the interacting components and enhanced our understanding of the regulatory landscape within the constructed ceRNA network through an insightful representation.

4.6 GO AND KEGG ENRICHMENT ANALYSIS

Gene Ontology (GO) is a knowledgebase that classifies genes based on their biological process (BP), cellular components (CC) and molecular function (MF), and provide a structured framework to understand the relationships between the genes. We used the graphical gene set enrichment, tool ShinyGO v0.80 (<http://bioinformatics.sdstate.edu/go/>) to perform the GO and KEGG enrichment analysis. In particular, the terms were set to “Homo Sapiens”, along with the FDR cut-off value to 0.05. The top 20 pathways in the were characterized based on -log₁₀-transformed False Discovery Rate (FDR) scores for obtaining more statistically-informed enrichment.

CHAPTER 5

RESULTS AND DISCUSSION

Non-small cell lung cancer (NSCLC) represents a dynamic field of oncology characterized by a decline in mortality rates, largely attributed to the advent of targeted therapies (*Kui et al., 2021*). However, the efficacy of these therapies across diverse smoking histories remains a key question, given their higher mutational burden in smokers. Despite the surging acclaim for targeted intervention, a gap persists in biomarker-driven therapy, necessitating a clear understanding of the genetic intricacies in NSCLC patients considering that non-smoker patients with targetable genetic alterations are relatively low (*Anna et al., 2022*). Such insights illuminate the potential for identifying novel druggable targets that can open up new avenues for precision medicine and the associated symptoms of resistance acquired with it (*Yuan et al., 2021*).

These advancements can, therefore, improve the development of unconventional treatment strategies for NSCLC patients. The interplay between the competing endogenous RNA (ceRNA) elements forms a complex regulatory network that plays a crucial role in cancer development and progression (*Huihui et al., 2021*). By competing for shared miRNAs, ceRNAs modulate each other's expression, influencing cellular processes that contribute to cancer initiation and metastasis (*Cai-Zhi et al., 2021*). Exploring the regulatory mechanisms governing ceRNAs is a critical area of research and understanding the intricate interplay among these ceRNA components could guide the development of novel therapeutic approaches, as these RNA elements hold promise as early cancer detection biomarkers, facilitating the personalized treatment strategies that focus on the unique molecular profile of individual NSCLC patients.

5.1 DIFFERENTIAL EXPRESSION ANALYSIS

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 (**Fig; 5.1, 5.2 and 5.3**). Subsequently, this initial list of DEGs was refined based on our research focus, forming the basis for further investigation. The UMAP plot (Uniform Manifold Approximation and Projection plot) and the Venn diagram, representing the DEGs, were also retrieved for visualization.

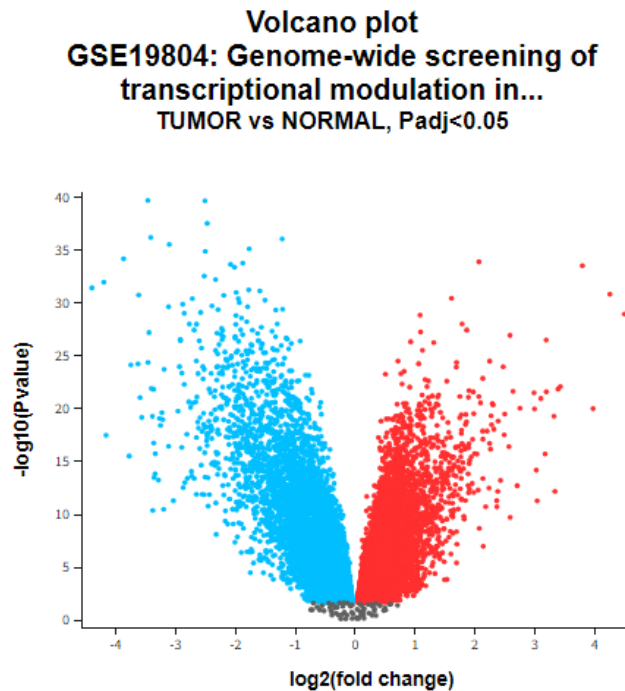


Fig 5.1. Volcano Plot of Differentially Expressed mRNAs; blue dots represent genes that are significantly downregulated, while red dots indicate genes that are significantly upregulated. The x-axis displays the log2 fold change, and the y-axis displays the negative logarithm of the p-value. Gene above the horizontal line are statistically significant ($p<0.05$)

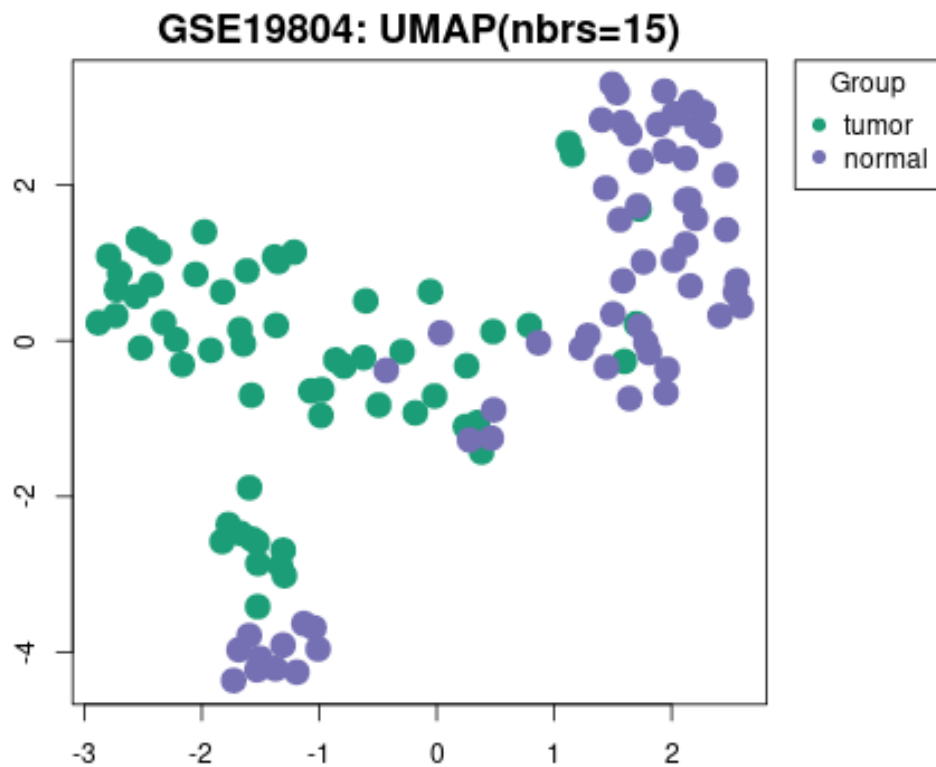


Fig 5.2. UMAP (Uniform Manifold Approximation and Projection) visualization of gene expression data from GSE19804 dataset. Each dot represents a single cell, colored according to the group it belongs to (tumor or normal). Green circles represent the cells from the tumor group and purple circles represent cells from the normal group

GSE19804: limma, Padj<0.05

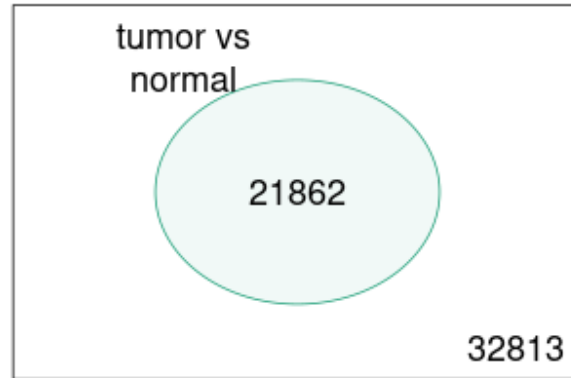


Fig 5.3. Venn diagram comparing the number of genes differentially expressed between tumor and normal samples, using the limma method with a p-value adjustment (Padj) less than 0.05. The diagram shows that there are 32,813 genes in the "normal" group and 21,862 genes in the "tumor vs normal" group, with an overlap between the two sets.

5.2 LITERATURE MINING

From the intensive literature analysis, we identified significant DEmiRNAs with a potential impact on immune regulation and 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 (*Nipin et al., 2021*), immune cell development (*Katharina et al., 2020*), interplay in cancer-immune talk (*Katerina et al., 2022*), 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.

5.3 SELECTION OF COMPONENTS FOR 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 including miRWalk, miRTarBase, LncCeRBase, and LncBase, and literature studies which also delved into the relationship between these molecules, were conducted and through the identification of potential relationships between the components miRNA/lncRNA and miRNA/mRNA, we curated a selection of 26 lncRNAs and 143 mRNAs for the finalization of the network.

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.

5.4 CONSTRUCTION OF ceRNA INTERACTION NETWORK

The constructed ceRNA network was then visualized using Cytoscape v3.10.0 software (*see Fig 5.4*). This analytical framework integrated the interacting components, providing an enriched perspective on the regulatory landscape within the established ceRNA network.

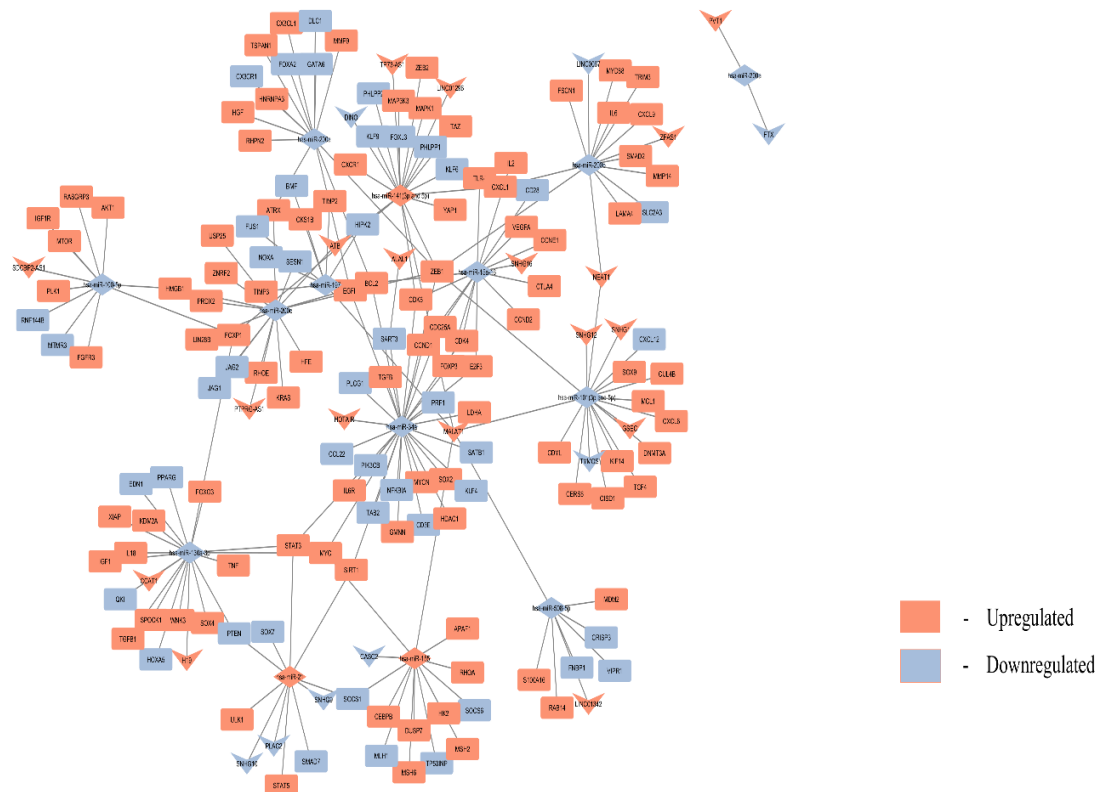


Fig 5.4. The ceRNA-Interaction Network in Cytoscape; shapes represent the RNA types (V-shape for lncRNAs, diamond for miRNAs, and round rectangle for mRNAs), and colors indicate the regulation type (red color represents upregulated, and blue color represents downregulated).

Within the network of 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 (Yang *et al.*, 2014). SIRT1 (Sirtuin 1) has been identified as a putative 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 (Munekazu *et al.*, 2008). A

study by *Lin et al., 2019*, 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. 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, 2023*. In accordance with findings by *Peng et al., 2023*, 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. 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 (*Dawn et al., 2013*). 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 (*Gregory et al., 2014*). 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.

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 (*Alejandro et al., 2020*), emerges as a frequently amplified oncogenic lncRNA, transcriptionally activated by tumor necrosis factor-kappa B (NF- κ B). Primarily functioning within the cytoplasm, ALAL1 exerts its influence on SART3, influencing their nuclear localization. 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 CDK4/6 (*Emily et al., 2019*). This mechanism represents a regulatory response to prevent uncontrolled proliferation, leading to a tumor suppressive effect in NSCLC cells. Our study suggests a novel hypothesis, stating that ALAL1 as a ceRNA component modulates miR-34a function indirectly, by targeting the SART3 gene. This could suggest how ALAL1 may inhibit cell cycle arrest, exacerbate uncontrolled proliferation, and facilitate tumor growth in NSCLC cells. 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 1), activating the NF- κ B pathway and cytokine production (*Xinying et al., 2016*). Its inverse correlation with TGF-beta expression influences response towards the immune checkpoint inhibitors (*Neda et al., 2021*). SOCS1 in NSCLC is known for inhibiting the FAK-dependent signaling pathway (*Mengmeng et al., 2017*). Additionally, CASC2 is also indicated in the tumor growth and poor survival in NSCLC (*Xin et al., 2018*). *Yuan et al.* has 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 (Ye *et al.*, 2023). This leads to the upregulation of SOCS1 expression, the target gene of miR-155, highlighting the ceRNA function of CASC2. This shared mechanism suggests significant implications in NSCLC, given their common targets.

5.5 GO AND KEGG ENRICHMENT ANALYSIS

Subsequent analysis incorporating GO and KEGG Enrichment Analysis performed revealed key pathways associated with the biological processes in NSCLC. The GO results of the three terms were highlighted in **Fig 5.5**, and these GO terms associated with the biological processes (**Fig 5.6**), cellular components (**Fig 5.7**), and molecular functions (**Fig 5.8**) were then visualized using a cnetplot. The GO terms were further analyzed to provide a detailed discussion on the significant biological processes associated with the differentially expressed genes. Additionally, the KEGG enrichment analysis highlighted the significance of key signaling pathways implicated in the pathogenesis of non-small cell lung cancer (**Fig 5.9 and Fig 5.10**).

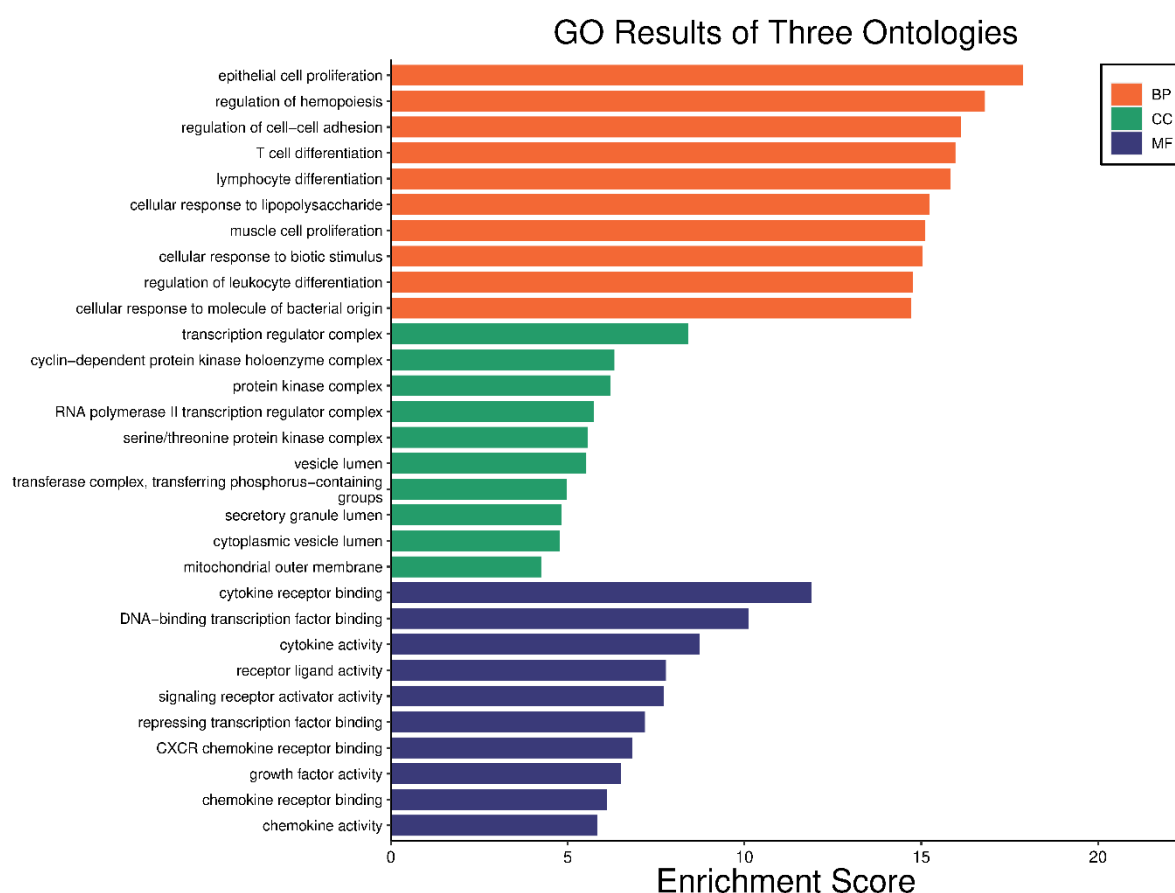


Fig 5.5. The image shows the GO results of three terms - biological process (BP), cellular component (CC), and molecular function (MF) - for various enriched biological processes and molecular functions. The x-axis represents the Enrichment Score, which indicates the level of enrichment for each GO term. The bars are color-coded based on the three ontologies: BP in orange, CC in green, and MF in purple. The figure provides a comprehensive overview of the key pathways and molecular mechanisms associated with the biological processes in the context of the study.

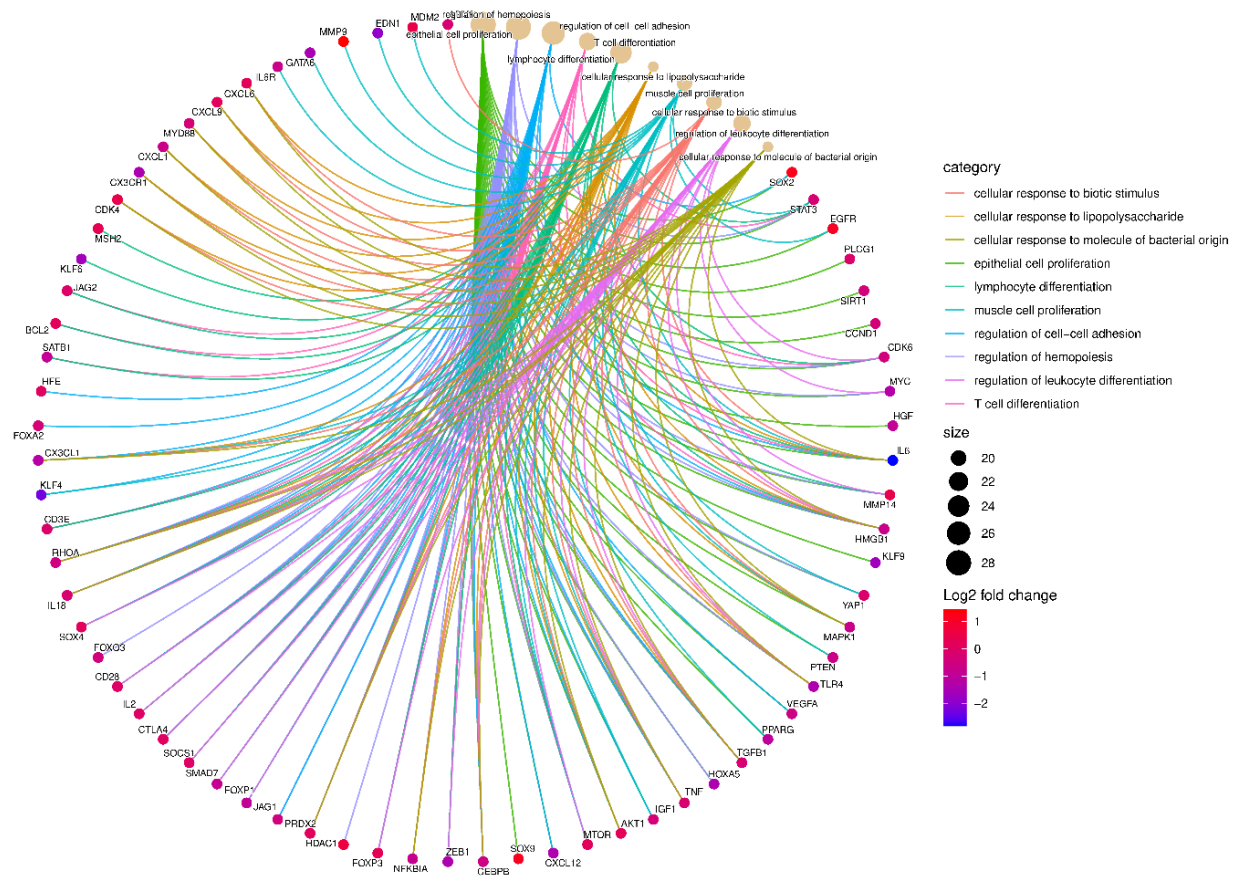


Fig 5.6. The figure presents a comprehensive cnetplot depicting the various biological processes associated with the target genes. The nodes represent different categories, with their size indicating the significance and the color gradient showing the log2 fold change in gene expression.

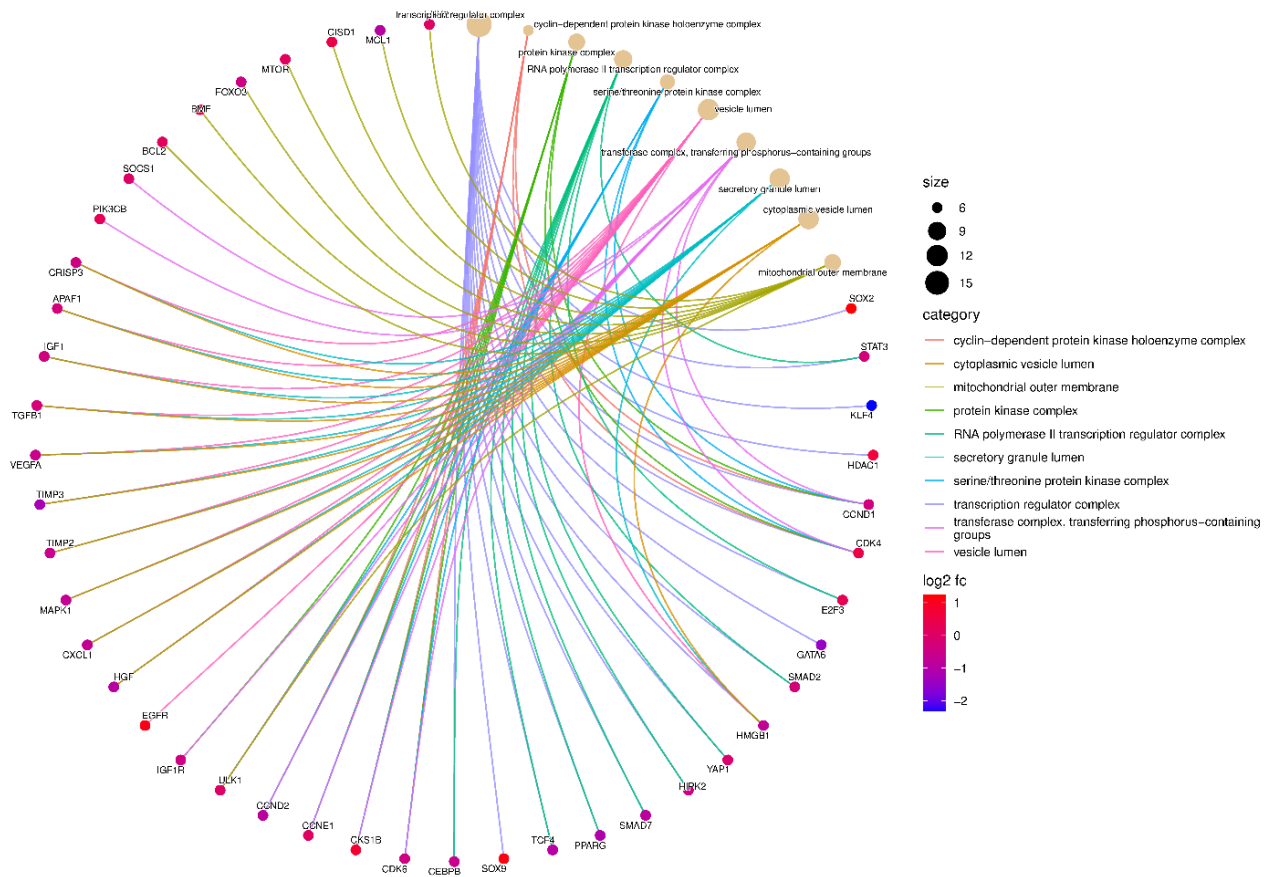


Fig 5.7. The figure presents a comprehensive cnetplot depicting the various cellular components associated with the target genes. The nodes represent different categories, with their size indicating the significance and the color gradient showing the log2 fold change in gene expression.

Table 5.1. Enriched GO Terms and their Associated Genes

Category	Term: Description	Genes
GO: Biological Processes	GO:0050673: Epithelial cell proliferation	SOX2/STAT3/EGFR/PLCG1/SIRT1/CCND1/CDK6/MYC/HGF/IL6/MMP14/HMGB1/KLF9/YAP1/MAPK1/PTEN/TLR4/VEGFA/PPARG/TGFB1/HOXA5/TNF/IGF1/AKT1/MTOR/CXCL12/SOX9/CEBPB
GO: Biological Processes	GO:1903706: Regulation of hemopoiesis	STAT3/ZEB1/NFKBIA/FOXP3/HDAC1/CDK6/MYC/MMP14/PRDX2/JAG1/HMGB1/YAP1/FOXP1/SMAD7/SOCS1/CTLA4/TLR4/IL2/CD28/FOXO3/SOX4/IL18/TGFB1/HOXA5/TNF/MTOR/CEBPB/RHOA
GO: Biological Processes	GO:0022407: Regulation of cell-cell adhesion	SOX2/CD3E/KLF4/FOXP3/CX3CL1/FOXA2/IL6/HFE/PRDX2/JAG1/HMGB1/SMAD7/SOCS1/CTLA4/IL2/CD28/VEGFA/SOX4/IL18/TGFB1/TNF/IGF1/AKT1/CXCL12/CEBPB/RHOA
GO: Biological Processes	GO:0030217: T cell differentiation	STAT3/ZEB1/CD3E/FOXP3/SATB1/BCL2/CDK6/IL6/PRDX2/JAG2/HMGB1/FOXP1/SMAD7/SOCS1/CTLA4/IL2/CD28/SOX4/IL18/MTOR/RHOA
GO: Biological Processes	GO:0030098: Lymphocyte differentiation	STAT3/ZEB1/CD3E/FOXP3/SATB1/BCL2/CDK6/IL6/MMP14/PRDX2/JAG2/HMGB1/KLF6/FOXP1/SMAD7/SOCS1/CTLA4/IL2/CD28/SOX4/IL18/MTOR/MSH2/RHOA
GO: Biological Processes	GO:0071222: Cellular response to lipopolysaccharide	NFKBIA/CDK4/CX3CL1/CX3CR1/CXCL1/MYD88/CXCL9/IL6/PRDX2/HMGB1/MAPK1/TLR4/IL18/TGFB1/TNF/AKT1/CXCL6/CEBPB/RHOA

GO: Biological Processes	GO:0033002: Muscle cell proliferation	IL6R/STAT3/EGFR/KLF4/CX3CL1/GATA6/MMP9/ L6/YAP1/MAPK1/FOXP1/PTEN/PPARG/EDN1/IL1 8/TNF/IGF1/AKT1/MTOR/MDM2
GO: Biological Processes	GO:0071216: Cellular response to biotic stimulus	NFKBIA/CDK4/CX3CL1/CX3CR1/CXCL1/MYD88/ CXCL9/IL6/PRDX2/HMGB1/MAPK1/TLR4/IL18/TG FB1/TNF/AKT1/CXCL6/CEBPB/APAF1/RHOA
GO: Biological Processes	GO:1902105: Regulation of leukocyte differentiation	ZEB1/FOXP3/CDK6/MYC/MMP14/PRDX2/HMGB1 /FOXP1/SMAD7/SOCS1/CTLA4/TLR4/IL2/CD28/S OX4/IL18/TGFB1/TNF/MTOR/CEBPB/RHOA
GO: Biological Processes	GO:0071219: Cellular response to molecule of bacterial origin	NFKBIA/CDK4/CX3CL1/CX3CR1/CXCL1/MYD88/ CXCL9/IL6/PRDX2/HMGB1/MAPK1/TLR4/IL18/TG FB1/TNF/AKT1/CXCL6/CEBPB/RHOA
GO: Cellular Component	GO:000566: Transcription regulator complex	SOX2/STAT3/KLF4/HDAC1/CCND1/CDK4/E2F3/ GATA6/SMAD2/HMGB1/YAP1/HIPK2/SMAD7/PP ARG/TCF4/SOX9/CEBPB
GO: Cellular Components	GO:0000307: Cyclin- dependent protein kinase holoenzyme complex	CCND1/CDK4/CDK6/CKS1B/CCNE1/CCND2
GO: Cellular Components	GO:1902911: Protein kinase complex	CCND1/CDK4/CDK6/CKS1B/ULK1/CCNE1/CCND 2/IGF1R
GO: Cellular Components	GO:0090575: RNA polymerase II transcription regulator complex	STAT3/E2F3/SMAD2/YAP1/HIPK2/SMAD7/PPAR G/TCF4/CEBPB

GO: Cellular Components	GO:1902554: Serine/threonine protein kinase complex	CCND1/CDK4/CDK6/CKS1B/ULK1/CCNE1/CCND2
GO: Cellular Components	GO:0031983: Vesicle lumen	EGFR/HGF/CXCL1/HMGB1/MAPK1/TIMP2/TIMP3/VEGFA/TGFB1/IGF1/APAF1/CRISP3
GO: Cellular Components	GO:0061695: Transferase complex, transferring phosphorus-containing groups	PIK3CB/CCND1/CDK4/CDK6/CKS1B/ULK1/SOCS1/CCNE1/CCND2/IGF1R
GO: Cellular Components	GO:0034774: Secretory granule lumen	HGF/CXCL1/HMGB1/MAPK1/TIMP2/TIMP3/VEGFA/TGFB1/IGF1/APAF1/CRISP3
GO: Cellular Components	GO:0060205: Cytoplasmic vesicle lumen	HGF/CXCL1/HMGB1/MAPK1/TIMP2/TIMP3/VEGFA/TGFB1/IGF1/APAF1/CRISP3
GO: Cellular Components	GO:0005741: Mitochondrial outer membrane	BCL2/BMF/ULK1/FOXO3/MTOR/CISD1/MCL1/HK2
GO: Molecular Functions	GO:0005126: Cytokine receptor binding	IL6R/STAT3/CCL22/PLCG1/CX3CL1/CXCL1/MYD88/CXCL9/IL6/SMAD2/SMAD7/IL2/VEGFA/IL18/TGFB1/TNF/CXCL12/CXCL6
GO: Molecular Functions	GO:0140297: DNA-binding transcription factor binding	STAT3/NFKBIA/KLF4/FOXP3/BCL2/GMNN/HDAC1/SIRT1/MYC/GATA6/SMAD2/HMGB1/FOXP1/HIPK2/PPARG/TCF4/SOX9/CEBPB

GO: Molecular Functions	GO:0005125: Cytokine activity	CCL22/CX3CL1/CXCL1/CXCL9/IL6/HMGB1/ IL2/VEGFA/EDN1/IL18/TGFB1/TNF/CXCL12/CXC L6
GO: Molecular Functions	GO:0048018: Receptor ligand activity	CCL22/CX3CL1/HGF/CXCL1/CXCL9/IL6/JAG1/JA G2/HMGB1/IL2/VEGFA/EDN1/IL18/TGFB1/TNF/IG F1/CXCL12/CXCL6
GO: Molecular Functions	GO:0030546: Signaling receptor activator activity	CCL22/CX3CL1/HGF/CXCL1/CXCL9/IL6/JAG1/JA G2/HMGB1/IL2/VEGFA/EDN1/IL18/TGFB1/TNF/IG F1/CXCL12/CXCL6
GO: Molecular Functions	GO:0070491: Repressing transcription factor binding	STAT3/BCL2/GMNN/HDAC1/MYC/GATA6/HMGB 1/PPARG
GO: Molecular Functions	GO:0045236: CXCR chemokine receptor binding	CX3CL1/CXCL1/CXCL9/CXCL12/CXCL6
GO: Molecular Functions	GO:0008083: Growth factor activity	HGF/CXCL1/IL6/JAG1/JAG2/IL2/VEGFA/TGFB1/I GF1/CXCL12
GO: Molecular Functions	GO:0042379: Chemokine receptor binding	STAT3/CCL22/CX3CL1/CXCL1/CXCL9/CXCL12/C XCL6
GO: Molecular Functions	GO:0008009: Chemokine activity	CCL22/CX3CL1/CXCL1/CXCL9/CXCL12/ CXCL6

Previously thought to be non-immunogenic, NSCLC was found highly responsive towards immune checkpoint inhibitors (ICIs). Even with the significant advancements, the immune-related mechanism in NSCLC remains unclear. Understanding these mechanisms such as the tumor microenvironment, innate and adaptive immune interaction and identifying the additional checkpoint targets, provide a groundwork for identifying responsive biomarkers in NSCLC (*Tina et al., 2021*). Thus, focusing on that, it was identified that, out of the top 30 GO terms, there were 19 GO terms which were substantially correlated with the immune regulation process, signifying their regulatory roles within NSCLC (**Table 5.1**).

GO:1903706, describes the regulation of hemopoiesis process, a biological process responsible for the generation of blood cells through the prolific proliferation of stem cells (*Madhumita and Leonard, 2013*). This regulatory mechanism occurs within the stromal environment and is facilitated by cell-cell interaction. In the context of our study, *Hong et al.* have identified a significant link between the process clonal hemopoiesis (CH) and the initiation of lung cancer, a finding of significant interest. Factors such as genetic variations and tobacco consumption contribute to the mutational landscape of clonal hemopoiesis which elucidates the associated risk of developing lung cancer (*Wei et al., 2022*). GO:0022407, depicts the regulation of cell-cell adhesion. Disruptions in cell-cell adhesion involving adherens junctions like E-cadherin contributes to invasive behavior (*Alisha et al., 2018*). Adhesion molecules influences malignancy and metastasis by driving motility and undergo functional shifts due to factors such as alterations in binding partners and interaction with integrins (*Heidi et al., 2013*). In NSCLC, the intercellular cell adhesion molecule-1 (ICAM-1) is identified in most of the histological subtypes of NSCLC, except for squamous cell carcinomas often lacking

ICAM-1 expression (Axel *et al.*, 2020). The focus on the complex interaction between cell adhesion, immune system and tumors, highlights the anti-tumor effort that primarily targets the immune cell's ability in engaging with the malignant cells and adhesion molecules, positioning them as a viable target for cancer immunotherapy (Heidi *et al.*, 2019). GO:0030217, describes the differentiation of T-cells; In NSCLC, T-cell differentiation is predominant determinant in shaping the immune environment, as elucidated in a study by Ghorani *et al.* The study revealed a critical association between Tumor Mutational Burden (TMB), known for its role as a secondary marker for genetic alterations in tumor cells, and the modulation of T-cells. Apparent changes in the T-cell population are bought by increased TMB. Interestingly, the phenotypic characteristics of these dysregulated T-cell subgroups are comparable to those of tumor neoantigen-responsive T-cells. This differentiation pattern is associated with poor patient outcomes, suggesting lower survival rates in lung cancer patients. T-cell differentiation is therefore evident as an initial factor in the pathophysiology of NSCLC (Ehsan *et al.*, 2020). GO:0030098, explains the regulation of lymphocytes differentiation. According to a study by Wasselius *et al.*, 1987, patients with NSCLC exhibit altered cellular immune function, as evidenced by a reduction in circulating lymphocytes, which further shows a subsequent reduction in T-helper and T-suppressor cells. Moreover, in advanced NSCLC, there was a significant decrease identified in B lymphocytes. Understanding this function of lymphocytes, particularly tumor infiltrating lymphocytes, is vital for the prognosis in NSCLC patients (Roy *et al.*, 2016). GO:1902105, describes the regulation of leukocyte differentiation; Leukocytes plays a crucial role in NSCLC. They serve as indicators of the disease through differential gene expression. The unique characteristics of leukocytes make them a potential diagnostic tool, offering insights into the early detection and potential therapeutic strategies for NSCLC (Limin *et al.*, 2021). One of the leukocytes, neutrophils is known to have both anti- and pro-tumor function, for e.g. Eosinophils. The prognostic effects of blood biomarkers including WBCs have been implicated in many cancer types including NSCLC (Sibille *et al.* 2021). GO:0071219, describes the cellular response to molecule of bacterial origin. In NSCLC, the cellular response to molecules of bacterial origin is a relevant aspect in the search for new therapeutic compounds. Bacterial-derived molecules, such

as those from plants, bacteria and fungi, were studied for their anti-cancer activities. These were known to trigger specific cellular responses such as anti-tumor effects. Understanding these bacterial-derived molecules and cellular response is necessary for the innovative treatment approaches with minimal side-effects (*Kamila et al., 2023*). GO:0005667, describes the transcription regulator complex, which is significant in the understanding of regulatory landscape within NSCLC. A study revealed crucial transcription factors including E2F3, FHL2 and so on, co-expressed in NSCLC. These factors were identified through a gene regulatory network (GRN) analysis, holding a potential as biomarkers for tumor development (*Beatriz et al. 2022*). GO: 0071216, depicts the cellular response to biotic stimuli. In response to biotic stimuli, cells activate intricate signaling pathways involving gene expression changes, immune response modulation, and cell communication. This dynamic cellular response is crucial for recognizing and defending against pathogens, thereby highlighting the interaction between the immune system and cellular processes in the face of biotic challenges (*Miao et al. 2021*). A study by *Menghao et al., 2020* highlighted the use of a nanoparticle in siRNA delivery for NSCLC. The nanoparticle enhanced the cellular response to biotic stimuli. This provided a strategy for targeted siRNA delivery to address NSCLC. Another study highlighted the role of immune cells such as M1 Macrophages, which in response to biotic stimuli, such as tumor-related antigens, gets activated. These changes in response to biotic stimuli holds promise in the field of personalized treatment for NSCLC patients (*Si-Fan et al., 2023*). Another GO term, GO:0071222 describes the cellular response to lipopolysaccharide (LPS). In the context of cancer, TLR4 activation, induced by LPS, has been implicated in promoting cancer growth and metastasis. Additionally, LPS-induced TLR4 signaling has been linked to adverse effects in various cancers, underscoring the intricate interplay between bacterial infections and immune responses in cancer development. A study has demonstrated the anti-inflammatory and anti-tumor effects in NSCLC cells when exposed to LPS, through a natural compound, which showed inhibition in cell growth, migration and modulation key markers (*Yen-sung et al., 2020*). The term GO:1902911 and GO:1902554 describes the protein kinase complex and serine threonine kinase complex respectively. The human genome encodes 538 protein kinases which plays a

crucial role in many cellular processes. In tumor cells, they are known for the initiation and progression of these abnormal cells (*Khushwant et al., 2018*). Many small-molecule kinase inhibitors have been developed for therapeutic effect against cancer. Many studies have highlighted the presence of Protein kinase C (PKC) that has been highly implicated in NSCLC. A study by Mohammed et al., has highlighted the role of PKC-alpha (PKC- α) that exhibits aberrantly high expression and regulation of mTORC1 and Akt signaling pathways in EGFR-mutant NSCLC cells. Inhibition of PKC- α in EGFR-mutant NSCLC cells leads to apoptosis, suggesting them as a potential target for personalized treatment for NSCLC (*Mohamed et al., 2019*). Akt a.k.a Protein Kinase B, a serine/threonine kinase, is a crucial component in regulating processes such as cell proliferation, growth and metabolism. The Akt pathway is frequently dysregulated in various cancers including NSCLC, and it plays a significant role in promoting tumor cell survival and growth (*Se-kyung et al., 2021*). The term GO:0031983 and GO:0060205 corresponds to the vesicle lumen and cytoplasmic vesicle lumen. In cancer, alterations in endocytosis contribute to dysregulated signaling. The cytoplasmic vesicle lumen, where internalized proteins undergo sorting for degradation or recycling is influenced by aberrant mechanisms driven by oncogenic factors like p53 and Ras, impacting cancer cell motility and receptor dynamics. Specifically in NSCLC, the cytoplasmic vesicle plays a crucial role in the endocytic regulation of receptor tyrosine kinases (RTKs) like EGFR and c-Met. Dysregulation of endocytosis-related mechanisms such as ubiquitination or degradation within the cytoplasmic vesicle, contributes to enhanced signaling and tumorigenesis (*Ira and Yosef, 2019*). The term GO:0034774 corresponds to the secretory granule lumen. Many studies have highlighted the significance of endocrine secretory granules and the regulatory influence of key factors in their formation within the lung cancer cells. GO:0061695 term defines the transferase complex, transferring phosphorus- containing groups. A study by *Zhixiao et al., 2021*, has implicated the significant enrichment of changes in cellular components (CC) related to the transferase complex and transferring- phosphorous-containing groups through the GO analysis study. This suggested a potential involvement of these molecular functions in the pathogenesis of lung adenocarcinoma, a subtype of NSCLC. The GO terms GO:0005126 and GO:0005125 describes the cytokine receptor binding

and cytokine activity respectively. In cancer, including NSCLC, cytokines are known to play a crucial role in modulating the tumor microenvironment. Cytokines and their receptors mediate signaling pathways that can either promote or inhibit tumor growth, angiogenesis, and metastasis. Dysregulation of cytokine signaling, in NSCLC, have shown its immunosuppressive properties, impacting the pro and anti-tumor responses. Several cytokines including IL-6/10, and IFNs influences tumor progression and also the elevated levels of pro-inflammatory cytokines, including TNF- α and chemokines, contributes to the complex microenvironment impacting the NSCLC growth and metastasis (*Yinghao et al., 2023*). Another two GO terms, GO:0048018 and GO:0030546, corresponds to receptor ligand activity and signaling receptor activator activity in cancer, receptor-ligand interactions play a pivotal role in regulating cellular characteristics. Aberrant activation of these signaling receptors, such as those associated with tyrosine kinase activity or seven-transmembrane domains, can lead to uncontrolled cell growth, survival and differentiation. Dysregulated receptor activator activity, influenced by ligands, contributes to oncogenic signaling pathways (*Carl et al., 2020*). A study by *Godin et al., 2007*, highlighted the role of Epidermal growth factor receptor EGFR in NSCLC by mediating the signaling functions. The activation of specific mutations was shown to heighten the EGFR activity, rendering NSCLC sensitive to tyrosine kinase inhibitors (TKIs). This understanding could contribute to the adverse clinical outcomes in TKI-resistant NSCLC. GO:0140297 and GO:0070491 describes the DNA binding transcription factors and Repressing transcription factor binding. In cancer, including NSCLC, DNA binding transcription factors influences critical cellular processes by regulating gene expression. These factors can either promote or suppresses the cancer progression. Dysregulation of these factors play a pivotal role in the molecular mechanisms underlying progression in NSCLC (*Beatriz et al., 2023*). Another significant GO terms, GO:0045236, GO:0042379, and GO:0008009 describes the CXCR chemokine receptor binding, Chemokine receptor binding, and Chemokine activity. NSCLC exhibits active angiogenesis and a complex microenvironment with neoplastic, stromal and inflammatory infiltrate cells. Chemokines and their receptors, in general, play a crucial role in lung tumorigenesis, progression, and metastasis (*Selma et al., 2015*). CXCRs (chemokine receptors),

particularly CXCR1 and CXCR2, play a crucial role in NSCLC by binding to chemokines like CXCL1, CXCL2, CXCL3, and CXCL8. These interactions contribute to the regulation of cancer cell proliferation, migration, and survival. Elevated expression of these chemokines and their receptors suggests their potential as biomarkers in NSCLC (Saumya *et al.*, 2022). The last term GO:0008083 describes the Growth factor activity. Growth factors play a crucial role in NSCLC by regulating various cellular processes such as proliferation, survival and invasiveness. The insulin-like growth factor (IGF) pathway, including IGF-1R, has been identified as a potential therapeutic target in NSCLC (Mary *et al.*, 2011). Additionally, other growth factors like epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) contribute to the complex signaling network in NSCLC, influencing tumor progression (Gillian *et al.* 2010, Yueshui *et al.* 2022, Xiaowei *et al.*, 2021 and Helen 2020).

KEGG Pathways Associated with NSCLC

19 KEGG pathways were extracted in this study, which were linked to the onset and advancement of NSCLC (**Table 5.2**).

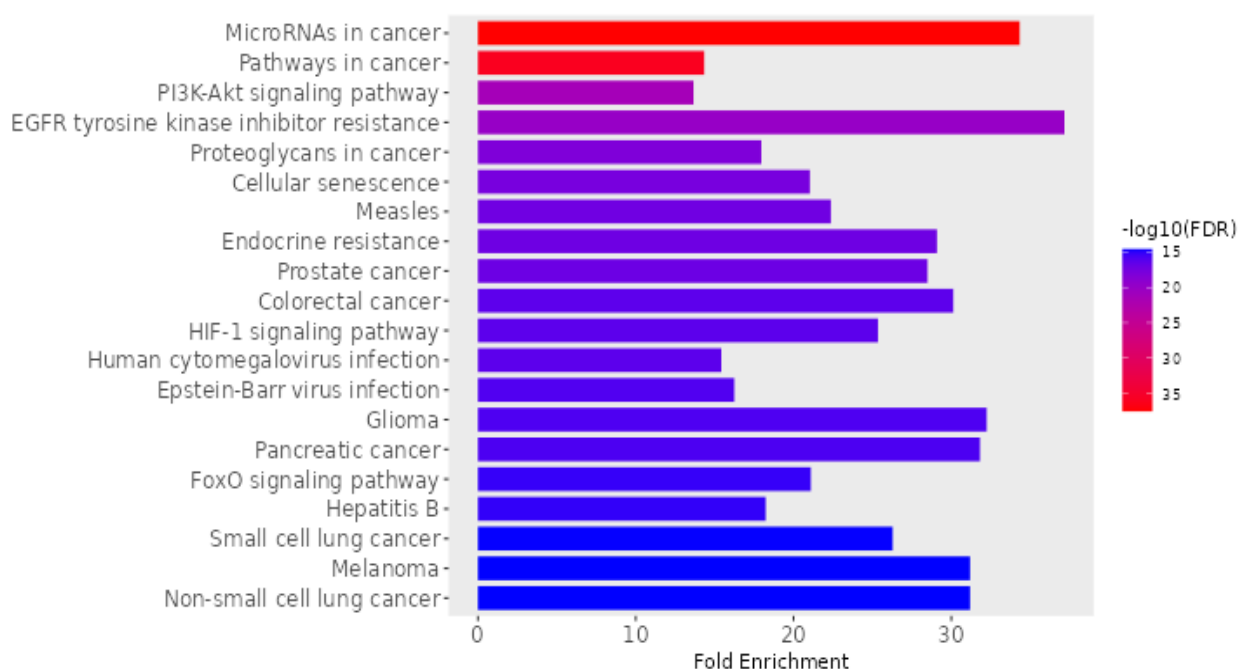


Fig 5.9. Top 20 enriched pathways from KEGG analysis. The y-axis shows the fold enrichment of microRNAs in that cancer type compared to normal tissue. The x-axis shows the false discovery rate (FDR) for the enrichment analysis.

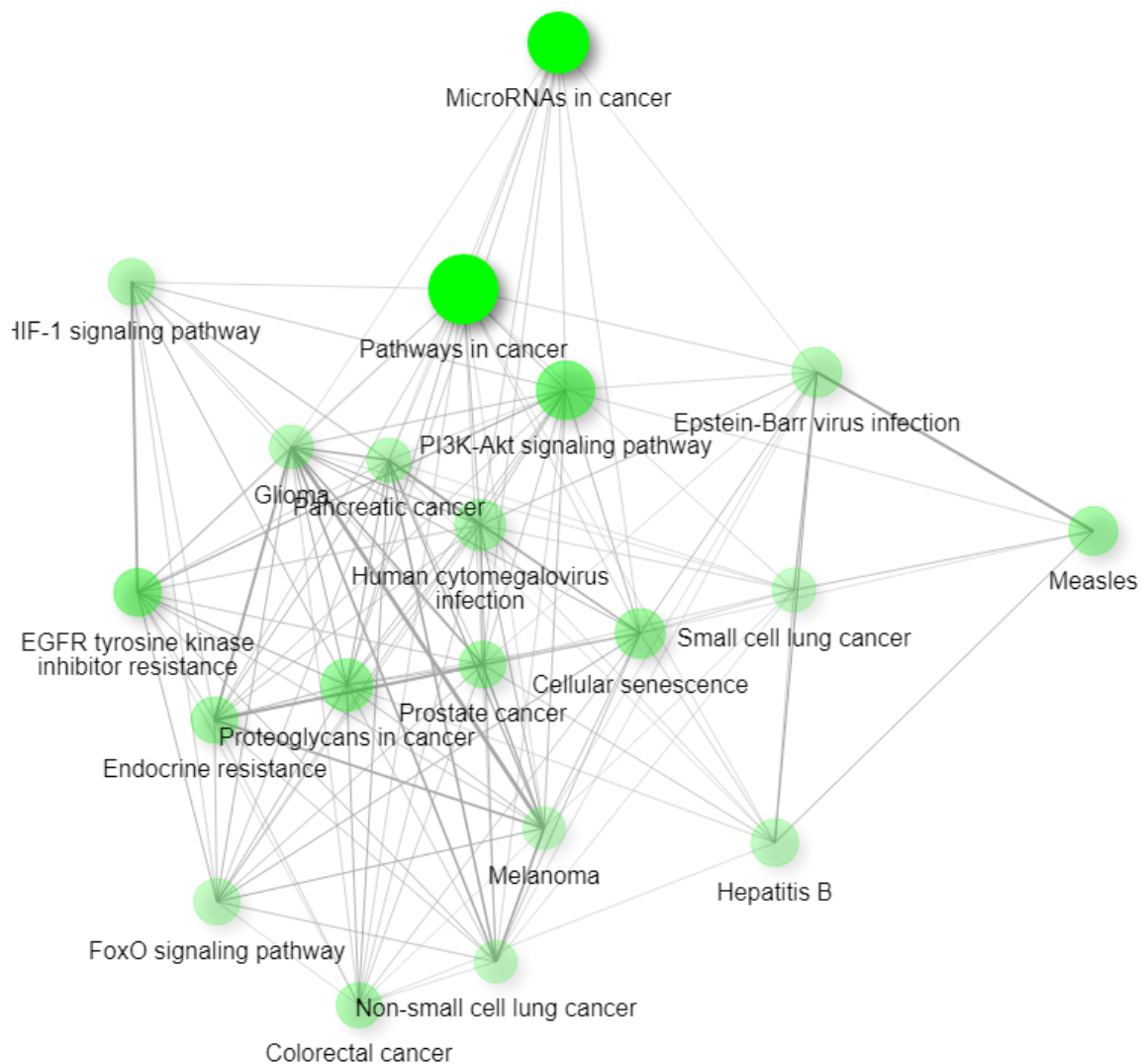


Fig 5.10. ShinyGO functional enrichment network for the target genes. Nodes represent enriched molecular functions. Size of node represents the number of genes involved in a function. Opacity relates to the significance of the enrichment. Edges between nodes represent overlap in gene membership, with thicker edges representing higher gene overlap between nodes.

Table 5.2. Top 20 Enriched Pathways from KEGG Analysis

TERM	DESCRIPTION	GENES
hsa05206	MicroRNAs in cancer	STAT3/ZEB1/EGFR/PLCG1/PIK3CB/BCL2/HDAC1/SIRT1/CCND1/CDK6/CDC25A/E2F3/MYC/MMP9/FSCN1/KRAS/ZEB2/MAPK1/FOXP1/BMF/TIMP3/PTEN/SOCS1/CCNE1/CND2/VEGFA/SOX4/MTOR/FGFR3/DNMT3A/MCL1/RHOA/MDM2
hsa04151	PI3K-Akt signaling pathway	IL6R/EGFR/PIK3CB/BCL2/CCND1/CDK4/CDK6/MYC/HGF/IL6/LAMA4/KRAS/PHLPP1/PHLPP2/MAPK1/PTEN/TLR4/IL2/CCNE1/CCND2/VEGFA/FOXO3/IGF1/AKT1/IGF1R/MTOR/FGFR3/MCL1/MDM2
hsa01521	EGFR tyrosine kinase inhibitor resistance	IL6R/STAT3/EGFR/PLCG1/PIK3CB/BCL2/HGF/IL6/KRAS/MAPK1/PTEN/VEGFA/FOXO3/IGF1/AKT1/IGF1R/MTOR/FGFR3
hsa05205	Proteoglycans in cancer	STAT3/EGFR/PLCG1/PIK3CB/CCND1/MYC/HGF/MMP9/SMAD2/KRAS/MAPK1/TIMP3/TLR4/VEGFA/TGFB1/TNF/IGF1/AKT1/IGF1R/MTOR/RHOA/MDM2
hsa04218	Cellular senescence	PIK3CB/SIRT1/CCND1/CDK4/CDK6/CDC25A/E2F3/MYC/IL6/SMAD2/KRAS/MAPK1/HIPK2/PTEN/CCNE1/CND2/FOXO3/TGFB1/AKT1/MTOR/MDM2
hsa05162	Measles	STAT3/CD3E/PIK3CB/TAB2/NFKBIA/BCL2/CCND1/CDK4/CDK6/MYD88/IL6/TLR4/IL2/CD28/CCNE1/CCND2/AKT1/APAF1
hsa01522	Endocrine resistance	EGFR/PIK3CB/BCL2/CCND1/CDK4/E2F3/MMP9/JAG1/JAG2/KRAS/MAPK1/IGF1/AKT1/IGF1R/MTOR/MDM2
hsa05215	Prostate cancer	ZEB1/EGFR/PIK3CB/NFKBIA/BCL2/CCND1/E2F3/MMP9/KRAS/MAPK1/PTEN/CCNE1/IGF1/AKT1/IGF1R/MTOR/MDM2
hsa05210	Colorectal cancer	EGFR/PIK3CB/BCL2/CCND1/MYC/SMAD2/KRAS/MAPK1/TGFB1/AKT1/MTOR/MLH1/MSH2/MSH6/RHOA

hsa04066	HIF-1 signaling pathway	IL6R/STAT3/EGFR/PLCG1/PIK3CB/BCL2/LDHA/IL6/MAPK1/TLR4/VEGFA/EDN1/IGF1/AKT1/IGF1R/MTOR/HK2
hsa05163	Human cytomegalovirus infection	IL6R/STAT3/EGFR/PIK3CB/NFKBIA/CCND1/CDK4/CDK6/E2F3/MYC/CX3CL1/IL6/KRAS/MAPK1/VEGFA/TNF/AKT1/MTOR/CXCL12/RHOA/MDM2
hsa05169	Epstein-Barr virus infection	STAT3/CD3E/PIK3CB/TAB2/NFKBIA/BCL2/HDAC1/CCND1/CDK4/CDK6/E2F3/MYC/MYD88/IL6/CCNE1/CCND2/TNF/AKT1/APAF1/MDM2
hsa05214	Glioma	EGFR/PLCG1/PIK3CB/CCND1/CDK4/CDK6/E2F3/KRAS/MAPK1/PTEN/IGF1/AKT1/IGF1R/MTOR/MDM2
hsa05212	Pancreatic cancer	STAT3/EGFR/PIK3CB/CCND1/CDK4/CDK6/E2F3/SMAD2/KRAS/MAPK1/VEGFA/TGFB1/AKT1/MTOR
hsa04068	FoxO signaling pathway	STAT3/EGFR/PIK3CB/SIRT1/CCND1/IL6/KRAS/MAPK1/PTEN/CCND2/FOXO3/TGFB1/IGF1/AKT1/IGF1R/PLK1/MDM2
hsa05161	Hepatitis B	STAT3/PIK3CB/TAB2/NFKBIA/BCL2/E2F3/MYC/MMP9/MYD88/IL6/KRAS/MAPK1/TLR4/CCNE1/TGFB1/TNF/AKT1/APAF1
hsa05222	Small cell lung cancer	PIK3CB/NFKBIA/BCL2/CCND1/CDK4/CDK6/E2F3/MYC/LAMA4/CKS1B/PTEN/CCNE1/XIAP/AKT1/APAF1
hsa05218	Melanoma	EGFR/PIK3CB/CCND1/CDK4/CDK6/E2F3/HGF/KRAS/MAPK1/PTEN/IGF1/AKT1/IGF1R/MDM2
hsa05223	Non-small cell lung cancer	STAT3/EGFR/PLCG1/PIK3CB/CCND1/CDK4/CDK6/E2F3/HGF/KRAS/MAPK1/FOXO3/ AKT1

1. *Pathways Describing Tumor Subtypes*: Among the 19 KEGG pathways, 10 KEGG pathways describe the regulatory network of a specific cancer subtype. KEGG pathway hsa05206 describes the common miRNA regulatory networks that transcends specific cancer subtypes. By focusing on miRNAs, which plays a crucial role in post-transcriptional gene regulation, the molecular features contributing to cancer initiation and progression can be uncovered. The intricate interplay between miRNAs and functional genes underscores the significance of pan-cancer analyses in understanding the common molecular pathways with implications for cancer migration, invasion, and overall progression. miRNA regulatory pathways play a significant pro- and anti-tumor role in NSCLC (*Sharif et al., 2022*).

Another KEGG pathway hsa05205, describes the proteoglycans in cancer. Proteoglycans play a significant role in cancer biology by contributing to various aspects of tumor growth, invasion, and metastasis. They interact with cell surface receptors, growth factors, and extracellular matrix components, influencing cell signaling pathways that regulate cell proliferation, migration, and survival. Additionally, proteoglycans can modify the tumor microenvironment, affecting immune cell infiltration, angiogenesis, and the development of drug resistance. Overall, proteoglycans influence the tumor progression and therapeutic responses by modulating cellular interactions, signaling pathways, and the tumor microenvironment (*Theresa et al., 2020*).

With respect to NSCLC, a study conducted by Gao et al. provided information about the central roles of TGF- β in NSCLC progression through their role in modulation of Decorin, a stromal proteoglycan produced by extracellular matrix (*Y Gao et al., 2019*). Another study by *Yi-Chieh et al.* provided revelations regarding Endocan's role as a receptor kinase ligand enhancer and its impact on EGFR-driven NSCLC progression suggest a potential connection to proteoglycans. Given the intricate molecular interactions within the NSCLC tumor microenvironment, including the involvement of proteoglycans in cancer pathways, understanding the regulatory axis of Endocan-EGFR sheds light on novel therapeutic opportunities to target proteoglycans-associated mechanisms in NSCLC, particularly in the context of TKI-resistance (*Yi-Chieh et al., 2020*)

2. *Pathways related to specific cancer subtypes*: In addition to their involvement in pan-cancer pathways, these molecular pathways have been linked with specific cancer subtypes. hsa05212 describes the regulatory network and pathways of pancreatic cancer. A study by *Hang et al.* have explored the relation of the specific biological processes associated with pancreatic cancer and NSCLC. The tumor associated factors and the functional pathways they participate in, have signified their relation. It has been identified that KRAS (Kirsten rat sarcoma viral oncogene homolog), TP53 (Tumor protein p53) have been reported to contribute to the progression of NSCLC (*Hang et al., 2016*).

KEGG pathway hsa05215 describes prostate cancer. It is known that the synergistic effects of β -catenin stabilization (present in Wnt pathway), KRAS activation, and PTEN (Phosphatase and tensin homolog) loss accelerate prostate cancer progression through increased mTORC1 (Mammalian target of rapamycin complex 1) activity. Moreover, APC loss (Adenomatous polyposis coli loss), which commonly occurs in prostate cancer, also collaborates with MYC overexpression and PTEN or p53 loss to enhance metastatic potential. Notably, APC mutations are also found in NSCLC, implying a common molecular pathway driving therapeutic resistance and disease progression in both malignancies (*Sarah et al., 2022*).

The KEGG pathway, hsa05210, describes the pathways associated with colorectal cancer. In colorectal cancer (CRC) and NSCLC, there were common dysregulated metabolic pathways with tumor progression, including CXC group of genes. These pathways, such as metabolism-related processes, underscore the interconnected nature of cancer development across different types, reflecting disruption in physiological metabolism during tumor advancement. Both CRC and NSCLC share dysregulated KEGG pathways related to cell cycle regulation, DNA replication, and cell division, indicating common molecular processes driving tumorigenesis in these cancers (*Chunyan et al., 2020 and Chunliang et al. 2019*).

In Glioma associated pathways (hsa05214), EGFR amplification and mutations are common, which leads to hyperactivation of downstream signaling cascades like PI3K-Akt, further promoting cell proliferation and survival. The EGFR pathway and PI3K-Akt pathway are known to be dysregulated in both Glioma and NSCLC. In NSCLC, EGFR mutation are key drivers, activating the PI3K-Akt pathway and contributing to tumorigenesis. Targeting these pathways with inhibitors like EGFR-tyrosine kinase inhibitors (TKIs) or PI3K inhibitors is a therapeutic strategy in both cancers, emphasizing shared molecular pathways despite their distinct tissue origins (*He-Fen et al., 2022*).

Small cell lung cancer (SCLC) and NSCLC are the main subtypes of lung cancer. A study by Yin et al. revealed pathways associated with SCLC (hsa05223) and NSCLC. There were crucial pathways involving cell cycle regulation, DNA replication, and mitotic spindle organization, highlighting common pathways driving lung cancer progression (*Yi yin et al., 2022*).

The pathways associated with the shortest path genes in NSCLC include signal transduction, cell cycle regulation, apoptosis, DNA damage repair, and cholesterol metabolism. In SCLC, the pathways are related to extracellular matrix (ECM) interactions, integrin signaling, cell cycle regulation, and apoptosis resistance (*Bi-Qing et al., 2013*).

Through KEGG analysis of NSCLC DEGs, one of the emerged significant pathways were identified were the Melanoma pathway (hsa05218), indicating potential molecular similarities between NSCLC and melanoma in terms of pathway activation. Both melanoma and NSCLC share mutations affecting major signaling pathways, including the MAPK (Mitogen-activated protein kinase) pathway.

In melanoma, the RAS/RAF/MEF/ERK pathway regulates vital cellular processes like growth, migration, and apoptosis. Similarly, In NSCLC, MAPK pathway dysregulation was identified, impacting cell proliferation, differentiation, and apoptosis, thus influencing tumor progression and prognosis. The common aberrant MAPK signaling in both cancers suggests potential biomarkers for predicting progression and

therapeutic targets, highlighting shared molecular mechanisms and therapeutic strategies between melanoma and NSCLC.

Both melanoma and NSCLC, also involve the PI3K pathway, crucial for regulating cell cycle, survival, and migration. Targeting this pathway with specific inhibitors shows promise for novel anticancer therapies in NSCLC, similar to approaches in melanoma treatment (*Jyoti et al. 2021, Rajesh et al., 2019 and Aaron 2020*).

3. *Signaling pathways associated with tumorigenesis:* In our analysis, the PI3K pathway (hsa04151) emerged as a significantly enriched KEGG pathway in NSCLC. This pathway is also implicated in other cancers like melanoma. The shared involvement of the PI3K pathway underscores a potential relationship and common signaling mechanisms between the cancer types. It further opens up avenues for further investigation into their interplay and strategies aimed at targeting this pathway. The PI3K-Akt-mTOR pathway plays a crucial role in NSCLC, contributing to tumor progression, resistance to EGFR-TKIs, enhanced cell survival, altered metabolism, and angiogenesis (*Jiang et al., 2022*).

Another KEGG pathway hsa04066, describes the HIF-1 α pathway is a critical survival mechanism in cancer, associated with metastasis, angiogenesis, poor prognosis, and so on. Despite its challenges, targeting HIF-1 α has shown promising progress in cancer therapy due its adaptive response to hypoxic stress and essential role in tumor biology (*Jonathan et al., 2017*). Hypoxia, a common feature in lung cancer, leads to resistance against conventional treatments. It is also known to promote metastasis. The activation of HIF-1 α signaling pathway under hypoxic conditions is pivotal in driving these effects, particularly in EGFR mutation-positive NSCLC where it contributes to the emergence of resistance cancer stem cells. Therefore, targeting this pathway could improve the sensitivity of lung cancer cells to EGFR-TKIs (*Qian et al., 2019*).

Another KEGG pathway hsa04068 describes the FOXO signaling pathway, which is integral to cellular regulation, acting as transcription factors that control gene expression related to crucial cellular functions such as differentiation, apoptosis, proliferation, DNA damage response, and oxidative stress management. Dysregulation of the FOXO proteins could thereby promote the progression of tumors. They play various roles within the normal cellular physiology and diseased state, with their activity regulated by post-translational modifications and interactions with various signaling pathways like PI3K-Akt signaling pathway. Activation of Akt phosphorylates FOXOs, leading to their cytoplasmic localization and inhibition of their tumor-suppressive functions (*Mohd et al., 2017*).

The FOXO signaling pathway plays a multifaceted role in NSCLC, influencing the tumor progression and metastasis. Histopathological studies link low FOXO expression to increased metastasis, while FOXO1 and FOXO3 are implicated in suppressing cell migration and metastasis in multiple malignancies. However, conflicting evidence suggests that high FOXO expression correlates with poor prognosis and enhanced metastasis in some cases. Genetic alterations involving FOXOs, such as chromosomal translocations in alveolar rhabdomyosarcoma and mixed lineage leukemia, result in oncogenic fusion proteins and FOXO locus haploinsufficiency, contributing to cancer development. Despite rare mutations, dysregulated FOXO expression and activity are predominant mechanisms in NSCLC, affecting key pathways and promoting cellular processes such as migration, invasion, and survival. Understanding the intricate role of FOXOs in NSCLC therefore provides insights into identifying potential targets to modulate the FOXO signaling to combat cancer progression (*Yannasittha and Eric, 2020*).

4. *Pathways that may participate in tumorigenesis:* Cellular senescence is a complex biological process characterized by irreversible growth arrest and plays a critical role in cancer and aging. It is known to play a protective role in preventing cancer initiation and maintaining tissue integrity, its accumulation effects, particularly through SASP (senescence associated secretory phenotype), can have detrimental consequences leading to age-related diseases and cancer progression (*Ruchi and Parmjit, 2021*).

In the context of NSCLC, KEGG pathway analysis performed in this study has shown the involvement of cellular senescence associated pathways (hsa04218). It is known to play a significant role in pathogenesis and progression of NSCLC, contributing to its complexity and treatment challenges. Key genes and pathways implicated in cellular senescence in NSCLC include TP53, CDKN2A, and telomerase, with mutations in these genes affecting DNA damage response, cell cycle regulation, and genomic stability. Additionally, senescent cells in NSCLC secrete pro-inflammatory factors collectively known as SASP, which includes cytokines like IL-6 and IL-8 associated with poor prognosis.

In NSCLC, the interplay between cellular senescence and the immune system, influence the tumor microenvironment dynamics and treatment outcomes. Senescent cells can promote immunosuppressive cell infiltration, leading to immune evasion and resistance to immunotherapy. However, inducing senescence can also promote anti-tumor immune responses and improve immunotherapy efficacy. Targeting SASP components and immune checkpoints, alongside specific drugs that eliminate senescent cells, emphasizes the need for further mechanistic understanding and biomarker validation to enhance clinical outcomes (*Huangkai et al., 2023*).

5. *Resistance based pathways affecting tumorigenesis*: NSCLC is influenced by mutations affecting various genes, including the EGFR, among others. The KEGG pathway hsa01521 describes the EGFR tyrosine kinase inhibitor (EGFR-TKI) resistance. These mutations contribute to the development and progression of NSCLC, influencing treatment responses. Many EGFR-TKIs have been effectively used for clinical treatment. However, patients eventually develop drug resistance, which is inevitable due to various mechanisms. These include the emergence of secondary mutations, activation of alternative pathways such as c-Met (cellular-mesenchymal to epithelial transition factor), HGF (hepatocyte growth factor), and AXL receptor tyrosine kinase, aberrance of downstream pathways including K-RAS mutations and loss of PTEN, impairment of the EGFR-TKIs-mediated apoptosis pathway and so on. Understanding these could present a strategy to overcome EGFR-TKIs resistance in NSCLC (*Carmelo et al., 2023*).

Resistance to EGFR-TKIs in NSCLC involves intrinsic and acquired mechanisms. Intrinsic resistance, characterized by immediate inefficacy of EGFR-TKIs, is often associated with non-sensitive EGFR mutations. These mutations alter the binding affinity of EGFR for TKIs, leading to reduced drug efficacy. HER2 (human epidermal growth factor 2) mutations, occurring in 2% of NSCLC patients, can confer EGFR-TKI resistance. HER3, another member of the HER family, is also identified in contributing TKI resistance by maintaining PI3K-Akt signaling pathway. Acquired resistance develops after prolonged EGFR-TKIs treatment and is mainly driven by secondary mutations, phenotypic transformations, activation of alternative pathways such as MET, IGF-1R (insulin-like growth factor 1 receptor), and so on. These lead to the reduced drug binding efficacy, thereby promoting cell survival and proliferation (*Florian et al. 2016*).

The KEGG pathway, hsa01522, describes the endocrine resistance. In breast cancer, various mechanisms contribute to endocrine resistance. These include loss of estrogen receptor (ER) expression, epigenetic changes like DNA methylation and histone deacetylation, mutations in ER, dysregulation of signal transduction pathways such as PI3K/Akt, NF- κ B (nuclear factor-kappa B), and so on. Additionally, miRNAs such as miR-200 family, also contributed to resistance.

To relate these mechanisms to NSCLC, we can consider the commonalities and differences in the context of endocrine resistance. While NSCLC and breast cancer are distinct in their tissue origins and specific signaling pathways involved, there are certain similar mechanisms which can be compared. For instance, in NSCLC, similar pathways like PI3K/AKT and EGFR signaling are known to contribute to resistance against targeted therapies, including TKIs. Similar to breast cancer, mutations in key signaling proteins, dysregulation of growth factor receptor, and alterations in apoptotic pathways can lead to resistance in NSCLC. Moreover, epigenetic changes such as DNA methylation and histone modifications have also been implicated in both breast cancer and NSCLC resistance mechanisms. These changes can affect gene expression patterns, including those related to drug sensitivity or resistance (*Rocio et al., 2012*).

6. *Infection-Related Pathways Affecting Tumorigenesis*: Recombinant measles virus (MeV) has emerged as a promising oncolytic virus in cancer therapy due to its genetic adaptability. MeV variants, tailored for specific tumor targets, exhibit potent oncolytic activity while maintaining safety profiles, as demonstrated in preclinical and clinical studies. The KEGG pathway, hsa05162, describes the Measles-associated pathway. In multiple myeloma and ovarian carcinoma, MeV-based therapies has shown significance in revolutionizing the cancer treatment by offering targeted and effective virotherapy options (Gemma et al., 2020).

In the context of lung cancer, the use of recombinant measles virus as an oncolytic agent is a promising approach. Studies have shown that this virus has anti-tumor effects on canine lung cancer cells, indicating its potential in virotherapy for lung cancer. The virus is designed to preferentially infect and amplify in tumor cells, leading to cell death and potentially inducing an anti-cancer immune response. Furthermore, certain immune-related genes led to the increased expression in response to the recombinant measles virus-based therapy, suggesting an activation of the immune system against tumor cells. Understanding the mechanisms of viral replication and the immune response in lung cancer cells can provide valuable insights for developing novel virotherapy strategies and improving treatment outcomes in both veterinary and human medicine (Kei et al., 2023).

Another KEGG pathway, hsa05163, describes the human cytomegalovirus- associated pathway. Human cytomegalovirus (HCMV) has garnered attention for its potential role in cancer progression, with studies revealing oncogenes in its genome linked to various cancers. Clinical trials are exploring HCMV as a therapeutic target, particularly in glioblastoma. However, understanding the precise mechanisms and specific genes contributing to cancer progression by HCMV remains a research challenge, highlighting the need for further investigation (Chuan et al., 2023).

Recent research has highlighted a potential association between HCMV and lung cancer, particularly in cases with specific gene mutations. The presence of HCMV has been observed in a notable percentage of lung cancer samples, suggesting a possible

role of this virus in lung tumorigenesis. Furthermore, studies have indicated higher levels of HCMV infection in lung adenocarcinomas with certain gene mutations compared to those without these mutations. This correlation underscores the need for further investigation into how HCMV may influence the development and progression of lung cancer, especially in the context of specific genetic alterations that could impact viral interactions within tumor cells (*Suzana et al., 2023*).

Certain viruses have been linked to lung cancer, including HCMV. Studies have shown a higher prevalence of HCMV infection in lung cancer samples compared to healthy individuals. The presence of HCMV in lung cancer cells could suggest a potential role in tumorigenesis, although the exact mechanisms are not fully understood. HCMV may interact with cellular signaling pathways, potentially promoting cancer cell growth and survival. However, further research is needed to elucidate the specific relationship between HCMV infection and lung cancer development (*Marie et al., 2023*).

The Epstein-Barr virus (EBV) associated pathway (hsa05169) was also identified as a significant pathway identified in our study. EBV has been associated with various types of cancer, including lung cancer. EBV belongs to the herpesvirus family and is transmitted through salivary contact. Following primary infection, EBV can establish a latent infection in memory B cells, potentially leading to reactivation during periods of stress or immunosuppression (*Pragnesh et al., 2022*). A study by *Chen Y et al. 2023*, investigated the potential association between EBV infection and lung cancer risk, to understand the development of lung cancer. By uncovering this association, this study hopes to provide new insights and targets for preventing, diagnosing, and treating lung cancer.

Another study by *Julia et al.* explored the interaction between EBV and lung cancer involving several potential mechanisms. They provided information about how tobacco smoke components could facilitate EBV latency establishment in lung epithelial cells, how it impairs the immune response, making lung epithelial cells more susceptible to EBV infection, furthermore, lung cancer cells may enhance EBV entry through increased expression of CD21, a receptor that could promote EBV infection. Overall,

these interactions between EBV, tobacco smoke, and so on, plays a complex role in the development and progression of lung cancer (*Julio et al., 2022*).

Our study also identified Hepatitis B (hsa05161) as a significant pathway. The relationship between hepatitis B virus (HBV) and lung cancer through various pathways, including mutagenesis of cancer-related genes, activation of cellular responses contributing to carcinogenesis, and epigenetic changes targeting tumor suppressor genes. HBV-related lung cancers often exhibit chromosomal alterations, mutations in key genes like p53, and activation of oncogenic pathways like WNT/ β -catenin (*Massimo and Jessica, 2016*).

The relationship between HBV infection and lung cancer has been studied to assess the risk of HBV reactivation in lung cancer patients receiving treatment, particularly with TKIs. Among patients with positive hepatitis B core antibodies (anti-HBc), HBsAg-positive individuals showed similar rates of HBV reactivation whether treated with chemotherapy or TKIs. However, HBsAg-negative TKI users had a lower risk of HBV reactivation. The main independent risk factor for HBV reactivation in TKI users was HBsAg positivity. These mechanisms could be explored for studying the progression of lung cancer, but further investigation is needed for understanding the prognosis of NSCLC (*Pro H-sin, 2023*).

Furthermore, to gain insights into the immune system's role in NSCLC, we focused only the pathways associated with the immune-related functions obtained from the GO analysis performed previously. The pathways were mainly related to immune regulation, cytokine modulation, cell dynamics, tissue repair, inflammation, and angiogenesis. This suggests the ceRNA network may influence various aspects of the immune response in NSCLC, potentially impacting anti-tumor immunity and immune evasion mechanisms.

Integration of the Immune-Infiltrating Cells and GO Enriched Terms associated with immune-related functions 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 have provided a deeper, more clarified perspective on the tumor microenvironment. *Zhao et al.* have identified the diverse immune cell populations within LUSC (*Jungang et al., 2020*). 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 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 (*P J Blair, et al., 1998*).

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 (*Lauren et al., 2015*). 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 (*Lindsey et al., 2020*). 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 (*Pui et al., 2017*). 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 (*Yu-Lin et al., 2018*). 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 play a significant role in modulating the inflammatory response (*Claudia et al., 2016*).

Neutrophils constitute another distinct category of immune cells present in LUSC tissues; their presence in the advanced T-cell stage indicates their crucial role in the immune microenvironment during the later stages of LUSC (*Christian et al., 2021*). 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 primary source of MMP-9 (matrix metalloproteinase-9). Elevated levels of MMP-9 expression correlate positively with the invasiveness 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 (Na Li, et al., 2021).

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 (Yan et al., 2023). Resting mast cell infiltration is markedly suppressed in NSCLC, and its inhibition is correlated with a subgroup at higher risk. The role of mast cells in NSCLC in general is ambiguous, with studies presenting diverse findings and emphasizing the need to define their prognostic value as therapeutic targets (Sowmya et al., 2021). CX3CL1 (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 (Dermot, et al., 2010). Moreover, some vascular endothelial growth factor-positive (VEGF-positive) lung adenocarcinomas were observed to trigger the migration of mast cells within the tumor microenvironment.

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 (Katarina et al., 2023). 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 (Fei et al., 2020). 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.

CHAPTER 6

SUMMARY AND CONCLUSIONS

This work highlights the key regulatory elements and intricate mechanisms governing non-small cell lung cancer (NSCLC) through the competing endogenous RNA (ceRNA) network. The in-depth analysis of the ceRNA network has the potential to advance future advancements in navigating the complexities of this disease.

The comprehensive literature search was utilized to predict the potential ceRNA interactions, revealing crucial interactions among the ceRNA components, particularly those involving a newly discovered long non-coding RNA (lncRNA) supported by limited prior research. These novel interactions present promising avenues for their participation in the processes associated with the tumor microenvironment.

However, the lack of direct experimental validation introduces a gap in the functional relevance of these associations. This limitation may restrict the depth of understanding regarding the underlying network dynamics. Despite this limitation, the interpretation of the identified pathways and interactions within the ceRNA network remains valuable. The key biomarker candidates, including lncRNAs, microRNAs (miRNAs), and their target genes, could serve as potential markers for early detection, diagnosis, prognosis, and therapeutic targeting of NSCLC, particularly those associated with immune system processes.

In conclusion, the findings presented in this study serve as a valuable resource, laying the groundwork for subsequent investigations, by providing a comprehensive understanding of the ceRNA network in the field of NSCLC research.

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