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DOKUZ EYLUL UNIVERSITY

IZMIR INTERNATIONAL BIOMEDICINE

AND GENOME INSTITUTE

INVESTIGATION OF THE ROLE OF NEDDYLATION PATHWAY IN THE PROCESS OF HEPATOCARCINOGENESIS

IŞIL KILIÇGÜN

MOLECULAR BIOLOGY AND GENETICS

MASTER DISSERTATION

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İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ

YÜKSEK LİSANS TEZ SAVUNMA SINAVI TUTANAĞI

Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı Moleküler Biyoloji ve Genetik Yüksek Lisans Programı 2020850015 numaralı öğrencisi Işıl Kılıçgün'ün, "Nedilasyon Yolağının Hepatokarsinogenez Sürecindeki Rolünün Araştırılması" konulu Yüksek Lisans tezini 07/09/2023 tarihinde yapılan savunma sınavı sonucunda başarılı olmuştur.

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ABBREVIATIONS

HCC: Hepatocellular Carcinoma

NASH: Nonalcoholic Steatohepatitis

PTM: Post-Translational Modification

UBL: Ubiquitin-Like Protein

NEDD8: Neural Precursor Cell-Expressed Developmentally Downregulated-8

NAE1: NEDD8 Activating Enzyme E1 Subunit 1

GDC: Genomic Data Commons

TCGA: The Cancer Genome Atlas

KEGG: Kyoto Encyclopedia of Genes and Genomes

CPTAC: Clinical Proteomic Tumor Analysis Consortium

RT-qPCR: Reverse Transcription-quantitative Polymerase Chain Reaction

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Investigation of the Role of Neddylation Pathway in the Process of Hepatocarcinogenesis

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ABSTRACT

Liver cancer remains a pressing global health challenge and stands as a leading cause of cancer-related deaths, primarily due to late diagnoses and limited therapeutic options. Hepatocellular carcinoma (HCC) represents the predominant form of liver cancer. The quest for effective and targetable biomarkers for HCC, coupled with a comprehensive understanding of its molecular mechanisms, holds promise for advancing treatment methodologies. One pivotal mechanism underpinning this process is post-translational modifications, with neddylation being a notable example. In this study, we meticulously analyzed the expression profiles of NEDD8 and the NEDD8 activating enzyme E1 subunit 1 (NAE1), both integral to the neddylation pathway, across independent HCC datasets. We subsequently discerned their associations with clinicopathological data and prognostic indicators. Notably, both genes exhibited elevated expression in tumor samples compared to their normal adjacent tissues. We then identified differential transcript profiles in patients based on the high and low expression of NEDD8 and NAE1. This was followed by pathway and functional analyses centered on patients with varying expression levels of key neddylation-associated molecules. Concluding our study, *in vitro* validation experiments were conducted for select molecules. Collectively, our findings posit that during hepatocarcinogenesis, the expression of NEDD8 and NAE1—central to the neddylation mechanism—undergoes significant alterations, potentially correlating with an adverse prognosis in HCC. Moreover, our results bolster the case for therapeutically targeting NEDD8 and NAE1 as a promising avenue for devising effective treatments.

Keywords: HCC, Neddylation, NEDD8, NAE1

Nedilasyon Yolağının Hepatokarsinogenez Sürecindeki Rolünün Araştırılması

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ÖZET

Karaciğer kanseri, özellikle geç tanı ve sınırlı tedavi seçenekleri nedeniyle, küresel sağlıkta acil bir sorun olarak karşımıza çıkmaktadır. Hepatosellüler karsinom (HCC), karaciğer kanserinin en yaygın formunu oluşturmaktadır. HCC için etkili ve hedeflenebilir biyobelirteçlerin araştırılması, birlikte moleküler mekanizmalarının kapsamlı bir şekilde anlaşılması, tedavi yöntemlerinin ilerletilmesi için umut vaat etmektedir. Bu sürecin temelinde yer alan mekanizmalardan biri post-translasyonel modifikasyonlardır ve nedilasyon bu örneklerden biridir. Bu çalışmada, nedilasyon yoluyla bütünleşik olan NEDD8 ve NEDD8 aktivasyon enzimi E1 alt birimi 1 (NAE1)'in ifade profillerini bağımsız HCC veri kümelerinde titizlikle analiz ettik. Ardından, bu genlerin klinikopatolojik verilerle ve prognoz göstergeleriyle ilişkilerini belirledik. Özellikle, her iki gen de tümör örneklerinde, normal komşu dokulara göre daha yüksek ifade gösterdi. Daha sonra, NEDD8 ve NAE1'in yüksek ve düşük ifadesine dayanarak hastalarda farklı transkript profillerini belirledik. Bu, anahtar nedilasyonla ilişkili moleküllerin değişen ifade seviyelerine sahip hasta verileri üzerinde yolak ve fonksiyonel zenginleştirme analizleriyle takip edildi. Çalışmamızı sonlandırırken, seçilen moleküller için *in vitro* doğrulama çalışmaları gerçekleştirildi. Toplu olarak, bulgularımız hepatokarsinojenez sırasında, nedilasyon mekanizmasının merkezinde yer alan NEDD8 ve NAE1'in ifadesinin önemli değişiklikler geçirdiğini ve potansiyel olarak HCC'de olumsuz bir prognozla ilişkilendirilebileceğini öne sürmektedir. Ayrıca, sonuçlarımız NEDD8 ve NAE1'in terapötik olarak hedeflenmesinin, etkili tedaviler geliştirmek için umut vaat eden bir yol olabileceğini güçlendirmektedir.

Anahtar Kelimeler: HCC, Nedilasyon, NEDD8, NAE1

1. GENERAL INFORMATION

1.1. Hepatocarcinogenesis

Globally, while liver cancer ranks sixth among the most frequent cancer types, it is the third in cancer-related death (Sung et al., 2021). It would appear that liver cancer will continue to be a serious health problem in the future because studies showed that by 2025, liver cancer will affect more than 1 million people per year. Although methods such as partial surgical resection of the liver and liver transplantation are the most commonly preferred treatment

options, these methods can only be applied to patients diagnosed at an early stage. There are different types of primary liver cancer, including hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), angiosarcoma, hemangiosarcoma and hepatoblastoma (Chidambaranathan-Reghupaty et al., 2021).

1.1.1. Hepatocellular Carcinoma (HCC)

HCC is the most common in primary liver cases, with a rate of 75% -85% (Sung et al., 2021). HCC includes five common processes; liver damage, chronic inflammation, fibrosis, cirrhosis, and HCC as showed in Figure 1.1. Alcohol consumption, viral infections including Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV), aflatoxin, and obesity can trigger chronic liver damage. In Asia and Africa, HBV is responsible for most HCC patients, so HBV, which is a DNA virus that can induce mutations in the host genome, is one of the important risk factors (Llovet et al., 2021). In the oncogenesis process, the HBx protein that is a viral regulator, plays a crucial role by altering several mechanisms including apoptosis, cell proliferation, and DNA repair (Desai et al., 2019). On the other hand, HBV can lead to non- cirrhotic HCC; for example, high viral loads can cause this situation (Desai et al., 2019). HCV, an RNA virus, causes the development of HCC less frequently than HBV, because most cases of HCV-related HCC usually require cirrhosis and advanced fibrosis (Balogh et al., 2016). The other risk factor is aflatoxin which is produced by fungal species. Aflatoxins, which are hepatotoxins, can be found in several agricultural crops (Balogh et al., 2016). The dominant aflatoxin, AFB1, acts as a mutagen to induce p53 mutations (Desai et al., 2019). Aflatoxin B1 and the HBV virus appear to have a synergistic effect on HCC and in this case the risk of HCC development is greatly increased (Chidambaranathan-Reghupaty et al., 2021). Alcohol consumption, which is one of the causes of not only liver cancer but also many other types of cancer, is another important factor. Alcohol can increase the risk of HCC by producing endotoxins, causing oxidative stress and inflammation (Desai et al., 2019). As indicated in Figure 1.1, factors such as obesity and type II diabetes mellitus may also result in HCC. With increasing obesity and diabetes, the incidence of Non-alcoholic fatty liver disease (NAFLD) has increased. NAFLD is a broad category of liver disease that consists of steatosis, NASH, fibrosis, and cirrhosis (Desai et al., 2019). Excessive accumulation of triglycerides in hepatocytes is observed in the early stages of NAFLD, but the progression of NAFLD to NASH and cirrhosis is a more complex process, and there are many hypotheses, the most common of which is that steatosis occurs first and then

sensitizes hepatocytes to inflammation (Chidambaranathan-Reghupaty et al., 2021). There are also factors for HCC development such as hereditary hemochromatosis and alpha 1-antitrypsin deficiency. In addition to these potential factors, males have 2 to 3 times higher risk for HCC incidence and mortality than females.

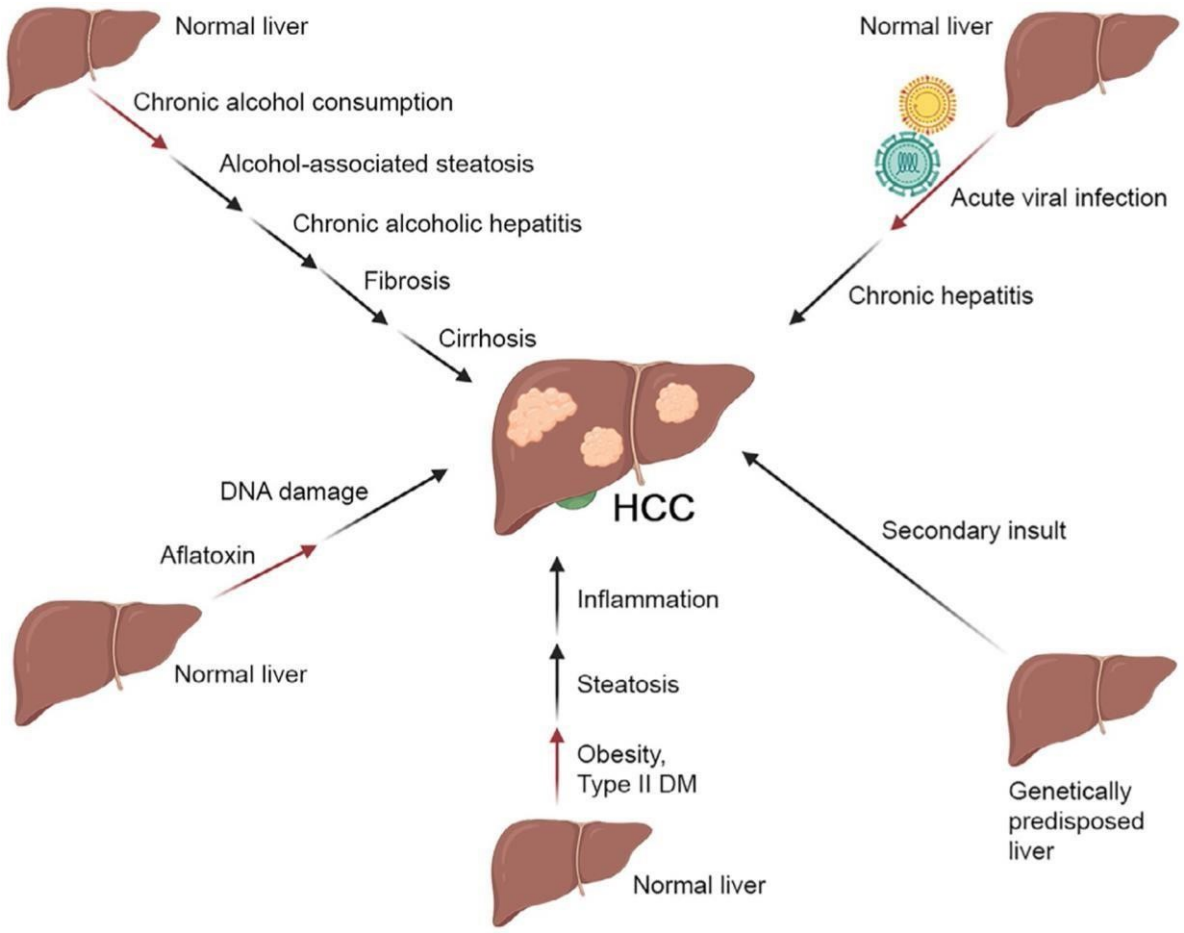


Figure 1.1. The main causes of HCC (Chidambaranathan-Reghupaty et al., 2021), under license number 5630180185414 (see Appendix).

The sequential accumulation of genetic and epigenetic changes is known to be important in HCC. At the molecular basis, HCC tumors have several somatic mutations. Some of these mutations may not be directly involved in the HCC process but some mutations that occur in putative driver genes activate signaling pathways that play an important role in HCC (Llovet et al., 2022). The most common mutation is *TERT* promoter mutations (Piñero, Dirchwolf and Pessôa, 2020). Telomere shortening causes genomic instability which is a hallmarks of cancer. However, high levels of telomerase activity may have a role in the development of hepatocarcinoma (Stroth et al., 2020). *CTNNB1* is the second most commonly altered gene in

HCC with 35% (Xu C et al., 2022). *CTNNB1* encodes β-catenin, which is a critical molecule in the WNT signaling pathway. The Wnt/β-catenin pathway has a major impact on liver homeostasis, regeneration and tumorigenesis (Xu C et al., 2022). Genomic studies have shown that 30% to 50% of tumors have aberrant activation of the Wnt/β-catenin cascade through inactivation of *CTNNB1* or WNT pathway inhibitors (AXIN1 and APC) (Llovet et al., 2021). *TP53*, a tumor suppressor gene, mutations is one of the other important mutations. *TP53* has a vital function in many mechanisms such as apoptosis and cell cycle regulation, so in general *TP53* mutations are seen in cases with poor prognosis in HCC. The chromatin remodeling complex component *ARID1A* mutation was frequently observed in the case, which may mean that this complex can trigger cancer development (Liu, Jiang and Guan, 2014). Epigenetic regulation, oxidative stress, the AKT-mTOR and MAPK pathways are also associated with HCC (Llovet et al., 2021). The other key mechanism is that HGF binds to and activates its specific receptor c-Met, which contributes to HCC development and metastasis (Wang et al., 2020).

Diagnosis and treatment planning for HCC is usually relied on laboratory tests and imaging studies which include ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) (Dimitroulis et al., 2017). Treatment after diagnosis is planned according to the stage of the disease. Many stage parameters such as metastasis, tumor number, tumor morphology, extrahepatic invasion, tumor size are used in various staging systems (Yim et al., 2018). There are other staging systems, but the Barcelona Clinic Liver Cancer (BCLC) staging system has become the standard in clinical management (Balogh et al., 2016). This system is critical in determining early stage patients who may be candidates for radiofrequency ablation (RFA) or resection, as well as intermediate and advanced stage patients who may be candidates for palliative therapies (Dimitroulis et al., 2017). Sorafenib also plays an important role in the treatment of HCC. Patients with advanced HCC but adequate liver function are candidates for sorafenib (Balogh et al., 2016).

The mechanism involved in the HCC process are still not fully elucidated today. By understanding the mechanisms involved in this process, prognostic and diagnostic markers will be elucidated (Yu et al., 2018). In this context, the interplay between transcriptional, post-transcriptional and post-translational regulation is important for cells to sustain homeostasis and activate alternative mechanisms and is the focus of current research (Olaizola et al., 2022).

1.2. Post-Translational Modifications

There are approximately 20,000 and 25,000 protein-coding genes in the human genome (International Human Genome Sequencing Consortium, 2004). However, the human genome has the potential to generate 1.8 million different types of protein (Jensen, 2004). Alternative splicing of transcripts, genomic recombination, and post-translational modifications are some of the sources of diversity in the protein derived from a single gene (Delgado et al., 2018). Post-translational modifications have a crucial role in mechanisms such as protein localization, activation, and degradation, and are key to the regulation of protein function. PTMs induce chemical modifications following polypeptide biosynthesis (Uversky, 2013). PTMs involve the addition of a modifying chemical group to one or more amino acid residues of a protein, or the addition of another protein to change the protein's properties (Deribe et al., 2010).

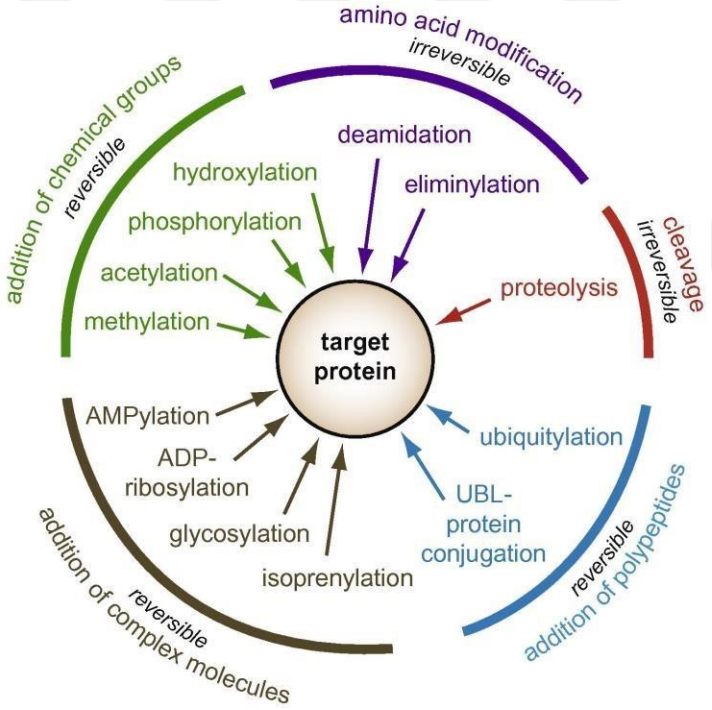


Figure 1.2. The variety and characteristics of post-translational modification mechanisms (Ribet and Cossart, 2010), under license number 5630180603933 (see Appendix).

Acetylation, phosphorylation, ubiquitination, ubiquitin-like proteins (UBLs) mediated post-translational modifications and methylation represent the most prevalent types of PTMs (Delgado et al., 2018). PTMs have been categorized according to different mechanisms, such as the addition of functional groups, as in phosphorylation, the binding of other proteins and

peptides, as in ubiquitination, and the modification of the chemical structure of amino acids (Uversky, 2013).

Ubiquitination and ubiquitin-like protein modifications are responsible for a vital regulatory mechanism in cellular processes (Jones et al., 2008). Ubiquitination is divided into three basic steps: activation, where ubiquitin is first activated by E1; conjugation, where it is conjugated to an E2 enzymes; and ligation, where E3 ligases form a bond with lysine residues of target proteins and the C-terminal glycine of ubiquitin (Deribe et al., 2010). The outcome of this process is a ubiquitin-substrate complex, and the ubiquitination reaction can be reversed by deubiquitinating enzymes (Guo, Rahimi and Tadi, 2013). As showed in Figure 1.2, ubiquitin-like protein (UBL) conjugation is also reversible. UBLs, which include neural precursor cell-expressed developmentally downregulated-8 (NEDD8), small ubiquitin-like modifiers (SUMO) and others are all structurally associated and are involved in a wide range of cellular mechanisms such as nuclear transport, proteolysis and autophagy (Delgado et al., 2018). Cullin ring ligases (CRLs), which can be activated by covalent binding of NEDD8 to cullin proteins by NEDD8 activating enzyme (NEA), are included in E3 ligases (Yang et al., 2019).

Dysfunction of PTM mechanisms has been linked to several diseases, as the play an important role in the protein regulation process. The elucidation of PTM mechanisms has paved the way for the identification of new biomarkers and targeted therapies to treat cancer (Sharma et al., 2019). Xu et al. (2018) established a database of 1,950 disease-associated PTMs in 749 proteins for 23 PTM types and 275 diseases, dramatically demonstrating the clinical impact of PTMs. In addition, they categorized the 23 PTM types and 275 diseases into 9 types of PTMs and 26 types of diseases. As showed in Figure 1.3a, phosphorylation, methylation and ubiquitination occupy a place in the top three. Neurological diseases have the highest percentage according to PTMs relationship at Figure 1.3b. PTMs have become popular in cancer research due to the recent understanding of their interactions with each other, as well as their individual elucidation (Sharma et al., 2019). Ubiquitination has a crosstalk with phosphorylation, so under some special conditions, phosphorylation can trigger ubiquitination, which can cause degradation (Sharma et al., 2019). Many of the enzymes involved in ubiquitination can also be drivers of cancer development. In addition, UBL modifications, which have a very similar mechanism with ubiquitination, have recently been an important subject of cancer research. Aberrant UBL modifications can be the trigger of various liver pathologies (Delgado et al.,

2018). In this context, there is a need for a better understanding of the NEDD8 conjugation pathway, known as the neddylation mechanism.

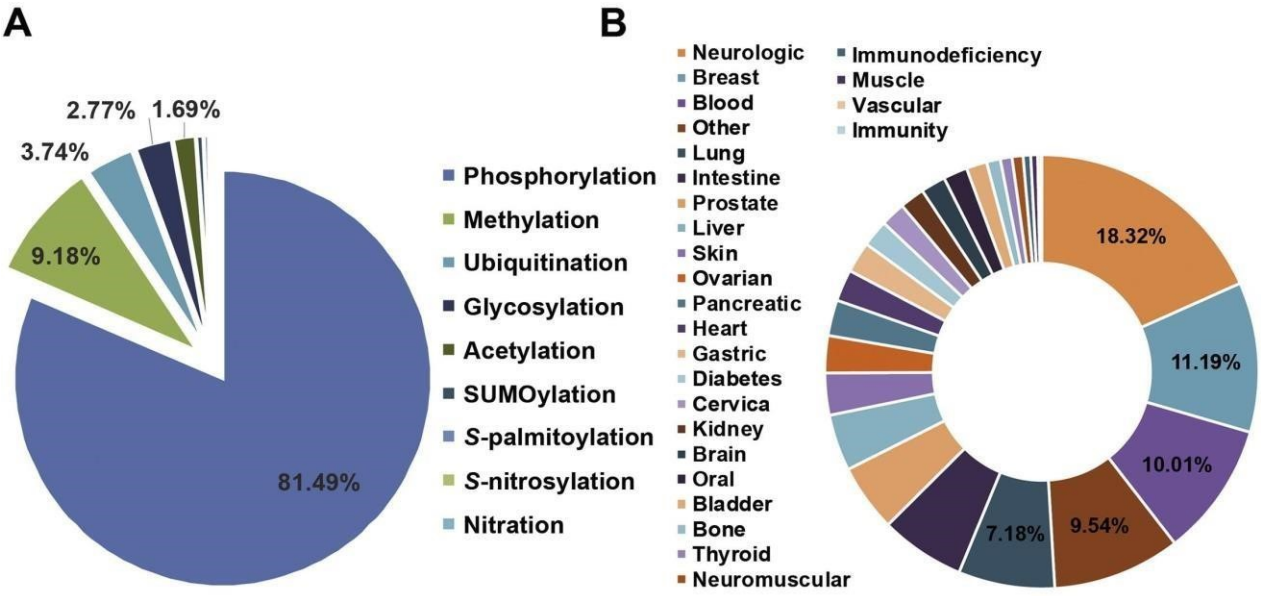


Figure 1.3. Distribution of PTMs and PTM-related diseases a) Percentage distribution of different PTMs associated with disease. b) Percentage of diseases grouped by tissue location according to their association with PTMs (Xu et al., 2018), under the terms of the Creative Commons CC-BY license.

1.3. Neddylation

The *NEDD8* gene was identified in 1992 (Kumar et al., 1992). *NEDD8* encodes a small ubiquitin-like protein with 80% homology to ubiquitin (Delgado et al., 2018). In the majority of eukaryotes, NEDD8 is conserved with a diverse expression profile (Delgado et al., 2018). In the first step of neddylation, NEDD8 precursor undergoes proteolytic processing to obtain mature protein, as shown in Figure 1.4. Deneddylase 1 (DEN1) can process the NEDD8 precursor form to reveal a C-terminal glycine residue (Enchev, Schulman and Peter, 2015). DEN1, a protease, is highly specific for NEDD8. After that, NEDD8 is adenylated by E1 NEDD8-activating enzyme (NAE) (Enchev, Schulman and Peter, 2015). NAE is specific for NEDD8 and is a heterodimer formed by amyloid-β precursor protein-binding protein 1 (APPBP1), known as NEDD8 activating enzyme E1 subunit 1 (NAE1), and ubiquitin-like modifier activating enzyme 3 (UBA3) proteins (Delgado et al., 2018). In the other step, he NEDD8-loaded NAE is directed to the E2 loading stage. Two enzymes, UBC12 and UBE2F,

act as E2 enzymes. They bind to NEDD8-loaded NAE and allow the transfer of NEDD8 to the E2 enzyme (Enchev, Schulman and Peter, 2015). In the following step, E3 ligases transfer NEDD8 from the E2 enzyme to its target protein. The E3 ligase is also responsible for the specificity of the neddylation substrate. All identified NEDD8 E3 enzymes, with the exception of SMURF1, are members of the RING subclass. Ring box protein 1 (RBX1), which can interact with UBC12 and UBE2F, and ring box protein 2 (RBX2), which can interact with UBE2F, are members of the RING family (Enchev, Schulman and Peter, 2015). In addition, there are non-RBX family NEDD8 E3 ligases such as c-CBL and MDM2 (Enchev, Schulman and Peter, 2015). The well-known substrate of NEDD8 is the cullin family of proteins, which can provide a molecular scaffold for the formation of cullin RING ligases (CRLs) (Olaizola et al., 2022). The final stage is deneddylation. Neddylation process is a reversible by action of deneddylases such as DEN1, COP9 (Delgado et al., 2018). As a result, the NEDD8-bound substrate dissociates while the E3 complex separates. This allows the neddylation process to restart.

Dysregulation of neddylation can cause many diseases, such as immunodeficiency, tumors, and neurodegenerative disorders (Yu et al., 2018). Neddylation of many substrates, including cullins, *MDM2*, and *p53*, plays a critical role in cancer. Because aberrant neddylation of CRLs change the ubiquitination of their substrate, it can lead to impaired DNA replication and cell cycle progression (Fernandez-Ramos and Martinez-Chantar, 2015). In the liver, the neddylation pathway is known to trigger the progression of fibrosis and is abnormal in HCC (Delgado et al., 2018). One of the oncoproteins called Hu antigen R (HuR) is neddylated in HCC (Yu et al., 2018). The other example is Akt and Liver Kinase B1 (LKB1), which are stabilized by the neddylation pathway and are involved in regulating energy metabolism in HCC (Yu et al., 2018). Moreover, the NEDD8-activating enzyme (NAE) inhibitor Pevonedistat (MLN4924) inhibited HCC growth, increased apoptosis, and induced autophagy (Yang et al., 2019).

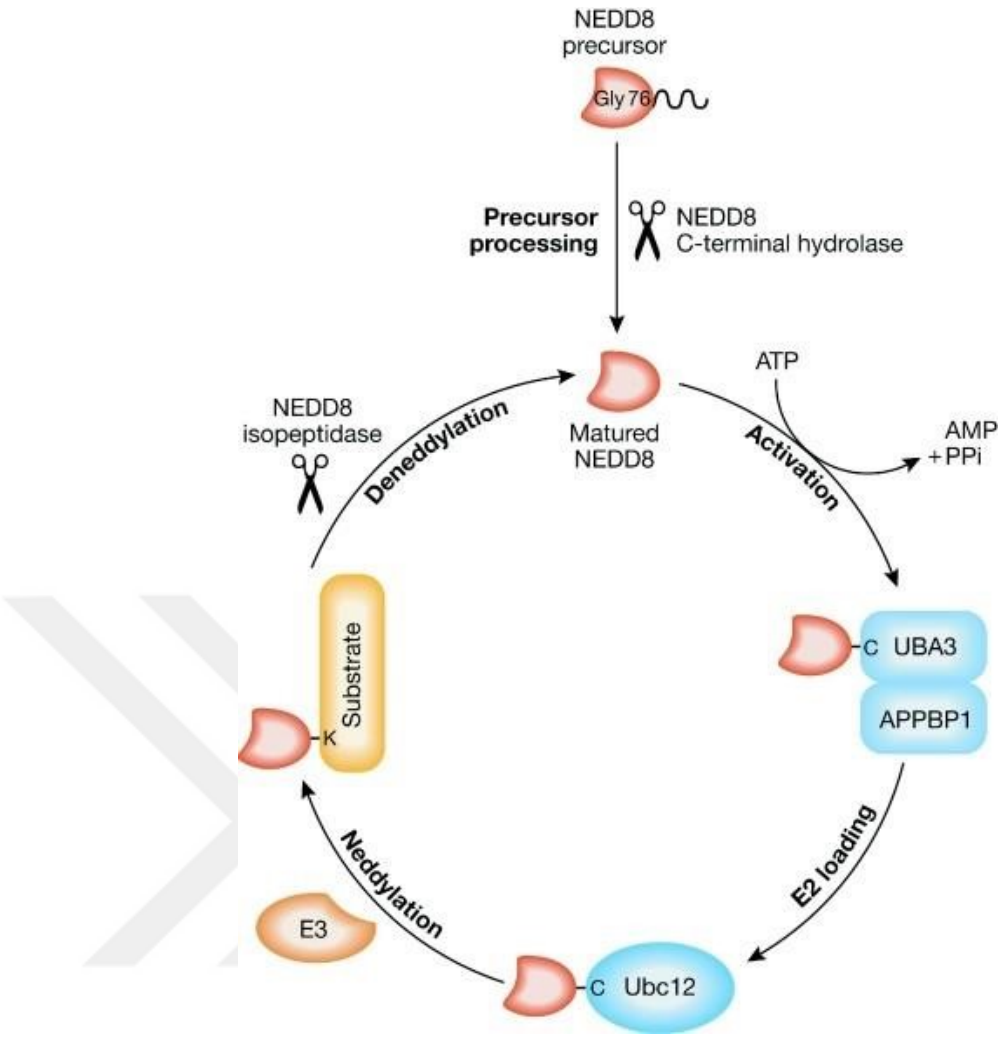


Figure 1.4. Overview of Neddylation (Rabut and Peter, 2008), under license number 5630180865771 (see Appendix).

Many signaling pathways, such as receptor tyrosine kinases (RTKs), can be affected by neddylation. In recent years, it has been revealed that the intracellular activity of RTKs is determined by neddylation. It has been reported that the epidermal growth factor receptor (EGFR) is neddylated by c-CBL, a RING finger E3 ubiquitin ligase, and this lead to increased ubiquitylation and endocytic internalization (Enchev, Schulman and Peter, 2015). Another RTK shown to be neddylated is transforming growth factor beta receptor II (TGFβRII). TGFβRII phosphorylation activates the c-CBL, and c-CBL can neddylate TGFβRII so stabilize and prolong its signaling (Enchev, Schulman and Peter, 2015).

c-Met RTK is essential in normal cell development and involved in cancer cell

growth/survival, motility/migration, invasion/metastasis, and angiogenesis (Organ and Tsao, 2011). Post-translational modifications detected in c-Met protein include proteolysis, phosphorylation, and glycosylation (Hu X et al., 2020; Zhang and Babic, 2016). However, it is not known whether c-Met receptor tyrosine kinase is a target of NEDD8. In order to find an answer to this question, our group examined c-Met neddylation status in HCC and it has been shown that c-Met is Neddylated upon HGF stimulation and c-Met inhibitor and neddylation inhibitor significantly suppress proliferation and migration ability of HCC cells (Unpublished data. Atabey,N.). In the light of this data, in this thesis project we examined the expression profile of NEDD8 and NEDD8-activating enzyme (NAE), the main molecules involved in the neddylation mechanism, in independent HCC datasets and determining its relationship with clinicopathological data and prognostic factors by bioinformatic approaches.

1.4. Computational Approaches

Bioinformatics is pivotal in the identification, interpretation, and categorization of biological data. While molecular computing has its roots deep in history, it was Leonard Adleman who first introduced the concept of a DNA computer and the DNA molecular biological calculation in 1994 (Liang et al., 2019). Another landmark in biology is the Human Genome Project, initiated by the US National Institutes of Health in 1990. The advancements in technology have since paved the way for more in-depth and comparative bioinformatics analyses. Multi-omics data, covering datasets like the genome, epigenome, transcriptome, proteome, and metabolome, has revolutionized system-level integration in medicine and biology (Subramanian et al., 2019).

Human diseases, including cancer and neurodegenerative disorders, are characterized by intricate and evolving interactions. Despite significant advancements at the molecular level, numerous aspects of these diseases remain enigmatic. Multi-omics data, which encompasses diverse datasets like genomics, epigenomics, transcriptomics, proteomics, and metabolomics, offers profound insights into the molecular underpinnings of diseases. This data not only deepens our understanding but also paves the way for innovative methods for early diagnosis, prevention, and treatment (Sun and Hu, 2016).

Merging multi-omics data with clinical insights is crucial for a holistic understanding of cellular processes. As the accumulation and processing of multi-omic data continue, platforms

like The Cancer Genome Atlas (TCGA), Cancer Cell Line Encyclopedia (CCLE), International Cancer Genomics Consortium (ICGC), and Clinical Proteomic Tumour Analysis Consortium (CPTAC) stand out as invaluable resources. For instance, TCGA offers profiles ranging from RNA-Seq and DNA-Seq to RPPA and Methylation for approximately 33 cancer types (TCGA; https://www.cancer.gov/ccg/research/genome-sequencing/tcga). Similarly, CCLE delivers intricate genetic profiles, including gene expression and copy number, for a vast array of human cancer cell lines (CCLE; https://sites.broadinstitute.org/ccle/). CPTAC provides proteomic data for selected TCGA cohorts, while ICGC offers resources like whole genome sequencing and genomic variation (Subramanian et al., 2019). These comprehensive datasets enable intricate analyses, fostering a deeper understanding of diseases at multiple levels. With the vastness of these datasets, the challenge is not just in collection but also in meaningful interpretation. The complexity of biological systems demands sophisticated analytical methods. This is where structured analysis techniques, particularly those related to gene expression, come into play. Their role in deciphering the intricate patterns and relationships within the data is indispensable.

Gene expression clustering is foundational in bioinformatics. Hierarchical clustering, which divides data into progressively smaller clusters, and partitioning, which segregates data into predetermined subsets, are both essential clustering methods (D'haeseleer, 2005). Over time, clustering algorithms have evolved to provide insights into gene behaviors under various systemic conditions (Oyelade et al., 2016). As these methods have matured, they've paved the way for more advanced analyses. The increasing volume and diversity of expression data have necessitated the development of more intricate analytical tools, especially when considering the variability across different tissues.

The proliferation of expression data from diverse tissues has catalyzed advancements in differential co-expression analysis. This is particularly crucial given the tissue-specific nature of gene expression and regulation. Notably, numerous genes associated with diseases exhibit irregular expression patterns (van Dam et al., 2017). Figure 1.5 delineates the core steps of co-expression network analysis. Initially, correlations between gene pairs are pinpointed within the expression data. These relationships are subsequently visualized as a network. The following phase centers on module definition, employing a variety of clustering techniques. The analysis can be tailored to specific needs, allowing for enrichment analysis, identification of regulatory networks, and the discovery of hub genes. A prominent tool in this domain is the Weighted Gene Correlation Network Analysis (WGCNA), renowned for discerning significant

connections between phenotypes and modules (van Dam et al., 2017). WGCNA employs hierarchical clustering, presenting gene modules in a dendritic format. Furthermore, the WGCNA R software package offers an extensive array of R functions, facilitating a myriad of tasks from network construction to data visualization and integration with third-party software (Langfelder and Horvath, 2008).

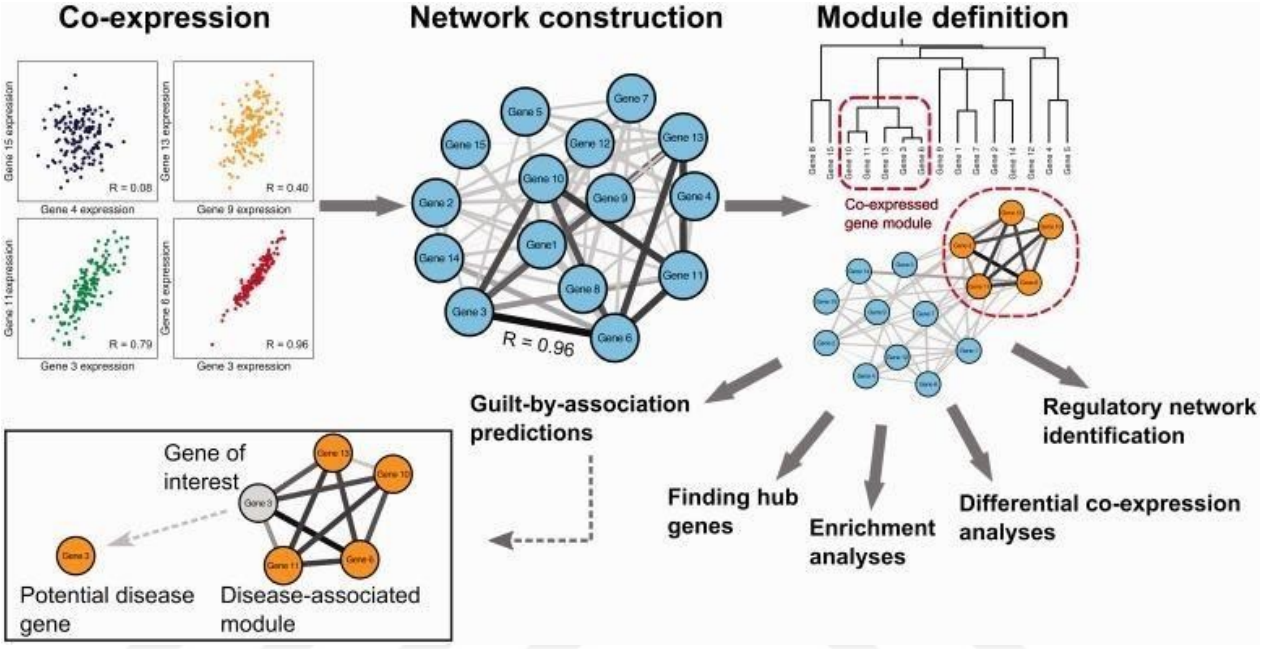


Figure 1.5. Workflow of a co-expression network analysis (van Dam et al., 2017), under the terms of the Creative Commons CC-BY license.

A vast array of databases and tools offer users a wealth of analysis capabilities, enabling in-depth exploration of publicly available datasets. In the realm of clinical research, the process of collecting patient samples and transforming them into datasets is intricate and time-intensive, yet of immeasurable value. Fortunately, these readily accessible tools streamline the process of tapping into extensive data libraries on various diseases, paving the way for enhanced analyses. Researchers can delve into aspects such as gene expression patterns and clinical features. Crucially, by juxtaposing normal and tumor samples, one can identify variations in genes and proteins. This comparative approach is instrumental in pinpointing prognostic markers, a pivotal aspect of cancer research. Detailed insights into the utilized databases are provided in the following paragraphs.

In recent years, the advent of advanced bioinformatics databases has revolutionized the field of genomics and molecular biology. Introduced in 2017, the Gene Expression Profiling

Interactive Analysis (GEPIA) has emerged as a pivotal tool in this domain (Tang et al., 2019). Its subsequent version, GEPIA 2, boasts enriched analyses, facilitating a broader spectrum of research capabilities. Notably, this platform was instrumental in analyzing the expression levels of NEDD8 and NAE1 across diverse cancer types, with a particular emphasis on overall survival analysis. For a more granular approach, the group cutoff in these survival analyses was adjusted using quartiles, centering the focus on the LIHC dataset.

Another significant contribution to the field is the Xena Browser, developed by the University of California, Santa Cruz (UCSC) (Goldman et al., 2020). This platform offers comprehensive data analysis and visualization services. Catering to large-scale cohort analyses, it also provides the flexibility for individualized research. Two primary datasets, GDC TCGA Liver Cancer and TCGA Liver Cancer, are available for exploration. The Genomic Data Commons (GDC), in collaboration with esteemed cancer research programs like TCGA, ensures the incorporation of the latest research methodologies. A notable achievement using this platform was the comparative visualization of NEDD8 and NAE1 gene expression levels between normal and tumor samples. This study further delved into clinical features using WGCNA analyses, identifying key parameters influencing liver cancer treatment and prognosis.

Further enriching the research landscape is the University of Alabama at Birmingham Cancer (UALCAN) data analysis portal (Chandrashekar et al., 2017). This platform offers swift access to a wealth of publicly available cancer OMICS data. For this study, the platform facilitated a comparison not only at the gene expression level but also at the protein expression level for NEDD8 and NAE1 between normal and tumor samples. Additionally, it enabled a comprehensive overall survival analysis.

Lastly, cBioPortal stands out with its multidimensional cancer genomics datasets, offering seamless access to molecular profiles and clinical features (Cerami et al., 2012). For the purpose of this research, the Liver Hepatocellular Carcinoma (TCGA, PanCancer Atlas) cohort was the primary focus. Extensive utilization of cBioPortal in work packages 1 and 2 allowed for a detailed exploration of how NEDD8 and NAE1 patterns manifest in clinical features. A meticulous grouping process, based on mRNA expression and RSEM, was employed. As depicted in Figure 2.2, this grouping was executed in two distinct manners: manual group generation and automated program-driven grouping. Subsequent analyses were conducted postsegregation based on high and low expression levels.

This thesis delves into the intricate roles of neddylation NEDD8 and the enzyme NAE1 in the development of hepatocarcinogenesis. By harnessing publicly available datasets, we aim to elucidate the mechanism of neddylation in hepatocellular carcinoma (HCC) and its interplay with pivotal candidate molecules. Our research undertook an exhaustive assessment of the neddylation pathway in HCC, leveraging data from expansive patient cohorts and independent studies.

Central to our investigation is the pressing question: "Do the expression levels of NEDD8 and NAE1, which are pivotal in regulating the neddylation mechanism, undergo alterations during hepatocarcinogenesis?" Our guiding hypothesis posits that changes in the transcription of NEDD8 and NAE1, key regulators of the neddylation process, correlate with a more aggressive phenotype and an unfavorable prognosis in HCC.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Liver Cancer Normal and Tumor Sample Numbers

In this study, *NEDD8* and *NAE1* gene expression levels from the databases in Table 2.1 were analyzed in normal and tumor samples. Samples from UALCAN are also available for protein expression analysis.

Table 2.1. List of databases used to compare *NEDD8* and *NAE1* gene expression levels

	Dataset	**Normal**	**Tumor**
UCSC Xena Browser	GDC TCGA Liver Cancer	50	371
	TCGA Liver Cancer	50	371
UALCAN	TCGA Liver Cancer	50	371
GEPIA 2	TCGA Liver Cancer	160	369
UALCAN	CPTAC	165	165

2.1.2 Clinical features obtained from cBioPortal

The number of samples used in three different categories in cBioPortal, namely Neoplasm Histologic Grade, Neoplasm Disease Stage American Joint Committee on Cancer Code and American Joint Committee on Cancer Tumor Stage Code, are shown in Table 2.2, Table 2.3, Table 2.4.

Table 2.2. Number of samples in the Neoplasm Histologic Grade

	Neoplasm Histologic Grade			
	G1	G2	G3	G4
NAE1 High	22	73	67	6
NAE1 Low	25	87	48	5
NEDD8 High	17	83	61	7
NEDD8 Low	30	77	54	4

*NEDD8/NAE1*_High	11	38	39	3
*NEDD8/NAE1*_Low	19	42	26	1

Table 2.3. Number of samples in the Neoplasm Disease Stage American Joint Committee on Cancer Code

	Neoplasm Disease Stage American Joint Committee on Cancer Code			
	Stage I	Stage II	Stage III	Stage IV
NAE1 High	75	36	45	4
NAE1 Low	86	41	32	1
NEDD8 High	86	42	35	3
NEDD8 Low	75	35	42	2
*NEDD8/NAE1*_High	46	19	23	3
*NEDD8/NAE1*_Low	46	18	20	1

Table 2.4. Number of samples in the American Joint Committee on Cancer Tumor Stage Code

	American Joint Committee on Cancer Tumor Stage Code				
	T0	T1	T2	T3	T4
NAE1 High	0	79	41	42	7
NAE1 Low	1	89	43	29	5
NEDD8 High	0	88	44	32	4
NEDD8 Low	1	80	40	39	8
*NEDD8/NAE1*_High	0	46	20	21	4
*NEDD8/NAE1*_Low	1	47	19	18	5

2.1.3 Cell lines from which cDNAs were obtained

cDNAs of 11 cell lines including SNU 182, SNU 387, SNU 423, SNU 475, SNU 449, HuH7, Mahlavu, SKHEP1, HEPG2, HEP40 and FOCUS were used. All cDNAs were prepared in our lab by Dehan Çömez & Gülsün Bağcı.

2.1.4 Primers

In this study, primers of *NEDD8*, *NAE1* and *RPL41* genes were used.

Table 2.5. List of Primers

	Forward Primer	**Reverse Primer**
RPL41	TCGGCACTTAGCATCATCAC	CATTCGCTTCTTCCTCCACTT
NEDD8	GTGAAGACGCTGACCGGAAA	CCAGGTGAAGGACTGAACCA
NAE1	ATGTCCACGAATTTTGCCGA	TTGATGACCTCTTGAGCAGCA

2.1.5 Equipment

Table 2.6. List of Equipment

Equipment	**Brand**	**Catalogue Number**
Vortex	Thermo Scientific	88880018
Centrifuge 5810 R	Eppendorf	5810 R
MicroCL 17R Microcentrifuge	Thermo Scientific	75002450
mySPIN™ 6 Mini Centrifuge	Thermo Scientific	75004061

2.2 Methods

Our research methodology was structured into distinct work packages (WPs) to ensure a systematic and comprehensive approach. In WP1, we utilized four primary databases: GEPIA2, Xena Browser, UALCAN, and cBioPortal. Our initial step involved probing the GEPIA2 database to ascertain the expression levels of NEDD8 and NAE1 across various cancer cohorts. This was followed by an overall survival analysis for both NEDD8 and NAE1. Using the Xena Browser, we discerned the expression levels of NEDD8 and NAE1 in both normal and tumor samples. UALCAN further facilitated our investigation by providing insights into the gene expression levels of NEDD8 and NAE1 in normal versus tumor samples.

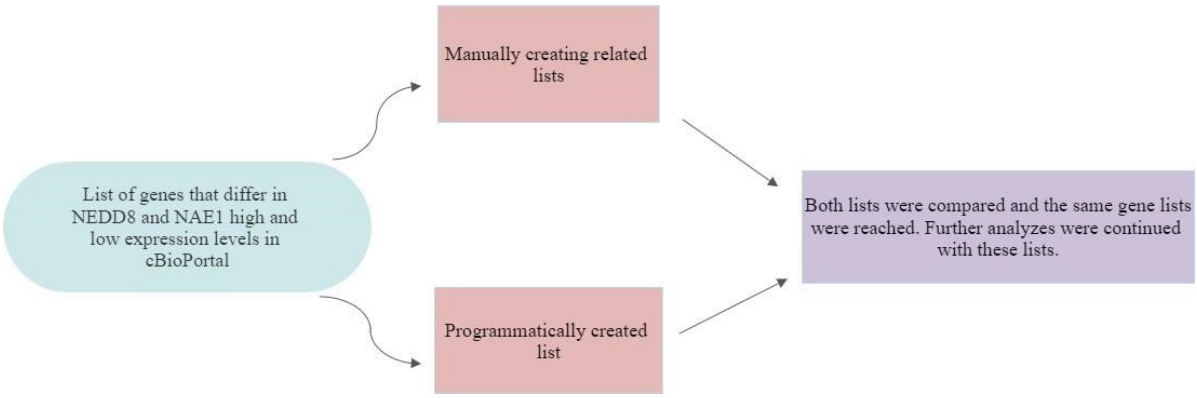


Figure 2.1. Overview of Work Package 2

WP2 was dedicated to identifying distinct transcript profiles in patients based on high and low expressions of NEDD8 and NAE1 and examining the potential correlations between these expression levels. Utilizing cBioPortal, we generated a list of genes that exhibited differential expression in patients with high and low levels of NEDD8 and NAE1. This list was curated using two distinct methodologies: manual curation and automated program-driven extraction. Subsequently, we categorized the genes into four groups: high expression of both NEDD8 and NAE1, low expression of both NEDD8 and NAE1, high expression of NEDD8 with low NAE1, and low expression of NEDD8 with high NAE1. A Venn diagram was employed to visualize and identify the genes that were consistently high or low in both NEDD8 and NAE1.

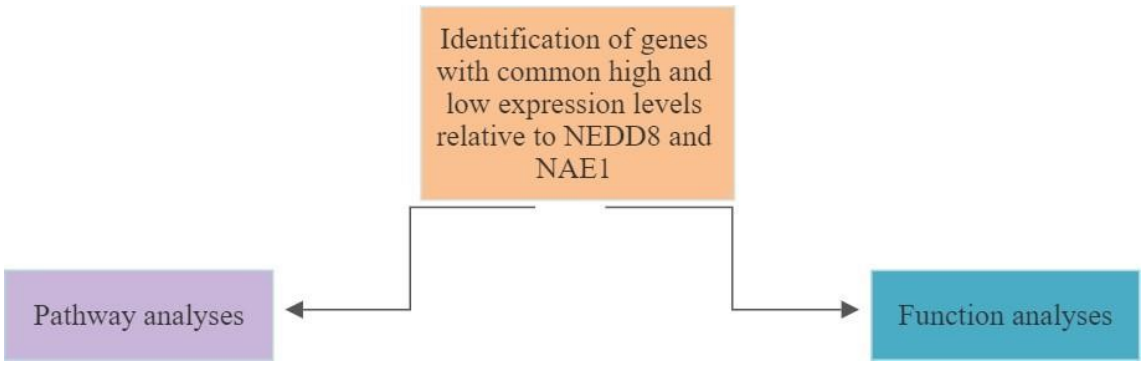


Figure 2.2. In Work Package 3, genes were identified as a result of cBioPortal analyses. Functional and pathway analyses were then performed.

WP3 was centered on the pathway and functional analysis of pivotal molecules linked with neddylation based on their expression levels. Our initial approach was to conduct a gene ontology enrichment analysis via R environment, which identified approximately 170 biological processes. During the coding phase, we established specific cutoffs for both p and q values to ensure meaningful results. Subsequently, we utilized the KEGG pathway database for pathway analysis, identifying associations with 319 distinct pathways. To further corroborate our findings, we employed the GeneCodis database for gene ontology biological process analysis, which revealed associations with 3,621 biological processes.

WP4 was bifurcated into in vitro and in silico validation segments: (i) *In Vitro* Validation: This phase involved RT-qPCR analysis. We utilized the Ampliqon RealQ Plus 2x Master Mix Green With ROX for the RT-qPCR reaction. A master mix solution was meticulously prepared for each primer, with 20ng of cDNA added to each well. After centrifuging the plate at 2,500 rpm for 3 minutes, we employed the ABI FAST 7500 qPCR instrument, as detailed in Topel et al. (Topel et al., 2021). The PCR program was set to 1 cycle of 15 minutes at 95ºC and 40 cycles of 30 seconds at 95 ºC, 30 seconds at 60 ºC, and 30 seconds at 72 ºC. (ii) *In Silico* Validation: This segment hinged on the Weighted Gene Correlation Network Analysis (WGCNA). We initiated by downloading and setting up the necessary packages, including WGCNA, in RStudio. After importing the expression data, we screened for excessive missing values and identified outlier samples. Phenotypic features were then integrated. The analysis progressed with network construction and module detection, culminating in the determination of the module-trait relationship, as depicted in Figure 3.10. Data from the Xena Browser, encompassing expression data and phenotype traits, were processed and loaded into R. Network construction was based on topological calculations, leading to the derivation of the module-trait relationship. Subsequent gene annotation was restricted to protein-coding genes. Pathway and functional analyses were once again conducted in R.

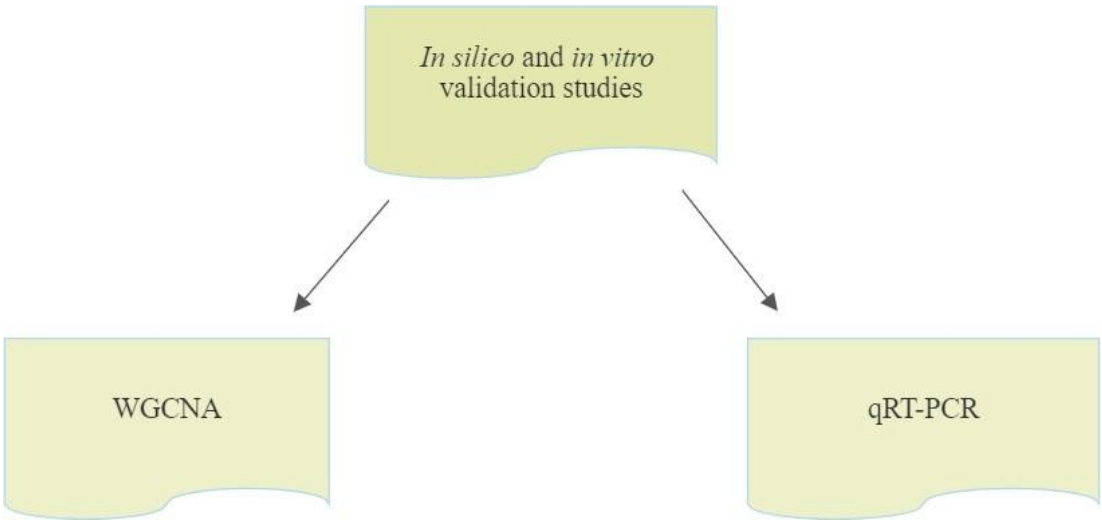


Figure 2.3. Overview of Work Package 4

3. RESULTS

3.1 Determination of the expression level of NEDD8 and NAE1 in various cancer cohorts

Upon examining the expression levels of NEDD8 and NAE1 across various cancer cohorts, we found that both genes exhibited significantly higher expression in tumor samples compared to normal samples in nearly all tested cohorts. The analysis results, obtained via GEPIA2, are depicted in Figures 3.1 and 3.2. In Figure 3.1, the cancer cohorts that have significant expression differences were indicated by the red color.

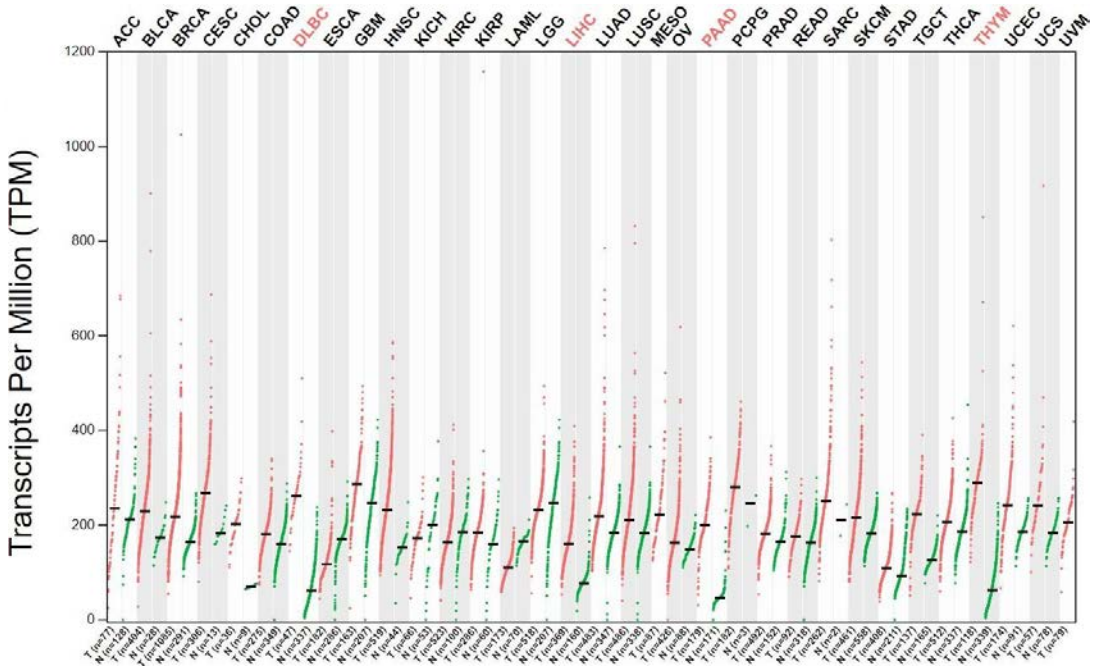


Figure 3.1. Pattern of *NEDD8* expression levels in tumor and normal samples in different cancer types in GEPIA2. There were 369 tumor samples (T) and 160 normal samples (N). Each dot represents expression of samples (dot plot). The analysis encompassed 33 cohorts from various cancer types. Cancer cohorts where *NEDD8* expression showed the most significant alterations between normal and tumor samples include: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC), Liver hepatocellular carcinoma ((LIHC), Pancreatic adenocarcinoma (PAAD), Thymoma (THYM).

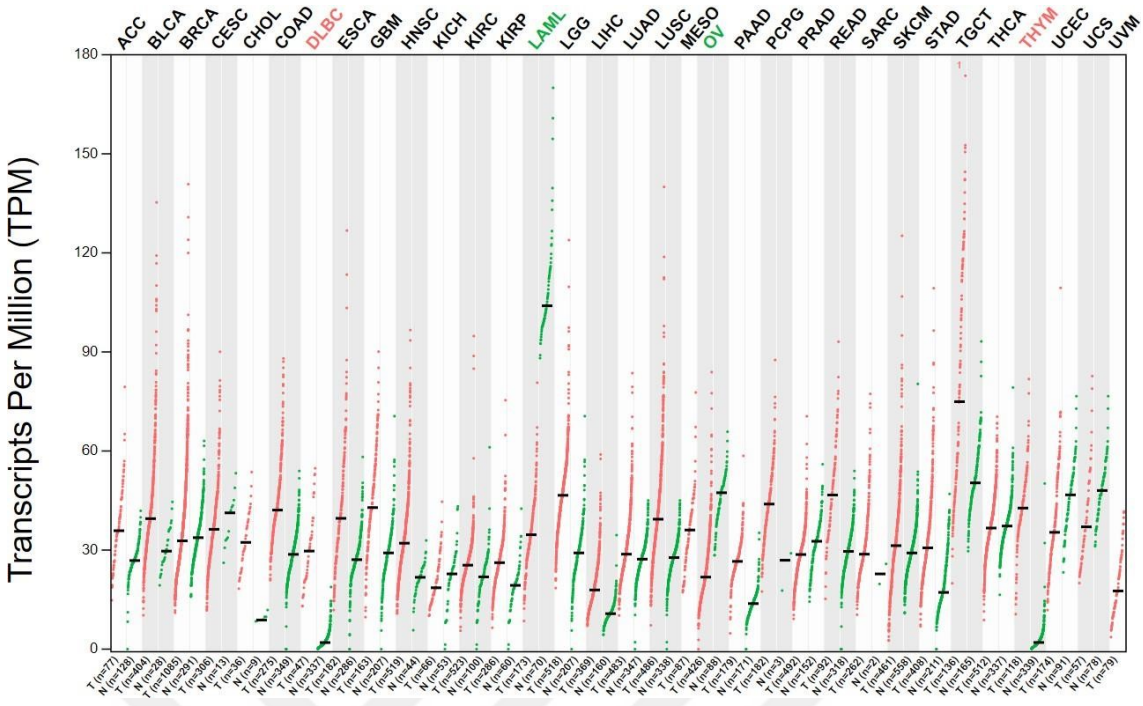


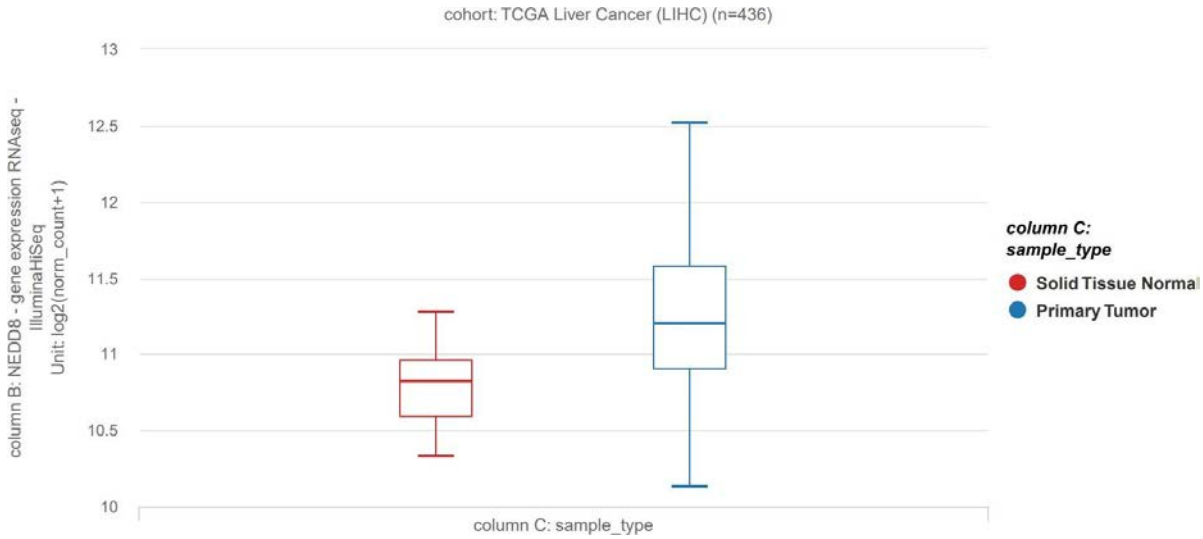
Figure 3.2. Pattern of *NAE1* expression levels in tumor and normal samples in different cancer types in GEPIA2. There were 369 tumor samples (T) and 160 normal samples (N). Each dot represent expression of samples (dot plot). The analysis encompassed 33 cohorts from various cancer types. Cancer cohorts where *NAE1* expression showed the most significant alterations between normal and tumor samples include: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC), Acute Myeloid Leukemia (LAML), Ovarian serous cystadenocarcinoma (OV), Thymoma (THYM).

3.2 Determination of the expression level of *NEDD8* and *NAE1* in liver cancer cohorts

Building on the previous procedure, we examined the differential expression of *NEDD8* and *NAE1* between normal and tumor liver cancer samples. Figure 3.3 and Figure 3.5 exhibit the gene expression profiles for both cohorts, as analyzed in the Xena Browser. Figure 3.3 shows that NEDD8 expression is significantly elevated in tumor samples compared to normal samples across both cohorts (Cohort 1: p= 3.236e-15, Cohort 2: p= 3.446e-8). Two plots were derived from the UALCAN database. *NEDD8* was analyzed on the basis of both gene expression and protein expression (Figure 3.4). The observed gene expression pattern of *NEDD8* in normal versus tumor samples aligns with previous findings, and this trend is mirrored at the protein level (p= 3.565e-04). Analyses from various platforms indicate that *NEDD8* expression is

significantly elevated in tumor samples (p= 1.624e-12, p= 3.565e-04 respectively). Figure 3.5 shows that *NAE1* gene expression is significantly higher in tumors than in normal samples for both cohorts from the Xena Browser (Cohort 1: p= 2.132e-16, Cohort 2: p= 2.434e-19). UALCAN results further confirm that *NAE1* is significantly upregulated in tumor samples at both the gene and protein expression levels (p= <1e-12, p= 1.569e-05 respectively). In conclusion, both *NAE1* and *NEDD8* are overexpressed at the transcript and protein levels in tumor tissues.

A



B

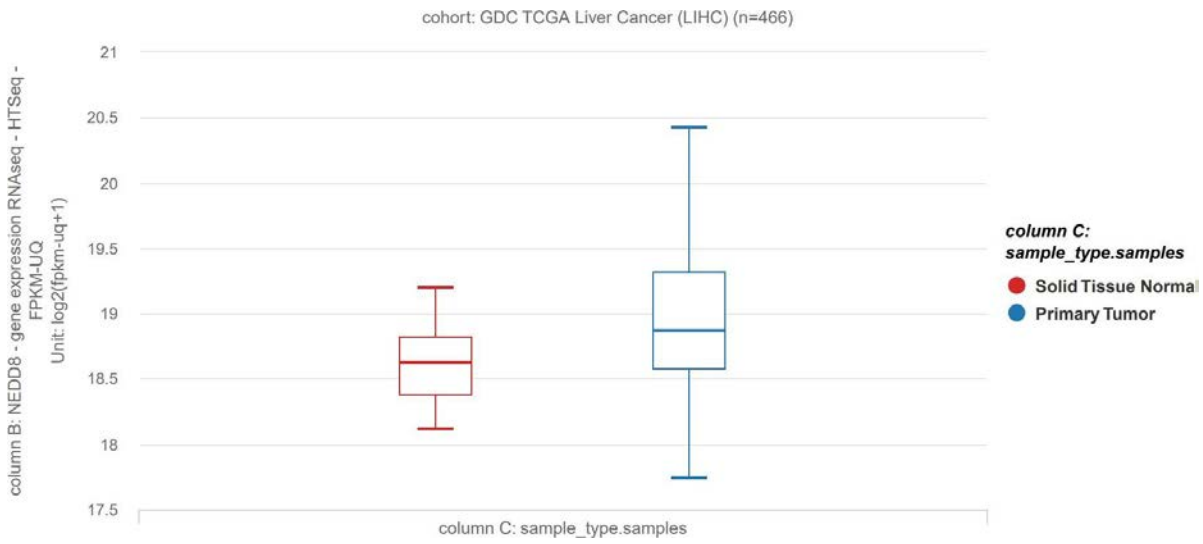
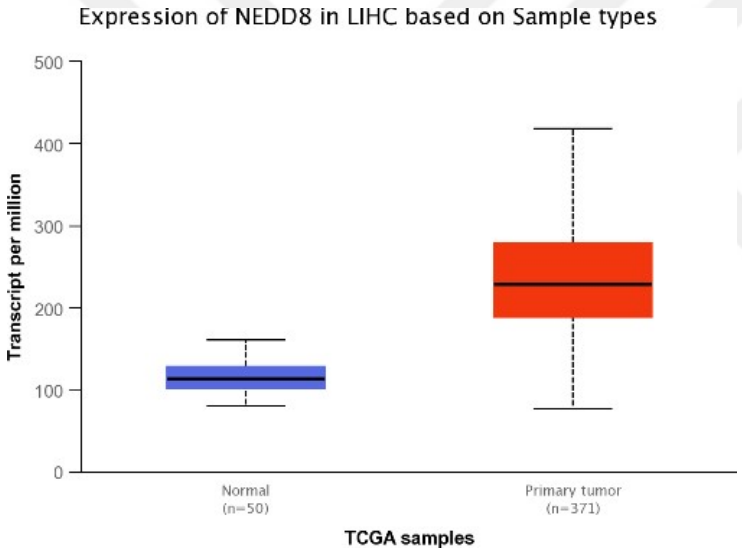


Figure 3.3. Showcases the expression levels of *NEDD8* in normal and tumor samples. Two distinct cohorts were sourced from the UCSC Xena Browser. **A.** The first cohort, derived from TCGA Liver Cancer, indicates that the solid tissue normal samples have a median *NEDD8* expression value of 10.8 (n=50), while the primary tumor samples have a median value of 11.2 (n=371). Welch's t-test/p = 3.236e-15 (t =-9.397). **B.** The second cohort, from GDC TCGA Liver Cancer, presents a median *NEDD8* expression value of 18.6 for solid tissue normal samples (n=50) and 18.9 for primary tumor samples (n=371). Welch's t-test/p = 3.446e-8 (t =-
6.020).

A



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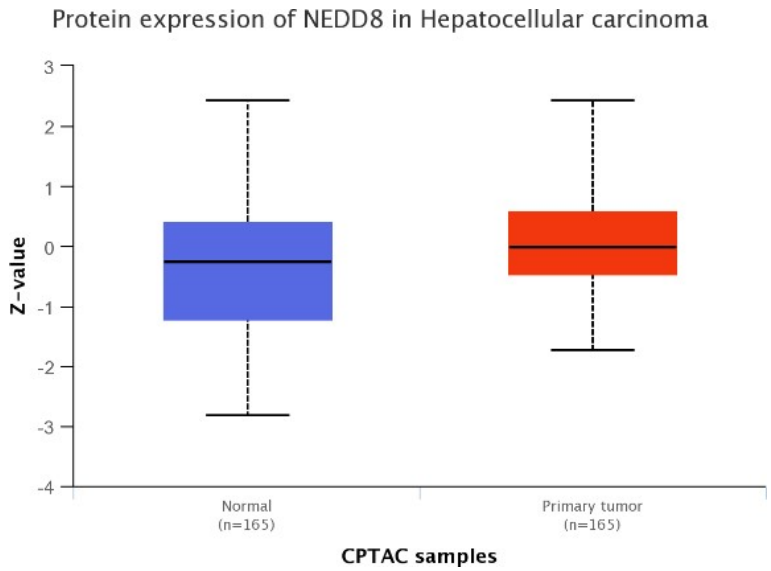
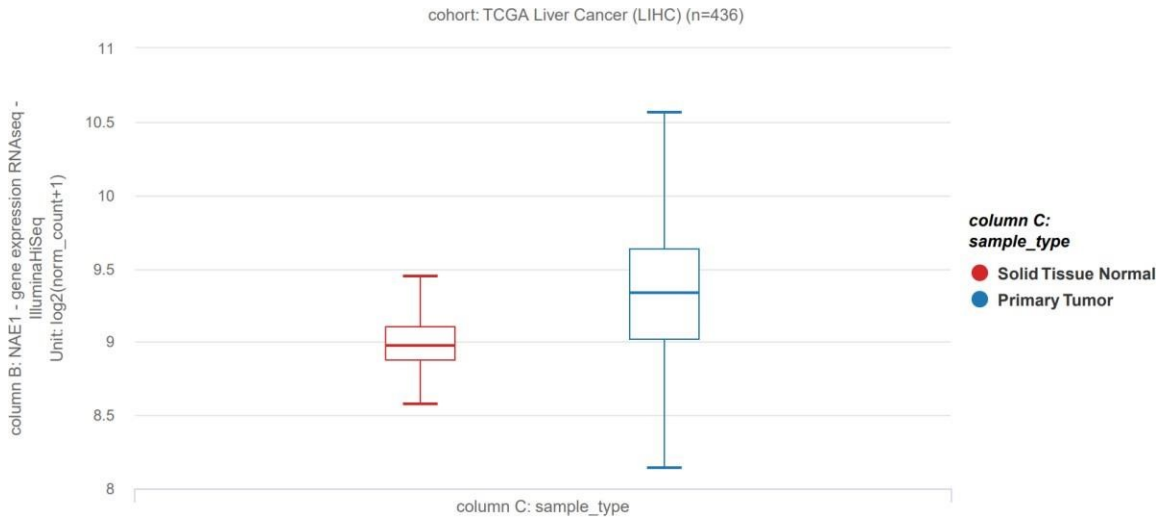


Figure 3.4. Analysis from the UALCAN database. **A.** The normal samples have a median *NEDD8* gene expression value of 114.001, while the primary tumor samples stand at 228.587. Statistical significance: 1.624e-12. **B.** When considering protein expression, the normal samples have a median value of -0.248, contrasting with the primary tumor samples that have a median value of -0.023. Statistical significance: 3.565e-04.

A



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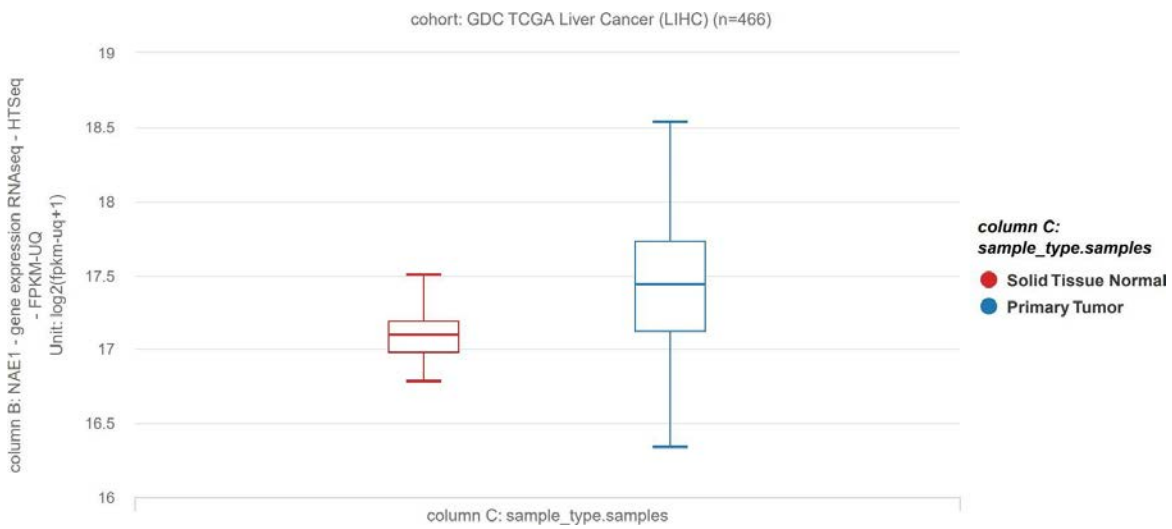
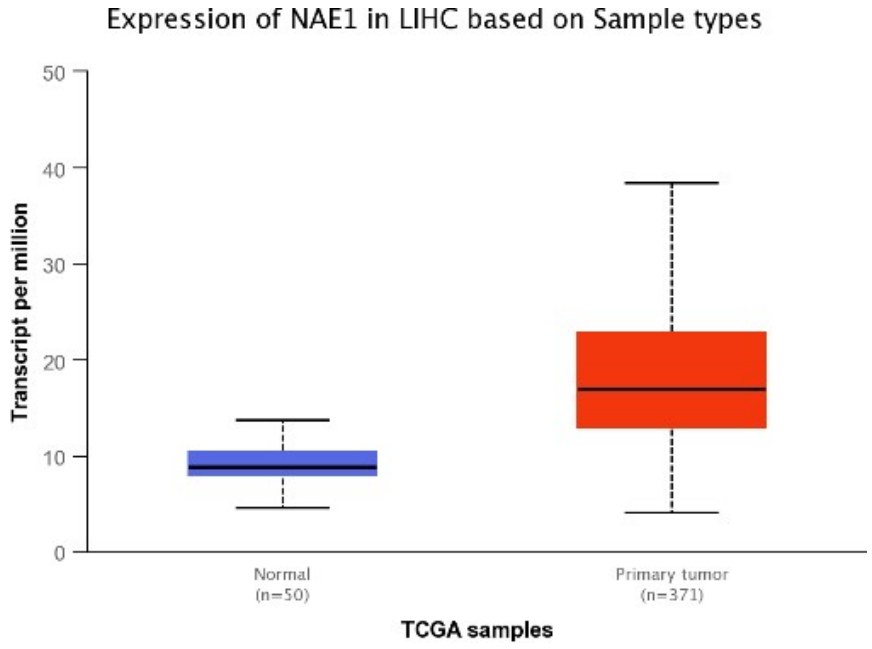


Figure 3.5. Showcases the expression levels of *NAE1* in normal and tumor samples. **A.** The first cohort from TCGA Liver Cancer shows a median value of 8.97 for solid tissue normal samples (n=50) and 9.34 for primary tumor samples (n=371). Welch's t-test/p = 2.132e-16 (t=-9.445). **B.** The second cohort from GDC TCGA Liver Cancer indicates a median *NAE1* expression value of 17.1 for solid tissue normal samples (n=50) and 17.4 for primary tumor samples (n=371). Welch's t-test/p = 2.434e-19 (t =-10.20).

A



B

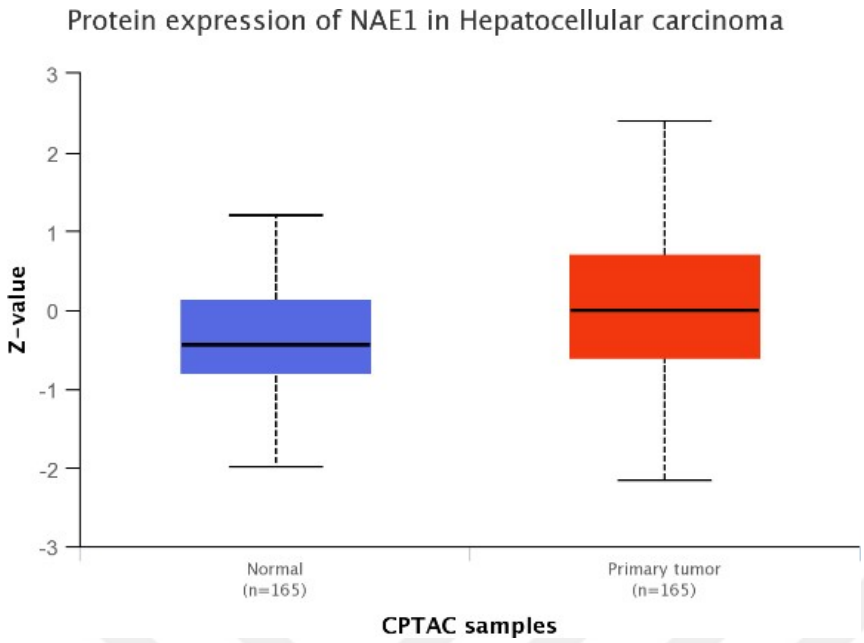


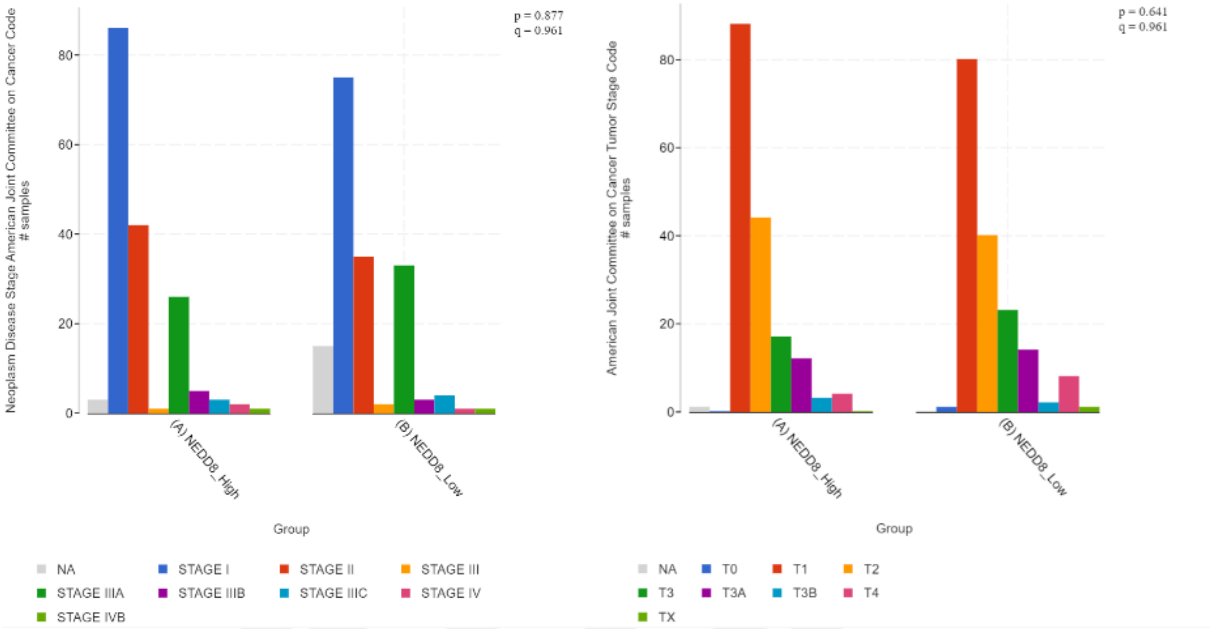
Figure 3.6. Analysis from the UALCAN database. **A.** The normal samples have a median *NAE1* gene expression value of 8.82, while the primary tumor samples exhibit a value of 16.893. Statistical significance: <1e-12. **B.** In terms of protein expression, the normal samples have a median value of -0.429, whereas the primary tumor samples have a median value of 0. Statistical significance: 1.569e-05.

3.3 Investigation of the relationship between *NEDD8* and *NAE1* with clinicopathological data

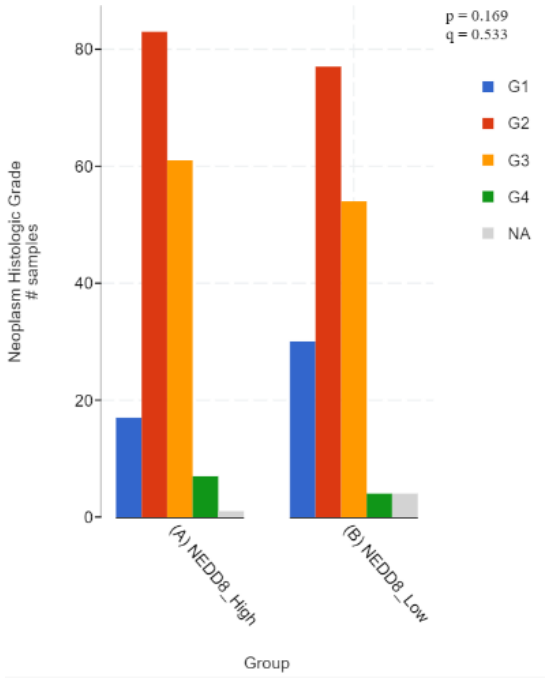
The cBioPortal offers extensive data on a myriad of clinical characteristics. We screened most of the clinical features associated with these characteristics, ultimately selecting three features for each gene: Neoplasm Disease Stage American Joint Committee on Cancer Code, American Joint Committee on Cancer Tumor Stage Code, and Neoplasm Histologic Grade, were selected for each gene. To explore potential correlations between *NEDD8* and *NAE1* expression levels and clinicopathological data, patients were categorized into two groups as *NEDD8*- or *NAE1*-high and *NEDD8*- or *NAE1*-low according to their median expression values obtained in cBioPortal. Our observations indicated no statistically significant differences in the expression levels of either NEDD8 (as shown in Figure 3.7) or NAE1 (as depicted in Figure 3.8) when compared with the tested clinical data ($p>0.05$). However, it was noted that patients in advanced stages of HCC were predominantly in the NAE1-High group. Subsequent analysis of clinical

data in patient groups with both simultaneous low and high expression levels of NEDD8 and NAE1 revealed no significant differences for any of the tested clinical data ($p>0.05$).

B


p = 0.877
q = 0.961
Neoplasm Disease Stage American Joint Committee on Cancer Code
samples
80
60
40
20
0
(A) NEDD8_High
(B) NEDD8_Low
Group
NA
STAGE I
STAGE II
STAGE III
STAGE IIIA
STAGE IIIB
STAGE IIIC
STAGE IV
STAGE IVB
p = 0.641
q = 0.961
American Joint Committee on Cancer Tumor Stage Code
samples
80
60
40
20
0
(A) NEDD8_High
(B) NEDD8_Low
Group
NA
T0
T1
T2
T3
T3A
T3B
T4
TX

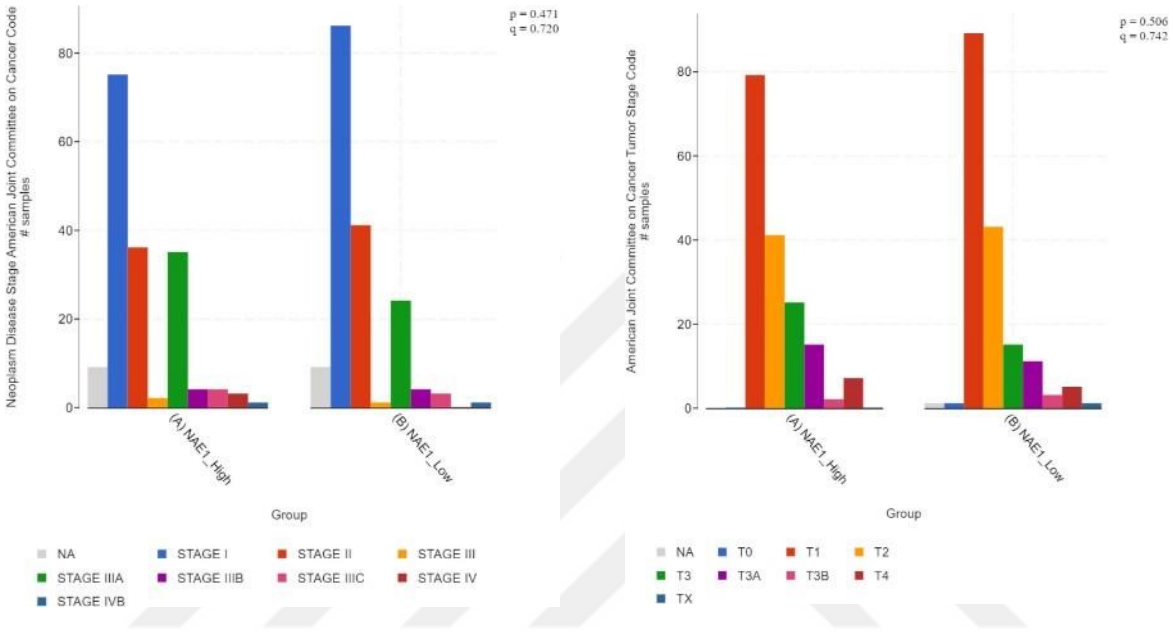
C


p = 0.169
q = 0.533
Neoplasm Histologic Grade
samples
80
60
40
20
0
(A) NEDD8_High
(B) NEDD8_Low
Group
G1
G2
G3
G4
NA

A

Figure 3.7. Visualization of data pertaining to *NEDD8* from cBioPortal, including **A,** Neoplasm Disease Stage American Joint Committee on Cancer Code, **B,** American Joint Committee on Cancer Tumor Stage Code, and **C,** Neoplasm Histologic Grade.

A **B**



C

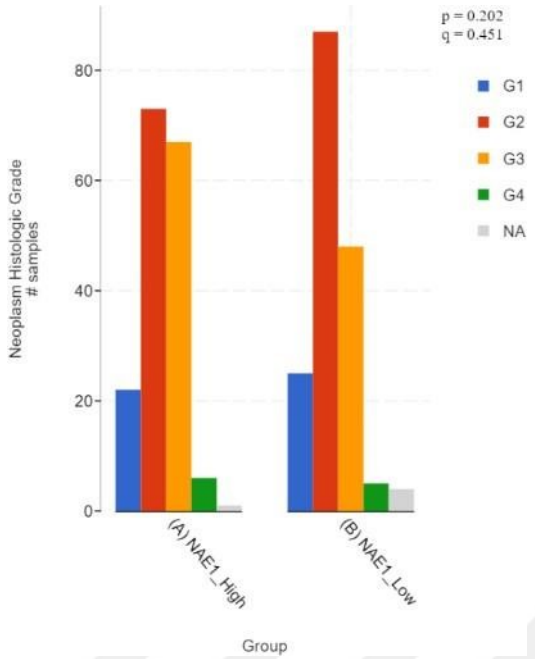
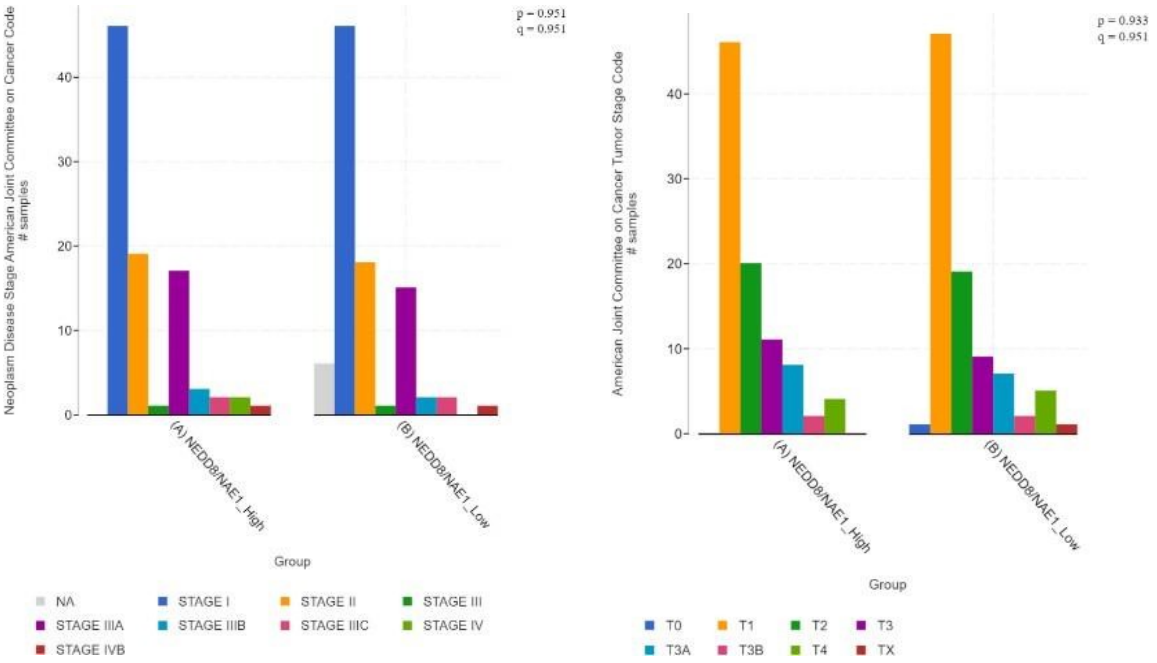


Figure 3.8. Visualization of data related to *NAE1* from cBioPortal, including **A,** Neoplasm Disease Stage American Joint Committee on Cancer Code, **B,** American Joint Committee on Cancer Tumor Stage Code, and **C,** Neoplasm Histologic Grade.

B



C

A

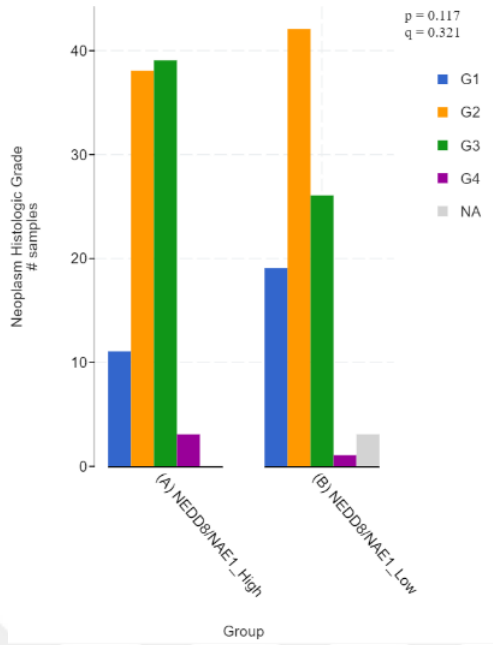
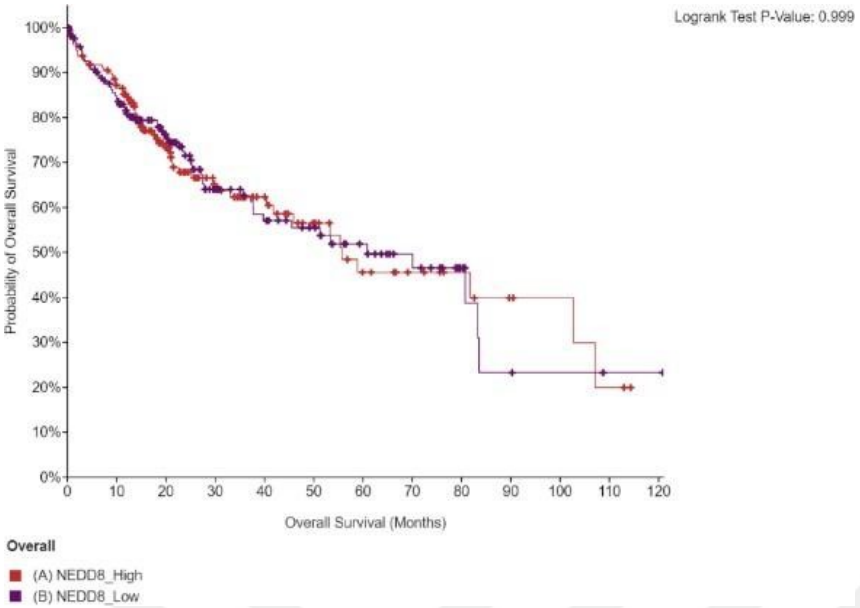


Figure 3.9. Visualization of data generated based on groups with both low and high expression levels of *NEDD8* and *NAE1* from cBioPortal, including **A,** Neoplasm Disease Stage American Joint Committee on Cancer Code, **B,** American Joint Committee on Cancer Tumor Stage Code, and **C,** Neoplasm Histologic Grade.

3.4 Investigation of *NEDD8* and *NAE1* in relation to prognostic factors

Survival analyses were performed across three different databases. Figure 3.10 illustrates that there's no statistically significant difference in the overall survival of patients in the NEDD8-High and NEDD8-Low groups. However, our data indicated that the overall survival of the NAE1-High group is significantly lower than NAE1-Low group (p=0.0378). In the UALCAN analyses, which were performed without any user intervention, a significant decrease in overall survival was observed in the NEDD8-High group (p=0.023) as depicted in Figure 3.11. A similar pattern was observed for *NAE1* in Figure 3.11 (p=<0.0001). In the GEPIA2 analyses, where groups were delineated by quartiles, the program automatically executed the survival analysis. A significant decrease in overall survival was confirmed for both the NEDD8-High and NAE1-High groups, with p-values of 0.0085 and 0.0016, respectively.



B

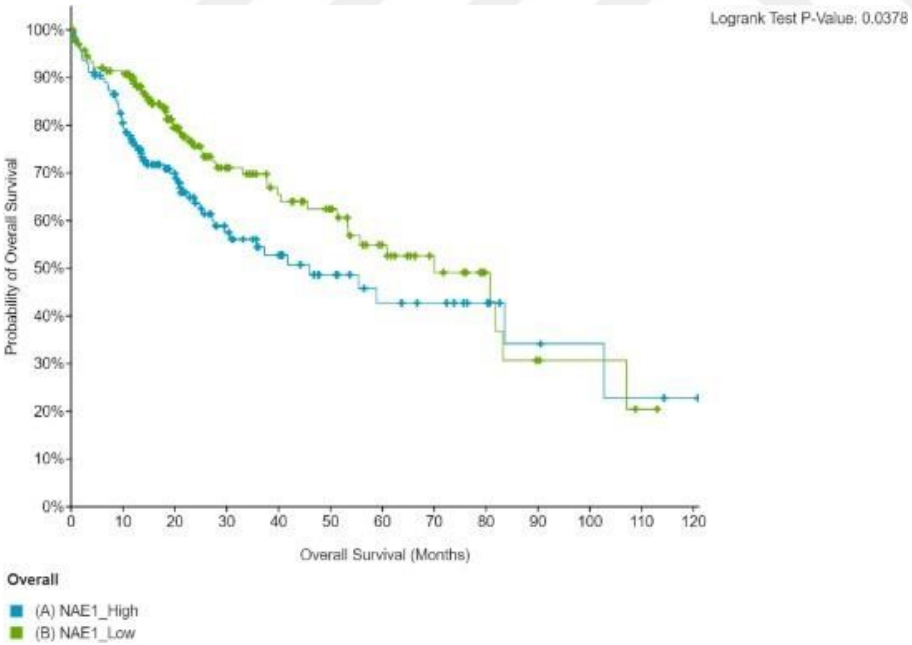


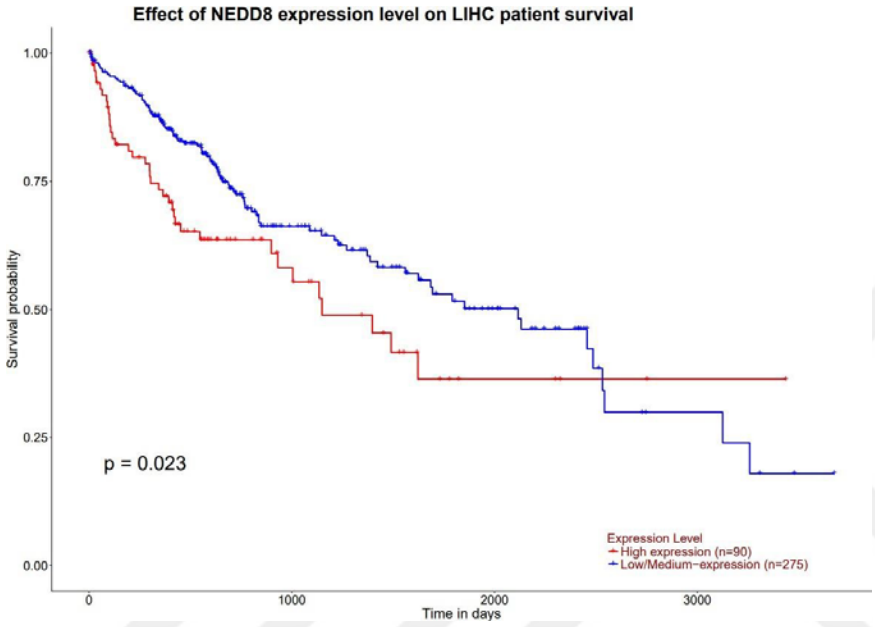
Figure 3.10. Overall survival analysis results from cBioPortal. **A.** Represents the low and high

NEDD8 expression groups. The NEDD8_High group consists of 168 samples, while the

A

NEDD8_Low group has 169 samples. **B.** Displays the groups with low and high NAE1 expression from cBioPortal. The NAE1_High group contains 168 samples, and the NAE1_Low group comprises 169 samples. For both genes, grouping was based on the median.

A



B

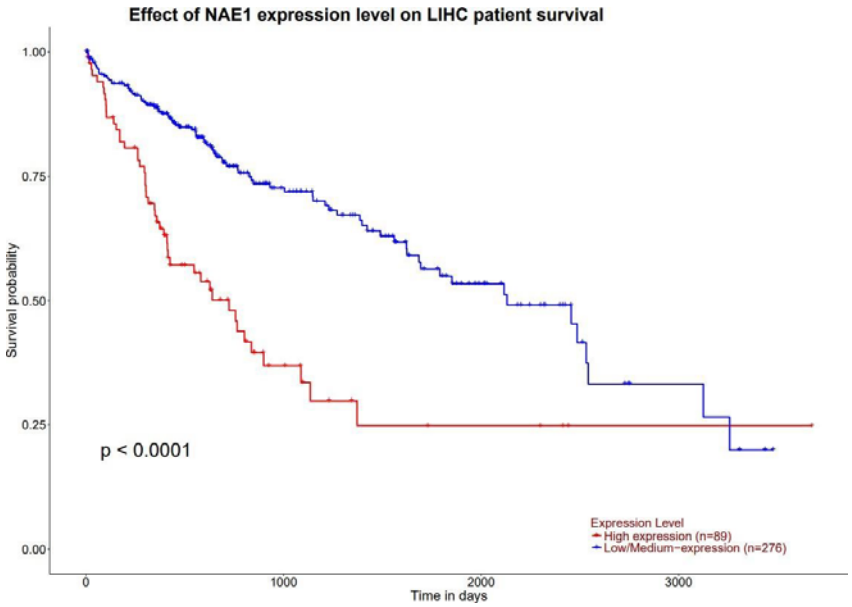


Figure 3.11. Overall survival analysis results from the UALCAN, with **A** focusing on NEDD8 and **B** on NAE1.

B

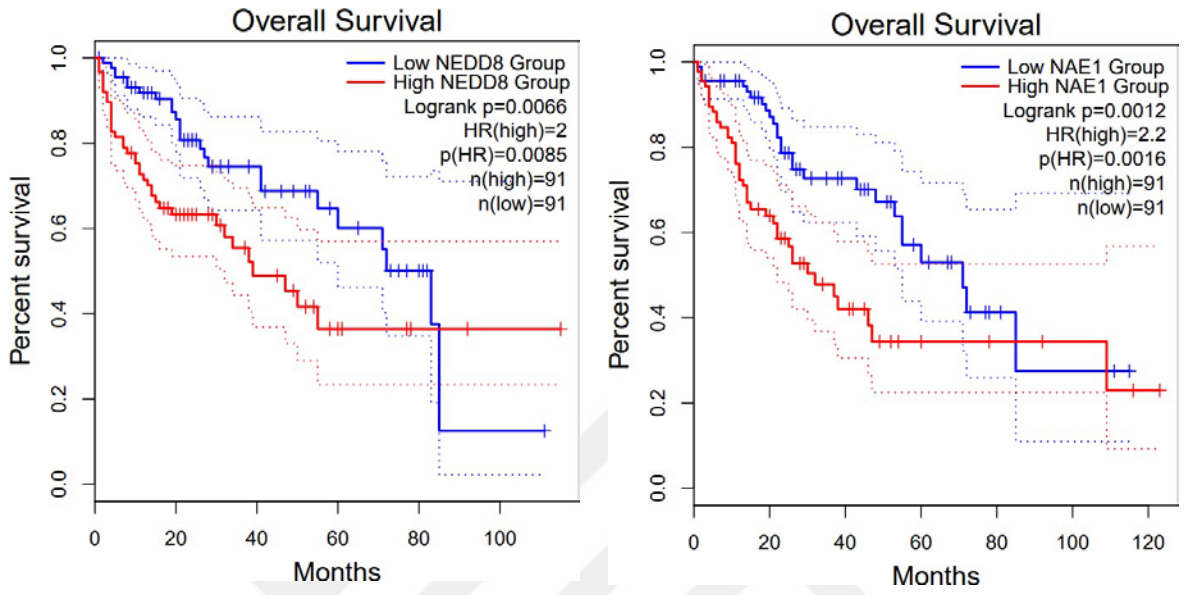


Figure 3.12. Present the survival analyses from GEPIA2. Both genes were analyzed by quartiles, with **A** detailing NEDD8 and **B** focusing on NAE1.

3.5 Identification of genes that differ in patients with high and low expression of *NEDD8* and *NAE1*

Initially, based on their *NEDD8* and *NAE1* expression levels in cBioPortal, samples were categorized into two groups. The variations in gene expressions are depicted in Figure 3.13. This figure, presented in the form of a volcano plot, indicates that the significance increases towards the top. In cBioPortal, significant genes associated with both *NAE1* and *NEDD8* were identified and selected.

A **B**

A

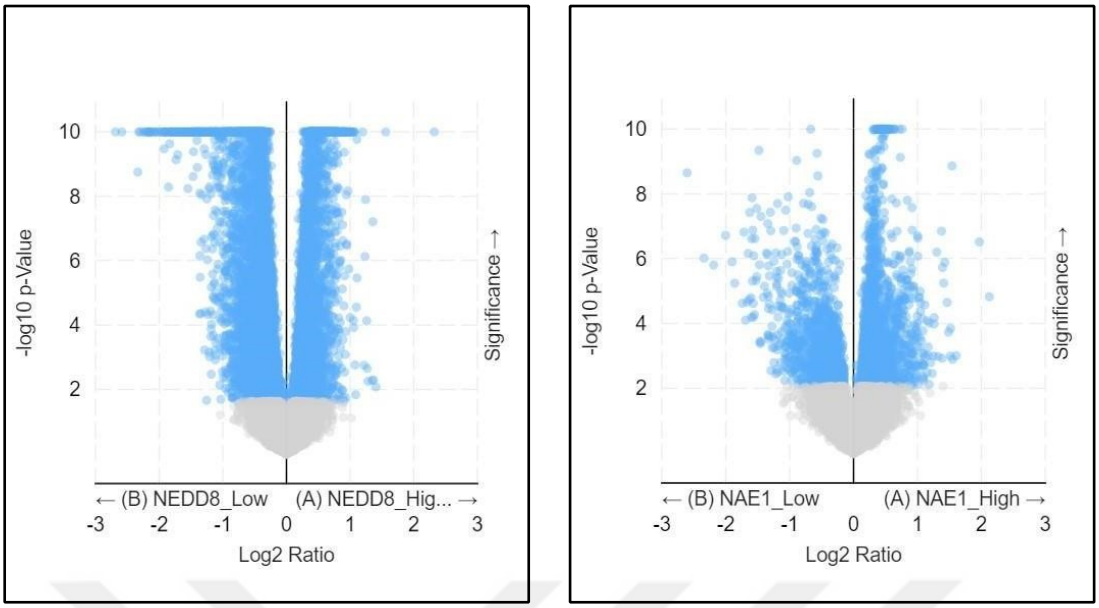


Figure 3.13. Using the RSEM (Batch normalized from Illumina HiSeq_RNASeqV2) values, the mRNA expression of both genes was categorized into two groups. Each dot in the figure represents an individual gene. **A** Represents *NEDD8*. **B** Depicts *NAE1*.

Subsequently, these genes were categorized based on their expression levels. To generate a list, genes with both low and high expression for *NEDD8* and *NAE1* were identified. Figure 3.14 illustrates that 675 genes exhibit a high expression pattern for both *NEDD8* and *NAE1*, whereas 415 genes demonstrate a low expression pattern.

A **B**



Figure 3.14. Representation of common genes with high and low expression for *NEDD8* and *NAE1*. **A.** Showcases the distribution of genes with high expression levels. **B.** Depicts the distribution of genes with low expression levels.

3.6 Pathway and function analyses according to expression differences in patients with high and low expression of important molecules

Pathway and functional analyses were conducted using the common genes identified at the intersections depicted in Figure 3.14. The results in Figure 3.15, sections A and B, stem from the Gene Ontology (GO) enrichment analysis executed within the R programming environment. Though both representations convey the same findings, they are visualized in two distinct formats: a bar plot and a dot plot. Given that significant cutoffs were established for both p and q values during the analysis, the outcomes are statistically meaningful. In Figure 3.15.C, the enrichment analysis was carried out using the GeneCodis program. Both sets of analyses indicate that the genes we derived from the common genes with low NEDD8/NAE1 and high NEDD8/NAE1 play a pivotal role in RNA metabolism.

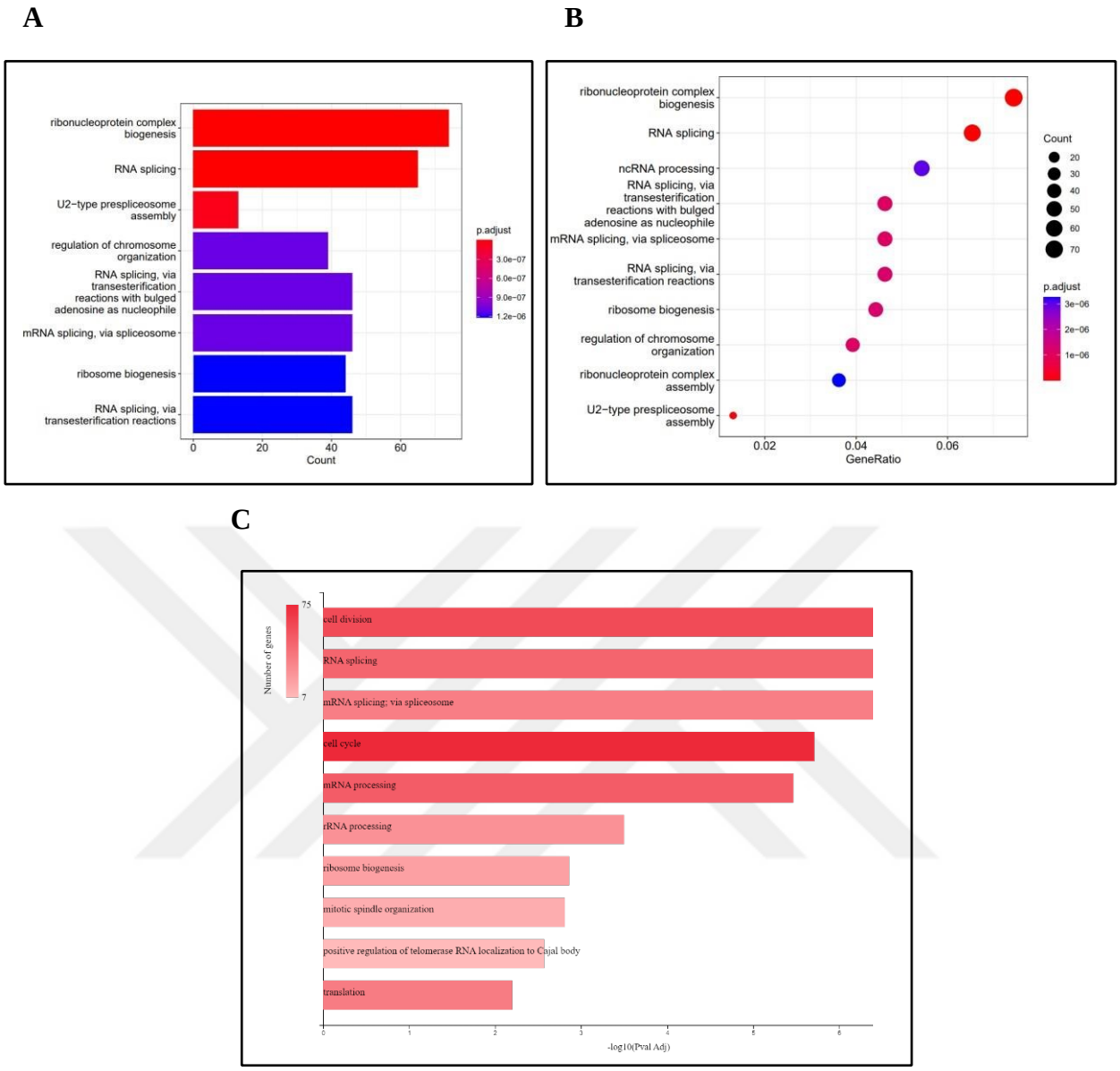


Figure 3.15. Enrichment analysis results for selected genes. **A, B** Depict the Gene Ontology enrichment analysis conducted via the R programming language. A significant cutoff was established for both p and q values during this analysis. **C** Represents the outcomes of the analysis executed on the GeneCodis platform.

Subsequent pathway analysis using the same list via the KEGG Pathway Database yielded data for 319 pathways, as depicted in Figure 3.16. Notably, hepatocellular carcinoma (HCC) is among these pathways, with 15 genes associated with the HCC pathway.

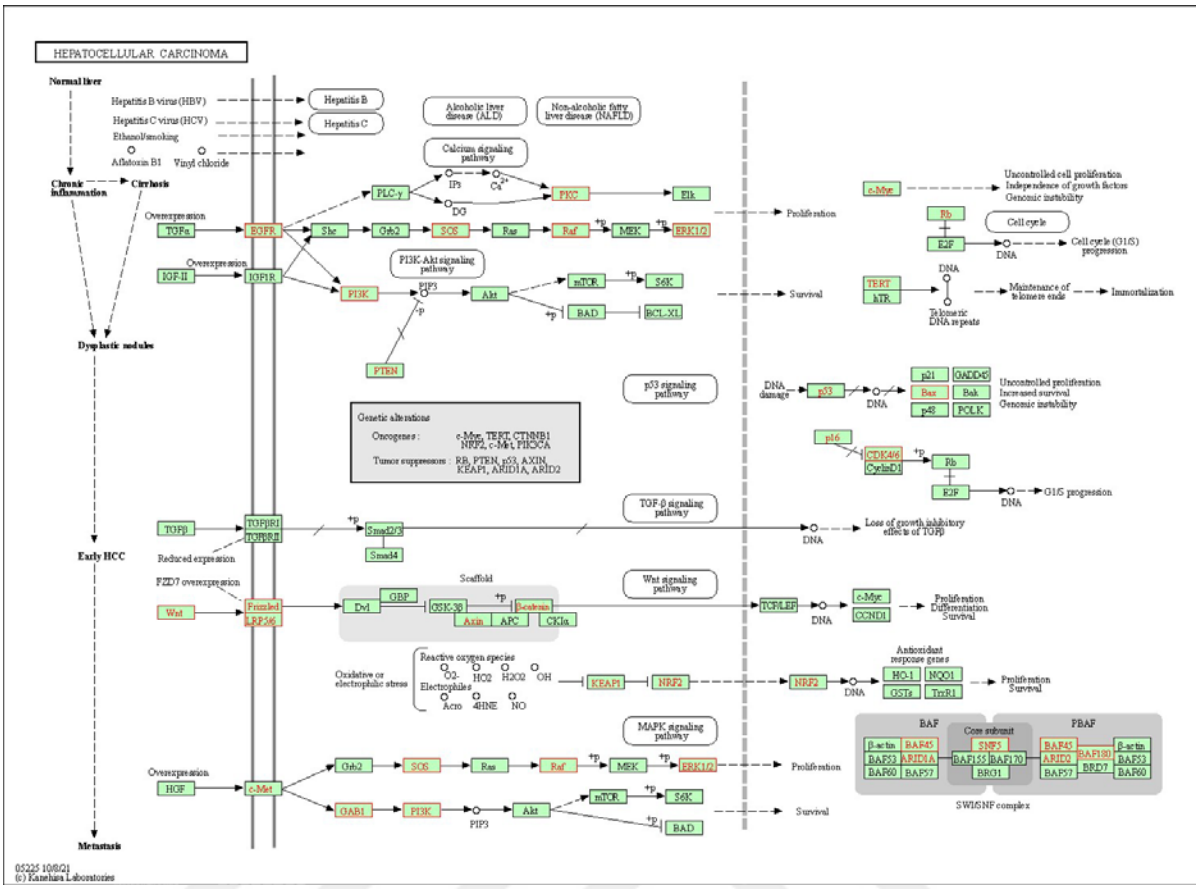


Figure 3.16. Pathway analysis results from the KEGG Pathway Database. The analysis revealed 319 distinct pathways. Notably, hepatocellular carcinoma (HCC) was among them, with 15 genes identified as associated with the HCC pathway.

3.7 Validation Studies

3.7.1. *In silico* validation of expression status of *NEDD8* and *NAE1* in different datasets by RStudio

To perform *in silico* validation analyses, we extracted two datasets from the Xena Browser. The first dataset represents the expression data of samples from the TCGA GDC Liver Cancer cohort. This dataset, labeled as TCGA-LIHC.htseq_fpkm-uq.tsv, comprises gene expression RNAseq data from 424 samples, encompassing both normal and tumor samples. Notably, this data remains unmanipulated. The second dataset from the Xena Browser pertains to phenotype trait data. These phenotype characteristics were also sourced from the GDC TCGA Liver Cancer cohort. Initially, all phenotype features were retrieved, and subsequently, the clinical features highlighted in Table 3.1 were selected from this comprehensive list.

Table 3.1. List of clinical features obtained from Xena Browser

Clinical Features Used for WGCNA Analysis from Xena Browser
1- adjacent_hepatic_tissue_inflammation_extent_type
2- albumin_result_lower_limit
3- bilirubin_lower_limit
4- bmi.exposures
5- ethnicity.demographic
6- fetoprotein_outcome_lower_limit
7- fibrosis_ishak_score
8- gender.demographic
9- history_of_neoadjuvant_treatment
10- lost_follow_up
11- morphology.diagnoses
12- neoplasm_histologic_grade
13- pathologic_M.
14- pathologic_N
15- pathologic_T
16- person_neoplasm_cancer_status
17- postoperative_rx_tx
18- post_op_ablation_embolization_tx
19- total_bilirubin_upper_limit
20- tumor_stage.diagnoses
21- vascular_tumor_cell_type
22- weight

The selection criteria were based on parameters influencing the treatment and prognosis of liver cancer. Our primary objective is to discern how gene expression profiles impact a patient's prognosis, treatment, and response to treatment. These parameters are pivotal in predicting the prognosis and treatment response in liver cancer patients. After gathering data for these 22 features, all data were numerically recoded in RStudio to prepare them for co-expression network analysis. The original data remained unaltered; only character-based features were numerically represented. In the phenotype data, recurrent and normal samples were excluded, focusing solely on the primary tumor, aiming to identify correlations pertinent to prognosis. Consequently, we have two prepared datasets. The subsequent step involves WGCNA analysis. Necessary packages, including the WGCNA package, are set up in R. Expression data are then imported, checked for excessive missing values, and outlier samples are identified. Phenotype

features are subsequently loaded. The analysis proceeds with network construction and module detection, culminating in determining the module-trait relationship, as illustrated in Figure 3.17.

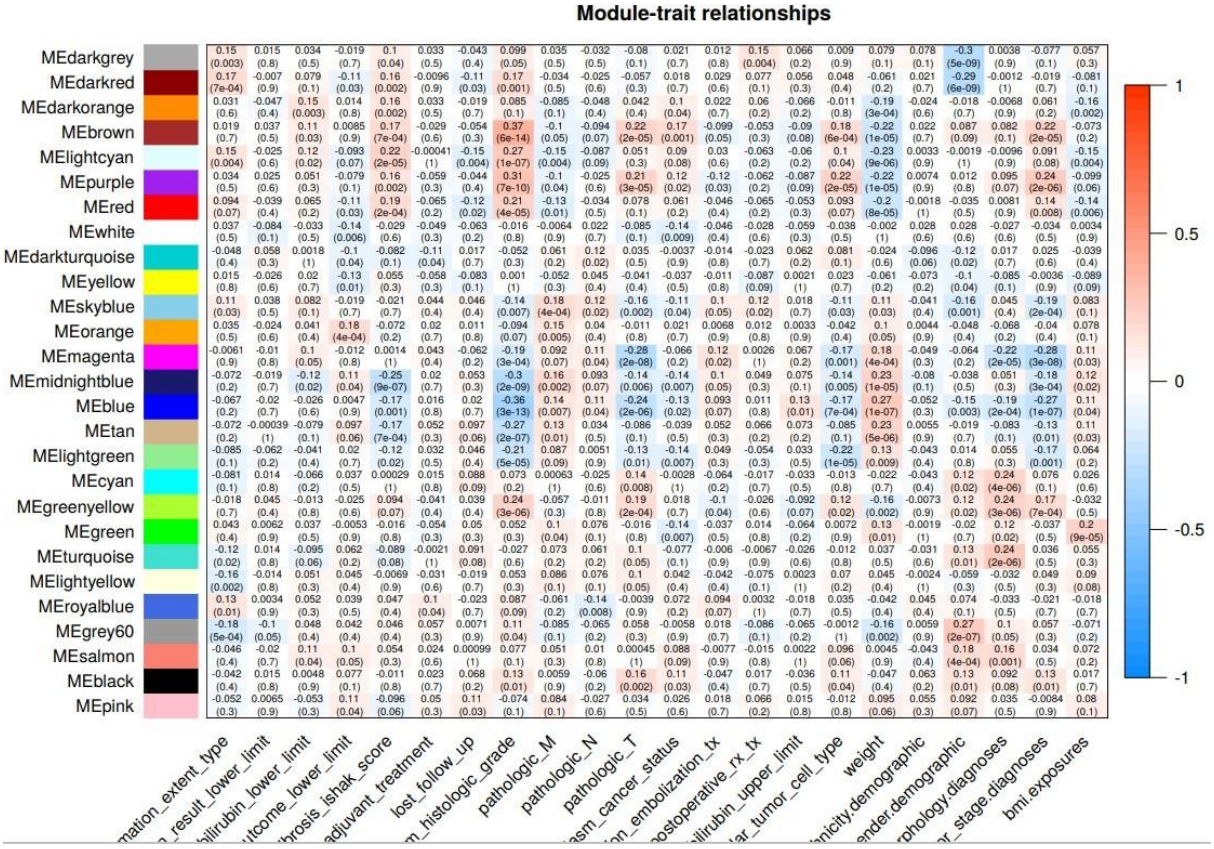


Figure 3.17. Illustrates the module-trait relationship. The gene expression data, sourced from Xena Browser, were segmented into subsets termed as modules. These modules were formed based on high correlation levels among specific gene groups. Each color depicted on the image's left side signifies a specific correlation strength. As the color shifts towards blue, it indicates an increasing inverse correlation, whereas a shift towards red denotes an increasing direct correlation. Clinical features are displayed on the horizontal axis.

Based on high correlation levels among specific gene groups, subclusters were formed. Each of these clusters was then analyzed to determine their correlation with the selected prognostic factors. Our analysis revealed that *NEDD8*, one of our genes of interest, was located in the 'yellow' cluster, while *NAE1* was situated in the 'purple' cluster.

No significant correlation was observed between the yellow module and any clinical features. However, the purple module exhibited a strong correlation with the neoplasm histological

grade. As depicted in Figure 3.18, there's a direct proportionality between the purple module and the neoplasm histological grade.



Figure 3.18. Illustrates the correlation of the purple module with the clinical feature of the neoplasm histological grade.

During the WGCNA analysis, a network topology analysis is also conducted. We use the network topology analysis of Figure 3.19 as a mathematical proof of our analysis. In subsequent steps, a soft threshold is set to determine co-expression similarity and adjacency, with power 8 being chosen. This power is the lowest at which the curve of the scale-free topology fit index plateaus upon reaching a high value, as depicted in Figure 3.19. Following the establishment of co-expression similarity and adjacency based on the network topology analysis results, the outcome of the hierarchical clustering calculation is displayed in dendrograms, which also merges with the color modules.

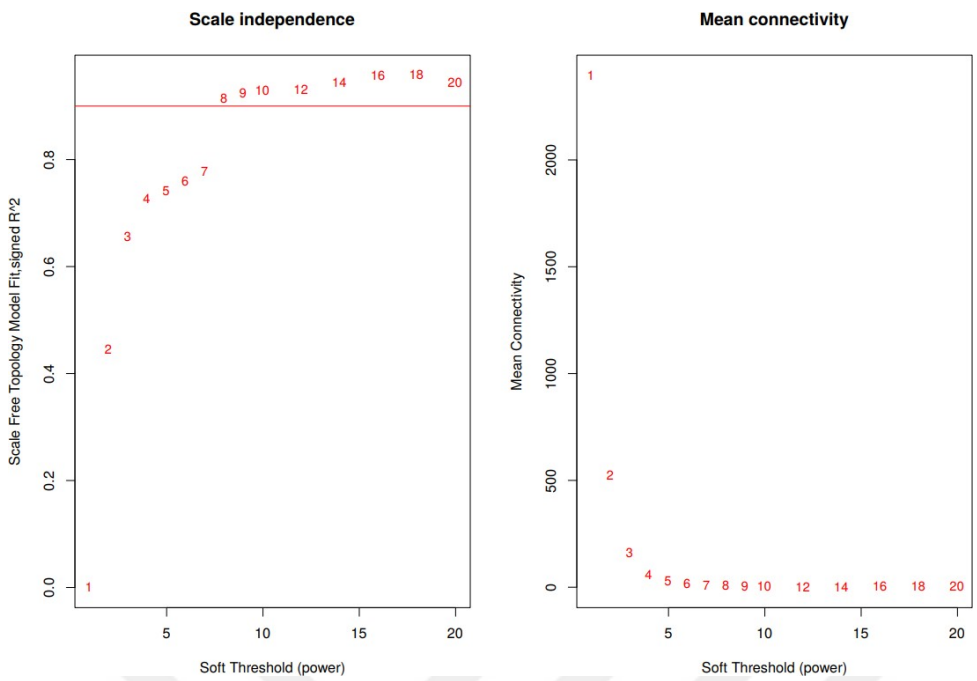


Figure 3.19. Presents the analysis of network topology.

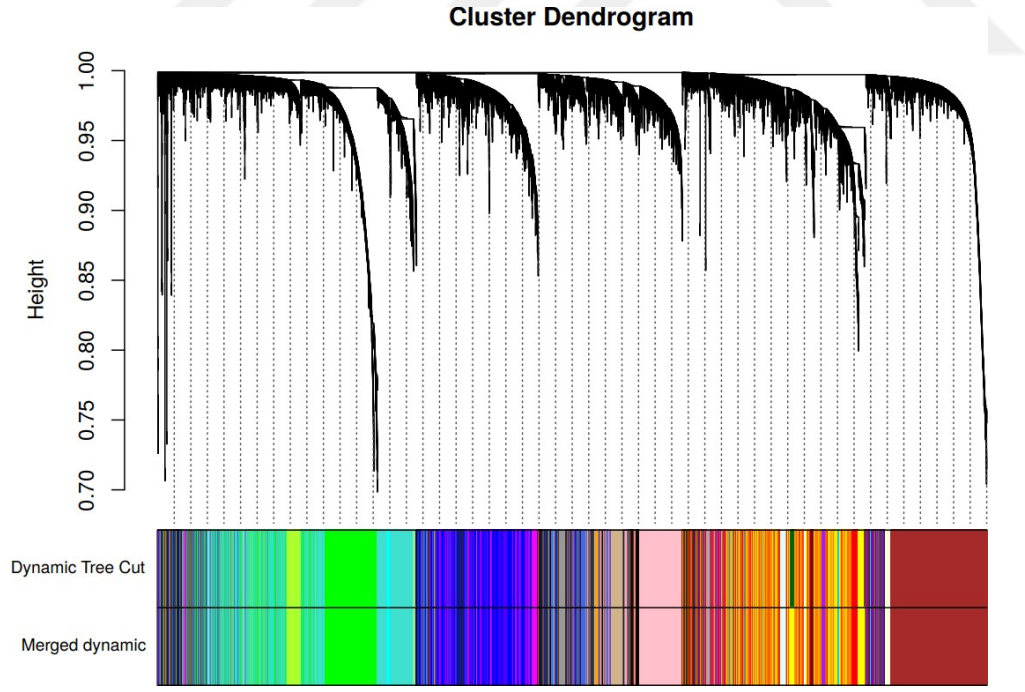


Figure 3.20. Depicts the clustering dendrogram of genes.

Figure 3.20 provides a simpler representation of the hierarchical distribution of genes, illustrating the similarity or distance between modules based on their expression profiles. Furthermore, the module-trait relationship revealed a striking similarity in the patterns of the brown, light cyan, purple, and red color modules concerning clinical features. An examination of the hierarchical distribution of the modules visualized in Figure 3.21 confirmed that these four modules exhibited high similarity to each other.

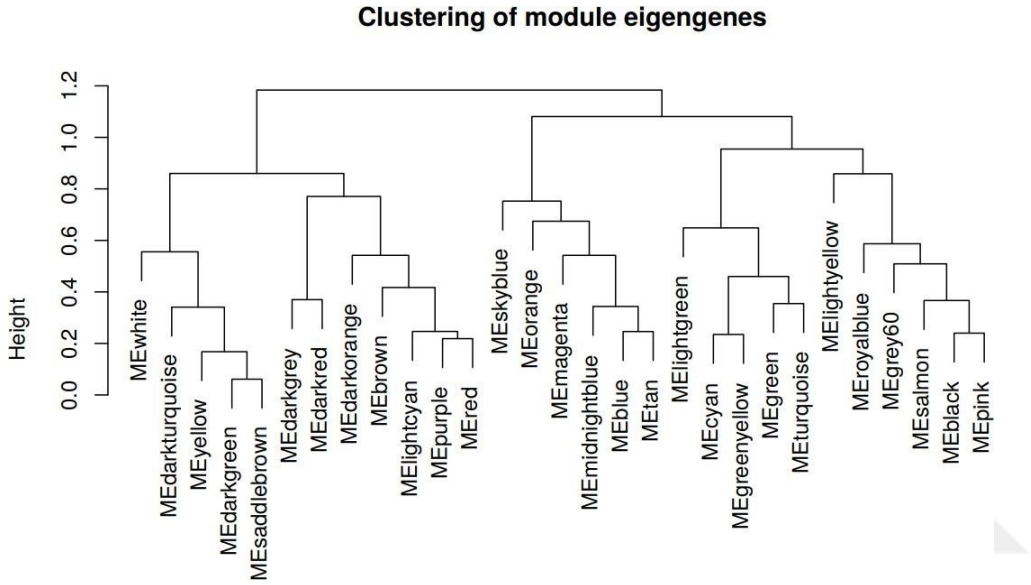


Figure 3.21. Illustrates the hierarchical distribution of gene modules.

Figure 3.22 exhibits the count of genes within each gene module. The yellow module, which encompasses NEDD8, has a higher gene count compared to the purple module that contains NAE1.

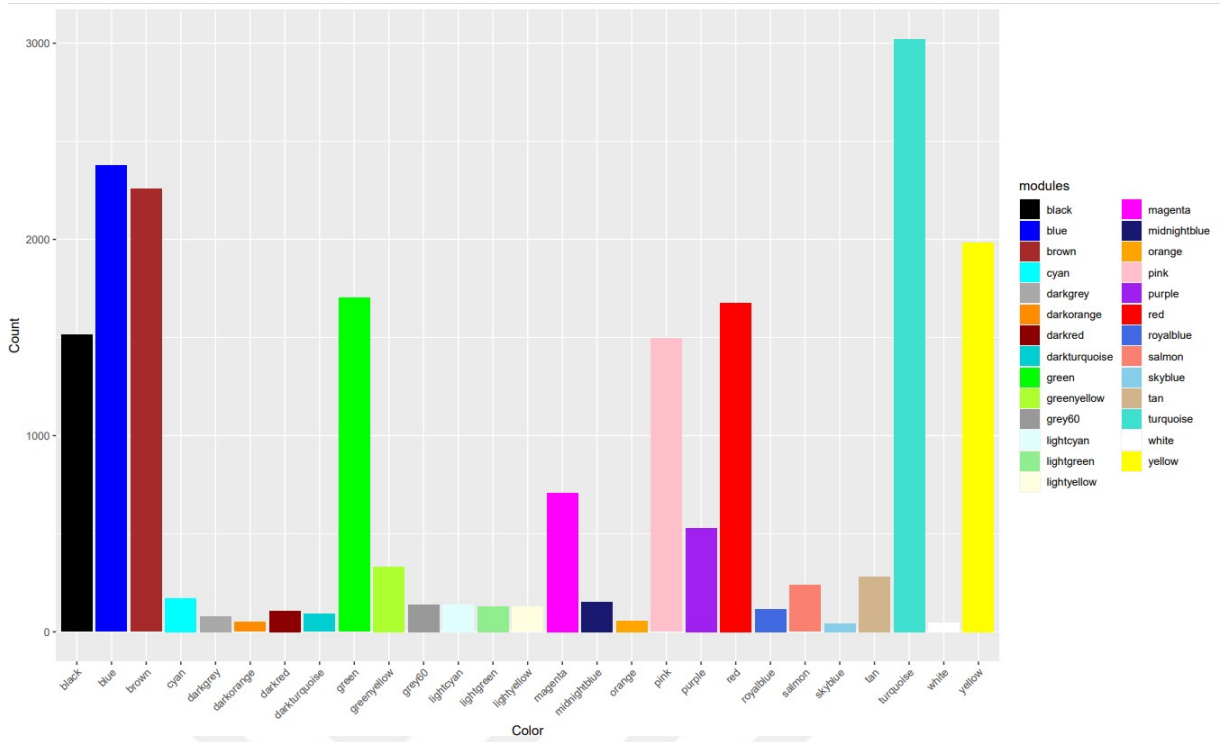


Figure 3.22. Illustrates the number of genes present in each module.

To better define the gene modules derived from the WGCNA analysis and gather more insights about the protein-coding genes, gene annotation was conducted. Given the extensive list of genes, which poses challenges for robust statistical analysis, we applied a filter to focus solely on protein-coding genes. Enrichment analysis was then performed on this refined list. The purpose of this analysis was to identify the biological processes associated with each module and to discern the processes more closely related to our genes of interest, *NEDD8* and *NAE1*. The enrichment analysis results for the yellow module, which contains *NEDD8*, are presented in Table 3.2. As this analysis was executed in R environment, a significance threshold was set for the p-value, ensuring that all listed processes have a statistically significant association with the yellow module.

Table 3.2. Enrichment Analysis Results for the Yellow Module

Description	pvalue
aerobic respiration	4.04E-42
oxidative phosphorylation	3.66E-41
cellular respiration	3.98E-41
mitochondrial gene expression	2.58E-39
mitochondrial translation	2.63E-38

mitochondrial respiratory chain complex assembly	4.69E-37
proton motive force-driven ATP synthesis	2.53E-33
energy derivation by oxidation of organic compounds	3.46E-33
ATP synthesis coupled electron transport	1.39E-31
mitochondrial ATP synthesis coupled electron transport	1.39E-31
proton motive force-driven mitochondrial ATP synthesis	2.33E-30
aerobic electron transport chain	1.93E-29
respiratory electron transport chain	2.01E-29
ATP biosynthetic process	1.28E-28
purine ribonucleoside triphosphate biosynthetic process	2.03E-27
purine nucleoside triphosphate biosynthetic process	3.26E-27
ribonucleoside triphosphate biosynthetic process	3.90E-27
electron transport chain	2.26E-26
nucleoside triphosphate biosynthetic process	5.94E-26
NADH dehydrogenase complex assembly	1.99E-24
mitochondrial respiratory chain complex I assembly	1.99E-24
ATP metabolic process	3.16E-22
purine ribonucleoside triphosphate metabolic process	2.58E-20
ribonucleoside triphosphate metabolic process	3.02E-20
purine nucleoside triphosphate metabolic process	8.10E-20
nucleoside triphosphate metabolic process	1.48E-18
purine ribonucleotide biosynthetic process	2.71E-18
mitochondrial electron transport, NADH to ubiquinone	5.84E-18
ribonucleotide biosynthetic process	1.45E-17
ribose phosphate biosynthetic process	6.33E-17
purine nucleotide biosynthetic process	7.77E-17
purine-containing compound biosynthetic process	1.52E-16
proton transmembrane transport	2.31E-16
nucleotide biosynthetic process	6.84E-15
nucleoside phosphate biosynthetic process	9.48E-15
purine ribonucleotide metabolic process	2.73E-14
cytochrome complex assembly	5.10E-11
macroautophagy	1.23E-10
protein targeting	7.72E-10
cell redox homeostasis	8.28E-10
mitochondrial cytochrome c oxidase assembly	2.21E-09

Description	pvalue
inner mitochondrial membrane organization	3.32E-09
regulation of mitochondrial gene expression	4.06E-09
mitochondrial electron transport, cytochrome c to oxygen	7.62E-09
establishment of protein localization to membrane	1.19E-08

regulation of mitochondrial translation	1.47E-08
respiratory chain complex IV assembly	4.09E-08
cristae formation	4.43E-08
mitochondrial membrane organization	4.65E-08
organelle disassembly	5.63E-08
mitochondrial electron transport, ubiquinol to cytochrome c	8.64E-08
vacuole organization	1.82E-07
mitochondrial transmembrane transport	4.70E-07
tRNA metabolic process	4.79E-07
protein targeting to mitochondrion	5.49E-07
mitochondrial transport	6.78E-07
protein insertion into membrane	9.79E-07
vesicle organization	1.06E-06
endosome transport via multivesicular body sorting pathway	1.12E-06
endoplasmic reticulum organization	1.87E-06
ubiquitin-dependent ERAD pathway	1.87E-06
protein insertion into ER membrane	1.89E-06
establishment of protein localization to mitochondrion	2.02E-06
tRNA processing	2.07E-06
mitochondrial respirasome assembly	2.36E-06
ERAD pathway	3.57E-06
protein folding	4.65E-06
protein localization to mitochondrion	4.94E-06
protein targeting to membrane	5.70E-06
protein insertion into ER membrane by stop-transfer membrane-anchor sequence	6.03E-06
lysosome organization	6.57E-06
lytic vacuole organization	6.57E-06
establishment of protein localization to organelle	7.21E-06
ribosome biogenesis	7.63E-06
response to endoplasmic reticulum stress	8.06E-06
multivesicular body sorting pathway	8.94E-06
tail-anchored membrane protein insertion into ER membrane	8.96E-06
regulation of protein catabolic process	1.16E-05
positive regulation of mitochondrial translation	1.42E-05
Golgi vesicle transport	1.46E-05
ncRNA processing	2.19E-05
vesicle budding from membrane	2.28E-05
virion assembly	2.36E-05

Description	pvalue
autophagy of mitochondrion	3.14E-05

mitochondrion disassembly	3.14E-05
endoplasmic reticulum to Golgi vesicle-mediated transport	3.48E-05
lipoprotein biosynthetic process	3.51E-05
regulation of proteasomal protein catabolic process	3.56E-05
protein localization to endoplasmic reticulum	3.89E-05
endosome to lysosome transport via multivesicular body sorting pathway	4.15E-05
quinone metabolic process	4.27E-05
protein lipidation	5.06E-05
endosome organization	5.06E-05
vacuolar acidification	5.29E-05
TORC1 signaling	7.38E-05
autophagosome maturation	9.05E-05
positive regulation of mitochondrial membrane potential	9.68E-05
lipoprotein metabolic process	0.000103951
autophagy of nucleus	0.00014481
cellular oxidant detoxification	0.000146292
melanosome assembly	0.000154393
sulfur compound metabolic process	0.000154482
peptidyl-cysteine modification	0.000178168
cellular response to increased oxygen levels	0.000180161
regulation of proteolysis involved in protein catabolic process	0.000186766
late endosome to vacuole transport	0.000211484
lysosomal lumen acidification	0.000225247
hydrogen peroxide metabolic process	0.00023108
COPII-coated vesicle budding	0.00024795
viral budding	0.000268763
intracellular pH reduction	0.000269762
melanosome organization	0.000270286
selective autophagy	0.00027887
regulation of generation of precursor metabolites and energy	0.000297097
pigment granule organization	0.000342291
cellular detoxification	0.000347638
retrograde protein transport, ER to cytosol	0.000361556
endoplasmic reticulum to cytosol transport	0.000361556
protein exit from endoplasmic reticulum	0.000380844
viral budding via host ESCRT complex	0.000446657
iron-sulfur cluster assembly	0.000479236
metallo-sulfur cluster assembly	0.000479236
TOR signaling	0.000486833
positive regulation of cellular respiration	0.000507168
ubiquinone metabolic process	0.000512114
regulation of intrinsic apoptotic signaling pathway	0.000538283

Description	pvalue
regulation of TORC1 signaling	0.000569422
vesicle targeting, to, from or within Golgi	0.000593565
regulation of proton transport	0.000609981
regulation of TOR signaling	0.000646353
respiratory chain complex III assembly	0.000652797
mitochondrial respiratory chain complex III assembly	0.000652797
protein targeting to ER	0.000689807
autophagosome organization	0.00074661
ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway	0.000747025
vacuolar transport	0.000752337
regulation of endosome size	0.000786842
tricarboxylic acid cycle	0.000809013
positive regulation of proteasomal protein catabolic process	0.00082782
positive regulation of protein catabolic process	0.000830171
mitochondrial RNA metabolic process	0.000994974
regulation of vesicle size	0.001021449
multivesicular body assembly	0.001032361
response to oxidative stress	0.001035041
regulation of lysosomal lumen pH	0.00108006
response to increased oxygen levels	0.00108006
protein insertion into mitochondrial inner membrane	0.001172061
positive regulation of membrane potential	0.001172061
positive regulation of proteolysis involved in protein catabolic process	0.001201888
protein palmitoylation	0.001303102
multivesicular body organization	0.001303102
intracellular protein transmembrane transport	0.00140474
establishment of protein localization to endoplasmic reticulum	0.00140474

The first ten biological processes from Table 3.2 are visualized in Figure 3.23, which is represented as a dot plot. Within the yellow module, energy metabolism is the dominant process. Although *NEDD8* is not directly mapped to a specific biological process in the gene enrichment analyses, it does not imply its irrelevance to the observed results. Given that *NEDD8* is part of the yellow module and shares a similar expression pattern with other genes therein, it suggests a potential association with these biological processes.

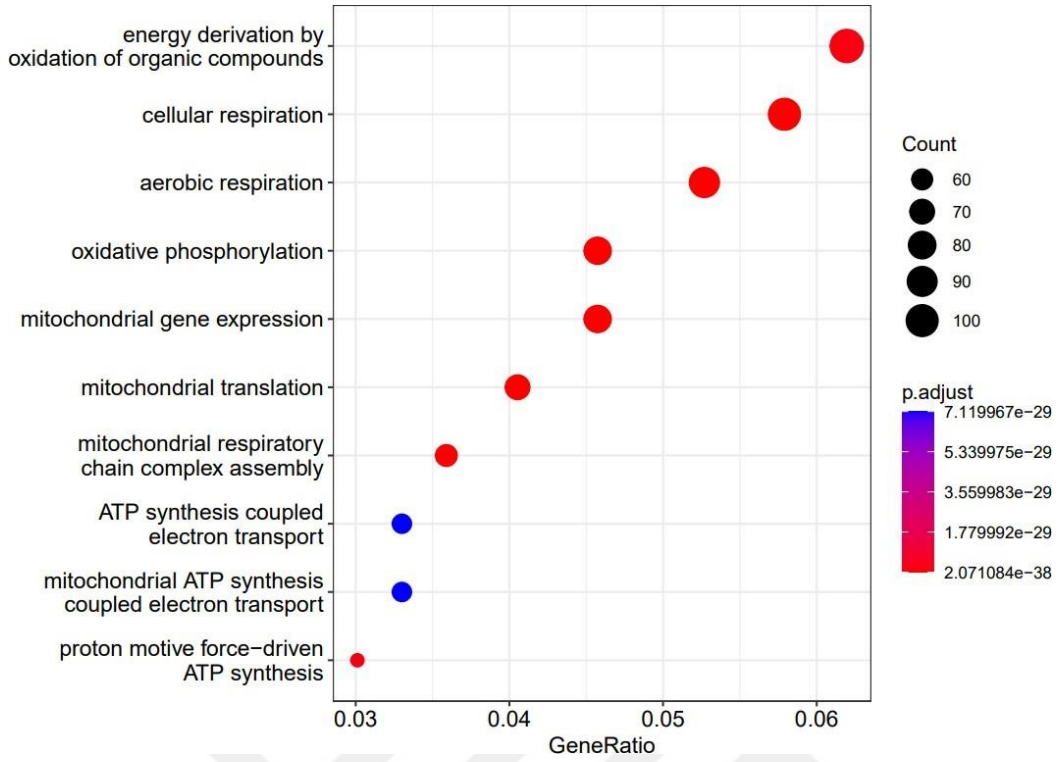


Figure 3.23. Illustrates the top ten biological processes within the yellow module. In this dot plot, the size of each dot corresponds to the number of genes linked to the respective biological process, while the color intensity varies based on the significance level of the p-value.

Subsequent analyses were conducted to derive KEGG pathway results using R. These results are depicted in Figure 3.24. Notably, the identification of non-alcoholic fatty liver disease aligns with the thesis hypothesis. Moreover, diseases closely associated with protein degradation emerge as dominant within this module.

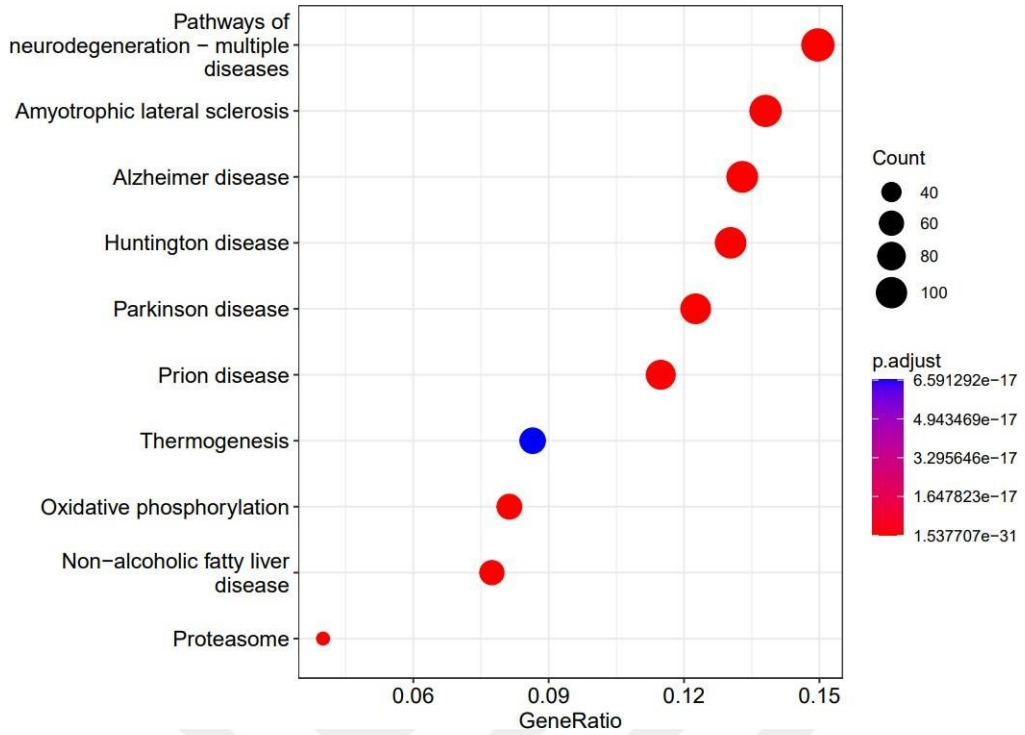


Figure 3.24. Presents the results from the KEGG Pathway analysis.

The purple module stands out as particularly significant due to its inclusion of *NAE1*. Enrichment analysis results for this module can be found in Table 3.3, highlighting notably impactful results related to cell fate. For a more visual representation of the purple module's enrichment analysis, refer to Figure 3.25, which displays the data in a dot plot format.

Table 3.3. provides a comprehensive list of biological processes associated with the purple module.

Description	pvalue
cell differentiation in spinal cord	1.11E-06
ventral spinal cord development	5.96E-06
central nervous system neuron differentiation	3.07E-05
neuron fate determination	3.50E-05
spinal cord motor neuron differentiation	3.70E-05

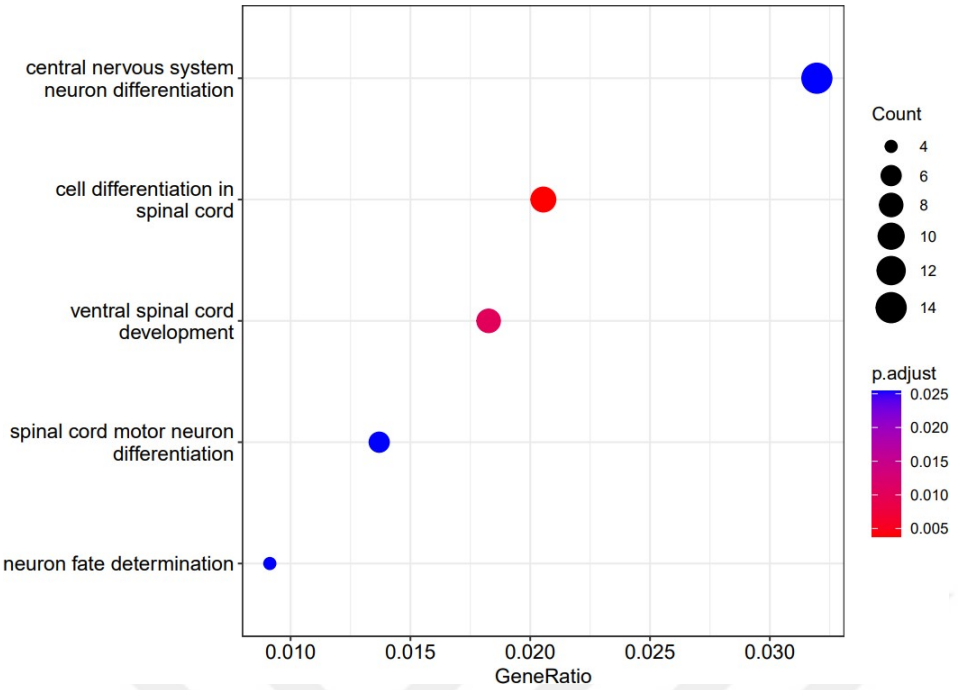


Figure 3.25. Provides a visualization of the biological processes associated with the purple module.

However, it is worth noting that the KEGG Pathway analysis results for the purple module could not be generated in RStudio due to an insufficient number of annotated genes.

3.7.2 *In silico* validation of expression status of *NEDD8* and *NAE1* in HCC cell line datasets

Subsequently, we investigated the mRNA expression levels of *NEDD8* and *NAE1* in HCC cell lines. Initially, we examined the expression pattern (measured in FPKM) using the Expression Atlas (CCLE) database, as visualized in Fig 3.26.

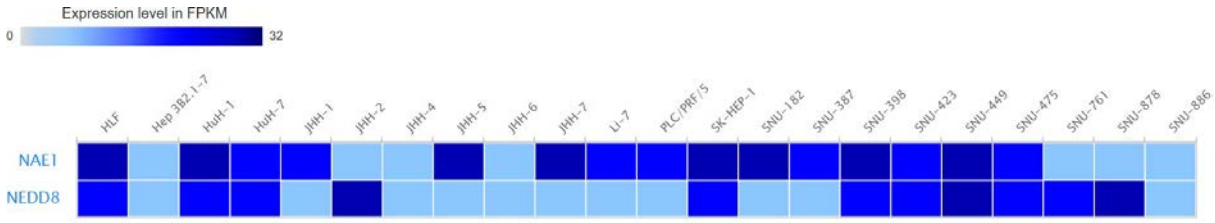
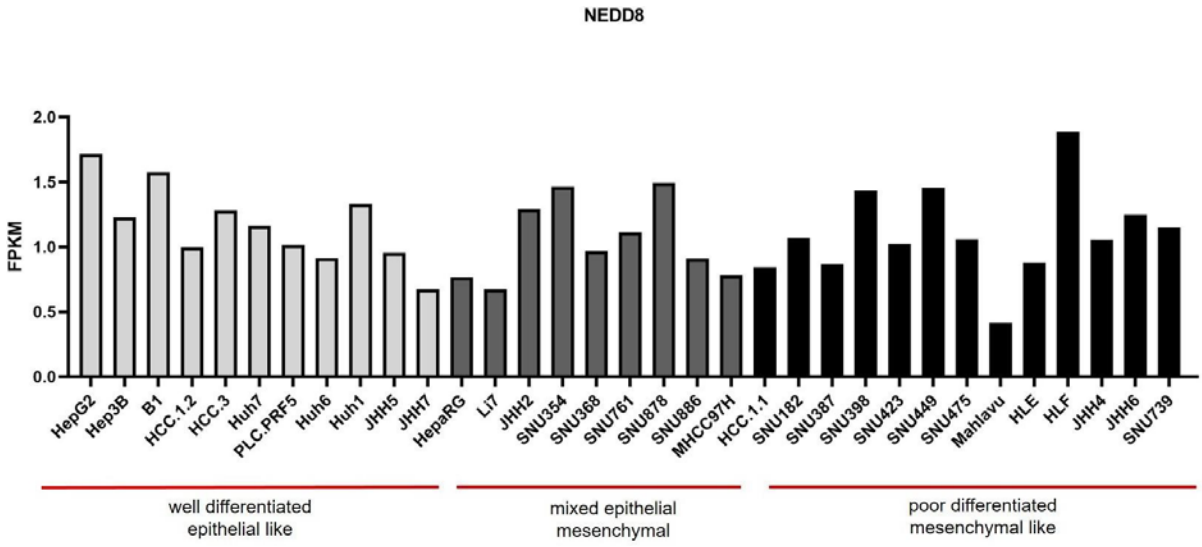


Figure 3.26. Presents the RNA-seq of HCC lines from the Cancer Cell Line Encyclopedia, with data units in FPKM.

Caruso et al. 2019 classified HCC cell lines based on their differentiation status using genomic, transcriptomic, and protein profile analyses into three categories: The CL1 group comprises hepatoblast-like cells with an epithelial phenotype, corresponding to the progenitor subclass of HCC patients, the CL2 subgroup consists of cell lines with a mixed epithelial-mesenchymal phenotype, while the CL3 subgroup encompasses mesenchymal-like cell lines with a poorly differentiated phenotype. Our analysis of the *NEDD8* and *NAE1* expression patterns according to this classification revealed that both NEDD8-High and NAE1-High cells predominantly belong to the CL3 subgroup. This observation aligns with our bioinformatic analyses of patient cohorts, which indicated that the expression levels of *NEDD8* and *NAE1* were higher in patients with lower overall survival rates and advanced HCC.

In light of these observations, we subsequently examined the expression levels of *NAE1* and *NEDD8* in the RNA-seq data (measured in FPKM) from the Liver Cancer Cell Line (LCCL) dataset as presented by Caruso et al. (2019). Figure 3.27 illustrates that the *NEDD8* expression is elevated in the poorly differentiated CL3 subgroup of cell lines. However, the *NAE1* expression does not exhibit a discernible correlation with the differentiation status of the tested cell lines.

A



B

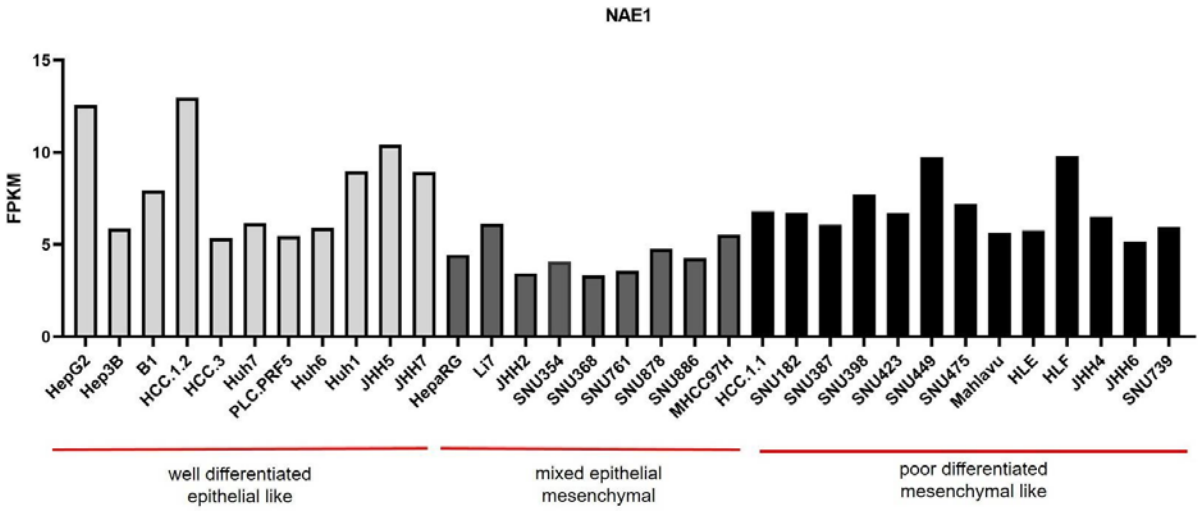


Figure 3.27. RNA-seq of HCC lines sourced from the LCCL dataset. The data is represented in FPKM units and was visualized using MS Excel. The red lines in the graphs indicate the median values. A For *NEDD8*, the median value is 1.05. B For *NAE1*, the median value is 6.08

3.7.3 *In vitro* validation of *NEDD8* and *NAE1* expression status of HCC cell lines by RT-qPCR

As a final step to validate our bioinformatics data, we assessed the NEDD8 and NAE1 expression status in 11 liver cancer cell lines available in our lab. These cell lines include SNU 182, SNU 387, SNU 423, SNU 475, SNU 449, HuH7, Mahlavu, SKHEP1, HEPG2, HEP40, and FOCUS.

A

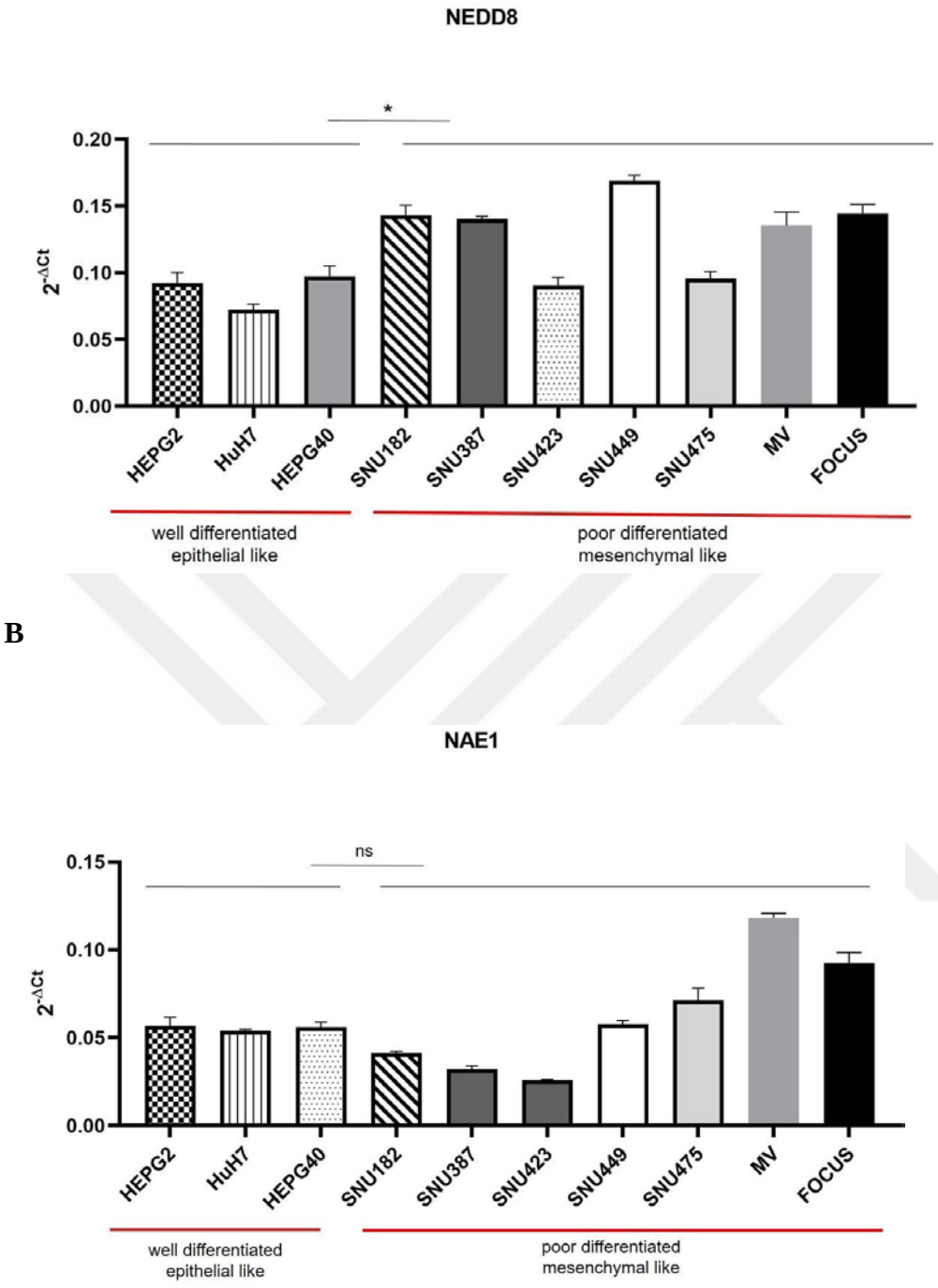


Figure 3.28. RT-qPCR results for NEDD8 and NAE1, visualized using GraphPad.

Consistent with the findings of Caruso et al. (2019), our RT-qPCR analysis revealed that HCC cell lines exhibiting high NEDD8 expression levels are predominantly poorly differentiated and fall within the CL3 subgroup.

4. DISCUSSION

Hepatocellular carcinoma (HCC) stands as a pressing health concern in contemporary medicine. Its alarmingly high mortality rate underscores the urgency of addressing this malignancy. The current therapeutic landscape for HCC remains limited, with Sorafenib emerging as a primary treatment option, albeit predominantly for advanced stages of the disease (Yang et al., 2019). This therapeutic limitation underscores the imperative need to identify potent and actionable biomarkers that can guide early diagnosis and targeted treatment strategies for HCC. A pivotal avenue of investigation in this context is the exploration of molecular mechanisms, particularly post-translational modifications (PTMs). PTMs represent intricate, dynamic, and often reversible processes that induce structural and functional alterations in proteins. These modifications can influence a protein's activity, stability, and cellular localization, with phosphorylation, glycosylation, and ubiquitination being some of the prominent examples (Piñero et al., 2020; Olaizola et al., 2022). Among these, ubiquitination and modifications involving ubiquitin-like proteins play a cardinal role in modulating cellular processes, including cell proliferation and differentiation (Jones et al., 2008). Neddylation, a distinct PTM, involves the conjugation of NEDD8, a member of the Ubiquitin-Like Protein (UBL) family, to target protein lysines (Yu et al., 2018). While neddylation is implicated in a myriad of cellular processes potentially linked to oncogenesis, its precise mechanistic role remains an enigma, warranting further exploration (Olaizola et al., 2022).

In our investigation, we delved into the roles and expression patterns of NEDD8 and its activating enzyme, E1 subunit 1 (NAE1), both pivotal components of the neddylation pathway. Our primary objective was to discern the expression dynamics of NEDD8 and NAE1 in normal versus tumor samples. Utilizing the GEPIA2 platform for an extensive analysis across various cancer cohorts, we observed a pronounced elevation in the expression levels of both NEDD8 and NAE1 in tumor samples compared to their normal counterparts. Notably, the significance of NEDD8's expression pattern in LIHC was among the top quartile. Subsequent evaluations using the Xena browser and UALCAN databases corroborated these findings, further emphasizing the heightened expression of these molecules in tumor samples. This trend was consistent at the protein level, as evidenced by our analysis in the UALCAN database, where both NEDD8 and NAE1 exhibited elevated protein expression in tumor samples, mirroring their gene expression patterns. The consistent and marked upregulation of NEDD8 and NAE1 in

tumor samples across multiple databases prompted us to postulate its potential prognostic implications in HCC. This hypothesis is further supported by literature, with Zhang et al. (2019) linking elevated expression of these molecules to a poorer prognosis in HCC patients.

Our subsequent endeavor was to discern the association of NEDD8 and NAE1 with specific clinical features. Utilizing the patient data available in cBioPortal, we categorized NEDD8 and NAE1 into two distinct groups based on their median expression levels. A comprehensive scan of numerous clinical features in cBioPortal yielded three features of significance: Neoplasm Disease Stage American Joint Committee on Cancer Code, American Joint Committee on Cancer Tumor Stage Code, and Neoplasm Histologic Grade. We further examined the variations in these clinical features across the NEDD8/NAE1 high and NEDD8/NAE1 low groups. A consistent pattern emerged for NAE1 across all three clinical features. The NAE1_High group exhibited an increased representation in advanced stages compared to the NAE1_Low group. While NEDD8's significance was less pronounced, it displayed elevated levels in stages 1 and 2, and also in advanced grades. Notably, the stage typically reflects tumor size and invasion, whereas the grade offers insights into differentiation. This observation aligns with the data from cell lines, and the gene enrichment results for NEDD8 resonate with proliferation genes. Furthermore, when both NEDD8 and NAE1 exhibit high expression, the disparity in grade becomes even more pronounced, especially in advanced stages G3+G4. In contrast, the group with low expression of both genes predominantly represented the early stages. This observation is in harmony with our pathway analyses, where altered genes in the KEGG database predominantly pertained to differentiation. Corroborating our findings, literature cites that MLN4924 inhibits cell proliferation in HCC cells (Yang et al., 2019), which aligns seamlessly with our clinical feature analyses.

In our exploration of survival outcomes, the combined NEDD8/NAE1 high and low groups did not yield a statistically significant p-value in the overall survival analysis. Intriguingly, when focusing solely on NAE1, a pronounced poor prognosis was evident in the NAE1 high group. This observation was further corroborated by survival analyses conducted using both GEPIA2 and UALCAN databases, where p-values were below the 0.05 threshold, indicating statistical significance. The consistent results for both NEDD8 and NAE1 bolster the hypothesis that these molecules may serve as prognostic biomarkers. It's worth noting that the limited customization options in cBioPortal, particularly when grouping by median, might have

influenced our findings. For a more comprehensive understanding, it would be prudent to analyze clinical characteristics using alternative databases in future studies.

Our investigations employing WGCNA highlighted biological processes associated with energy metabolism in the yellow module, where NEDD8 is situated. This aligns with literature suggesting that MLN4924-induced neddylation inhibition modulates mitochondrial energy metabolism in cancer cells (Zhou et al., 2019). This congruence underscores the potential significance of this mechanism, especially within the context of HCC. Our in vitro experiments with cell lines further revealed that NEDD8 expression was markedly elevated in poorly differentiated cell lines. This observation, in tandem with our pathway analysis highlighting genes associated with differentiation, paints a consistent picture. Moreover, the elevated expression of NEDD8 in advanced tumor grades, which reflect the differentiation status, further cements its potential role in tumor progression.

5. CONCLUSION

Our findings robustly indicate that the expression levels of both NEDD8 and NAE1 undergo a significant upregulation during the progression of hepatocarcinogenesis. Delving deeper into pathway and functional analyses, we discerned a notable association between these molecules and a plethora of genes implicated in HCC. Intriguingly, within this gene set, we identified genes pivotal to cellular differentiation and others that serve as central nodes in expansive cellular networks. These observations were further corroborated through our cell line studies. Furthermore, the WGCNA analyses underscored the significance of energy metabolism in relation to NEDD8 and emphasized cell fate processes for NAE1. Collectively, these insights position both NEDD8 and NAE1 as compelling candidates for prognostic biomarkers in HCC.

6. PERSPECTIVES

Moving forward, it is imperative to conduct more in-depth analyses involving broader cohorts. There's also a significant value in examining expression data, as this would add a richer layer of insight to our current understanding. Moreover, during the network analyses that delineate the module-trait relationships, we discerned several modules mirroring the patterns of the yellow and purple modules. These newly identified modules undoubtedly merit further exploration.

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8. APPENDIX

8.1 Curriculum Vitae (CV)

PERSONAL INFORMATION

Date of Birth/Place: 04/01/1996 / Istanbul

Phone: (0090) 539

E-mail Address:

EDUCATION

2020-2023: M.Sc. in Molecular Biology and Genetics (100% English), GPA: 3,50/4,00

DOKUZ EYLUL UNIVERSITY/IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

2015-2020: B.Sc. in Molecular Biology and Genetics (30% English), GPA: 3,25/4,00

ISTANBUL UNIVERSITY

2010-2014: DR. KEMAL NACI EKSI ANATOLIAN HIGH SCHOOL, GPA:79,17/100

RESEARCH EXPERIENCE

September 2020-January 2022: Lymphoid Cancer Genomics, Dokuz Eylul University/Izmir

International Biomedicine and Genome Institute

August 2019-September 2019: Medical Genetics, Istanbul Medical Faculty

January 2018-February 2018: Laboratory of Proteomics, Faculty of Science, Istanbul University

PERSONEL SKILLS

Turkish (Native), English (Advance), German (Beginner)

COMPUTER SKILLS

Bioinformatics Tools, R Programming, MS Office Applications

LABORATORY SKILLS

Cell Culture Techniques, Bacterial Transformation, Colony PCR, RT-qPCR, Weighted Gene Co-Expression Network Analysis, Statistical Methods for Bioinformatics, The databases used in bioinformatics (cBioPortal, Xena Browser, GEPIA 2, DAVID, NCBI-GEO, KEGG Pathways, GeneCodis, etc.), RNA/DNA Isolation from Cell Lines, To process and interpret RNA-seq and RPPA data, Performing gene enrichment analysis in R, Performing pathway and funtion analysis in R.

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Toplantı Tarihi	28/09/2022	**Toplantı Günü**	: Çarşamba
Toplantı Sayısı	12	**Toplantı Saati**	: 10:30

Sayın Prof. Dr. Safiye Neşe ATABEY,

2022-036 Protokol No'lu; sorumlusu olduğunuz "Nedilasyon Yolağının Hepatokarsinogenez Sürecindeki Rolünün Araştırılması" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini ederiz

Prof. Dr. H.

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