RESEARCH ARTICLE

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In Silico Analysis Revealed a Role for *NUSAP1* in Lung Adenocarcinoma through E2F1/hsa-let-7b-5p/lncRNA-TMPO-AS1

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Abstract

Objectives: Nucleolar and spindle-associated protein 1 (*NUSAP1*) is crucial for chromosomal segregation and spindle assembly, its expression correlates with high morbidity and mortality rates, necessitating better understanding of prognosis-related networks. **Methods:** The study used databases like KM Plotter, TNMplot, UALCAN, OncoMX, GEPIA2, OncoDB, ENCORI, TIMER 2.0, CancerSEA, miRNet, CellTracer, TISIDB, GSCA, and the Enrichr database to analyze *NUSAP1* expression in lung cancer tumors and normal tissues. **Results:** The *NUSAP1* gene is overexpressed in both adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC). However, LUAD has a poor prognosis for overall survival (OS) (HR = 1.94), first progression (FP) (HR = 1.96), and post-progression survival (PPS) (HR = 1.48), while LUSC showed no significant data. High *NUSAP1* expression is significantly associated with adenocarcinoma smoker patients. The study also found a strong correlation between lncRNA-TMPO-AS1 overexpression and poor OS prognosis in LUAD smokers, a negative relationship between miRNA hsa-let-7b-5p and TMPO-AS1/E2F1/*NUSAP1* expression, and a positive correlation between S and M phase cell cycle checkpoints, tumor infiltrating CD4 immune cells, and *NUSAP1* expression in lung adenocarcinoma. **Conclusion:** Smokers with lung adenocarcinoma have worse prognoses due to higher E2F1, *NUSAP1*, and TMPO-AS1 levels, possibly due to TMPO-AS1 sponge formation with hsa-let-7b-5p.

Keywords: *NUSAP1*- Lung Adenocarcinoma- ceRNA Network- Prognosis- Smokers

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Introduction

Worldwide, lung cancer holds the top most rank among all cancers in terms of patient mortality rate, making it the most prevalent cause of fatal deaths [1]. As per studies, smoking cigarettes is the primary risk indicator for lung cancer, supported by strong evidence. Compared to someone who has never smoked, a lifetime smoker is up to three to four times more likely to develop lung cancer [2, 3]. Based on the clinicopathological characteristics of lung cancer, it is broadly classified into two: Non-small-cell lung cancer (NSCLC) (comprising 85%) and Small-cell lung cancer (SCLC) (comprising 15%). The NSCLC is further subclassified into: Lung Adenocarcinoma (LUAD) (arising in the epithelial cells of the lungs) and Lung Squamous cell carcinoma (LUSC) (in between the airway tract) [4]. In addition, LUAD is the more complex and fatal type of lung cancer with a high rate of reoccurrence and poor prognosis. To deepen the understanding of cancer and its pathophysiology, the study of proteomics and genomics plays a critical role [5]. In recent years, conventional diagnostic treatments such as tissue sample biopsy have been replaced with the serum/fluid biopsy that includes extraction of the fluids from the body (contains samples of blood, saliva, urine, etc.) for the ease of diagnosing therapies in lung cancer [6].

Lung cancers often have poor prognosis due to the lack of early diagnostic markers in the market. While several biomarkers have been identified and used as treatment therapies, their clinical utility remains limited. Mutation-based biomarkers, such as KRAS, MET, ROS1, PDL1, EGFR, and ALK, have limitations such as their lack of sensitivity and specificity at the tumor site, producing false positives, and failing to detect all mutations in cancer cells [7]. This raises the need for novel gene expression-based biomarkers. One solution involves understanding the role of gene expression in the cell cycle, which regulates mRNA expression at the genomic level. The Nucleolar and spindle-associated protein 1 (*NUSAP1*) gene is a key regulator in the cell cycle at the G2/M transition checkpoint [8]. Elevated *NUSAP1* expression has been associated with leading tumor formation in various cancers, including liver [9], breast [10], prostate [11], gastric [12], and bladder cancer [13]. *NUSAP1* dysregulation has also

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been observed in epigenetic alteration, such as DNA methylation, which can stimulate chromosomal and genetic instability, leading to transcriptional activation of oncogenes [14]. A study aimed to determine the regulatory mechanism or competitive endogenous RNA (ceRNA) network working behind *NUSAP1* dysregulation and the influence of non-coding RNAs (miRNA and lncRNA) on its expression. The findings showed that *NUSAP1*'s and lncRNA TMPO-AS1's elevated expression in lung adenocarcinoma smoker patients, along with hsa-let-7b-5p's down expression, was associated with poor prognosis. A transcription factor, E2F1, was found to be associated with all molecules and may cause dysregulation, leading to chromosomal instability and aggressive tumor growth. Further analysis into cell cycle regulation, apoptosis mechanism, and tumor microenvironment was also conducted. The study suggests that the *NUSAP1*/E2F1/TMPO-AS1/hsa-let-7b-5p axis is crucial for lung adenocarcinoma progression, and this regulatory ceRNA network may be a potential target for therapeutic intervention.

Materials and Methods

Expression Analysis of NUSAP1

The study used the UALCAN (The University of Alabama At Birmingham Cancer Data Analysis Portal) [15] database to study the gene expression of the *NUSAP1* gene across TCGA cancers, which was validated by using TIMER 2.0 (Tumor Immune Estimation Resource) [16], TNMplot [17], and OncoMX [18] databases. Further, the differential expression analysis of *NUSAP1* in cases of normal vs. lung cancer was carried out using the UALCAN, ENCORI (Encyclopedia of RNA Interactomes) [19], GEPIA2 (Gene Expression Profiling Interactive Analysis) [20], and TCGAnalyzeR (The Cancer Genome Altas Analyzer) [21] databases, along with gene expression based on the patient's smoking history and gender using UALCAN. Next, protein expression analysis was done for both LUAD and LUSC using The Human Protein Atlas [22] database. Also, methylation analysis of *NUSAP1* was done using the UALCAN and MethMarkerDB [23] databases.

Survival Analysis

The Kaplan-Meier Plotter [24] was utilized for determining the prognostic significance of *NUSAP1* in lung cancer cases. The analysis included all three types of survival status: overall survival (OS), first progression (FP), and post-progression survival (PPS), along with their histological subtypes- Adenocarcinoma and Squamous cell carcinoma, along with the smoking status of the patients. The "Gene symbol, and the Affy id: *NUSAP1*; 219978_s_at" were used for the analysis.

The transcription regulation and Heterogeneity analysis

To determine the transcriptional factors (TFs) regulating the *NUSAP1* gene, the Enrichr [25] database was utilized. Next, the survival analysis of the selected transcriptional factor was performed across all three types of survival status, OS, FP, and PPS (Gene symbol; Affy ID: E2F1; 2028_s_at), using the KMplotter database. Further, the correlation value between *NUSAP1* and the TF was found out using the ENCORI, TIMER2.0, and OncoDB [26] databases. The differential expression of the TF in LUAD was studied using the ENCORI, UALCAN, and OncoDB databases. Furthermore, to determine the heterogeneity associated with *NUSAP1* in LUAD, the Enrichr database was used, and the top expressed genes were selected for further analysis. The correlation between *NUSAP1* overexpression and the co-expressed genes was analysed using the TIMER [27] and ENCORI databases.

Competitive Endogenous RNA (ceRNA) Regulatory Network Analysis

To determine the regulatory effect of non-coding RNAs (miRNA and lncRNA) having on mRNA, the miRNet [28] database was used to find out the *NUSAP1*- associated miRNAs in LUAD. Further, the correlation between the selected miRNA and *NUSAP1* was found out using the ENCORI and CancerMIRNome [29] databases, along with the correlation with TF using ENCORI. The prognostic significance of the miRNA in LUAD was evaluated using the KM Plotter and CancerMIRNome databases. Lastly, the miRNA differential expression was analysed using CancerMIRNome, UALCAN, and ENCORI. Next, the study evaluated the lncRNA associated with *NUSAP1* using the Enrichr database, the top associated lncRNA were further analysed using UALCAN and correlated using the ENCORI databases. The miRNet database was used for network analysis of lncRNA/*NUSAP1*/TF/miRNA, and further correlation analysis was done using ENCORI. The survival analysis and lncRNA differential expression analysis in LUAD were carried out using the KM Plotter and UALCAN, and ENCORI databases, respectively.

NUSAP1's Role in Biological Processes, Cell Cycle and Tumor Infiltrating Immune Cells (TIICs)

The role of *NUSAP1* in different biological processes was found out using the CancerSEA [30] database. Further, to determine *NUSAP1*'s role in different phases of the cell cycle, Cyclebase3.0 [31] and ENCORI databases were utilized. *NUSAP1*'s correlation with cell cycle checkpoints was analyzed using ENCORI database. Also, the apoptotic genes that can be targeted in the case of *NUSAP1* overexpression in LUAD were analysed using the Harmonizome [32] database, and its further analysis was carried out using the ENCORI, OncoDB, KM Plotter, and UALCAN databases. Lastly, the association of TIICs with *NUSAP1* in LUAD was analyzed using TISIDB (Tumor Immune System Interaction Database) [33] and TIMER2.0.

Results

NUSAP1: Pan-cancer and Differential Expression Analysis

The previous literature has suggested the role of *NUSAP1* as an oncogene in several cancer cases. And hence, the study first analyzed the *NUSAP1*'s expression across all TCGA cancers using the UALCAN database.

The results showed *NUSAP1*'s overexpression in almost all the cancers except for a few, as shown in Supplementary Figure 1A. More research using the TIMER 2.0 and TNMplot databases showed similar *NUSAP1* overexpression in pan-cancer, as shown in Supplementary Figures 1B and C. To further corroborate the results, the OncoMX database was used to analyze *NUSAP1* expression across pan-cancer, and very interestingly, the results showed *NUSAP1* being highly expressed in lung cancer cases with a fold change of 3.13, as shown in Supplementary Table 1. Hence, the further analysis of *NUSAP1* in lung cancer, which comprises of LUAD and LUSC, was carried out using several databases. The differential expression analysis of *NUSAP1* in both subtypes was analyzed using the UALCAN database. The results showed *NUSAP1*'s significant (P = 1.6e-12) overexpression in both LUAD and LUSC, with a fold change of 8.7 and 9.6, respectively, as shown in Figures 1A–B. Similarly, in the ENCORI and GEPIA2 databases, Figure 1C-E showed a consistent pattern of overexpression in both LUAD and LUSC, with a fold change of 6.5 and 11.9, respectively. To strengthen our study, a transcriptome-based database, TCGAnalyzeR, was used which gave insights into the expression profile of *NUSAP1* in normal vs. tumor samples based on single-cell RNA sequencing data, and it was found that *NUSAP1* significantly overexpresses in both LUAD and LUSC with a log fold change of 2.4 and 3.6, respectively, as shown in Figure 1F-G.

After analyzing *NUSAP1* expression at a gene expression level, our study then established the *NUSAP1*'s protein expression level and Immuno-histocompatibility (IHC) in both healthy normal cells and tumor cells using The Human Protein Atlas database, and we found strong *NUSAP1* intensity in tumor cells as compared to healthy normal cells as shown in Figure 1H-J. For a complete analysis, epigenetic modifications associated with *NUSAP1* dysregulation was studied, and using the UALCAN database, we found that *NUSAP1* gets hypomethylated in the case of LUAD with a significant P value of 1.01e-03, the same was validated using the MethmarkerDB database, and the results showed consistent hypomethylation as shown in Figure 1K-M. The UALCAN database also showed hypermethylation of *NUSAP1* in the case of LUSC but with an insignificant P value (6.4e-02), as shown in Supplementary Figure 2A.

Prognostic Significance of NUSAP1

We analyzed the survival status of lung cancer patients associated with *NUSAP1*'s expression by using the KM Plotter database. The survivality in terms of three statuses were evaluated, namely overall survival (OS) (HR = 1.71, CI = 1.52–1.93, P <1e-16), first progression (FP) (HR = 1.71, CI = 1.44–2.03, P = 4e-10), and post-progression survival (PPS) (HR = 1.57, CI = 1.27–1.94, P = 2e-05), as shown in Figure 2A-C. The elevated hazard ratios and significant p values confirm that *NUSAP1* functions as an oncogene in lung cancer. Furthermore, the comparison between the low expression cohort and the high expression cohort as mentioned in Table 1 shows that suppressing or inhibiting *NUSAP1* in lung cancer can lead to a good prognosis and improved patient outcome. The study next investigated the survival chances based on the histological classification of lung cancer. The *NUSAP1*'s expression level in LUAD and LUSC across all three survival parameters was evaluated, and the results revealed that *NUSAP1* overexpression was significantly linked to poor prognosis in LUAD: OS + LUAD (HR = 1.94, CI = 1.63-2.31, P = 4.9e-14), FP + LUAD (HR = 1.96, CI = 1.6-2.4, P = 3.6e-11), and PPS + LUAD (HR = 1.48, CI = 1.16-1.88, P = 0.0015), as shown in Figure 2D-F. Further analysis in the LUSC subtype showed insignificant results for all survival statuses: OS + LUSC (HR = 1.09, CI = 0.89-1.32, P = 0.41), FP + LUSC (HR = 1.14, CI = 0.76-1.71, P = 0.53), and PPS + LUSC (HR = 1.75, CI = 0.97-3.18, P = 0.062) as shown in Figure 2G-I. And, hence, it can be said that *NUSAP1* can be considered a poor prognostic classifier for lung adenocarcinoma.

Next, considering that the smoking history of a patient also affects their survivality, the study evaluated the *NUSAP1* expression and survival status in LUAD smokers using the UALCAN and KM Plotter databases, respectively. As shown in Supplementary Figure 2B-C, the *NUSAP1* expression level was found to be elevated in smoker patients as compared to normal and non-smokers, along with overexpression in males. Similarly, a multivariate survival analysis including all three parameters along with LUAD smokers showed consistent *NUSAP1* overexpression associated with poor survival status: OS + LUAD+ Smoker (HR = 1.76, CI = 1.35-2.3, P = 2.1e-05); FP + LUAD+ Smoker (HR = 1.54, CI = 1.19-2, P = 0.0011); and PPS + LUAD+ Smoker (HR = 1.42, CI = 1.05-1.92, P = 0.021) as shown in Figure 2J-L and Table 1. As mentioned in the same table, multivariate analysis with other parameters such as non-smokers, male and female didn't show a consistent pattern. The study suggests that the overexpression of *NUSAP1* in lung adenocarcinoma smoker patients is linked to poor prognosis and survival rate, and it could serve as a potential prognostic biomarker for high-risk patients.

The Transcriptional Regulation and Heterogeneity Analysis

The molecular expression of genes requires the involvement of several regulators. Any change in the regulatory mechanism can regulate stemness, tumor progression, metastasis, and anticancer drug resistance. The key regulators involved in the expression of the *NUSAP1* gene were analyzed using the Enrichr database. The top 5 transcription factors, as mentioned in Supplementary Table 2, were found to be associated with *NUSAP1*, out of which E2F1 was at the top with a P value of 0.0019. E2F1 is a member of the E2F family that plays a critical role in cell growth and proliferation during the cell cycle. The E2F family consists of 8 family members, mainly responsible for the transcriptional expression of the gene. To validate the results obtained from the Enrichr database, all the 8 E2F family members were evaluated for their prognostic significance in lung cancer and its subtypes using the KM Plotter database. As mentioned in Supplementary Table 3, out of the 8 only E2F1, F2F4, E2F7, and E2F8 showed significant differences in the

Table 1. Survival Analysis of *NUSAP1* Gene in Lung Cancer

S.NO.	Gene	Index	Patient Number	Hazard Ratio	CI	Log(P)	High expression cohort (months)	Low expression cohort (months)
1	*NUSAP1*	OS	2166	1.71	1.52-1.93	<1e-16	47	96.2
		FP	1252	1.71	1.44-2.03	4.00E-10	45.08	164
		PPS	477	1.57	1.27-1.94	2.00E-05	9	21.22
2		OS/LUAD	1161	1.94	1.63-2.31	4.90E-14	52	116
		FP/LUAD	906	1.96	1.6-2.4	3.60E-11	11	31.61
		PPS/LUAD	376	1.48	1.16-1.88	0.0015	11	27.07
3		OS/LUSC	780	1.09	0.89-1.32	0.41	49	63
		FP/LUSC	220	1.14	0.76-1.71	0.53	11	16.33
		PPS/LUSC	51	1.75	0.97-3.18	0.062	7	11
4		OS/LUAD/ SMOKER	546	1.76	1.35-2.3	2.10E-05	62	96
		FP/LUAD/ SMOKER	516	1.54	1.19-2	0.0011	44	89
		PPS/LUAD/ SMOKER	245	1.42	1.05-1.92	0.021	10.81	25.79
5		OS/LUAD/ SMOKER/ MALE	319	1.84	1.28-2.62	7.00E-04	24	47.77
		FP/LUAD/ SMOKER/ MALE	299	1.76	1.25-2.49	0.0012	40	102
		PPS/LUAD/ SMOKER/ MALE	137	1.26	0.84-1.88	0.27	12.9	21
6		OS/LUAD/ SMOKER/ FEMALE	227	1.93	1.29-2.88	0.0011	52	116
		FP/LUAD/ SMOKER/ FEMALE	217	1.24	0.83-1.86	0.29	55	84
		PPS/LUAD/ SMOKER/ FEMALE	108	1.69	1.08-2.66	0.021	8.7	37
7		OS/LUAD/ NONSMOKER	192	3.19	1.64-6.18	0.00029	49	96
		FP/LUAD/ NONSMOKER	189	2.86	1.69-4.85	4.50E-05	22	69
		PPS/LUAD/ NONSMOKER	69	1.33	0.71-2.5	0.37	24.47	23.81
8		OS/LUAD/ NONSMOKER/ MALE	31	11.47	1.43-92.12	0.0038	-	-
		FP/LUAD/ NONSMOKER/ MALE	30	4.7	1.28-17.22	0.01	-	-
		PPS/LUAD/ NONSMOKER/ MALE	-	-	-	-	-	-
9		OS/LUAD/ NONSMOKER/FEMALE	161	2.44	1.2-4.96	0.011	52	96
		FP/LUAD/ NONSMOKER/FEMALE	159	2.66	1.49-4.76	0.00058	24.1	69
		PPS/LUAD/ NONSMOKER/FEMALE	56	1.07	0.53-2.18	0.84	25.92	27.2

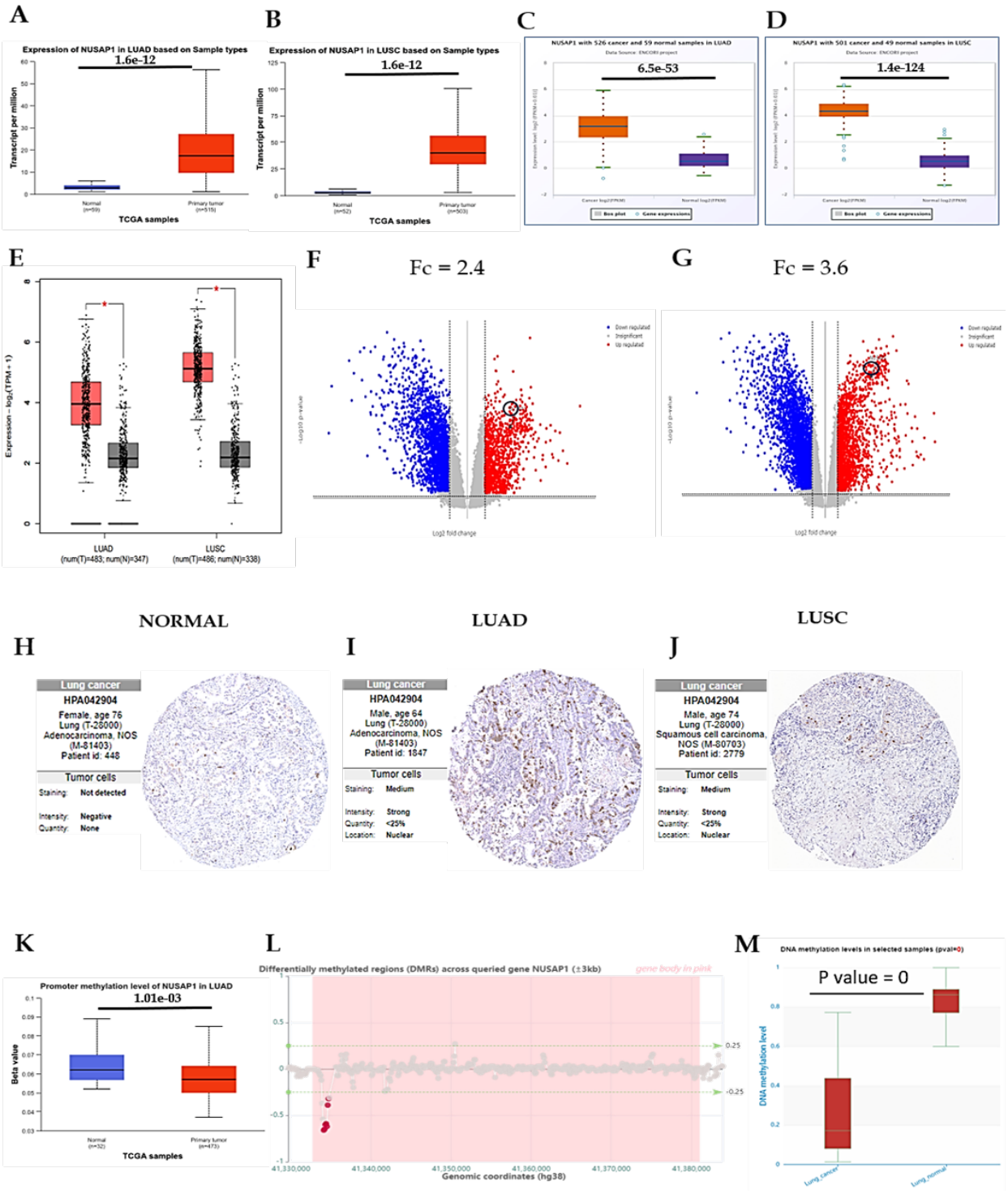

Figure 1. Differential Expression of *NUSAP1* in Lung Cancer (A–G), mRNA expression was analyzed in normal lung tissue and primary tumors. The UALCAN database showing (A) LUAD (normal n = 59, tumor n = 515), (B) LUSC (normal n = 52, tumor n = 503), using ENCORI: (C) LUAD (normal n = 59, tumor n = 526), and (D) LUSC (normal n = 49, tumor n = 501). GEPIA2 showing (E) adenocarcinoma (normal N = 347, tumor T = 483) and squamous cell carcinoma (normal N = 338, tumor T = 486). TCGAnalyser database showing (F) adenocarcinoma (Fc = 2.4) and (G) squamous cell carcinoma (Fc = 3.6). Protein expression analysis of NUSAP1 in lung cancer (H-J), in comparison with normal vs. tumor cells in LUAD and LUSC, (I) LUAD tumor and (J) LUSC tumor. (K-M) Methylation of NUSAP1 expression in LUAD was analyzed using (K) UALCAN (normal n = 32, tumor n = 473) and (L-M) MethmarkerDB.

survival spans of the lung cancer patients. On further histological analysis on overall survivality, it was seen that only E2F1, E2F4, and E2F8 were significantly associated with LUAD patients and none with LUSC, as shown in Supplementary Table 4. To further downstream, the analysis was performed based on the classification of

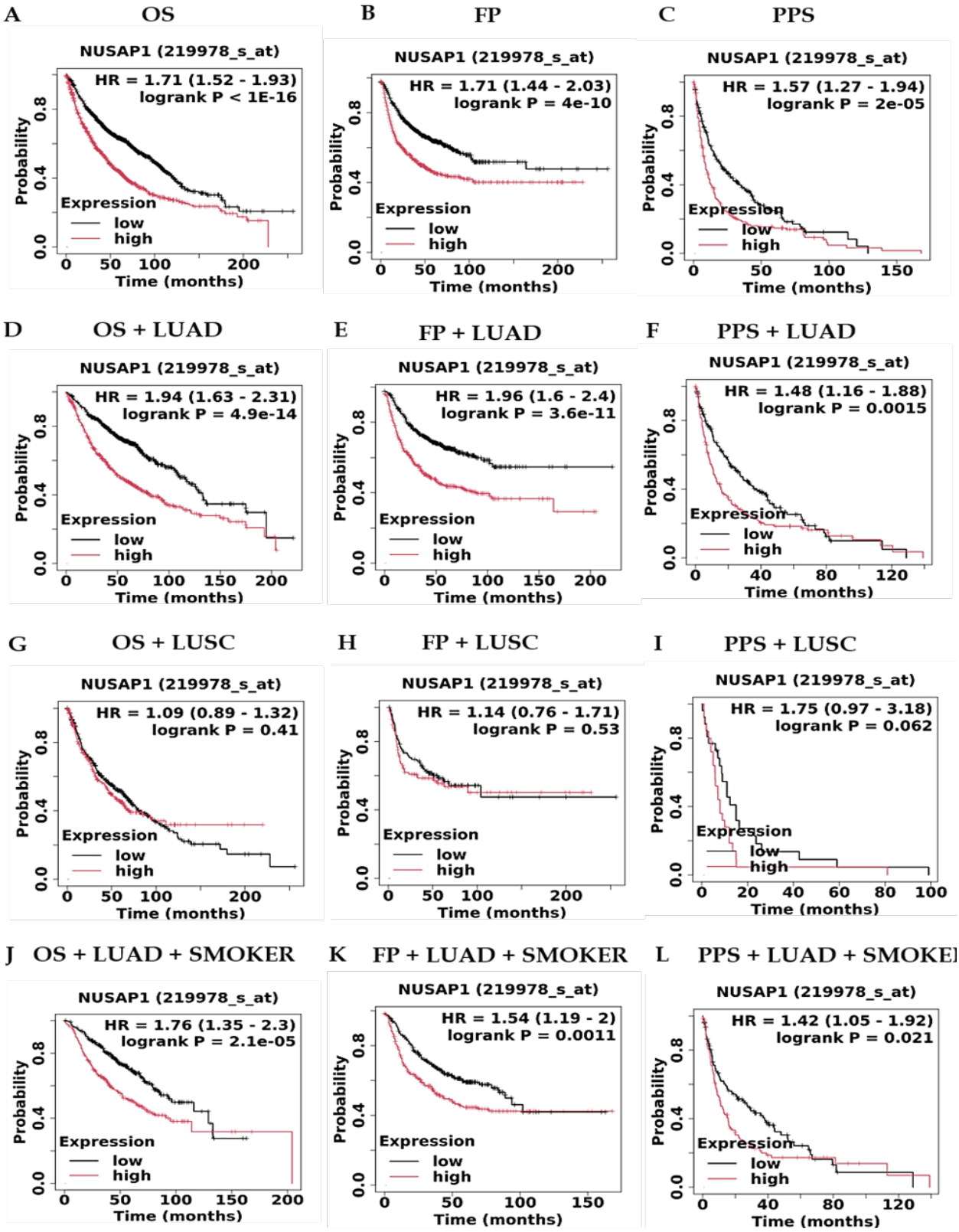

Figure 2. Survival Analysis of *NUSAP1* Overexpression by Using KM Plotter in Cases of (A) Overall survival (OS) (n = 2166), (B) First progression (FP) (n = 1252), (C) Post progression survival (PPS) (n = 477), (D) OS + LUAD (n = 1161), (E) FP + LUAD (n = 906), (F) PPS + LUAD (n = 376), (G) OS + LUSC (n = 780), (H) FP + LUSC (n = 220), and (I) PPS + LUSC (n = 51), (J) OS + LUAD + Smoker (n = 546), (K) FP + LUAD + Smoker (n = 516), (L) PPS + LUAD + Smoker (n = 245).

stages and gender, the data, as presented in Supplementary Tables 5 and 6, revealed that only E2F1 was significantly associated with poor prognosis, and most importantly, E2F1 was found to be strongly associated with LUAD smoker patients, as shown in Supplementary Table 7. Figure 3A-C shows the survival plots obtained using the KM Plotter database for the survival status of E2F1 in cases of overall survival (OS) (HR = 1.45, CI = 1.28-1.63,

P = 1.1e-09), OS + LUAD (HR = 1.53, CI = 1.29-1.81, P = 1.1e-06), and patients who have smoked in the past + OS + LUAD (HR = 1.63, CI = 1.25-2.12, P = 0.00026). After establishing the prognostic significance of E2F1 with poor prognosis in case of lung adenocarcinoma smoker patients, correlation analysis of *NUSAP1* vs. E2F1 was carried out using ENCORI, TIMER2.0, and OncoDB databases. The significant linear plots with the regression values: R = 0.677, Rho = 0.71, and R = 0.3243, respectively, as shown in Figure 3D-F, show strong positive correlation between both, validating E2F1 to be a key regulator of *NUSAP1* gene expression in LUAD conditions. Further, differential expression analysis of E2F1 was done in normal vs. LUAD samples using multiple datasets like ENCORI, UALCAN, and OncoDB as shown in Figure 3G-I, and the box plots obtained show significant upregulation of E2F1 in tumor samples, along with in cases of smoker males (Supplementary Figure 2D-E).

Cancer being a heterogenous disease is not caused by the dysregulation of a single gene, and hence a heterogenous model consisting of *NUSAP1*'s top correlated genes were identified using the Enrichr database. The top 10 co-expressed genes, namely: B*UB1, BUB1B, DTL, HJURP, HMMR, KIF11, NCAPG, RRM2, TOP2A,* and *TPX2*, were further evaluated using the TIMER, and ENCORI databases in LUAD condition.

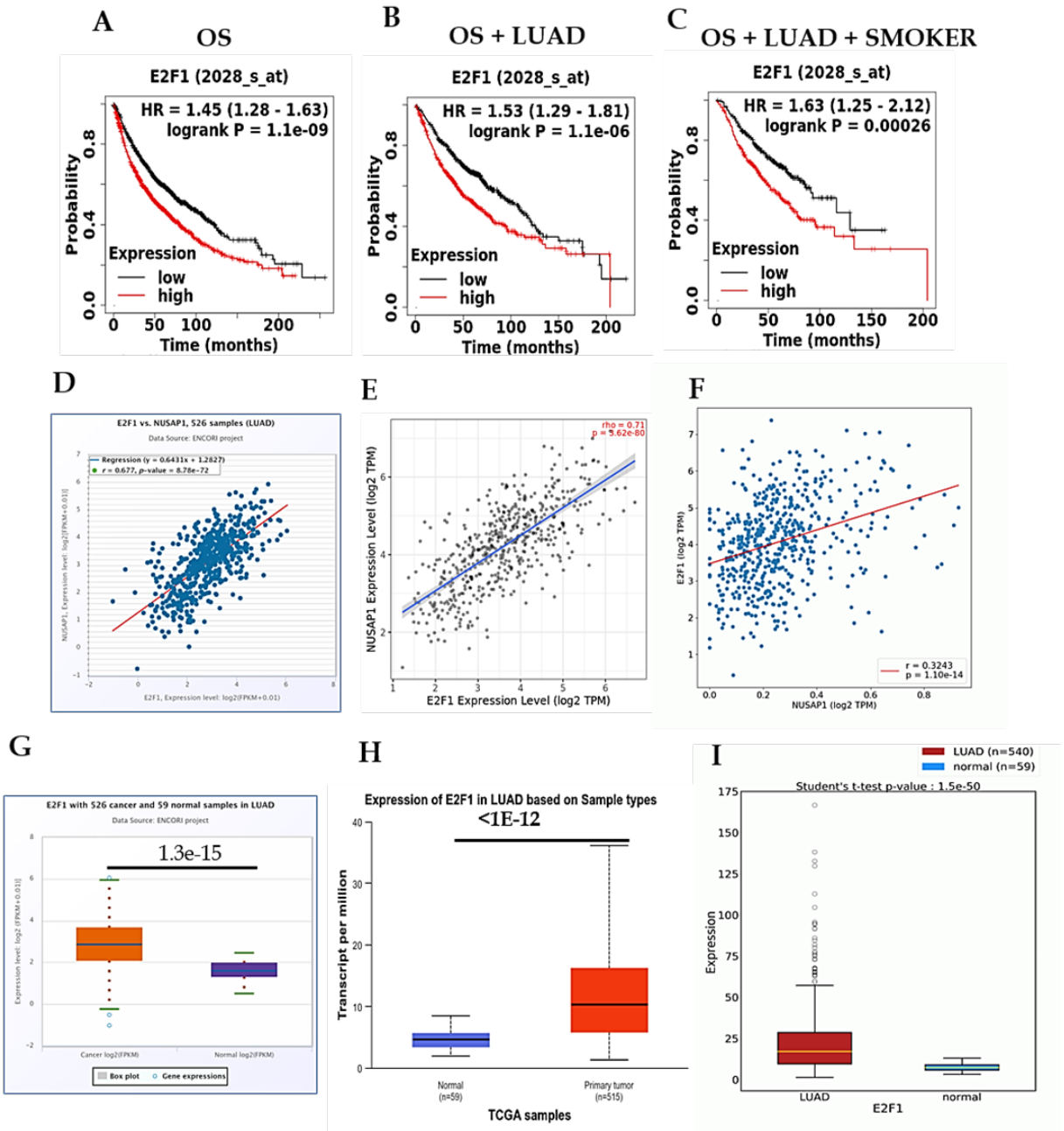

Figure 3. Analysis of Transcription factor E2F1 Associated with *NUSAP1* Overexpression. Survival analysis of E2F1, using KM plotter in cases of (A) OS (n = 2166), (B) OS + LUAD (n = 1161), (C) OS + LUAD + Smoker (n = 546). Correlation expression analysis between E2F1 and NUSAP1 using (D) ENCORI (n = 526), (E) TIMER2.0, and (F) OncoDB datasets. Differential expression of E2F1 in normal lung tissue and primary tumors in LUAD using (G) ENCORI (normal n = 59, tumor n = 526), (H) UALCAN (normal n = 59, tumor n = 515), and (I) OncoDB (normal n = 59, tumor n = 540).

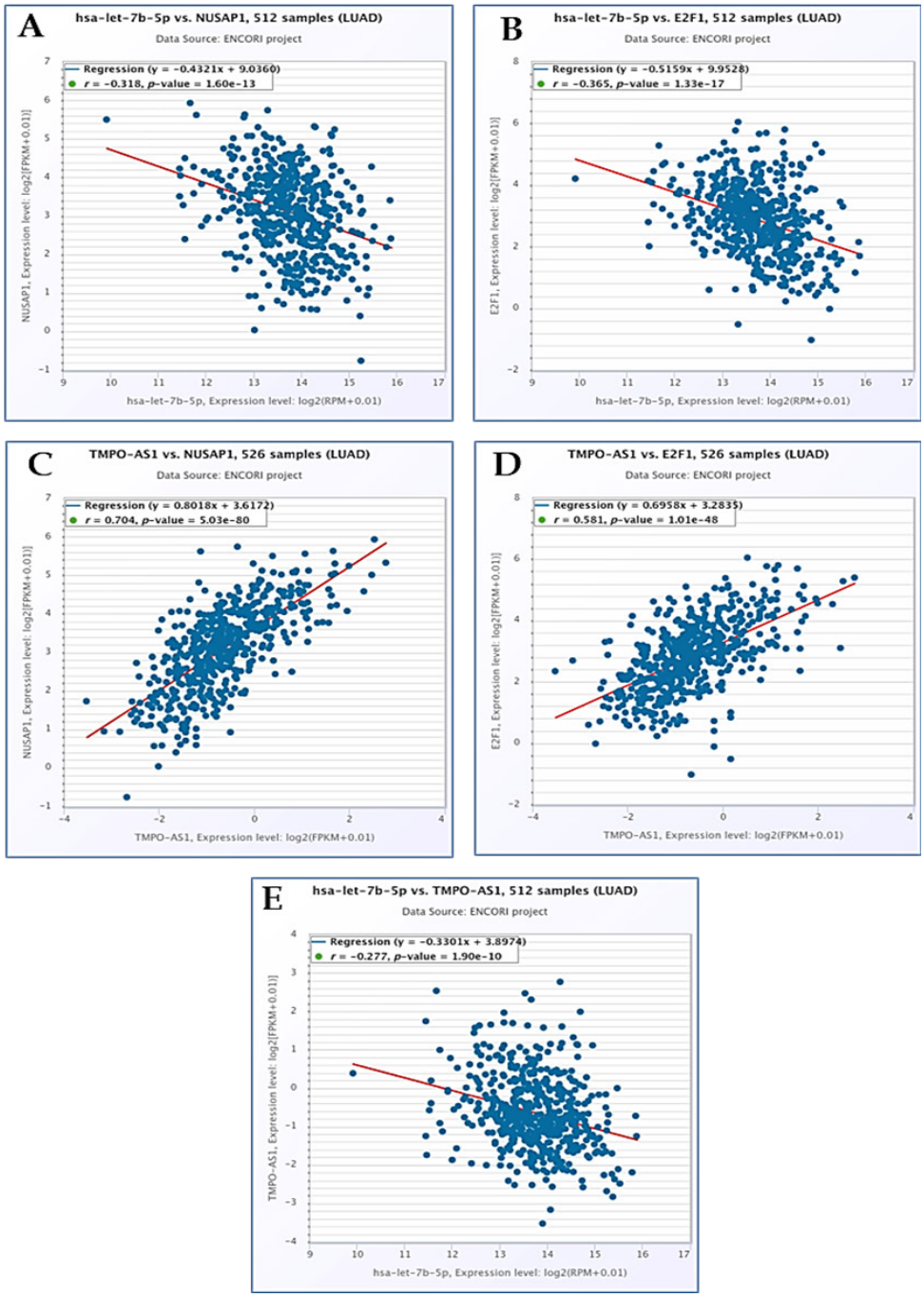

Figure 4. Correlation Expression Analysis in LUAD Cancer, Using the ENCORI Database (A) hsa-let-7b-5p vs. NUSAP1 (n = 512), (B) hsa-let-7b-5p vs. E2F1 (n = 512), (C) TMPO-AS1 vs. NUSAP1 (n = 526) (D) TMPO-AS1 vs. E2F1 (n = 526) (E) hsa-let-7b-5p vs. TMPO-AS1 (n = 512).

The correlation analysis done using the two databases showed significant linear plots, indicating these genes have a strong positive correlation with *NUSAP1*, suggesting their potential involvement in similar oncogenic pathways and processes. Supplementary Figure 3A-J represents data obtained from the TIMER database with R values = 0.88, 0.94, 0.82, 0.88, 0.81, 0.91, 0.90, 0.88, 0.87, and 0.89, respectively. Similarly, Supplementary Figure 3K-T shows the correlation values obtained using the ENCORI database (R = 0.89, 0.94, 0.83, 0.89, 0.82, 0.90, 0.91, 0.89, 0.88, and 0.89, respectively). The strong correlation values provide a strong basis for future evaluation of these genes in lung adenocarcinoma cases.

competitive endogenous (ceRNA) RNA regulatory Network Analysis for NUSAP1

The regulatory mechanism comprises complex networks, like coding and non-coding regions of RNA (mRNA and lncRNAs) that compete for their binding with the shared miRNA, on which the entire molecular mechanism and gene expression rely. In normal healthy tissue, the binding of miRNA with the mRNA takes place, which controls the gene expression by repressing it. However, in cancerous conditions, lncRNA suppresses

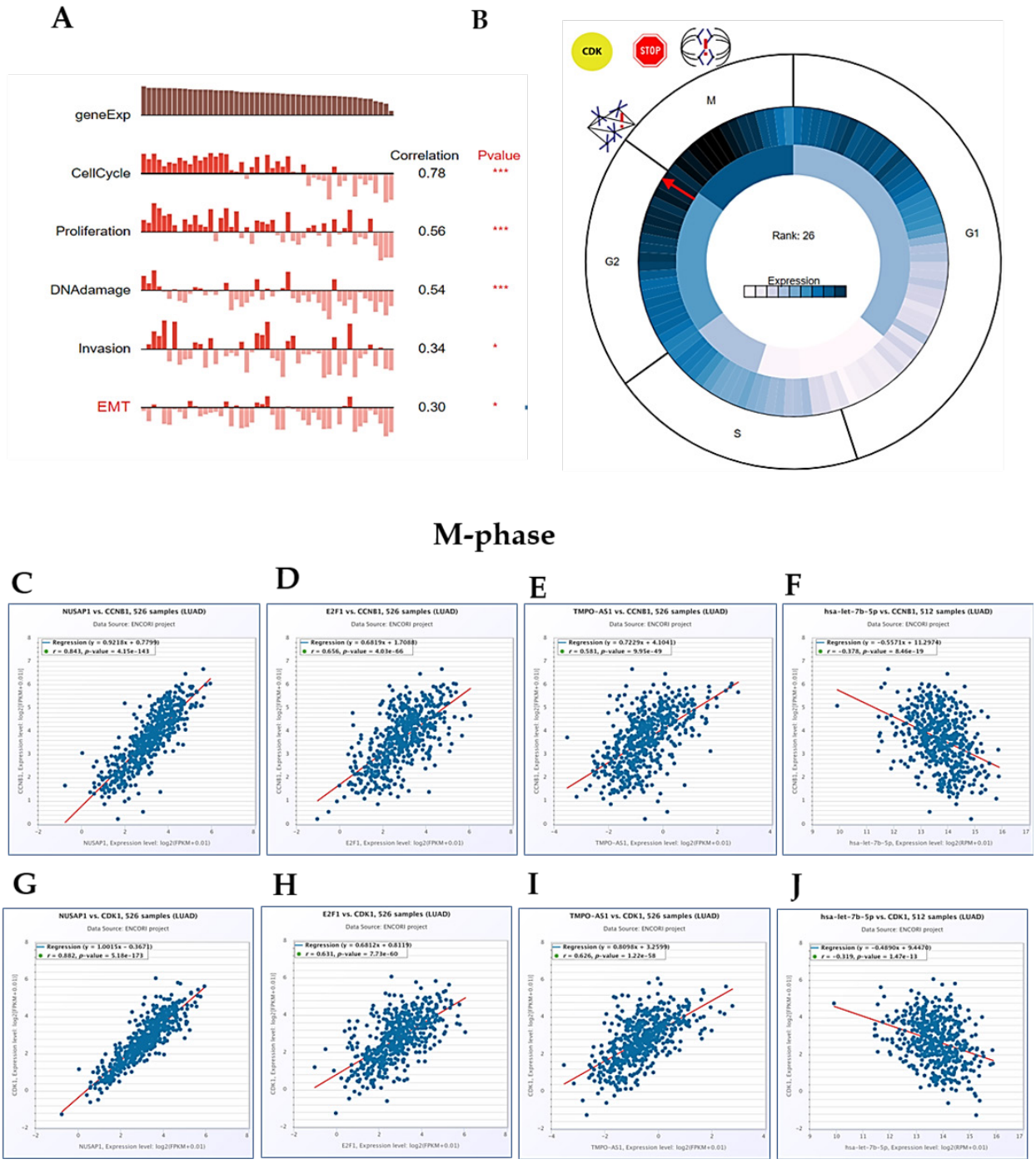

Figure 5. Biological Processes and Proliferation Mechanism Analysis. (A) Using the CancerSEA database to find out the NUSAP1 role in various biological processes. (B) Using the CycleBase database to find out the role of NUSAP1 in the cell cycle. Correlation expression analysis of NUSAP1/E2F1/TMPO-AS1/hsa-let-7b-5p with CDK and Cyclins checkpoints of the cell cycle in LUAD, using the ENCORI database (C) NUSAP1 vs CDK1 (n = 526), (D) E2F1 vs CDK1 (n = 526), (E) TMPO-AS1 vs CDK1 (n = 526), (F) hsa-let-7b-5p vs CDK1 (n = 512), (G) NUSAP1 vs CyclinB (n = 526), (H) E2F1 vs CyclinB (n = 526), (I) TMPO-AS1 vs CyclinB (n = 526), (J) hsa-let-7b-5p vs CyclinB (n = 512).

this regulatory activity by sponging miRNA, resulting in the constant overexpression of the mRNA. To analyze this regulatory expression, firstly we investigated the miRNAs associated with *NUSAP1* and E2F1 using the miRNet database and found that particularly 4 miRNAs: hsa-mir-24-3p, hsa-let-7g-5p, hsa-mir-1-3p, and hsa-let-7b-5p were associated with them, however, further analysis using the miRNet concentric circle feature showed only hsa-let-7b-5p to be in close proximity with *NUSAP1* as shown in Supplementary Figure 4A. To further validate this proximity, the correlation analysis was carried out using the ENCORI and CancerMIRNome databases, which showed a strong negative association between hsa-let-7b-5p and *NUSAP1* with R values of -0.318 and -0.382, respectively, as shown in Figure 4A and Supplementary Figure 4B. Also, the association between hsa-let-7b-5p and E2F1 observed using the ENCORI database showed a negative correlation (R = -0.365) as shown in Figure

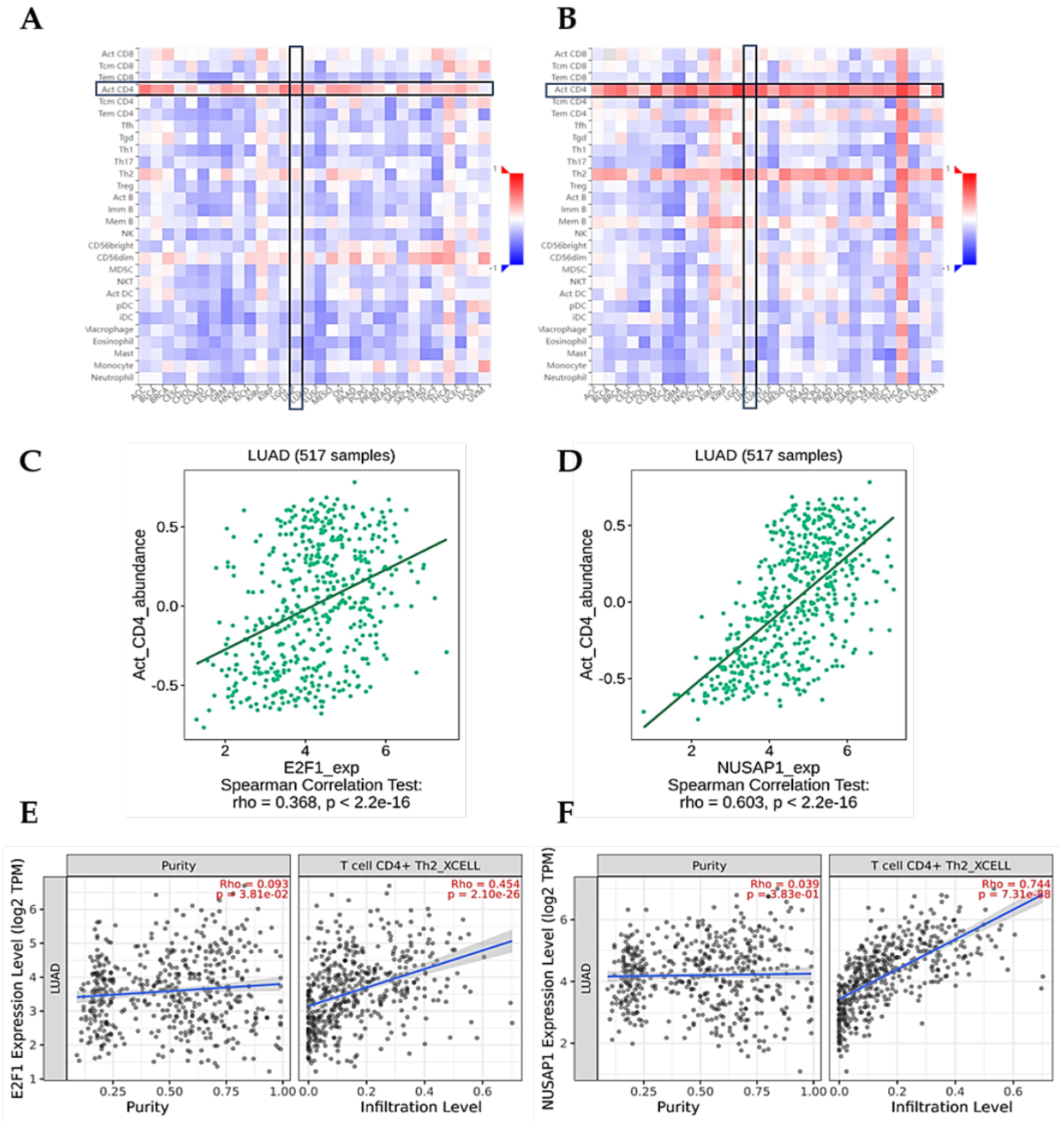

Figure 6. Tumor Infiltrating Immune Cells. (A-B) Heatmap showing various immune cells. (C-F) Correlation analysis between CD4 vs. E2F1 and CD4 vs. NUSAP1, using (C-D) TISIDB and (E-F) TIMER2.0 databases, respectively.

4B. hsa-let-7b-5p has been suggested as a prognostic molecule for various other cancers, and to determine its significance in LUAD, the study visualized the survivality and its accuracy to predict true positive results. As shown in Supplementary Figure 4C, the KM Plotter database was used to determine the survivality, and very interestingly, low expression of let-7b-5p (HR = 0.71, CI = 0.53-0.95, P = 0.021) was associated with poor LUAD prognosis. Also, area under the curve (AUC) analysis was carried out using the CancerMIRNome database, and the AUC value of 0.89 indicates the high probability of the let-7b-5p being able to distinguish between true positives and false positive results, making it a reliable molecule in LUAD cases (Supplementary Figure 4D). Further, we analyzed the differential expression of hsa-let-7b-5p in LUAD using the CancerMIRNome, UALCAN, and ENCORI databases. The overall results showed a significant down-expression of hsa-let-7b-5p in tumor samples as compared to normal, as shown in Supplementary Figure 4E-G. Also, significant low expression of let-7b-5p in smoker patients was found using UALCAN (Supplementary Figure 4H). Furthermore, a pan-cancer analysis performed using a transcriptomic database, TACCO, showed the highest negative fold change (-3.27) in the case of lung cancer as compared to other TCGA cancers, listed in Supplementary Table 8.

Next, the study focuses on determining the lncRNA associated with *NUSAP1*, and using the Enrichr database, it was found that TMPO-AS1 was the topmost lncRNA to be significantly (P = 1.344e-20) correlated with *NUSAP1*,

as shown in Supplementary Table 9. To further validate, the miRNet database was used to analyze the network between TMPO-AS1, *NUSAP1*, E2F1, and hsa-let-7b-5p, and as shown in Supplementary Figure 5A, a direct interaction was found between all the molecules. A correlation analysis performed using the ENCORI database showed strong positive interactions between TMPO-AS1 and *NUSAP1* (R = 0.704), TMPO-AS1 and E2F1 (R = 0.581), along with a negative correlation between TMPO-AS1 and hsa-let-7b-5p (R = -0.277), as shown in Figure 4C-E. The negative correlation between TMPO-AS1 and hsa-let-7b-5p indicates the fact that TMPO-AS1 works as sponging factor for hsa-let-7b-5p, inhibiting its mRNA regulatory effect in LUAD conditions. The survival analysis of TMPO-AS1 in LUAD smoker patients using KM Plotter as shown in Supplementary Figure 5B-C showed high expression of TMPO-AS1 associated with poor OS + LUAD (HR = 2.16, CI = 1.69-2.76, P = 4.1e-10) and OS + LUAD + smoker (HR = 2.34, CI = 1.41-3.88, P = 0.00066) prognosis. Further, differential expression analysis of TMPO-AS1 in normal vs. tumor using the ENCORI and UALCAN databases showed significant upregulation of TMPO-AS1's expression in LUAD samples along with smoker patients, as shown in Supplementary Figure 5D-F.

Thus, our findings revealed that miRNA hsa-let-7b-5p and lncRNA TMPO-AS1 are strongly associated with *NUSAP1* and E2F1 in LUAD smoker patients and can be considered for future research. We could also use these molecules as a potential biomarker for targeting lung adenocarcinoma expression based on this ceRNA network, to guide clinical interventions.

NUSAP1's Role in Biological Processes, Cell Cycle and Tumor Infiltrating Immune Cells (TIICs)

The dysregulation of a gene affects various biological processes, which was analyzed using CanserSEA database, as shown in Figure 5A, *NUSAP1* dysregulation in LUAD greatly affects cell cycle (R = 0.78), Proliferation (R = 0.56), DNA damage (R = 0.54), Invasion (R = 0.34), and EMT (R = 0.30). On further analysis of *NUSAP1* expression in different cell cycle phases using Cyclebase 3.0, its expression was found to be associated with the G2/M transition phase (Figure 5B). The key regulators of the cell cycle are known as Cycle dependent kinases (CDKs) and Cyclins that ensure its proper regulation and progression. Cyclins and CDKs works in complex at different phases: Cyclin D with CDK 4 and 6 during G1 and early S phase, Cyclin E with CDK 2 during late G1 and S phase, Cyclin A with CDK 2 during late S to G2phase and Cyclin B with CDK1 during late G2 and M phase. Our study investigated the correlation between all CDKs, and Cyclins as shown in Supplementary Table 10 and found *NUSAP1*, E2F1, TMPO-AS1, and hsa-let-7b-5p dysregulation significantly associated with only S phase and M phase regulators. The dysregulation observed in the S phase (Supplementary Figure 6A-H) where DNA synthesis occurs suggests that these molecules may influence the accurate replication and integrity of the genetic material. Whereas in the M phase (Figure 5C-J) which encompasses mitosis and cytokinesis, the dysregulation of *NUSAP1* which is essential for spindle formation and chromosome segregation, can lead to disruption of cell cycle progression, contributing to genetic instability and uncontrolled cell proliferation of LUAD cells. Also, the above stated factors are known as the hallmarks of cancer and can contribute to the poor prognosis.

After observing a positive correlation between *NUSAP1* and cell cycle regulators the study investigated the molecules that are involved in the apoptosis process, which can be upregulated to potentially target and reduce the proliferation rate. A total 81 apoptotic genes were found using Harmonizome database as listed in Supplementary Table 11. As established in the above study upregulating hsa-let-7b-5p can also be associated with reducing the aberrant activities, and hence the apoptosis genes must positively correlate with it. As shown in the same Supplementary Table 11 only a few out of 81 genes namely FAS, PIK3R1, PIK3CG and PPP3CA were found to be in strong positive association with let-7b-5p. On further, correlation analysis with TMPO-AS1 and *NUSAP1* only PPP3CA (Protein Phosphatase 3, catalytic subunit, alpha isozyme) was found to strongly negatively associated with both the target genes with R value -0.327 and -0.218 respectively as shown in Supplementary Table 12 and Supplementary Figure 7A-B. The positive correlation value found between hsa-let-7b-5p vs. PPP3CA was R = 0.258 as shown in Supplementary Figure 7C. Further validation of the previous result was also performed using the OncoDB database, a correlation analysis of PPP3CA gene with *NUSAP1* (R = -0.128) and TMPO-AS1 (R = -0.194), showed a negative correlation, as shown in Supplementary Figure 7D-E respectively.

Next, analysis of PPP3CA was performed, using the KM Plotter (Aff Id: 202457_s_at) it was observed that its downregulation was associated with poor prognosis, in the cases of overall survival (HR = 0.75, CI = 0.67-0.85, P = 2.8e-06), OS + LUAD (HR = 0.69, CI = 0.58-0.81, P = 1.6e-05), and OS + LUAD + Smoker (HR = 0.75, CI = 0.58-0.97, P = 0.03) as shown in Supplementary Figure 7F-H. Further, differential expression analysis also showed significant down expression of PPP3CA in tumor as compared to normal samples, using UALCAN, ENCORI and OncoDB, as shown in Supplementary Figure 7I-K.

Further, the TISIDB database is crucial for determining cancer treatment efficacy and patient prognosis. A study investigated the relationship between *NUSAP1* expression and E2F1 transcription factor with the TIICs using TISIDB. A heatmap was created with various TIICs, including T follicular helper cells, effector memory CD8 cells, regulatory T cells, activated CD4 T cells, NK cells, mast cells, memory B cells, monocytes, neutrophils, eosinophils, and macrophages as shown in Supplementary Figure 6A-B, and a strong correlation was found between Act_CD4 cells vs. E2F1 (R = 0.368) and *NUSAP1* (R = 0.603) in LUAD as shown in Figure 6C-D. Further validation using TIMER2.0 database also showed a strong positive correlation between CD4 + Th2 immune cells vs. E2F1 (R = 0.4) and *NUSAP1* (R = 0.7) as shown in Figure 6E-F.

These findings suggest that targeting *NUSAP1* expression could be a potential therapeutic strategy

for lung cancer treatment. By suppressing *NUSAP1* overexpression, it may be possible to inhibit invasion, proliferation, metastasis, and epithelial-mesenchymal transition, ultimately improving patient outcomes. This highlights the importance of further research and development of targeted therapies specifically aimed at *NUSAP1* inhibition.

Discussion

Cancer is a major public health issue worldwide, with increasing numbers of new cases and deaths each year. Cancer immunotherapy, based on immune checkpoint blockade, has revolutionized the treatment landscape across multiple tumor types, even as a first-line clinical treatment [34]. However, not all tumor patients benefit greatly from or respond to immunotherapy. It is critical to identify and characterize novel biomarkers for tumor immunotherapy or immunomodulation to develop precise immunotherapy strategies and achieve more durable responses. Lung adenocarcinoma (LUAD) is a prevalent malignancy with a high death rate and a low 5-year survival rate of only 15-20% [35]. The understanding of LUAD's onset and progression remains underexplored. Nucleolar and spindle-associated protein 1 (*NUSAP1*) is a crucial protein involved in chromosome separation, spindle assembly, and cell cycle regulation [36]. It is abnormally elevated in various cancers, including liver, breast, prostate, gastric, and bladder cancer [37]. High *NUSAP1* expression is associated with a poor prognosis for prostate [11] and breast [38] cancer. *NUSAP1* controls pathways such as Wnt/β-catenin, Hedgehog, PI3K/AKT, and Hippo-Yap1 [39–42]. It also takes part in the process of responding to DNA damage in cells. However, its role in LUAD development remains elusive. Dysregulation of *NUSAP1* can disrupt these pathways, promoting tumor growth and metastasis [43]. Various cancers associate high *NUSAP1* levels with proliferation, invasion, and metastasis, while cervical cancer patients associate low expression with a poor prognosis [44].

In this study, we elucidate the oncogenic role of *NUSAP1*, with a particular focus on its expression and prognostic significance in LUAD. We show strong proof that *NUSAP1* is significantly overexpressed in LUAD, which leads to a poor prognosis and lower survival rates in patients, especially smokers, by using multiple databases to do in-depth analyses. We initially established the overexpression of *NUSAP1* across various cancers using several databases, confirming its widespread oncogenic potential. Immunohistochemistry (IHC) analysis using the Human Protein Atlas database showed that *NUSAP1* was more intense in tumor cells than in healthy cells. This further reinforces its role in tumor growth. We also explored epigenetic modifications, which revealed hypomethylation of *NUSAP1* in LUAD, potentially contributing to its overexpression. We performed survival analyses to determine how important *NUSAP1* is for predicting lung cancer survival. Notably, LUAD patients with high *NUSAP1* expression had a HR of 1.94 for OS, 1.96 for FP, and 1.48 for PPS, which means they had a significantly higher chance of having a negative outcome. In contrast, the survival analysis in LUSC showed no significant correlation, highlighting the specificity of *NUSAP1*'s prognostic value in LUAD. Considering the impact of smoking on LUAD, the study evaluated *NUSAP1* expression and survival status in smoker patients. Smoker patients showed elevated *NUSAP1* expression, and multivariate survival analysis confirmed its association with poor prognosis in this subgroup. These findings suggest that *NUSAP1* could serve as a valuable biomarker for high-risk LUAD patients, particularly smokers. Gulzar et al. [45] and Chiu et al. [46] investigated the transcriptional regulation of *NUSAP1* and identified E2F1 as a key regulator. The strong link between E2F1 and a negative prognosis in LUAD, especially in smokers, and its positive relationship with *NUSAP1* expression highlight its part in controlling *NUSAP1*. This highlights the potential therapeutic targeting of the E2F1-*NUSAP1* axis in LUAD. Recently researchers have focused on identifying a competitive endogenous RNA network to determine the role of *NUSAP1* in tumor proliferation and invasion, such as in a study conducted by Li et al., (2023) they identified that a lncRNA, LINC01393 promotes glioblastoma cell proliferation, migration, and invasion through the miR-128-3p/*NUSAP1* axis [47]. Additionally, Zhang et al. [48] reported that linc00689 binds to miR-129-5p, leading to *NUSAP1* overexpression and silencing this lncRNA suppressed the growth and invasion of osteosarcoma cells. Similarly, a competitive endogenous RNA (ceRNA) analysis helped us learn more about the regulatory network of *NUSAP1* in lung adenocarcinoma cases. It showed that hsa-let-7b-5p was strongly linked to both *NUSAP1* and E2F1. The high prognostic value and low level of hsa-let-7b-5p in LUAD indicate its potential as a therapeutic target. Additionally, the study found that TMPO-AS1, a long noncoding RNA, saturates hsa-let-7b-5p, leading to *NUSAP1* overexpression. The study added to the evidence that *NUSAP1*, E2F1, hsa-let-7b-5p, and TMPO-AS1 work together to control things by showing that high levels of TMPO-AS1 are linked to a bad outlook in LUAD smokers. The study also examined the biological processes impacted by *NUSAP1* dysregulation, discovering significant connections with cell cycle regulation, proliferation, DNA damage, invasion, and the epithelial-mesenchymal transition (EMT). *NUSAP1*'s association with the G2/M transition phase and its interaction with cell cycle regulators such as cyclins and CDKs underscores its crucial role in cell cycle progression. Finding that PPP3CA is a possible apoptotic gene that is positively correlated with hsa-let-7b-5p makes the therapeutic potential of targeting this regulatory network even stronger. Lastly, the study looked at the area around the tumor and found a strong link between *NUSAP1*/E2F1 expression and immune cells that enter the tumor (TIICs). The fact that *NUSAP1* is positively linked to activated CD4+ T cells and CD4+ Th2 immune cells suggests that it might be able to change the immune system in LUAD, opening up new treatment options. Potential therapeutic strategies targeting *NUSAP1* in LUAD could involve using small-molecule inhibitors to disrupt the *NUSAP1*-E2F1 interaction. Additionally, we could employ miRNA mimics or lncRNA inhibitors to restore the

expression of hsa-let-7b-5p, thereby reducing *NUSAP1* levels. Researchers could also explore immunotherapy approaches, leveraging the altered immune landscape associated with *NUSAP1* to enhance anti-tumor immune responses. Researchers could check how well miRNA mimics worked by looking at the levels of hsa-let-7b-5p and *NUSAP1* in cells that had been treated with them using quantitative PCR and Western blot analysis. Additionally, researchers could employ functional assays like cell proliferation, apoptosis, and invasion assays to evaluate alterations in cellular behavior. Finally, in vivo studies using LUAD xenograft models could provide insights into the therapeutic potential and overall impact on tumor growth and progression.

In conclusion, this study reveals *NUSAP1*'s oncogenic role in lung adenocarcinoma (LUAD), indicating its potential as a prognostic biomarker and therapeutic target. It reveals the E2F1-*NUSAP1* regulatory axis and the ceRNA network involving hsa-let-7b-5p and TMPO-AS1, where TMPO-AS1 functions as a molecular sponge, regulating hsa-let-7b-5p and thereby regulating *NUSAP1* expression. Future research should focus on developing targeted therapies against *NUSAP1* and its regulatory network for improved patient outcomes.

Author Contribution Statement

RN: Conception, study design, critical reading, and intellectual assessment of the manuscript Study design, and preparation of the manuscript. SN: Study design, and preparation of the manuscript. PV: Study design, and preparation of the manuscript.

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Ethical Clearance

Since this study exclusively utilized publicly available online databases for data extraction and analysis, ethical clearance was not required as per institutional guidelines.

Availability of Data

The data for this in-silico study were sourced from publicly accessible databases. The respective links and references for these datasets are provided the methodology section for transparency and reproducibility.

Conflicts of interest

The authors declare that they have no competing interests.

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