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Research article

Identification of the prognostic and therapeutic values of cyclin E1 (*CCNE1*) gene expression in Lung Adenocarcinoma and Lung Squamous Cell Carcinoma: A database mining approach

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HIGHLIGHTS

- *CCNE1* gene is highly expressed in LUAD and LUSC tissues
- *CCNE1* gene is differentially methylated in LUAD and LUSC tissues
- *CCNE1* overexpression is associated with the poor overall survival of LUAD and LUSC patients

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ABSTRACT

Cyclin E1 (*CCNE1*) is a protein-coding gene that belongs to the Cyclin family of genes which controls the G1/S phase transition of the cell cycle. Previously, its abnormal expression pattern has been examined and found to be correlated with ovarian and breast cancer progression. Herein, we exploited a bioinformatics and database mining strategy to unveil the therapeutic and prognostic significance of *CCNE1* gene expression in Lung Adenocarcinoma (LUAD) and Lung Squamous Cell Carcinoma (LUSC). *CCNE1* gene was reported to be highly expressed in LUAD and LUSC tissues. Its promoter and coding sequences were reported to be aberrantly methylated in LUAD and LUSC tissues than in normal tissues. Moreover, around 12 somatic mutations (frequency: 0.7%) were recorded in the *CCNE1* coding region from different studies involving LUAD and LUSC patients' whole genome sequences. The *CCNE1* gene expression was also correlated with LUAD and LUSC patients' overall and disease-specific survival. Immune infiltration analysis revealed the association between *CCNE1* gene expression and the abundance of numerous immune cells (i.e., T cells and B Cells) infiltration in LUAD and LUSC patients. Two previously known genes involved in oncogenic processes i.e., *CDC45* and *PDCD5* were identified as the most highly co-expressed genes of *CCNE1* in LUAD and LUSC tissues. Altogether, the *CCNE1* gene and its transcriptional and translational products may serve as a prognostic or therapeutic target in the diagnosis and treatment of LUAD and LUSC patients. The scientific findings of this study should assist in translating *CCNE1* into clinical practice for lung cancer diagnosis and treatment.

1. Introduction

Cancer, the abnormal and invasive growth of cells, ranks as one of the leading causes of death globally [1]. Worldwide, cancer incidence and mortality are rapidly increasing with disastrous consequences on healthcare systems [2]. In recent years, lung cancer has been identified as the second most commonly diagnosed cancer with an estimated 2.2 million new cases and the most fatal cancer with an estimated 1.8 million deaths in 2020 [2]. Unfortunately, these figures are predicted to rise even further in the long-term as factors contributing to lung cancer such as tobacco smoking are growing particularly in developing nations such as China, India and Russia [3]. Among the various types of lung cancers,

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approximately 85% of cases are non-small cell lung cancer (NSCLC) and the rest 15% of cases belong to small cell lung cancer (SCLC). Lung Adenocarcinoma (LUAD) and Lung Squamous Cell Carcinoma (LUSC) are the main subtypes of NSCLC in which patients show poor survival rates as approximately 75% of patients progress to advanced cancer stages at the time of diagnosis [4]. However, the medical support of LUAD and LUSC patients is often interrupted by the lack of proper diagnostic and therapeutic measures. Having said that, early detection of lung cancer can significantly improve the prognosis for patients. To illustrate, the UK Office for National Statistics demonstrated in their 2013–2017 database that patients diagnosed with stage I lung cancer had a 1-year survival rate of 85–89% compared to 17–21% in patients diagnosed with stage IV [5]. Thus, an effective prognostic marker and therapeutic target that can aid in the early detection and treatment of LUAD and LUSC patients is essential to reduce the burden on the healthcare system.

Cyclins are regulatory proteins involved in various phases of the cell cycle for modulating catalytic subunits known as cyclin-dependent kinases (CDKs). Together, they form distinct complexes that help ensure that chromosomal DNA is doubled, repaired and segregated properly from the parent cells to the daughter cells [6]. Amongst the major classes of mammalian cyclins, E-type cyclins involving Cyclin E1 and Cyclin E2 form complexes with Cyclin-dependent kinase 2 (CDK2) to regulate the cell cycle progression from G1-phase into S-phase [7]. Cyclin E1 (*CCNE1*), encoded by the *CCNE1* gene, can be overexpressed in many instances resulting in premature entry to S-phase. Consequently, there is greater stress at replication forks along with double-strand DNA breaks repaired by homologous recombination pathway, low DNA replication rate and deletions in the genome [8, 9]. Additionally, continual expression of Cyclin E1-CDK2 complex can lead to inhibition of mitotic exit resulting in misalignment of chromosomes in metaphase, polyploidy and mis-segregation. Furthermore, it can result in hyper-phosphorylation of Ser 18 residue of Centromere Protein A (CENPA) which interferes with normal centromere formation and causes mitotic defects [9].

Overexpression of *CCNE1* has been associated with certain types of cancers such as breast cancer, ovarian cancer, endometrial cancer and bladder cancer [9, 10, 11, 12, 13]. Moreover, greater expression along with somatic mutation of *CCNE1* indicates poor prognosis in specific cancer subtypes. For example, poor overall survival (OS) is associated with *CCNE1* amplification in patients with recurrent triple-negative breast cancer [14]. Similarly, overexpression of *CCNE1* is associated with a higher risk cancer-specific death in high-grade serous carcinoma (HGSC) patients [15]. Overexpression of the *CCNE1* gene has also been shown to increase the oncogenic activities of NSCLC cells [16]. Therefore, *CCNE1* expression has been suggested to be a promising prognostic biomarker and therapeutic target fordifferent cancer subtypes. In this study, we analyzed the prognostic and therapeutic potential of *CCNE1* expression in LUAD and LUSC tissues through a bioinformatic approach. We investigated the DNA methylation patterns of *CCNE1* promoter, mutations, the correlation between *CCNE1* expression and survival rate, the correlation between *CCNE1* expression and clinical manifestations, the association between *CCNE1* expression and immune cell infiltration, and co-expression of genes with *CCNE1* in LUAD and LUSC patients (Figure 1). The experimental outcome of this study will assist in further clinical development of *CCNE1*-based diagnostic and therapeutic measures for LUAD and LUSC diagnosis and treatment.

2. Materials and methods

2.1. Differential CCNE1 gene expression analysis in normal and cancerous lung tissues

Different web-based tools, i.e., Oncomine (www.oncomine.org/resource/), UALCAN (http://ualcan.path.uab.edu/) and GEPIA2 (http://gepia2.cancer-pku.cn/) were utilized to optimize the differential expression of *CCNE1* gene across cancerous lung and corresponding normal tissues [17, 18, 19]. The curated, outliers eliminated and normalized TCGA LUAD and TCGA LUSC data were utilized for the differential *CCNE1* expression analysis. The parameter values were kept at default during the analysis (i.e., $|Log2FC|>1$, $P < 0.01$), and the control and test variables were subjected to one-way-ANOVA in the GEPIA2 server and student's t-test in UALCAN server. Finally, we retrieved comparative images of the histology samples delineating differential

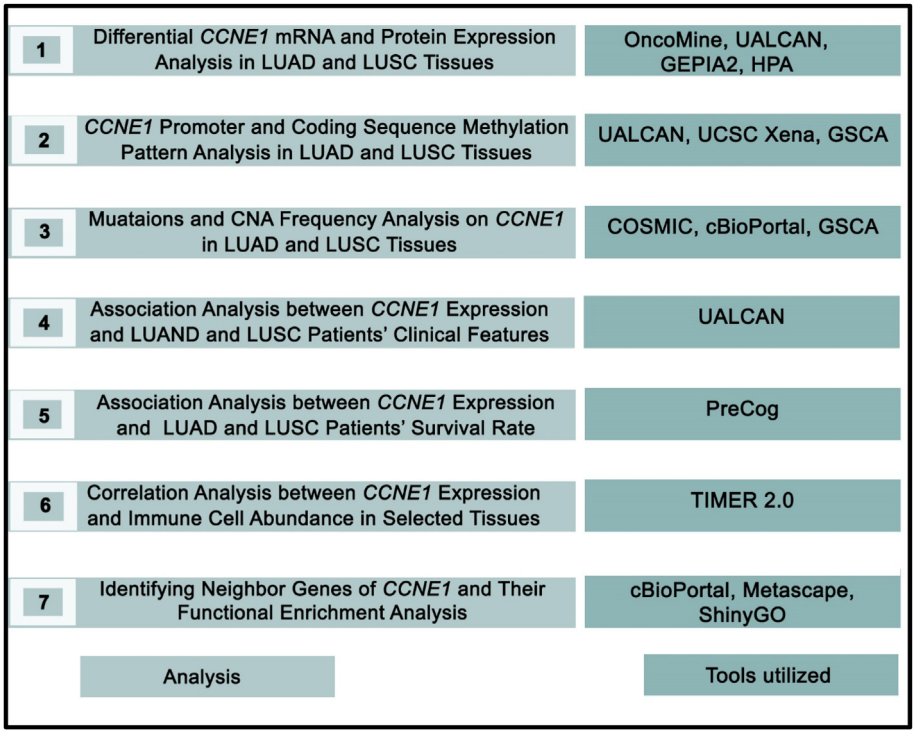

Figure 1. Summary of the strategies and tools utilized and stepwise procedure exploited in the overall study.

protein level expression of the *CCNE1* gene between normal and LUAD and LUSC tissues from the Tissue and Pathology modules of the HPA server (https://www.proteinatlas.org/) [20].

2.2. Determination of the CCNE1 coding region and promoter methylation status in LUAD and LUSC tissues

The *CCNE1* gene was quired in the UALCAN database to evaluate any variation in the promoter methylation pattern between normal and LUAD and LUSC tissues using the methylation data from TCGA cohorts. The hypothesis of the experiment was validated by performing a students' t-test. After that, we utilized the UCSC Xena browser to identify the gene body methylation status of the *CCNE1* gene in LUAD and LUSC tissues, where TCGA LUAD (n = 706) and LUSC (n = 626) samples were selected as the basis sample set [21]. The coding region methylation pattern of the *CCNE1* gene was evaluated utilizing the Illumina Human Methylation 450k data while keeping other parameters default in the UCSC Xena browser. Lastly, the differences in the methylation pattern of the *CCNE1* gene in cancerous and normal lung tissues were evaluated from the mutation module of the GSCA server (http://bioinfo.life.hust.edu.cn/GSCA/#/) [22]. The impact of *CCNE1* methylation on its mRNA expression in LUAD and LUSC tissues was also determined from the GSCA server.

2.3. Examining the somatic mutation and copy number alteration frequency in CCNE1 gene LUAD and LUSC samples

At first, the *CCNE1* gene was queried in the COSMIC online database (https://cancer.sanger.ac.uk/cosmic), which provides a collective overview of the somatic mutation of different genes in human cancers. We searched the *CCNE1* gene against the mutation information available for LUAD and LUSC which are susceptible to cancer [23]. Thereafter, the cBioPortal (https://www.cbioportal.org/) online server was explored to measure the mutation and copy number alteration (CNA) frequency in the *CCNE1* gene across different LUAD and LUSC studies. cBioPortal is an open-resource and online platform for translational research that assists in the multidirectional analysis of a particular gene and aids in the exploration of the genomic and epigenomic variations in the gene of particular interest [24]. A total of 11 LUAD and LUSC studies spanning more than 3,000 patients and 3,100 samples including those submitted by TCGA, OncoSG, CPTAC and MSKCC were selected to explore the mutation and copy number alteration frequency in the *CCNE1* gene. Lastly, the mutation module of the GSCA server was again investigated to discover the association between CNAs present in the *CCNE1* gene and its mRNA level expression in LUAD and LUSC tissues.

2.4. Association analysis between CCNE1 expression and different survival rates of LUAD and LUSC patients

The correlation between *CCNE1* expression and LUAD and LUSC patients' survival rates was established by investigating the PRECOG server (https://precog.stanford.edu/) [25]. The PRECOG server is a repository of genomic profiles of cancer patients that allows its users to identify the correlation of a prognostic gene expression with patients' survival. The choice of the test was a log-rank t-test between differentially *CCNE1*-expressing patients (50% cutoff value of the mean). The association between *CCNE1* gene expression and Overall Survival (OS) and Disease-Specific Survival (DSS) of LUAD and LUSC patients was explored in this step by analyzing the observed hazard ratio (HR) and considering a $p < 0.05$ threshold to be significant.

2.5. Demonstration of the association between the clinical manifestation of LUAD and LUSC patients and CCNE1 expression

The correlation between the *CCNE1* expression profile at the mRNA level and different clinical characteristics of LUAD and LUSC patients was evaluated using the TCGA LUAD and LUSC samples available in the UALCAN server. Differential expression patterns of *CCNE1* based on individual cancer stages, patients' gender, age, race, smoking habit, nodal metastasis status, and T53 mutation status were analyzed and reported. The experiment was performed utilizing the server's default parameters and subjecting the variables in between test and control samples to student's t-test, and results obtained with a p-value of <0.05 were considered statistically significant.

2.6. Analyzing the association between CCNE1 gene expression and abundance of immune cell infiltration in LUAD and LUSC patients

The association between *CCNE1* gene expression and the abundance level of tumor-infiltrating immune cells was determined using the TIMER 2.0 server (http://timer.cistrome.org/) [26]. The TIMER 2.0 server allows the establishment of any correlation between specific gene expression and immune cell infiltration across a diverse set of cancer. Our gene of interest was searched using the immune module of the server and the association between *CCNE1* gene expression and CD8+ T Cell, CD4+ T Cell, B Cells, Natural Killer (NK) cells, and Macrophage, were determined for LUAD and LUSC based on p-value cutoff and correlation coefficient. The mode of association was then retrieved from the server as a scatter plot representation.

2.7. Identification of the neighbor genes Co-expressed with CCNE1 in LUAD and LUSC tissues and their functional enrichment analysis

The genes that are co-expressed with *CCNE1* in LUAD and LUSC tissues were identified by the cBioPortal web browser utilizing the TCGA (Firehose Legacy) database. In the first step, the most highly co-expressed genes of *CCNE1* were identified in LUAD and LUSC samples. Then top 50 co-expressed genes were used for their pathway activity analysis as a cluster from the ShinyGO server (http://bioinformatics.sdstate.edu/go/) [27]. Thereafter, the top 50 co-expressed genes were used to prepare an enrichment protein-protein interaction (PPI) network based on their function from the Metascape server (https://metascape.org/gp/index.html) [28].

3. Results

3.1. Expression analysis of CCNE1 gene in cancerous and normal lung tissues

The expression pattern of the *CCNE1* gene was analyzed only in cancerous lungs and normal tissues using three different servers. The OncoMine server showed that *CCNE1* is highly expressed in both LUAD (n = 45, p = 2.01e-12) and LUSC (n = 27, p = 4.00e-12) tissues (Figures 2a and 2b). LUAD primary tumor samples (n = 515) were also found to express high levels of the *CCNE1* gene (p=<1e-12) compared to the normal lung tissues (n = 59) from the TCGA database integrated into the UALCAN server (Figure 2c). Additionally, LUSC samples (n = 503) also showed *CCNE1* overexpression (p = 1.62e-12) in comparison with the normal lung tissue samples (n = 52) (Figure 2d). Finally, the GEPIA2 analysis revealed the similar pattern of *CCNE1* overexpression in both LUAD and LUSC samples (Figures 2e and 2f). Thereafter, the immunohistochemistry (IHC) analysis of the *CCNE1* protein expression in LUAD and LUSC tissues was carried out. *CCNE1* protein showed moderate staining against the antibody used in both LUAD and LUSC samples compared to no staining in the normal tissues indicating the higher protein level expression of *CCNE1* in those affected cells compared to the healthy tissues (Supplementary Figure S1a, S1b and S1c).

3.2. Examination of the promoter and coding sequence methylation patterns in CCNE1 gene in LUAD and LUSC tissues

The methylation status of the *CCNE1* gene coding promoter in LUAD and LUSC samples was determined from the UALCAN online-based tool. No significant association and observable difference for *CCNE1* promoter methylation pattern were observed between normal lung and LUAD

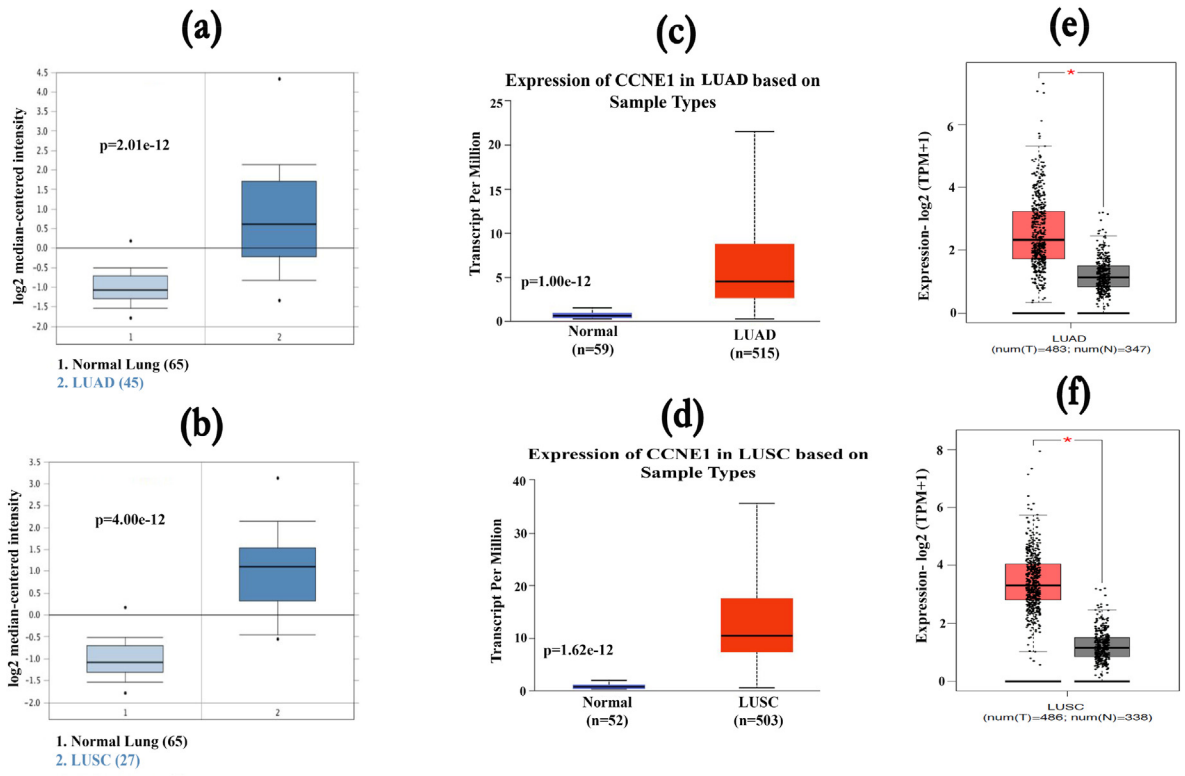

Figure 2. The expression analysis of *CCNE1* gene expression in LUAD and LUSC tissues from OncoMine (a, b) UALCAN (c, d) and GEPIA2 (e, f) servers. The red and black box represent cancer and normal samples, respectively, for the results obtained from GEPIA2 server (e, f). *CCNE1* gene was found to be highly expressed in both LUAD and LUSC tissues compared to their normal counterparts.

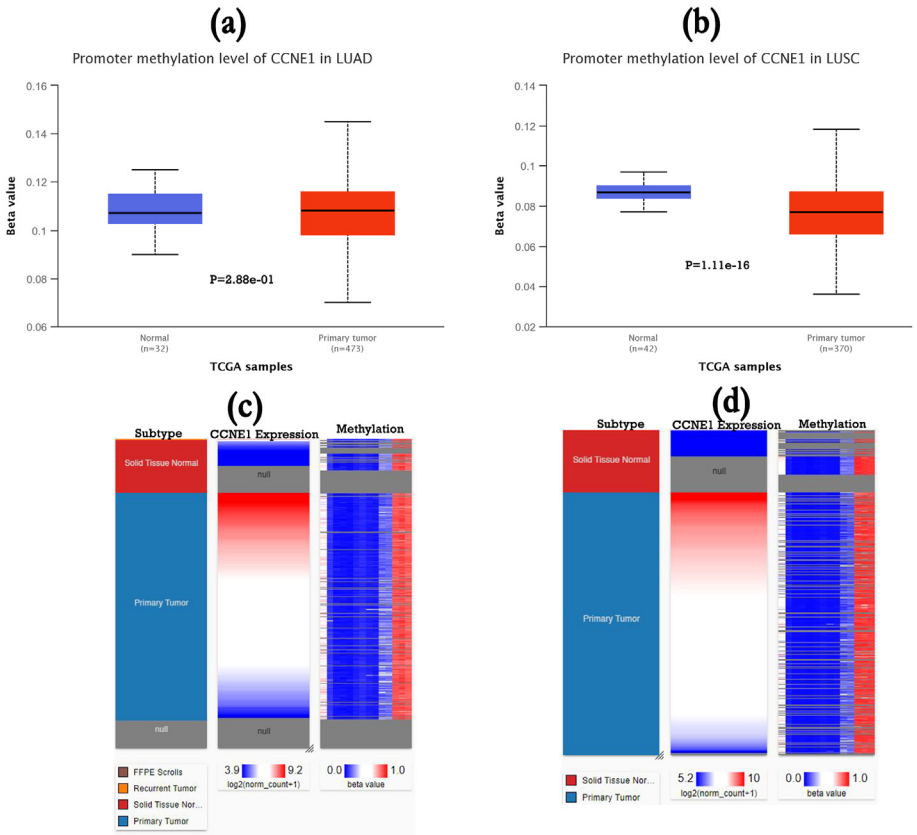

Figure 3. The promoter methylation pattern of the *CCNE1* gene in LUAD (a) and LUSC (b) tissues. Beta value cut-offs in the range of 0.7 to 0.5 indicates hypermethylation, and 0.3–0.25 indicates hypo-methylation. The coding sequence methylation pattern of the *CCNE1* gene in LUAD (c) and LUSC (d) tissues. *CCNE1* promoter was found to be hypo-methylated in LUSC tissues. The coding sequence of the *CCNE1* gene follows a common pattern of methylation. Beta Value Cutoff: 0: unmethylated, 0.5: hemimethylated (50% methylation), 1: hypermethylated.

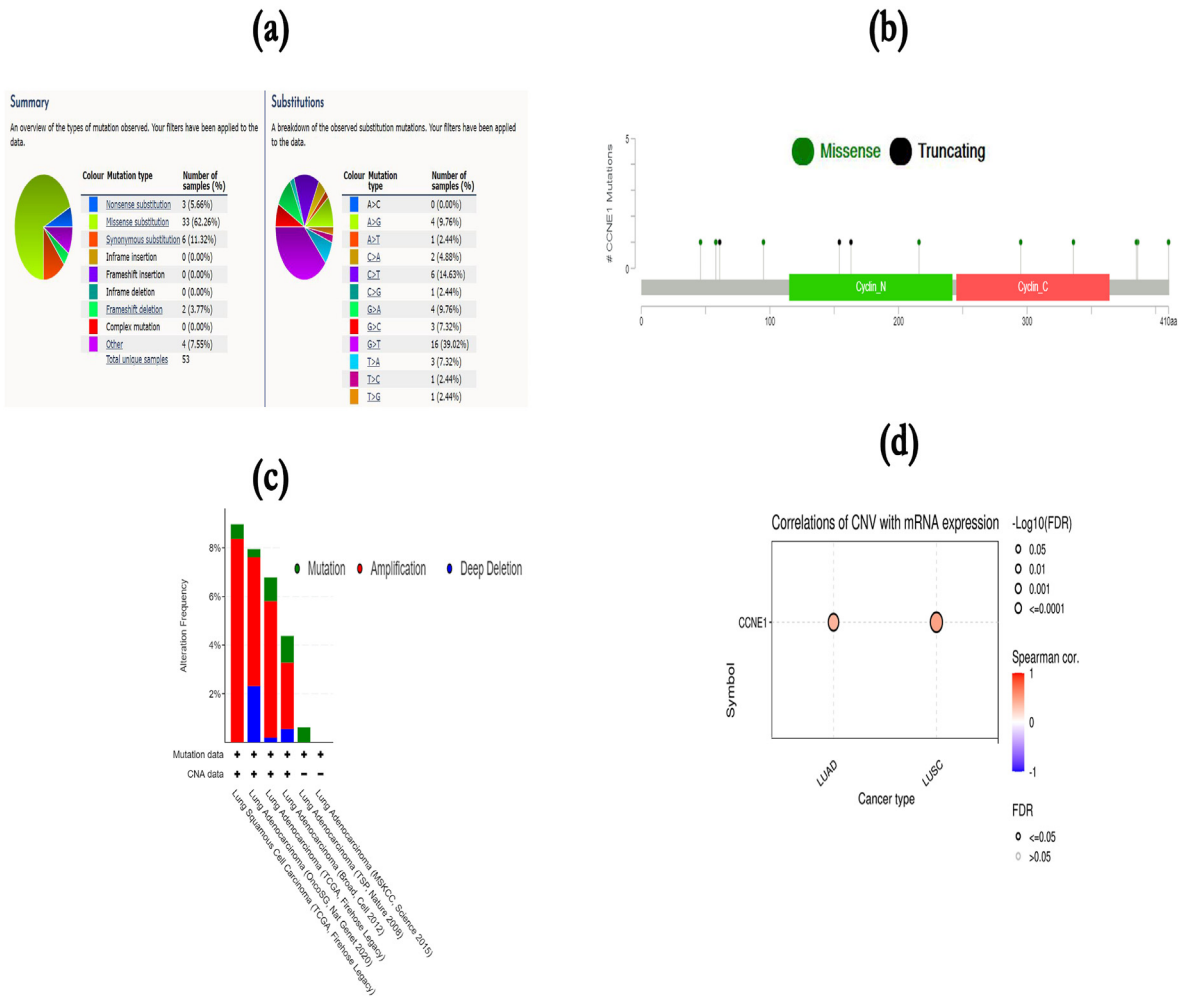

Figure 4. The result of mutation analysis from the COSMIC. Pie chart reflecting the distribution of different mutation and substitution categories in *CCNE1* gene in LUAD and LUSC tissues (a). The lollipop diagram of the mutational landscape in *CCNE1* protein sequence in different LUAD and LUSC samples (b). The bar diagram represents the distribution of mutations and CNAs present in the *CCNE1* gene across different LUAD and LUSC studies (c). Bubble plot representation of the association between CNAs in the *CCNE1* gene and its level of expression in LUAD and LUSC tissues (d).

tissues (p = 2.88e-01) (Figure 3a). On the contrary, the *CCNE1* gene body was reported to be less methylated in LUSC tissues and a significant presence of hypomethylated promoter of *CCNE1* in LUSC tissues was recorded (p = 1.11e-16) (Figure 3b). In terms of coding sequence methylation pattern, *CCNE1* gene coding DNA sequence showed a similar pattern of methylation both in LUAD and LUSC samples and a larger portion of the coding sequence at the 5′ end and a little downstream to the 5′ end remains unmethylated or less methylated (Figures 3c and 3d). A little chunk of the *CCNE1* coding gene at the 3′ end was found to contain hypermethylated regions as evidenced by an elevated level of the beta value indicated in the red color landscape. Thereafter, the methylation difference analysis between normal lung and LUAD and LUSC tissues from the GSCA served revealed that the *CCNE1* gene is less methylated in the test samples compared to the control which might be attributed to the larger less methylated 5' end of the coding sequence (Supplementary Figure S2a). Subsequently, the effect of *CCNE1* methylation on its mRNA expression level in LUAD and LUSC tissues was determined and unsurprisingly it was noticed that the hypomethylation was associated with high *CCNE1* expression in those cancerous tissues (Supplementary Figure S2b).

3.3. Identification of the mutational landscape in CCNE1 gene across different LUAD and LUSC samples

The COSMIC analysis showed the presence of multiple mutations across the *CCNE1* coding sequence in LUAD and LUSC tissues (Figure 4a). Among the mutations, missense substitution (n = 33, 62.26%) was the most prevalent form of mutation followed by synonymous substitution (n = 6, 11.32%) across the selected cancerous samples. Nonsense substation (3) and frameshift deletion (4) were also found to be present in the *CCNE1* coding region of LUAD and LUSC patients. The cBioPortal analysis revealed that the *CCNE1* gene contains a total of 12 mutations with a somatic mutation frequency of 0.7% across LUAD and LUSC samples. Among these, 9 mutations were nonsynonymous point mutations and 3 other mutations were reported to be truncating (Figure 4b) (Supplementary Table S1). As in par with the previous finding, missense mutation was the most common form in the selected studies. The copy number alteration (CNA) frequency suggested that amplification accounted for the most CNA across LUAD and LUSC data from the TCGA databases whereas mutation and deep deletion are responsible for a minor contribution (Figure 4c). The association analysis between the observed CNAs in the *CCNE1* gene and its expression at the mRNA level in LUAD and LUSC tissues reported that the CNA events in our gene of interest were positively associated with the mRNA level expression in those selected cancerous tissues (p < 0.05, Cor:>0) (Figure 4d).

3.4. Correlation between CCNE1 gene expression and LUAD and LUSC patients' survival

The survival analysis aids in a better understanding of the clinicopathological stage of the patients and thus helps in formulating diagnostic and

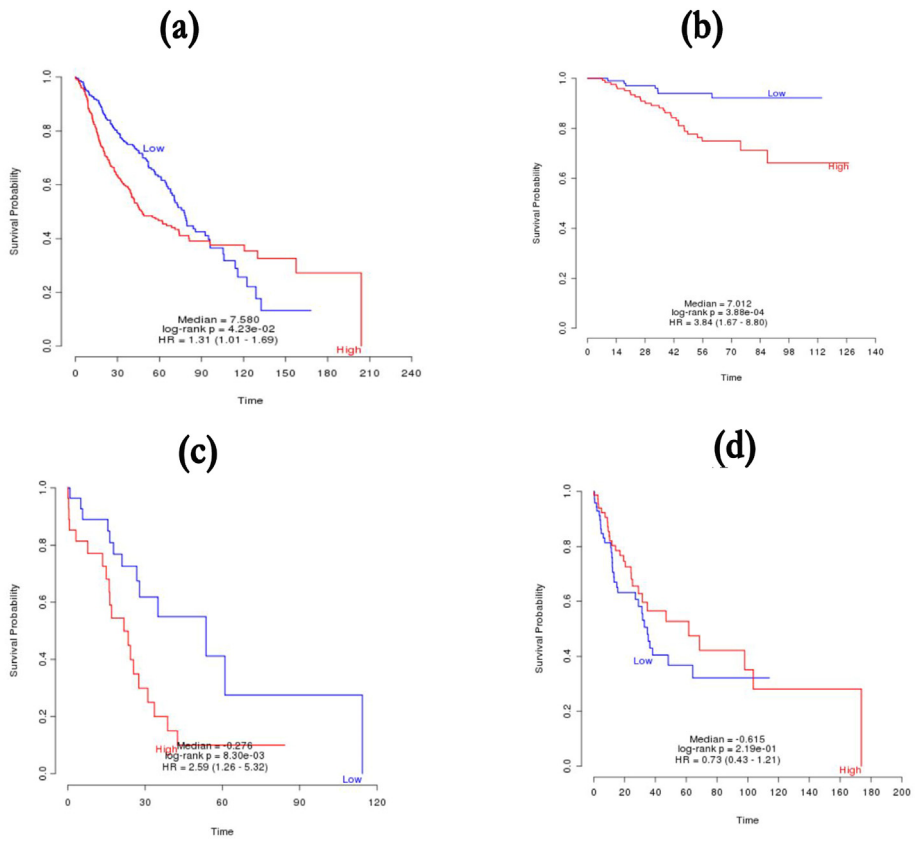

Figure 5. The survival analysis of the association between *CCNE1* gene expression and OS in LUAD (a) and LUSC (c) and DSS in LUAD (b) and LUSC (d) patients. *CCNE1* gene overexpression was negatively correlated with LUAD and LUSC patients' OS. Whereas its overexpression was negatively correlated with LUAD patients' DSS and positively with LUSC patients' DSS.

therapeutic measures. The survival analysis showed that the *CCNE1* gene expression is correlated with LUAD and LUSC patients' survival rates. The analysis report suggested that the *CCNE1* overexpression is significantly and negatively correlated with the OS of LUAD patients (p = 4.23e-02, Hazard Ratio (HR):1.31) (Figure 5a). A similar significant correlation was also observed for *CCNE1* overexpression in LUAD patients in terms of Disease-Specific Survival (DSS) (p = 8.30e-03, HR:2.59), reflecting that high *CCNE1* expression is the cause of worsening DSS (Figure 5b). Alongside, the highly *CCNE1* expressing LUSC patients were predicted to have a shorter OS compared to low *CCNE1* expression groups (p = 3.88e-04, HR:3.84) (Figure 5c). On the contrary, though no significant correlation was found between *CCNE1* expression and LUSC patients' DSS, the lower p-value and HR (p = 2.19e-01, HR:0.73) reflected that the *CCNE1* overexpression is positively correlated with the DSS of LUSC patients (Figure 5d). These results suggest that the *CCNE1* transcriptional and translational products may serve as prognostic marker over the clinical course of LUAD and LUSC patients.

3.5. Association between CCNE1 gene expression and clinicopathological factors in LUAD and LUSC samples

The association between *CCNE1* gene expression and clinicopathological characteristics of LUAD and LUSC patients was determined from the UALCAN server. The *CCNE1* gene overexpression was found to be correlated with LUAD patients' age (Figure 6a). It was observed that the gene is highly expressed at an early ages and gradually decreases over time. The male LUAD patients express more *CCNE1* genes than the female patients (Figure 6b). Asian LUAD patients express more *CCNE1* mRNAs followed by African-American and Caucasian patients. Also, the expression of the *CCNE1* gene increases gradually in relation to individual cancer stages (Figure 6d). The overexpression of the *CCNE1* gene was also found to be correlated with advancing nodal metastasis stage in LUAD patients (Figure 6e). Smoking habit was also correlated with *CCNE1* gene overexpression (Figure 6f). Moreover, *CCNE1* gene expression was found to be upregulated more in TP53 protein mutant patients compared to those in which TP53 protein is non-mutant (Figure 6g).

On the contrary, the LUSC patients showed a lower level of *CCNE1* gene expression at the early stage followed by a marginal increase and then a reduction in expression again (Figure 7a). Female and male LUSC patients express a similar level of *CCNE1* gene but in a higher volume compared to the normal samples (Figure 7b). In contrast to the LUAD patients, the *CCNE1* gene is highly expressed in African-American LUSC patients followed by Caucasian and Asian patients (Figure 7c). A consistent and similar level of *CCNE1* gene overexpression is observed in LUSC patients throughout different cancer stages (Figure 7d). Moreover, the gene overexpression increases gradually with advancing metastasis status and decreases to a greater value at the last stage (Figure 7e). As in par with the LUAD patients, *CCNE1* gene overexpression is also correlated with patients smoking habits and TP53 mutation status (Figures 7f and 7g).

3.6. Association between CCNE1 gene expression and immune cell infiltration level in LUAD and LUSC patients

The *CCNE1* gene was inspected for its transcriptional association with the abundance of immune cells in LUAD and LUSC patients. Significant positive association was found between the abundance of CD8+ T Cell (Cor: 0.128, p = 3.17e-23), Macrophage (Cor: 0.119, p = 7.99–03), NK Cell (Cor: 0.126, p = 5.04e-03) and *CCNE1* gene expression in LUAD patients (Figure 8a). Moreover, a significant negative association was discovered between B Cell (Cor: -0.173, p = 1.6e-04) infiltration and *CCNE1* gene expression in LUAD patients. However, no significant

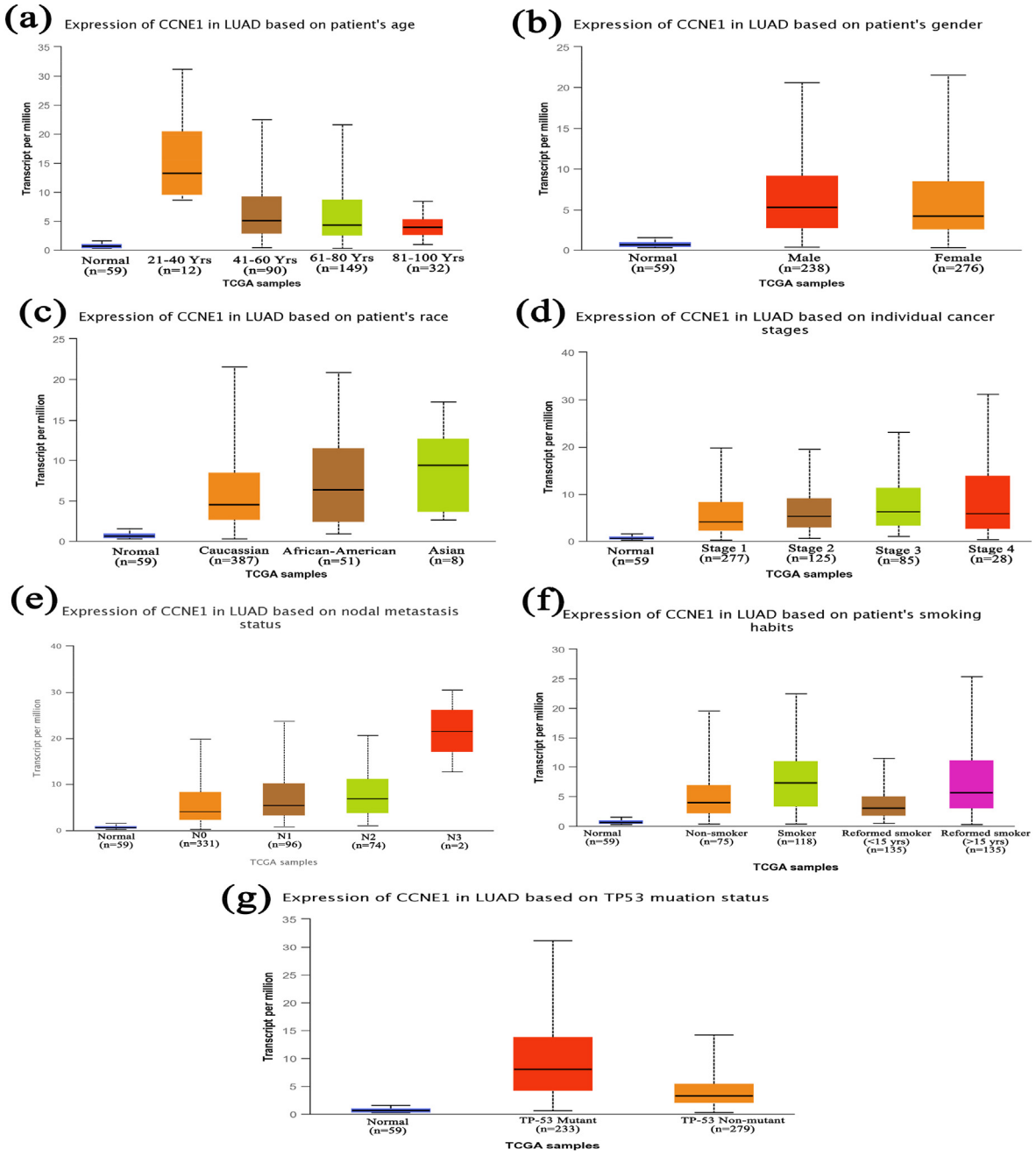

Figure 6. The association between *CCNE1* gene overexpression and LUAD patients' clinicopathological features i.e., age (a), gender (b), race (c), cancer stage (d), nodal metastasis status (e), smoking habit (f) and TP53 mutation status (g) (p=<0.05 for all characteristics). Normal: Samples collected from the adjacent normal tissues of the cancer specimens (Source: The Cancer Genome Atlas).

correlation was observed between *CCNE1* gene expression and CD4+ T cell abundance in LUAD patients (p = 8.02e-01). On the other hand, *CCNE1* gene expression was negatively associated with CD8+ T Cell (Cor: -0.156, p = 6.25e-04) in LUSC microenvironment whereas a positive correlation was observed in LUAD patients. NK Cell (Cor: -0.141, p = 2.02e-03) infiltration levels also showed positive correlation with *CCNE1* expression in LUSC tissues (Figure 8b). In contrast to LUAD tissues, LUSC patients were predicted to show a negative correlation between *CCNE1* expression and Macrophage (Cor: -0.154, p = 7.65e-04) infiltration levels. However, though the LUAD patients showed a positive association between B Cell abundance level and *CCNE1* expression, no significant correlation was observed between B Cell (and also CD4+ T cell) infiltration level and *CCNE1* gene expression in LUSC patients (Figure 8b). Overall, macrophage and CD8+ T Cells were reported to show a stronger correlation with *CCNE1* expression levels in LUAD and LUSC patients.

3.7. Analysis of the genes Co-expressed with CCNE1 in LUAD and LUSC tissues and their pathway activity

The *CCNE1* gene was investigated to identify its positively co-expressed genes in LUAD and LUSC patients from the TCGA database. Cell Division Cycle 45 (*CDC45*) was found to be the most highly co-expressed gene in LUAD tissue samples (cor:0.81, p = 9.31e-54) (Supplementary Figure S3a). In LUSC tissues, *CCNE1* gene was reported to be most highly co-expressed with Programmed Cell Death 5 (*PDCD5*) gene (cor:0.47, p = 5.28e-11) (Supplementary Figure S3b). All the significant

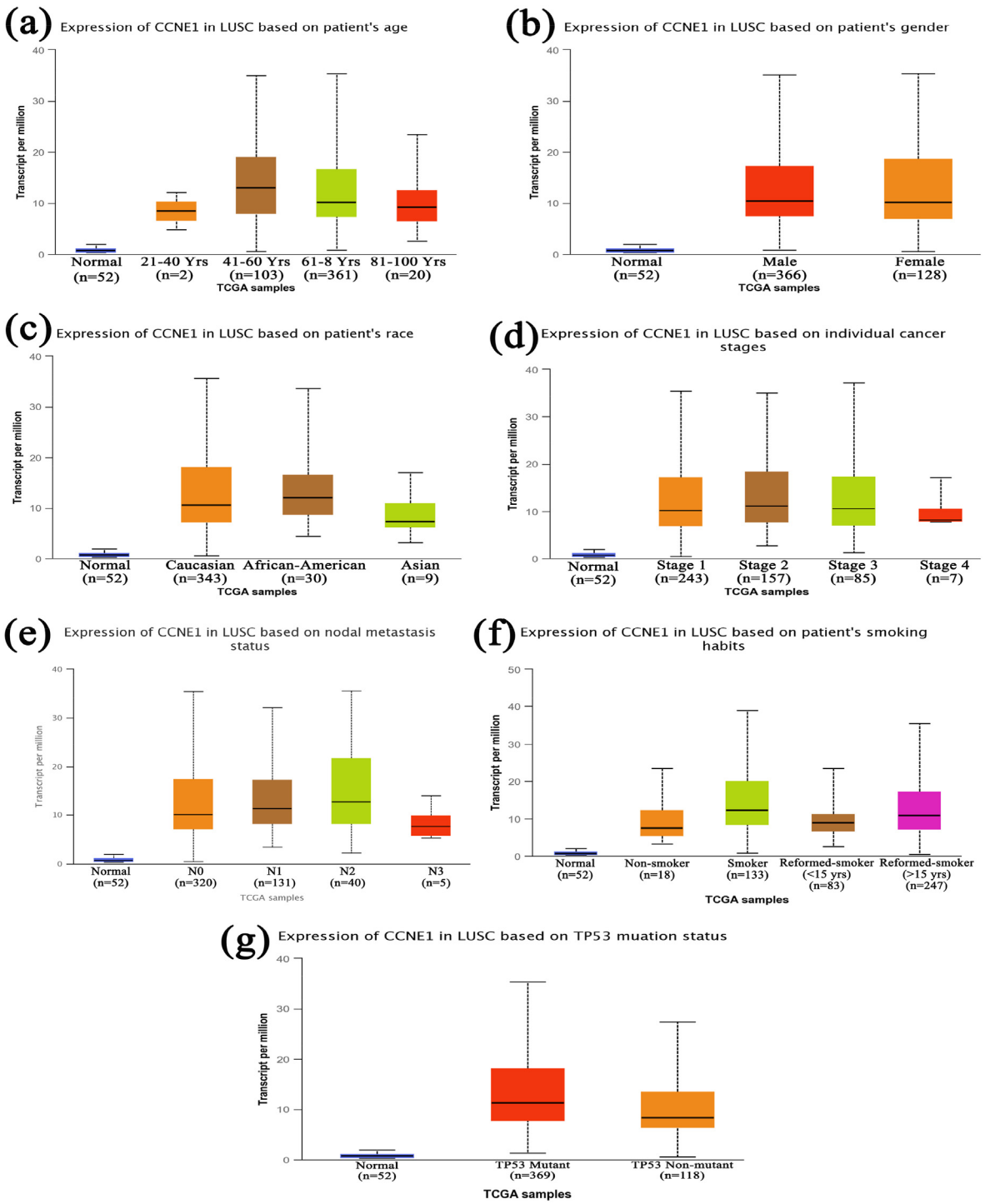

Figure 7. The association between *CCNE1* gene overexpression and LUSC patients' clinicopathological features i.e., age (a), gender (b), race (c), cancer stage (d), nodal metastasis status (e), smoking habit (f) and TP53 mutation status (g) (p=<0.05 for all characteristics). Normal: Samples collected from the adjacent normal tissues of the cancer specimens (Source: The Cancer Genome Atlas).

positively co-expressed genes (Cor:>0.02, $p < 0.05$) of *CCNE1* in LUAD and LUSC tissues can be found in Supplementary File 2 and 3, respectively. Then the top 50 co-expressed genes in LUAD and LUSC tissues were selected for pathway activity analysis. The pathway analysis suggested that the top 50 co-expressed genes of *CCNE1* in LUAD and LUSC tissues are mostly involved in maintaining cell cycle, cell cycle processes and regulating mitotic cell division (Figures 9a and 9b). Thereafter, the co-expressed genes were used to form an enrichment PPI network. The enrichment network of the co-expressed genes also suggested that the cluster is mainly responsible for the maintenance of different phases of the cell cycle, mitotic cell division, spindle organization and organelle fission in LUAD and LUSC tissues (Figures 9c and 9d).

4. Discussion

In this study, the prognostic and therapeutic values of the *CCNE1* gene in LUAD and LUSC have been explored using comprehensive bioinformatics analysis and a database mining approach. Differential expression

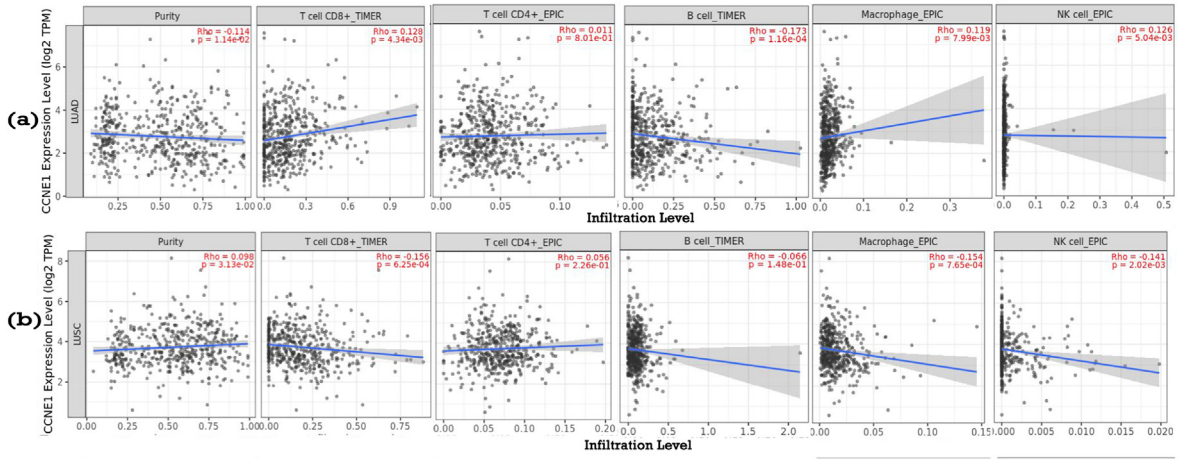

Figure 8. Scatter plot representation of the association between *CCNE1* gene expression and different immune cell infiltration in LUAD (a) and LUSC (b) tissues. *CCNE1* gene expression was found to show distinct and varying degrees of association between different immune cell infiltrations in LUAD LUSC tissues.

analysis of different genes can help in the identification of pivotal roles of particular genes in the oncogenic development of healthy cells and subsequent growth [29]. We found that the *CCNE1* gene is overexpressed in LUAD and LUSC tissues at the mRNA level suggesting the possible involvement of the *CCNE1* gene in the initiation of lung cancer and its exacerbation. Previously, *CCNE1* gene overexpression has been linked to influencing the tumorigenicity of NSCLC [30]. An abnormal pattern of *CCNE1* gene expression has also been found to be associated with triple-negative breast cancer and ovarian cancer exacerbation [31, 32]. Moreover, *CCNE1* gene expression was also found to be higher in LUAD and LUSC tissues at the protein level by inspecting IHC images. Therefore, the *CCNE1*-based IHC staining may assist in LUAD and LUSC diagnosis, evaluating whether the tumor is benign or malignant and measuring the response to any *CCNE1*-targeted therapy.

Aberrant promoter methylation of particular genes from sputum, urine and plasma can serve as a predictive marker for lung cancer and aid in the early-stage diagnosis of LUAD and LUSC patients in a non-invasive manner [33, 34]. Though no observable difference was recorded for *CCNE1* promoter between LUAD and normal tissues in our experiment, the hypomethylation pattern of *CCNE1* promoter in LUSC tissue may provide insightful information on LUSC prognosis and serve in methylation-sensitive diagnosis. Again, the overall *CCNE1* gene coding sequence was observed to be differentially methylated in LUAD and LUSC tissues when compared to the adjacent healthy tissues which should assist in the screening of NSCLC patients in a non-invasive manner. Additionally, the distinct promoter methylation pattern of the *CCNE1* gene in LUAD and LUSC tissues is supposed to assist in the further stratification of LUAD and LUSC patients. Apart from this, the aberrant methylation pattern of particular genes is one of the major epigenetic driving factors for cancer development and progression [35], and hence DNA methylation has become a promising target in cancer treatment, especially in personalized patient care, with strategies like DNA methyltransferase inhibitors. Given that the *CCNE1* promoter and coding sequence is differentially methylated in LUAD and LUSC tissues, its abnormal methylation pattern should help in future *CCNE1*-based epigenetic clinical decision-making. Alongside, promoter hypomethylation is associated with the high transcriptional activity of different genes [36] which might be the reason for high *CCNE1* expression in LUSC patients. However, the overexpression of the *CCNE1* gene in LUAD patients given the similar pattern of promoter methylation when compared to normal samples might be attributed to the factors like high upstream transcriptional signals, however, such assumptions require further laboratory investigation.

Somatic driver mutations and CNAs play pivotal roles in cancer development and growth by changing the dosage at which a gene product is produced in normal tissues and CNAs affect such activities to a greater extent compared to other point mutations [37]. In our study, we found evidence of multiple amplification events on the *CCNE1* gene across different LUAD and LUSC studies signifying its impact on the expression level of the *CCNE1* gene. In support of such a hypothesis, we later discovered that *CCNE1* CNAs are positively associated with its mRNA level expression in LUAD and LUSC tissues suggesting its possible involvement in cancer development given its major functional role in controlling the cell cycle. Thus, the *CCNE1* gene and its downstream products appear to be a promising target in LUAD and LUSC treatment. Importantly, the altered expression level of *CCNE1* caused from genetic variations may also indirectly influence the tumorigenic transformation of healthy lung cells if it is interconnected with oncogenes or tumor-suppressor genes within its interaction network though such speculation requires further molecular level investigation. Recently, the amplification of *CCNE1* has been shown to be associated with poor survival of triple-negative breast cancer patients [38]. Moreover, the presence of missense mutations in protein-coding genes can result in the translation of nonfunctioning, abnormally functioning or entirely no protein and influence the oncogenic transformation of healthy cells [39]. The sign of multiple nonsynonymous mutations in the *CCNE1* gene across different LUAD and LUSC samples signifies that the resulting defective protein coded by this gene can promote abnormal cell cycle activity in those affected patients.

The survival analysis of this study reported that the *CCNE1* gene overexpression may predict LUAD patients' poor OS and DSS and LUSC patients' poor OS. Previously, *CCNE1* gene overexpression was proven to be associated with poor survival of ovarian, bladder and breast cancer patients [40]. Therefore, the *CCNE1* expression might guide the stratification of high-risk lung cancer patients, tracking the patients throughout the entire disease state and recommending management measures. The clinicopathological characteristics and *CCNE1* gene overexpression correlation analyses revealed that both LUAD and LUSC patients express high levels of *CCNE1* gene across different cancer and nodal metastasis stages irrespective of their age and sex. This along with the survival analysis suggest that the *CCNE1* gene and its translational products may serve as an important diagnostic measure for *CCNE1*-based early-stage LUAD and LUSC diagnosis, determination of cancer cell fitness and identifying the metastatic potential of the affected cells. Additionally, the TP53 mutant LUAD and LUSC patients were found to express more *CCNE1* mRNAs compared to the TP53 non-mutant patients. Thus, the presence of a mutant *TP53* gene along with a high level of *CCNE1* gene expression might provide more insightful prognostic information on LUAD and LUSC patients, however, further laboratory research is warranted for that purpose.

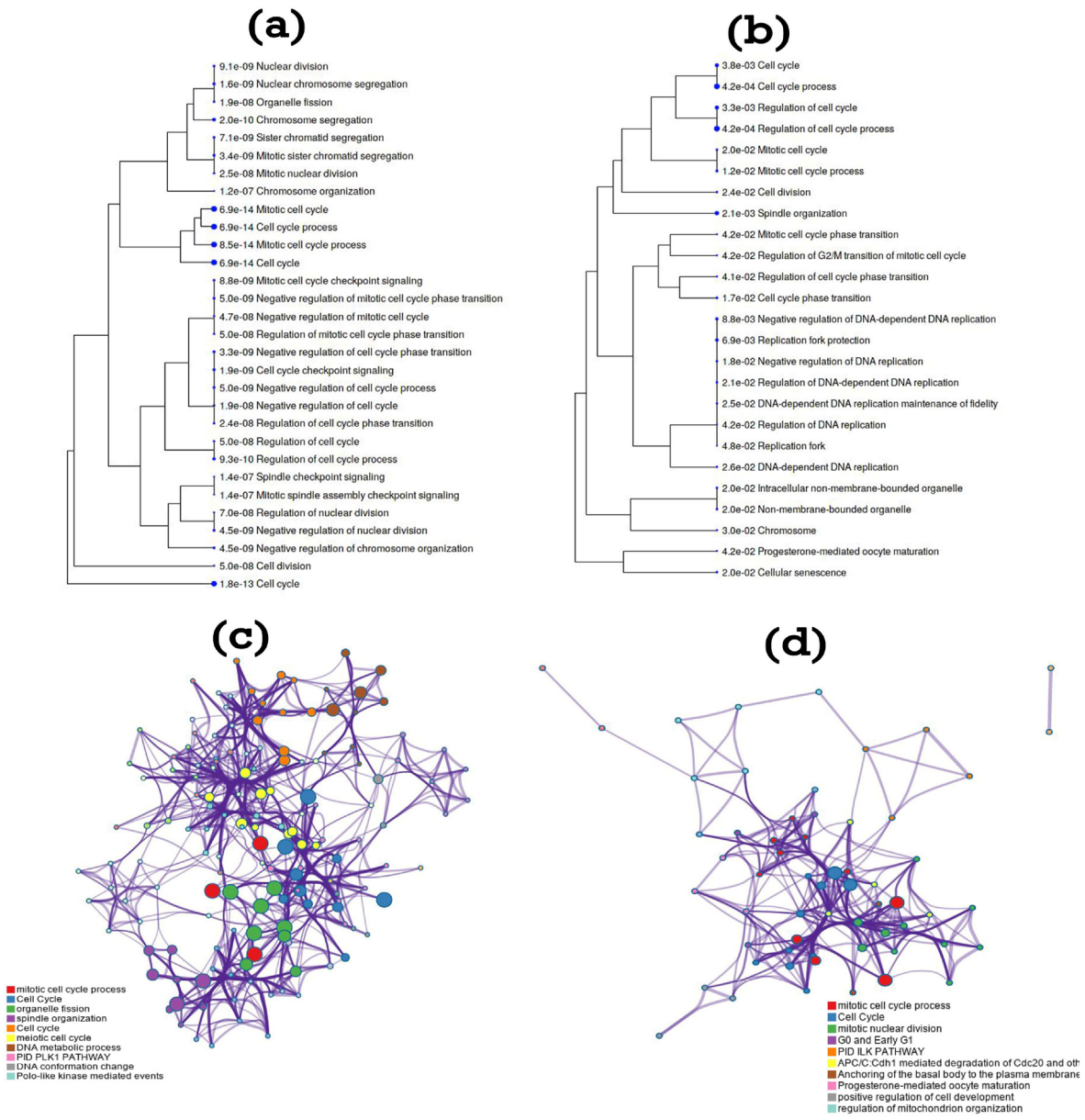

Figure 9. The pathway activity tree of co-expressed genes of *CCNE1* gene in LUAD (a) and LUSC (b) tissues (Size of blue circles corresponds to the volume of a term among the genes and the numeric values are representing the p-value). Pathway enrichment network terms of the co-expressed gene in LUAD (c) and LUSC (d) tissues (Color represents the function and the circles represent the nodes, functionally related nodes are present closely).

Tumor-infiltrating immune cells can worsen the tumor growth and development in the cancer microenvironment by inducing systemic inflammation and the activity of immune cells in the microenvironment is sometimes controlled by particular gene expression [41]. Thereby, immune infiltration analysis can help in keeping track of cancer patients over the clinical stages and thus aid in accurate diagnosis and treatment. The *CCNE1* gene expression was found to be both positively and negatively correlated with several immune cells including B Cells, CD8+ T Cells, CD4+ T Cells, Macrophages and NK cells. Therefore, the impairing level of *CCNE1* expression by the presence of any mutation in the coding sequence can alter the immune cell activity in LUAD and LUSC patients. Moreover, such an immune cell population may also aid in *CCNE1*-based dual diagnosis or combinatorial therapeutic approaches (i.e., additional immunotherapy) in LUAD and LUSC patients.

The gene co-expression analysis revealed that *CCNE1* is highly co-expressed with *CDC45* and *PDCD5* in LUAD and LUSC tissues respectively. The *CDC45* gene has been proposed to be an oncogene for NSCLC development recently [42]. Moreover, *CDC45* has recently been reported as a predictive prognostic marker for colorectal cancer [43]. Low content of *PDCD5* product in the serum of lung cancer patients has been linked to cancer aggravation and lesions [44]. These pieces of evidence further strengthen the fact that the *CCNE1* expression may also play oncogenic roles in LUAD and LUSC patients and hence could serve as a therapeutic target since the co-expressed genes are functionally related.

Thereafter, the functional enrichment analysis of the co-expressed genes of *CCNE1* in LUAD and LUSC patients revealed that most of the co-expressed genes were responsible for maintaining cell cycle, controlling cell cycle processes, spindle organization and nuclear division. Deregulation in any of these biological processes can result in the tumorigenic transformation of a healthy cell [45].

Overall, the experimental findings of this study suggest that the *CCNE1* gene and its transcriptional products are differentially expressed in LUAD and LUSC tissues. They possess several different mutations and differential levels of promoter and coding sequence methylation patterns

in LUAD and LUSC samples compared to their normal counterparts. Their expression pattern is disproportional across various LUAD and LUSC stages. Moreover, *CCNE1* gene expression is correlated with poor LUAD and LUSC patients' overall survival. Altogether, the *CCNE1* gene and its transcriptional and translational products may serve as a diagnostic and therapeutic target for LUAD and LUSC. The association between *CCNE1* expression and abundance of different immune cells in LUAD and LUSC patients and functional enrichment analysis of its co-expressed genes should guide the further clinical development of *CCNE1*-based diagnostic markers and therapeutic interventions for these two NSCLC subtypes. This study should assist and uphold future efforts on LUAD and LUSC screening and treatment based on *CCNE1* which is currently underway by the authors.

Declarations

Author contribution statement

Md. Asad Ullah: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Maisha Farzana: Analyzed and interpreted the data; Wrote the paper.

Md. Shariful Islam; Ripa Moni; Umme Salma Zohora: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammad Shahedur Rahman: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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