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Research article

Analysis of the interlink between glucose-6-phosphate dehydrogenase (G6PD) and lung cancer through multi-omics databases

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ABSTRACT

Glucose-6-Phosphate Dehydrogenase (G6PD) is a crucial enzyme that executes the pentose phosphate pathway. Due to its critical nodal position in the metabolic network, it is associated with different forms of cancer tumorigeneses and progression. Nonetheless, its functional role and molecular mechanism in lung cancer remain unknown. The present study provides intricate information associated with G6PD and Lung Cancer. Varieties of public datasets were retrieved by us, including UALCAN, TCGA, cBioPortal, and the UCSC Xena browser. The data obtained were used to assess the expression of G6PD, its clinical features, epigenetic regulation, relationship with tumour infiltration, tumour mutation burden, microsatellite instability, tumour microenvironment, immune checkpoint genes, genomic alteration, and patient's overall survival rate. The present study revealed that the G6PD expression was correlated with the clinical features of lung cancer including disease stage, race, sex, age, smoking habits, and lymph node metastasis. Moreover, the expression profile of G6PD also imparts epigenetic changes by modulating the DNA promoter methylation activity. Methylation of promoters changes the expression of various transcription factors, genes leading to an influence on the immune system. These events linked with G6PD-related mutational gene alterations (FAM3A, LAG3, p53, KRAS). The entire circumstance influences the patient's overall survival rate and poor prognosis. Functional investigation using STRING, GO, and KEGG found that G6PD primarily engages in hallmark functions (metabolism, immunological responses, proliferation, apoptosis, p53, HIF-1, FOXO, PI3K-AKT signaling). This work provides a wide knowledge of G6PD's function in lung cancer, as well as a theoretical foundation for possible prognostic therapeutic markers.

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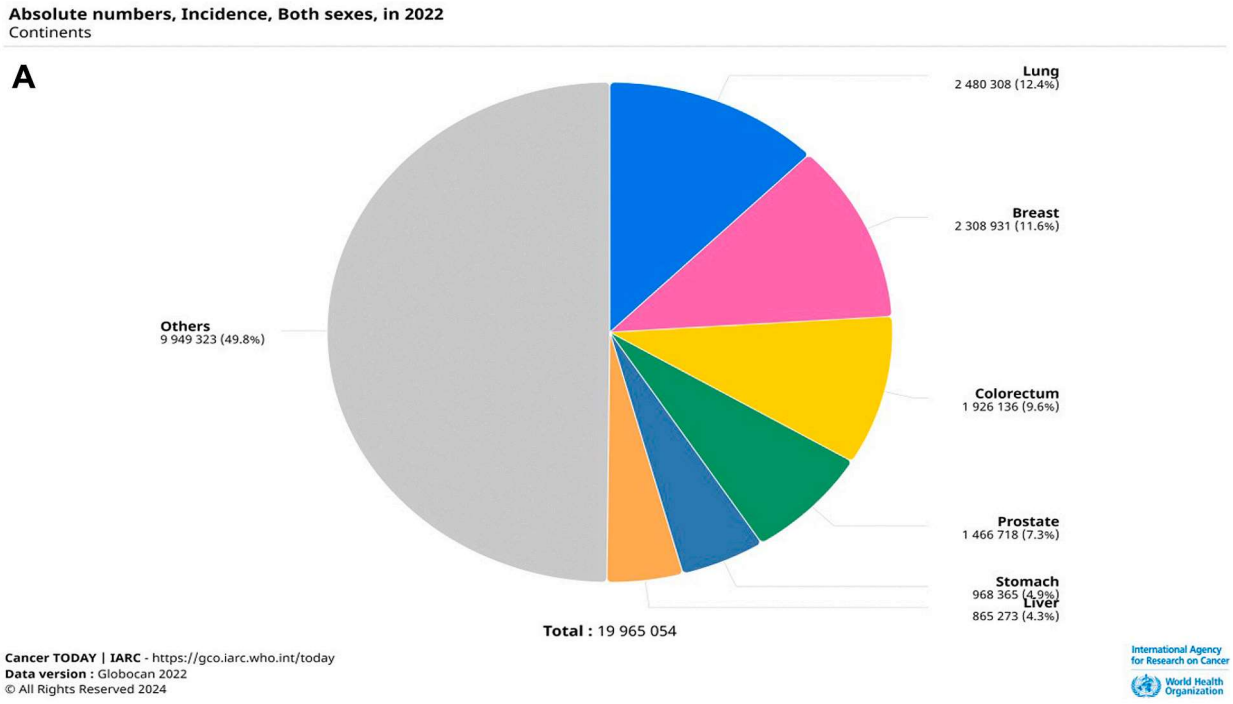
Abbreviations:

G6PD	Glucose-6-Phosphate Dehydrogenase
PPP	Pentose Phosphate Pathway
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
TCGA	The Cancer Genome Atlas
GTEx	Genotype-Tissue Expression
UCSC	University of California Santa Cruz
GEO	Gene Expression Omnibus
GEPIA	Gene Expression Profiling Interactive Analysis
TPM	Transcripts Per Million
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
ACC	Adrenocortical carcinoma
BLCA	Bladder Urothelial Carcinoma
BRCA	Breast invasive carcinoma
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	Cholangio carcinoma
COAD	Colon adenocarcinoma
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma
ESCA	Esophageal carcinoma
GBM	Glioblastoma multiforme
HNSC	Head and Neck squamous cell carcinoma
KICH	Kidney Chromophobe
KIRC	Kidney renal clear cell carcinoma
KIRP	Kidney renal papillary cell carcinoma
LGG	Brain Lower Grade Glioma
LIHC	Liver hepatocellular carcinoma
MESO	Mesothelioma
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PRAD	Prostate adenocarcinoma
PCPG	Pheochromocytoma and Paraganglioma
READ	Rectum adenocarcinoma
SARC	Sarcoma
SKCM	Skin Cutaneous Melanoma
STAD	Stomach adenocarcinoma
THCA	Thyroid carcinoma
THYM	Thymoma
TGCT	Testicular Germ Cell Tumours
UCEC	Uterine Corpus Endometrial Carcinoma
UCS	Uterine Carcinosarcoma
UVM	Uveal Melanoma
TMB	Tumour Mutational Burden
MSI	Microsatellite Instability
ICP	Immune Checkpoints
CD27	Cluster of Differentiation 27
CD28	Cluster of Differentiation 28
CD40	Cluster of Differentiation 40
ICOS	Inducible T-cell Co-stimulator
BTLA	B and T Lymphocyte Attenuator
CD276	Cluster of Differentiation 276
CTLA4	Cytotoxic T-Lymphocyte-Associated protein 4
IDO1	Indoleamine 2,3-dioxygenase-1
IDO2	Indoleamine 2,3-dioxygenase-2
LAG3	Lymphocyte Activation Gene-3
VTCN1	V-set domain-containing T-cell activation inhibitor 1
SIGLEC7	Sialic acid-binding Immunoglobulin-type Lectin 7
SIGLEC9	Sialic acid-binding Immunoglobulin-type Lectin 9
MLH1	DNA mismatch repair protein Mlh1 or MutL protein Homolog 1
MSH2	DNA mismatch repair protein Msh2 or MutS Homolog 2
MSH6	MutS Homolog 6
PMS2	Mismatch repair endonuclease PMS2

1. Introduction

Metabolism is a key event that occurs in every single living cell of the body for the sustainability of life. Anabolism and catabolism are a set of chemical reactions associated with the biological macromolecules (carbohydrate, protein, lipid, and nucleic acid). The complex cascade reactions are decisive for the cell's fate and harmonized regulation of metabolic networks and are essential for normal cellular functions. Genetics, age, physical activity, diet, hormones, and environmental factors affect metabolic regulation. Metabolic alterations are linked with the compromised functional and mechanistic consequences. One of the finest examples is an influx of



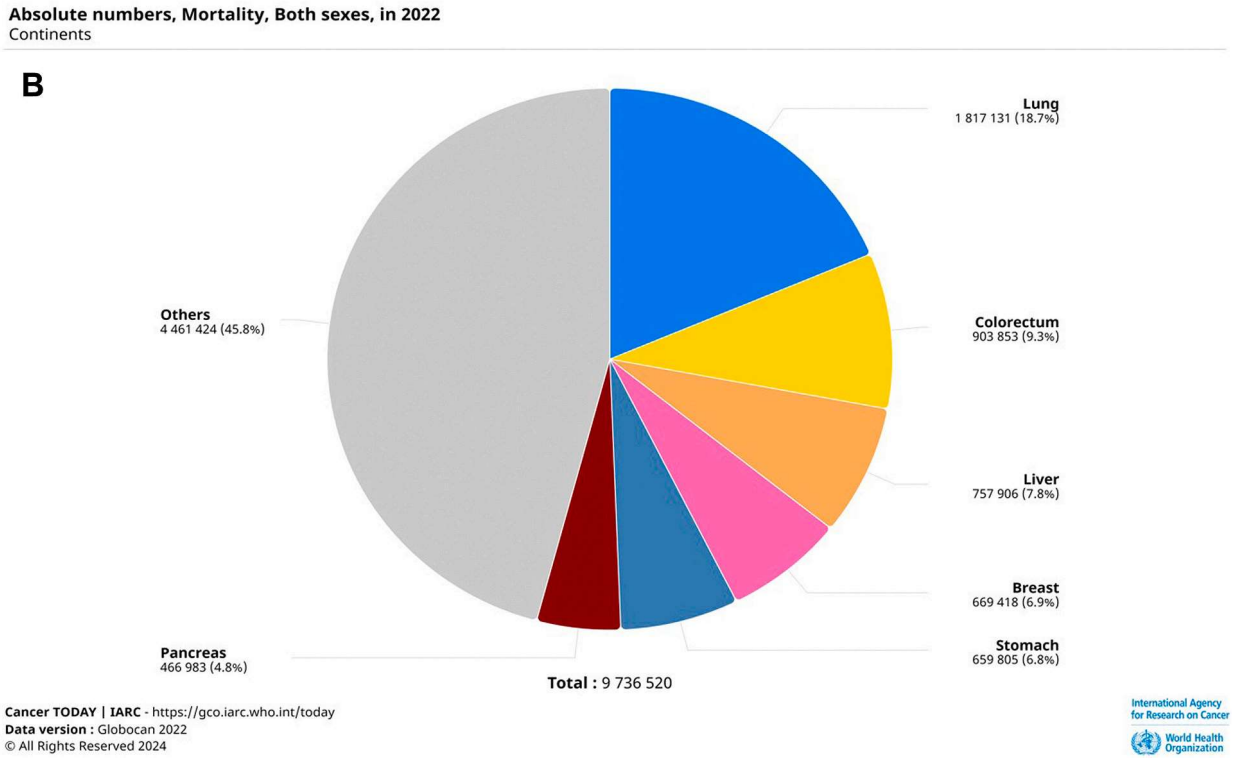


Fig. 1. A pie chart shows the estimated number of new cases diagnosed (A) and deaths in 2022 (in males and females of all ages) due to different types of cancer (B) [Data source: Globocan 2022, Graph production: Global Cancer Observatory (GCO) (https://gco.iarc.who.int/todayhttp://gco.iarc.fr/)].

glucose via glucose transporters (GLUTs). Glycolysis occurs under the normal influx of glucose while a high influx of glucose tends to operate the pentose phosphate pathway (PPP). PPP is a metabolic path that yields NADPH and ribose-5-phosphate, and the alteration of this pathway (Glucose metabolism) is considered one of the hallmarks of cancer [1,2]. PPP has been implicated in several aspects of cancer progression, encompassing invasion, metastasis, and resistance to therapeutic interventions [3]. Glucose-6-phosphate dehydrogenase (G6PD) is a rate-limiting enzyme in the oxidative branch of PPP. G6PD expression is elevated in several types of malignancies, as cancer cells become more dependent on G6PD-mediated NADPH generation than normal cells due to increasing metabolic needs and oxidative stress [4–8].

The increasing frequency of cancer diagnoses in both developed and developing economies is emergent as a global issue, posing a substantial threat to human health and imposing a considerable financial burden on global public health systems [9]. According to data from 185 countries around the world, cancer is now the first leading cause of death with a significant global public health burden [10]. In terms of incidence, lung cancer is the most frequent type of cancer, accounting for 12.4 % (Fig. 1A). Furthermore, the Globocan Report 2022 shows that the mortality rate is the highest at 18.7 % for all forms of cancer (Fig. 1B). Lung cancer is categorized into two types: non-small cell lung cancer (NSCLC; diagnosed in 80 % of cases) and small cell lung cancer (SCLC; diagnosed in 20 % of cases). There are several therapies for lung cancer, including radiation, chemotherapy, targeted therapy, immunotherapy, and surgery, but sadly they all have a lot of adverse effects. Drug and radiation resistance in cancer cells has lately emerged as a key concern. Because carcinogenesis is so complex, pan-cancer analysis is commonly used in tumour research to reveal the similarity and heterogeneity of multiple tumour genes and biological processes. It also provides insight into cancer treatment and prevention. The Cancer Genome Atlas (TCGA) is commonly used to discover particular functional genes, facilitating in-depth cancer gene research [11,12].

In this work, we attempted to compile all available evidence about the biological involvement of G6PD in lung cancer. We systematically examined, characterized, and explored the relationship between G6PD expression and clinical features, as well as epigenetic regulation by promoter methylation. We also investigated the relationship of G6PD with immune infiltration, tumour mutational burden, microsatellite instability, immune checkpoints genes, mutation, and survival analysis of patients along with gene set enrichment analysis. The study explores the probable molecular mechanism of G6PD in lung cancers (LUAD and LUSC) and its clinical prognosis. Findings from this study also contribute a theoretical foundation for the development of new drugs to treat lung cancer with G6PD inhibitors.

2. Materials and methods

2.1. Acquisition of data and processing

The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/) has sequencing data for genes discovered in tumours and surrounding normal tissues from more than 30 cancers. The Genotype-Tissue Expression (GTEx) database provides information on gene expression changes across tissues [13]. The University of California Santa Cruz (UCSC) data portal (https://xenabrowser.net/datapages/) was utilized to access pan-cancer patient RNA-Seq and clinical data from the TCGA database, as well as datasets from the GEO database (https://www.ncbi.nlm.nih.gov/gds) as a data supplement to conduct lung cancer G6PD analysis [14].

2.2. Analysis of G6PD expression profiles in lung cancer

The TCGA database was utilized to investigate the expression profile of G6PD in tumour and normal tissues from over 30 different malignancies. Furthermore, the "Gene_DE" module of the TIMER 2.0 database (https://cistrome.shinyapps.io/timer) was used to assess the variations in gene expression levels between distinct tumour tissues and their neighbouring normal tissues [15]. GEPIA2 (http://gepia2.cancer-pku.cn/) was utilized to undertake tissue-wise gene expression analysis in two distinct lung cancer types [16]. To construct the box plot, a single gene analysis module was adopted, with a P cut-off value of 0.05 and a log2FC cut-off value of 1, as well as the option "Match TCGA normal value". The data (counts) were converted to transcripts per million (TPM) format and normalized using log2 (TPM+1). Log2 (TPM+1) data were utilized for logarithmic scaling to generate box plots.

2.3. Correlation between G6PD expression and clinical features of lung cancer

To assess the relationship between G6PD expression and clinical characteristics of lung cancer (LUAD and LUSC), UALCAN (The University of Alabama at Birmingham CANCER Data Analysis Portal) (http://ualcan.path.uab.edu/) was used [17]. We investigated a variety of clinical factors, including tumour types, cancer stages, race, gender, age groups, smoking behaviours, histological subtypes, and nodal metastases of cancer.

2.4. Epigenetic regulation by promoter methylation

The UALCAN-based online tool (https://ualcan.path.uab.edu/) assesses epigenetic control of gene expression via promoter methylation. The UALCAN database was used to examine G6PD DNA methylation [17].

2.5. Immune infiltration, ICP, MSI analysis

The "Immune Association Gene" tool in TIMER 2.0 to investigate the link between G6PD level and immune cell infiltration in both

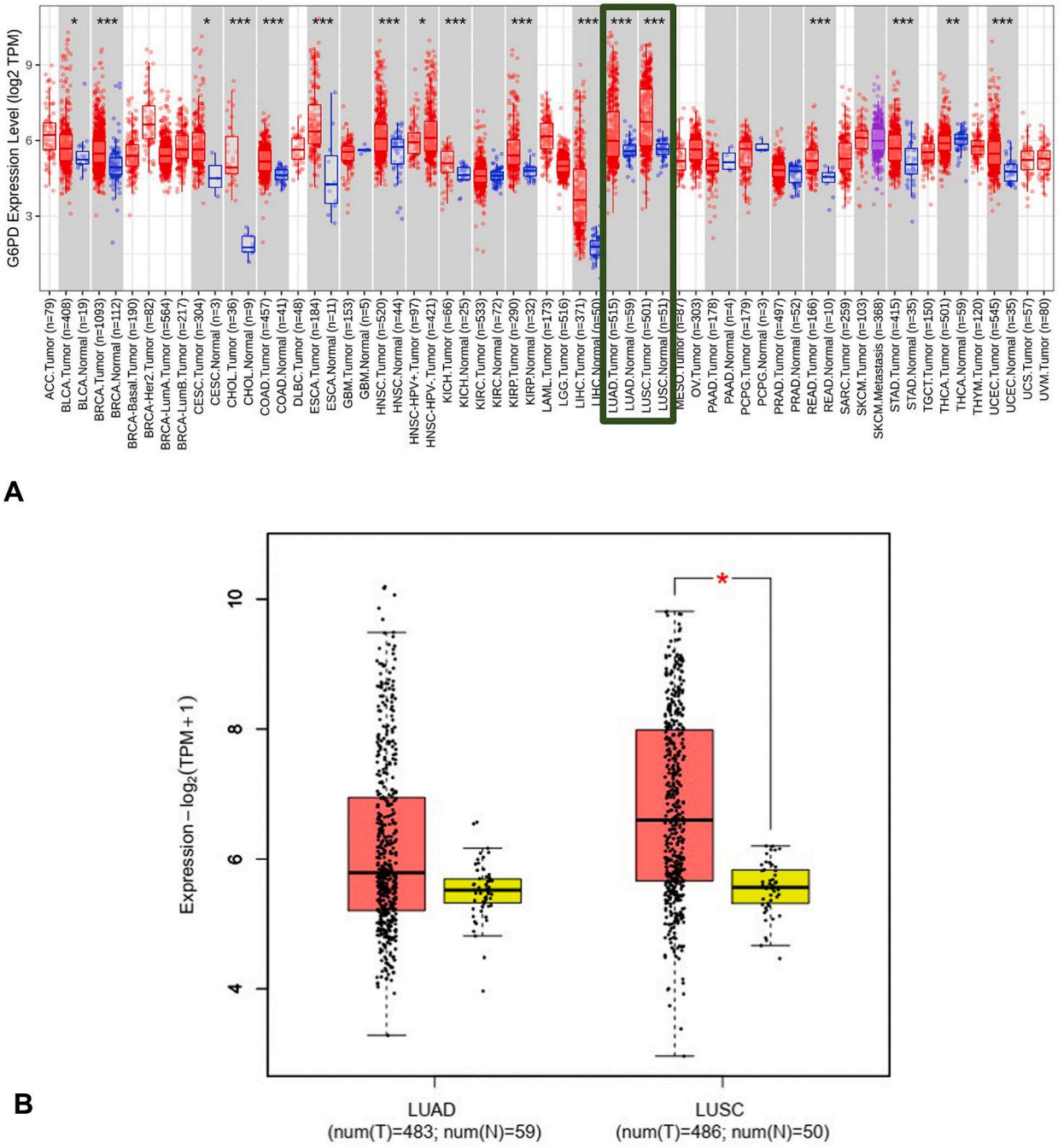


Fig. 2. Expression profile of G6PD in distinct types of cancers from the cancer genome atlas (A) the differential expression profile of human G6PD gene between tumor and adjacent normal tissues of the different cancer types from TCGA database in Gene_DE module of TIMER 2.0 in the form of the box plot. The statistical significance computed by the Wilcoxon test is annotated by the number of stars (*p < 0.05, **p < 0.01, ***p < 0.001). The expression data were normalized through log2 conversion. The box plot in blue represents normal samples and the red box plot indicates tumour samples. The green rectangle box represents the highest expression of G6PD in two different types of lung cancer (LUAD and LUSC) among the different cancers (B) Differences in G6PD expression between two different types of lung cancer from the TCGA database and normal samples from the GEPIA2.0 database. Red colour indicates tumour samples while yellow shows normal samples. All expression data were normalized through log2 (TPM+1) conversion. (*P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

types of lung cancers (LUAD, LUSC) [15]. We considered all T cells, B cells, macrophages, fibroblasts, and NK cells for the immune filtration study. XCELL algorithm was used to estimate the immune infiltration. The obtained results are depicted in the form of heat maps. TISIDB is a website for gene- and tumour-immune interaction (http://cis.hku.hk/TISIDB/index.php) [18]. G6PD gene expression was analysed in several immune subtypes, including C1 (wound healing), C2 (IFN-γ dominant), C3 (inflammatory), C4 (lymphocyte depletion), C5 (immunologically quiet), and C6 (TGF-β dominant). TMB and MSI are two crucial biomarkers in the tumour microenvironment [19,20]. Based on the literature, 14 ICP genes were selected including stimulatory (CD27, CD28, CD40, ICOS) and inhibitory checkpoint molecules (BTLA, CD276, CTLA4, IDO1, IDO2, LAG3, VTCN1, SIGLEC7, and SIGLEC9) [21]. To explore the correlations between G6PD expression, immune checkpoint-related genes, and MSI genes (MLH1, MSH2, MSH6, and PMS2) in human lung cancers (LUAD, LUSC) within the TCGA cohort, we utilized the "Gene_Corr" module in the TIMER 2.0 database [15]. This allowed us to generate a heat map showing the statistical significance with ρ value after purity adjustment via Spearman's correlation analysis. The Pearson correlation of marker genes was further calculated [22].

2.6. G6PD, mutation and patients' survival

cBioPortal for Cancer Genomics v6.0.0. has been used to evaluate the correlation between G6PD, mutation and survival of patients (https://www.cbioportal.org/). The cancer type summary module is used to estimate the prevalence and type of gene mutations in TCGA tumours. To filter, we utilized the 'TCGA Pan-Cancer Atlas Studies for LUAD and LUSC module' and entered 'G6PD' as the query term. G6PD gene variant characteristics such as frequency, mutation type, and mutation location were chosen. Using the 'Mutations' module, the mutation site information may be studied in the protein structure's schematic diagram. The UALCAN-online tool revealed a relationship between G6PD expression and TP53 mutation status in LUAD and LUSC. The mutation has an impact on the person's overall survival rate when compared to people with changed G6PD expression and those without. The overall survival rate data was analysed and obtained from the cBioPortal database.

2.7. Gene set enrichment analysis

A G6PD protein-protein interaction (PPI) network was attained through the STRING database (https://cn.string-db.org/) [23]. To build a protein-protein interaction (PPI) network, the following parameters were used: Protein name: G6PD, Species: Homo sapiens. The minimum needed interaction score is the highest confidence (0.900). The meaning of network edges is evidence. Active interaction sources include experiments, databases, co-expression, neighbourhood, gene fusion, and co-occurrence. The maximum number of interactors that can be employed is 100 for shell 1 and 150 for shell 2. The PPI was then visualized using Cytoscape software (version 3.10.1), and hub genes were identified using the MCODE plugin. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were then performed.

3. Results and discussion

3.1. High G6PD expression associated with different cancers

G6PD is the first rate-limiting enzyme in the oxidative branch of the PPP, producing ribose and the reducing equivalent NADPH to sustain growth and development [5,24,25]. Aberrant changes to G6PD alter the functioning of PPP which changes NADPH levels and leads to inhibition of normal cell proliferation. A decrease in G6PD activity impacts embryonic and organismal development, and deviant activation of the PPP or G6PD is associated with tumorigenesis [4,7,4,26–53].

G6PD expression was shown to be greater in tumour samples than in normal samples across all kinds of TCGA malignancies (Fig. 2A). Fig. 2A's screening profile revealed that lung malignancies [LUSC] had the greatest expression of G6PD when compared to other types of cancers such as BLCA, BRCA, CESC, CHOL, COAD, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LIHC, READ, SARC, STAD, and UCEC. Comparison of G6PD expression with the tumour samples also follows the same trend and it was the highest in lung cancer, especially in LUSC (Fig. 2A). Upon examining the TIMER 2.0, it was discovered that the expression of G6PD was considerably elevated in various types of cancer, including BLCA, BRCA, CHOL, COAD, ESCA, HNSC, KICH, KIRP, LIHC, LUAD, LUSC, READ, STAD, and UCEC when compared to their normal counterparts (Fig. 2A). Taken together, the results imply that G6PD is abnormally expressed in several malignancies and may play an important role in tumour pathogenesis. These findings motivate us to further research the involvement of G6PD in lung cancer. Fig. 2B shows a substantial difference ($^*P < 0.05$) in the expression of G6PD between the TCGA tumour and normal samples. Expression-level variations differ between LUAD and LUSC. Rapidly growing cancer cells have evolved myriad mechanisms to activate G6PD to support the cellular requirements for NADPH production and synthesis of fatty acids and nucleic acids. These aberrant G6PD profiles in malignancies drive us to dwell upon lung cancer to elucidate its role.

3.2. Correlation between the G6PD expression and clinical features of lung cancer

Looking specifically at lung cancer, we evaluated the effect of G6PD expression in LUAD and LUSC. The expression of G6PD is much higher in primary tumours than in normal cells (Fig. 3A). Metabolism plays an important role in the initiation and development of cancer. G6PD plays a pivotal role in the metabolic network [26]. Even in the presence of oxygen, cancer cells prefer glycolysis to oxidative phosphorylation. This is referred to as aerobic glycolysis, or the Warburg effect [54]. A second reason for preferring glycolysis is to avoid the production of reactive oxygen species invariably produced during oxidative metabolism. Cancer cells

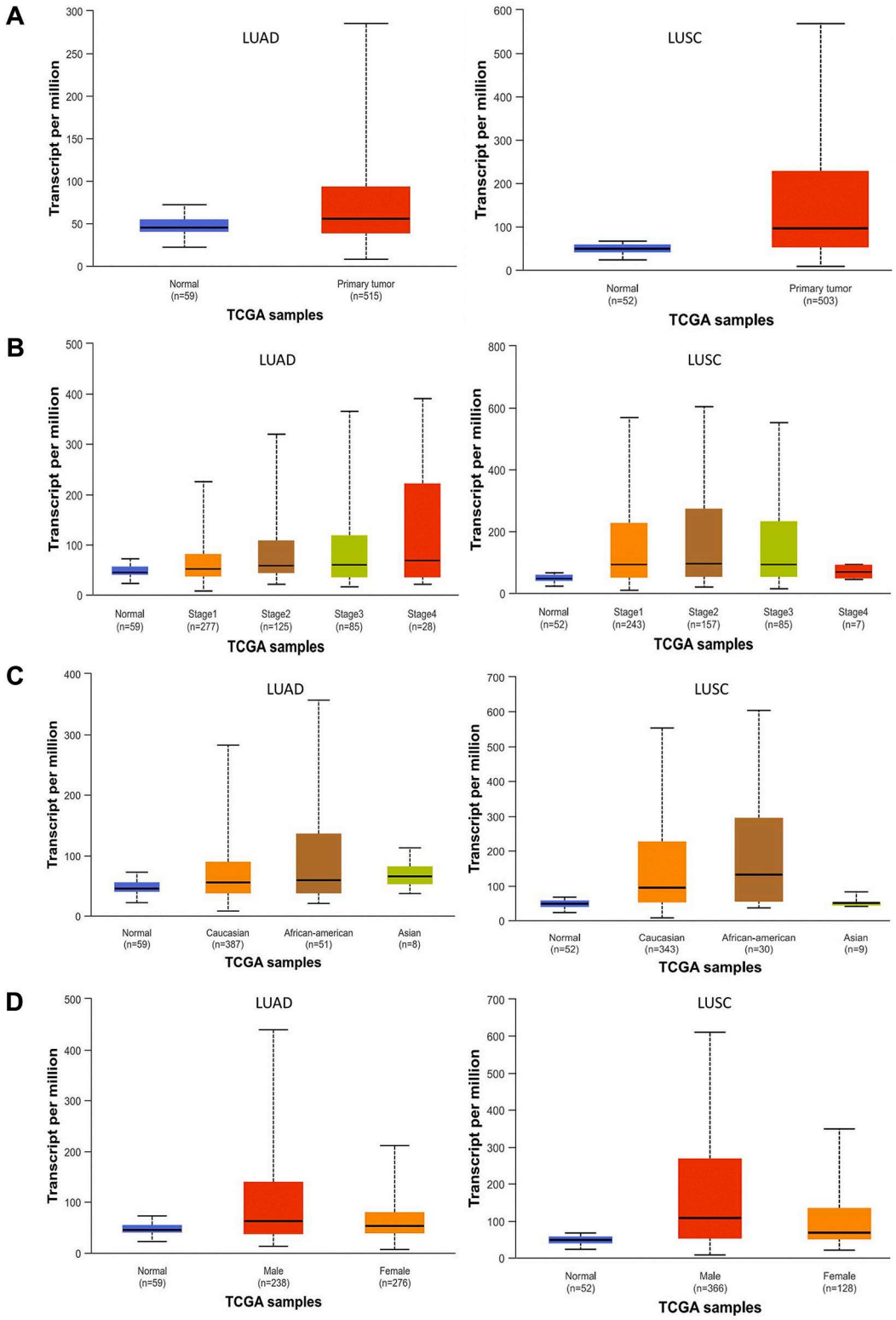


Fig. 3. A positive correlation between G6PD expression and the clinicopathological aspects of lung cancer (LUAD and LUSC) based on the (A) sample type (B) different stages (C) patient's race (D) gender (E) age (F) smoking habit (G) histological subtypes, and (H) nodal metastasis. Abbreviations used in the graph stated as following: Not Otherwise Specified (NOS), Mixed subtype (Mixed), Lung Clear Cell Adenocarcinoma (Clear cell), Lung Bronchioloalveolar Carcinoma Non-mucinous (LBC- Non-mucinous), Lung Solid Pattern Predominant Adenocarcinoma (Solid Pattern

Predominant), Lung Acinar Adenocarcinoma (Acinar), Lung Bronchioloalveolar Carcinoma Mucinous (LBC-Mucinous), Mucinous (Colloid) Carcinoma (Mucinous Carcinoma), Lung Papillary Adenocarcinoma (Papillary), Lung Mucinous Adenocarcinoma (Mucinous), Lung Micropapillary Adenocarcinoma (Micropapillary), Lung Signet Ring Adenocarcinoma (Signet Ring), Lung Basaloid Squamous Cell Carcinoma (Basaloid), Lung Papillary Squamous Cell Carcinoma (Papillary), Lung Small Cell Squamous Cell Carcinoma (Small Cell), No regional lymph node metastasis (N0), Metastases in 1–3 axillary lymph nodes (N1), Metastases in 4–9 axillary lymph nodes (N2), Metastases in 10 or more axillary lymph nodes (N3).

consume more glucose than normal cells. Employing this pathway is advantageous to cancer cells because of the quick synthesis of ATP via glycolysis for cell multiplication, as well as the build-up of glycolytic intermediates for growth. This gives cancer cells the ability to outcompete normal cells even in poor situations. This distinct trait is attributed to the upstream regulation of G6PD by oncogenic and/or metabolic regulators. The expression of G6PD increases dramatically as the cancer advances from stage 1 to stage 3 for LUAD and LUSC, but in stage 4 the expression pattern varies for LUSC (Fig. 3B). The data on individuals self-identifying as Caucasian, African American, and Asian were also analysed. Caucasian and African American populations expressed G6PD at greater levels than Asians. Lung cancer is prevalent in these demographics (Fig. 3C). The most complete report in the United States is based on data from the Surveillance, Epidemiology, and End Results Program (SEER), which revealed that the yearly incidence of lung cancer was higher among blacks (51.9 per 100,000), followed by whites (51.4 per 100,000) over the previous five years (2016–2020) (https://seer.cancer.gov/statistics-network/explorer/). Fig. 3D shows that both males and females are more likely to develop lung cancer due to increased G6PD levels. Males have a greater incidence rate (56.4 per 100,000) than females (45.3 per 100,000) (https://seer.cancer.gov/statistics-network/explorer/). Age is crucial in maintaining good health and immunity to illnesses. The age data were classified into five groups: normal and tumour (21–40 years, 41–60 years, 61–80 years, and 81–100 years). The chance of G6PD expression increases with age and based on G6PD expression, individuals over the age of 40 are more likely to develop LUAD and LUSC (Fig. 3E). SEER report stated that in the last 5 years, the incidence rate has increased with age as observed between 40 and 64 years (41.3 per 100,000), 65–74 years (242.8 per 100,000), and above 75 years (349.4 per 100,000) (https://seer.cancer.gov/statistics-network/explorer/) [55].

G6PD deficiency is a genetic disorder with a high prevalence in certain ethnic groups such as African, Mediterranean, and Southeast Asian which likely evolved as a protective form of malaria (https://www.ncbi.nlm.nih.gov/books/NBK470315/). The Mediterranean variant of G6PD (G6PD-Mediterranean) is one of the most common and severe forms of G6PD deficiency which is associated with various health outcomes, including a reduced probability of certain cancers [56]. And if these G6PD mutations are more prevalent in specific ethnic groups, they might exhibit different survival outcomes when affected by cancer. More likely, patients from ethnic groups with a high prevalence of protective G6PD variants might benefit from therapeutic strategies compared to those from groups with higher-risk variants.

Tobacco smoking is the most significant and frequent risk factor for lung cancer [57,58]. Cigarette smoke includes about 7000 compounds, including more than 60 known carcinogens and other toxicants linked to chronic illnesses. Although only around one in every nine smokers develops lung cancer, long-term smokers are predicted to have a 10- to 30-fold higher risk than lifetime non-smokers [59]. Surprisingly, in both forms of lung cancer, the patient's smoking propensity is not related to G6PD expression levels (Fig. 3F).

Fig. 3G illustrates that LUAD has more histological subtypes than LUSC, with a varied expression of G6PD in both LUAD and LUSC also between subtypes. In LUAD, the histological subtypes NOS, Mixed, Acinar, Papillary, Micropapillary, and Mucinous revealed significant alterations in G6PD expression, but in LUSC, the NOS and Basaloid subtypes showed larger changes. Metastasis is one of the key events associated with the cancer spread in the body and, interestingly the degree of G6PD expression in LUAD and LUSC varies with the metastatic stage. The expression level altered dramatically between the N3 and N2 stages (Fig. 3H). G6PD expression in LUAD is linked with the cancer's advanced stages, lymph node metastases, poor differentiation, pleural invasion, vascular invasion, and lymphatic invasion. Furthermore, G6PD-positive patients with overexpression at the invasive front had considerably worse survival rates than G6PD-negative patients [60].

3.3. *Alteration of DNA methylation and the expression of G6PD*

DNA methylation is an epigenetic change found in the genome that plays an important regulatory role in transcriptional processes and is critical for proper cellular development [61,62]. Methylation is an essential process related to chromatin structure and dynamics; extensive methylation in DNA results in the creation of heterochromatin [63]. Epigenetic alterations, such as DNA methylation, have been shown to play an important role in cancer cells' immunological tolerance and could lead to genetic diversity [64–66]. G6PD promoter methylation levels were much greater in LUSC than in LUAD (Fig. 4A).

Methylation of the promoter may vary in distinct stages and grades of cancer along with the race and age of patients. Moreover, it can change different histological subtypes of the tumour alongside the type of cancer. Less or high methylation sometimes changes the expression patterns of the gene in diverse types of lung cancer. Fig. 4B depicts the hypermethylated genes associated with LUAD and LUSC and the involvement of genes varies between them. In almost every kind of cancer, several genes are methylated inappropriately and inactivation of tumour suppressor genes by DNA hypermethylation is considered to be an early and frequent feature of lung cancer [67,68]. T-box transcription factor 15 (TBX15) expression was shown to be considerably upregulated in a range of malignancies, including lung cancer, and was associated with a poor prognosis [69,70]. In adddition, different transcriptional factors from the Homeobox family (HOXA, HOXD) and Zinc finger proteins (ZIC, ZNF) are mostly related to hypermethylation and associated with more aggressive form of lung cancer cells [71,72]. Somatostatin receptors (SSTR) are G protein-coupled receptors (GPCRs) that are found in tissues throughout the body, including many cancers [73]. SSTRs block adenylate cyclase, limit calcium influx, increase p53

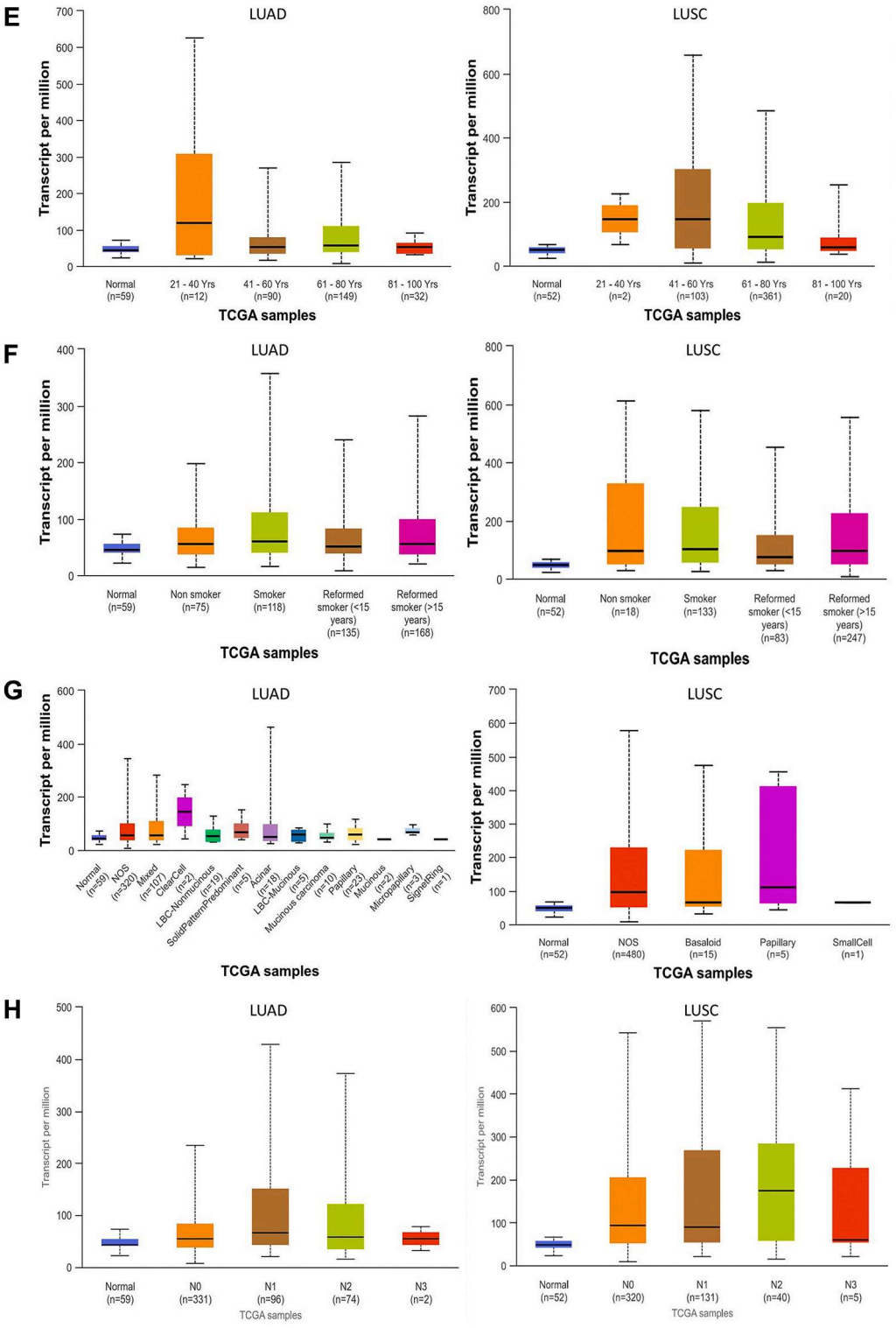


Fig. 3. (*continued*).

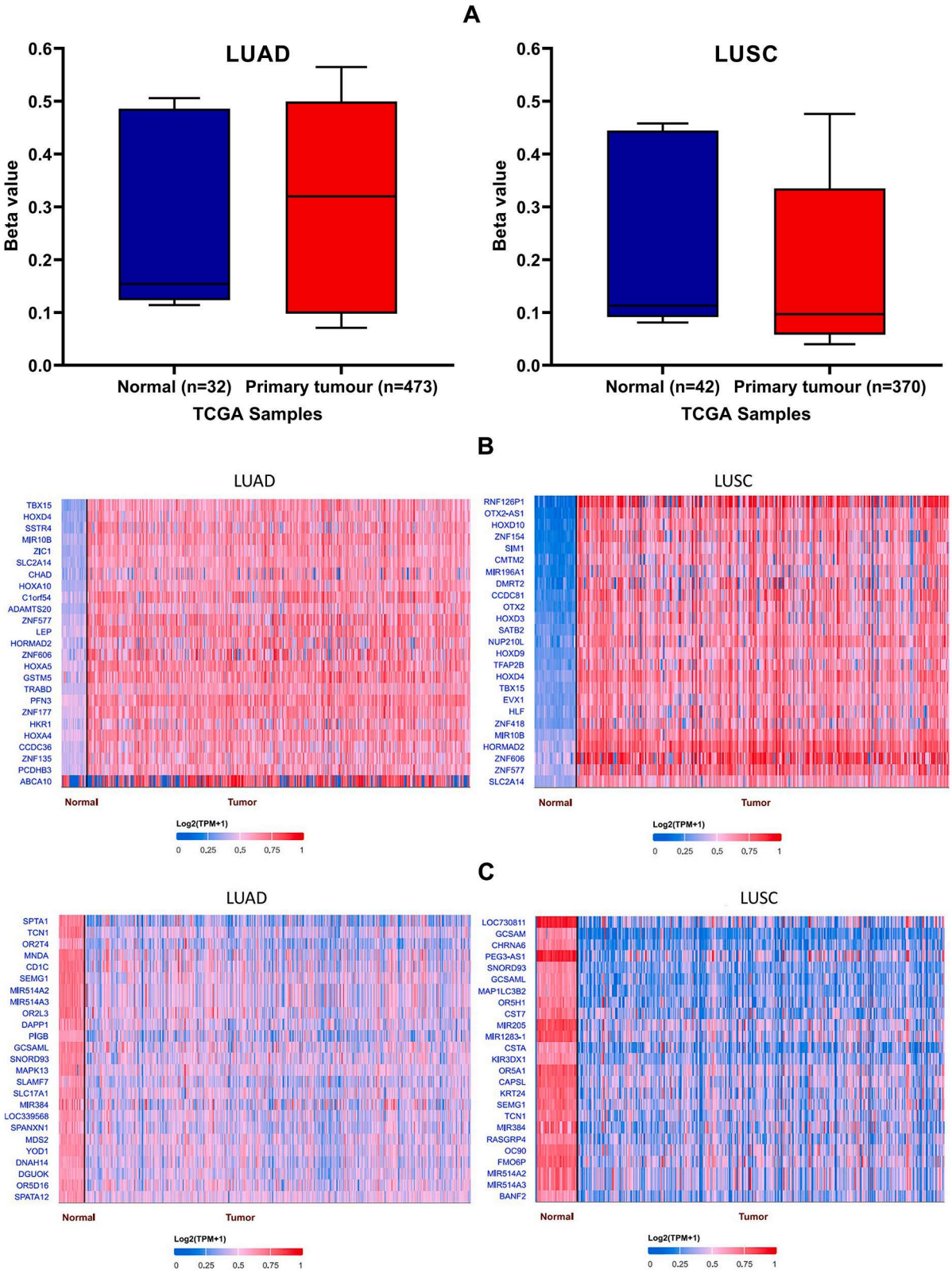


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Fig. 4. DNA promoter methylation alters the expression of G6PD (A) Deviant promoter methylation status of G6PD observed in LUAD and LUSC primary tumour samples compared to normal. The X-axis shows the type of cells, and the Y-axis depicts the beta value. Beta value is the indication mark based on the level of DNA methylation. It ranges from 0 to 1, where 0 represents (unmethylated), up to 0.1–0.3 (hypomethylated), 0.5–0.7 (hypermethylated), and 1 represents (fully methylated) (B) The heat map represents the expression profile of genes with hypermethylated promoters in LUAD and LUSC tumours compared to normal cells, (C) the heat map indicates the involvement of genes with the hypomethylated promoters in LUAD and LUSC.

expression and Bax to trigger apoptosis, and impact ERK1/2 and AKT to reduce cell proliferation [74,75]. Overall, DNA methylation influences the binding of transcription factors, resulting in altered gene expression. Transcobalamin 1 (TCN1) is a possible prognostic biomarker that corresponds with immunological infiltrates in lung adenocarcinoma, while Spectrin Alpha Erythrocytic 1 (SPTA1) is associated with poor overall survival rates [76,77].

3.4. Immune infiltration analysis and G6PD expression correlation with tumour mutational burden (TMB), microsatellite instability (MSI), and immune checkpoints (ICP) genes

The kind of immune cells entering tumours influences the likelihood of cancer recurrence [78]. The Tumour Microenvironment (TME) consists of important components including the extracellular matrix and a variety of invading immune cells, including regulatory T cells, B cells, neutrophils, macrophages, natural killer cells, and dendritic cells. Thus, we investigated the link between G6PD expression and immune infiltration in LUAD and LUSC malignancies. In most tumours, G6PD expression was linked to immune cell infiltration and the level of expression correlates favourably with the majority of tumours. G6PD was highly expressed in T cells (CD8, CD4 Th1, and Th2), B cells, NK cells, and macrophages in LUAD and LUSC (p-value <0.05, Spearman $r > 0$). In LUAD, G6PD is highly expressed in B-naïve cells, neutrophils, and dendritic cells. G6PD is expressed more strongly in LUSC by CD4 (non-regulatory, memory), memory B cells, and mast cells (Fig. 5A). Taken together, these findings suggest that G6PD may be implicated in tumour microenvironment and immune responses.

We examined G6PD mRNA expression in several immunological subtypes using molecular typing as described by Thorsson et al., 2019 [79]. In LUAD (p-value = 1.71e-04) and LUSC (p-value = 4.66e-06), there was a substantial difference in G6PD expression features across C1 (wound healing), C2 (IFN-γ dominant), C3 (inflammatory), C4 (lymphocyte depletion), C5 (immunologically quiet), and C6 (TGF-β dominant) subtypes (Fig. 5B).

Subsequently, the immune infiltration impacts the expression pattern of various ICP genes. And interestingly, ICP genes were found to positively correlate with the G6PD expression in numerous tumour types including OV, PAAD, LIHC, LGG, GBM, COAD, READ, PRAD, and KIRC, etc. In addition, G6PD expression was shown to be positively correlated with CD274, and CD40 in LUSC and CD276 in LUAD. A negative correlation was found between BTLA, CD27, CTLA4, and CD28 (p-value ≤ 0.05, Spearman $r < 0.2$) (Fig. 5C). These findings imply that G6PD may coordinate the activation of these ICP genes across many pathways, making it a potential target for immunotherapy in lung cancer.

The emergence of immune checkpoint inhibitors (ICIs) during the last decade has quickly revolutionized the landscape of lung cancer treatment and made it manageable for certain patients with advanced and metastatic stages [80]. TMB has a reputation for better-predicting treatment outcomes and helps direct ICI clinical applications. MSI is thought to be one of the most influential elements in the onset and progression of cancer as well as in the immunotherapy responses [81]. Microsatellite Instability-High (MSI-H) has been identified as a third potentially independent predictive biomarker for ICIs after TMB [82]. MSI-H is a hypermutator phenotype found in malignancies with a defective mismatch repair system (dMMR), primarily owing to mutations in the mismatch repair genes MLH1, MSH2, MSH6, or PMS2 [83]. MSI-H/dMMR accumulates somatic mutations and generates "non-self" neoantigens, which increases the host's anti-tumour immune response [84]. MSI-H was found in more than 30 cancer types, with an overall prevalence of 3.72 %; however, in lung cancer, the frequency was as low as 0.36–0.4 % [85,86]. As a result, the relationship between G6PD expression and MSI was studied in LUAD and LUSC. It has been observed that G6PD expression is linked favourably with the MSH2, MSH6, and PMS2 genes in both LUAD and LUSC (p-value ≤ 0.05, Spearman $r > 0$), but negatively with the MLH1 gene (Fig. 5D).

3.5. Correlation between G6PD, mutation and patients' survival

The G6PD gene is genetically diverse, located on chromosome X's high-density region XQ28, which encodes G6PD. The G6PD gene is 18 kb long, with 13 exons and 12 introns. G6PD is a major regulatory enzyme in the PPP pathway, which is active in both normal and malignant cells. The status of G6PD gene change in human lung tumours from the TCGA cohort was investigated further in the cBioPortal database. Mutation, copy number variation, and structural variation all contribute to G6PD alterations. So far, more than 400 biochemical and genetic variations of G6PD have been found [87]. In LUAD, 25 (4.42 %) of 566 patients showed a G6PD gene change (Fig. 6A). In LUSC, 3.9 % (19 instances) of the 487 cases modified their G6PD profile. The alterations are the result of mutation, profound deletion, and amplification. Deep deletion is about half (0.35 %) of the LUSC (0.75 %), while amplification contributes somewhat more (3 %) in the LUAD than in the LUSC (2.05 %). The mutation rate is lower in LUAD (1.06 %) than in LUSC (1.23 %). Fig. 6B depicts the mutation count on the y-axis against the type of lung cancer (LUAD and LUSC). The mutation handles the copy number and structural variations in the G6PD gene.

Somatic mutation frequency is 1.1 % in the G6PD (Fig. 6C). The increased rate of variants of unknown (or uncertain) significance (VUS) observed in lung cancer patients and considered for genomic profiling of LUAD [88]. Most somatic mutations are missense

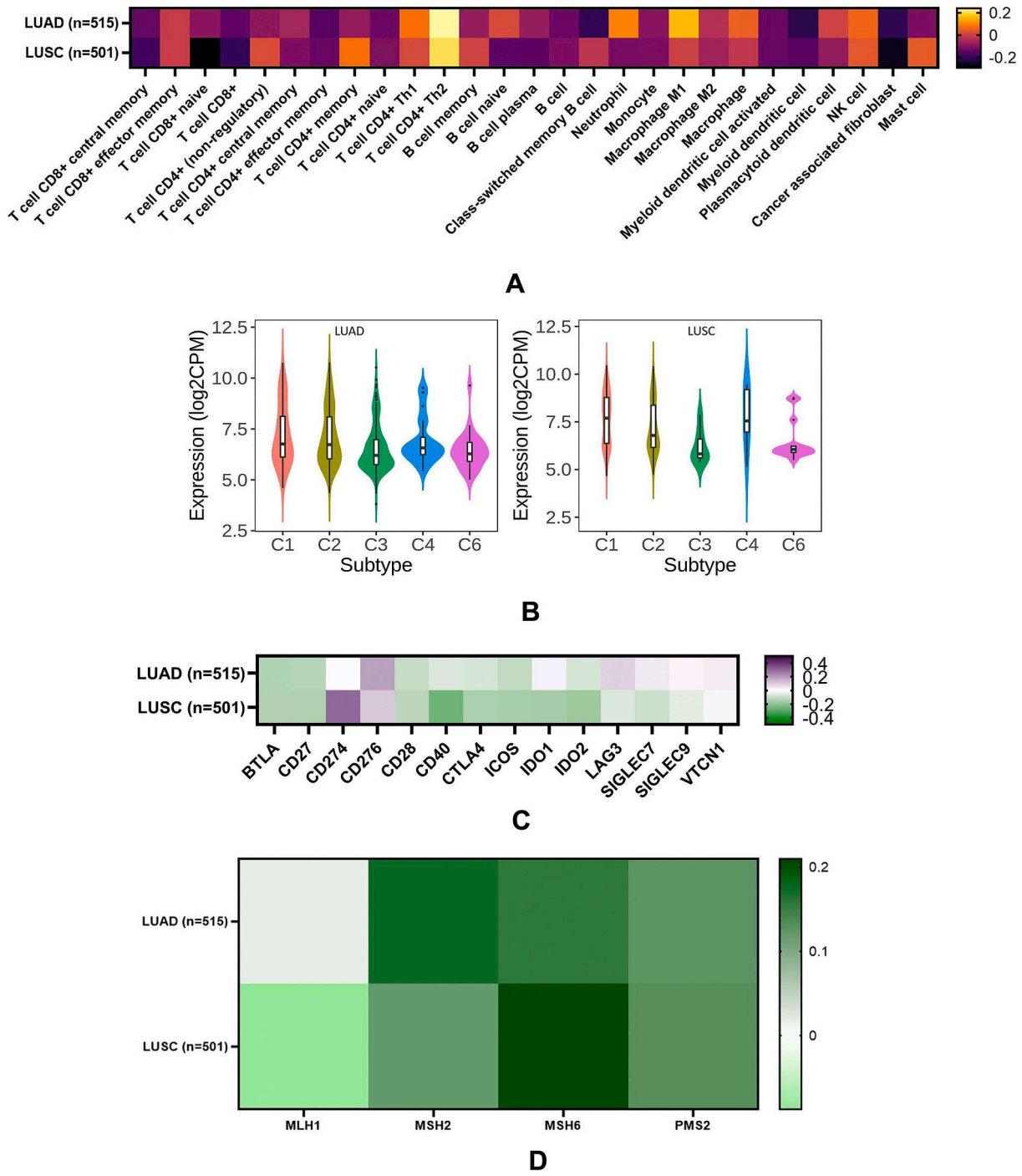

Fig. 5. Linking of G6PD expression with the immune system (A) The heat map depicts the positive and negative correlation of Immune infiltration and G6PD expression by XCELL algorithm (B) The violin plot shows the strong positive association between the G6PD mRNA expression in different immune subtypes of LUAD and LUSC (C) The heat map represents the relationship of Immune Check Point genes with the expression of G6PD in LUAD and LUSC (D) Association of G6PD expression with the MSI genes.

mutations with unknown significance and are found in both N and C regions of the G6PD. Truncating mutation with unknown significance is also found in the C terminus region of G6PD. This truncating mutation can be found in any type of Nonsense, Nonstop, Frameshift deletion, Frameshift insertion, or Splice site (Fig. 6C). Fig. 6D shows the 3D structure of the G6PD with the mutation types.

Genomic variations in G6PD affect the various metabolism-regulating signals. FAM3A, SLC10A3, L antigen family member 3 (LAGE3), Plexin A3 (PLXNA3), and Ubiquitin-like 4 A protein (UBL4A) are the most alerted genes in terms of frequency (70 %) with a

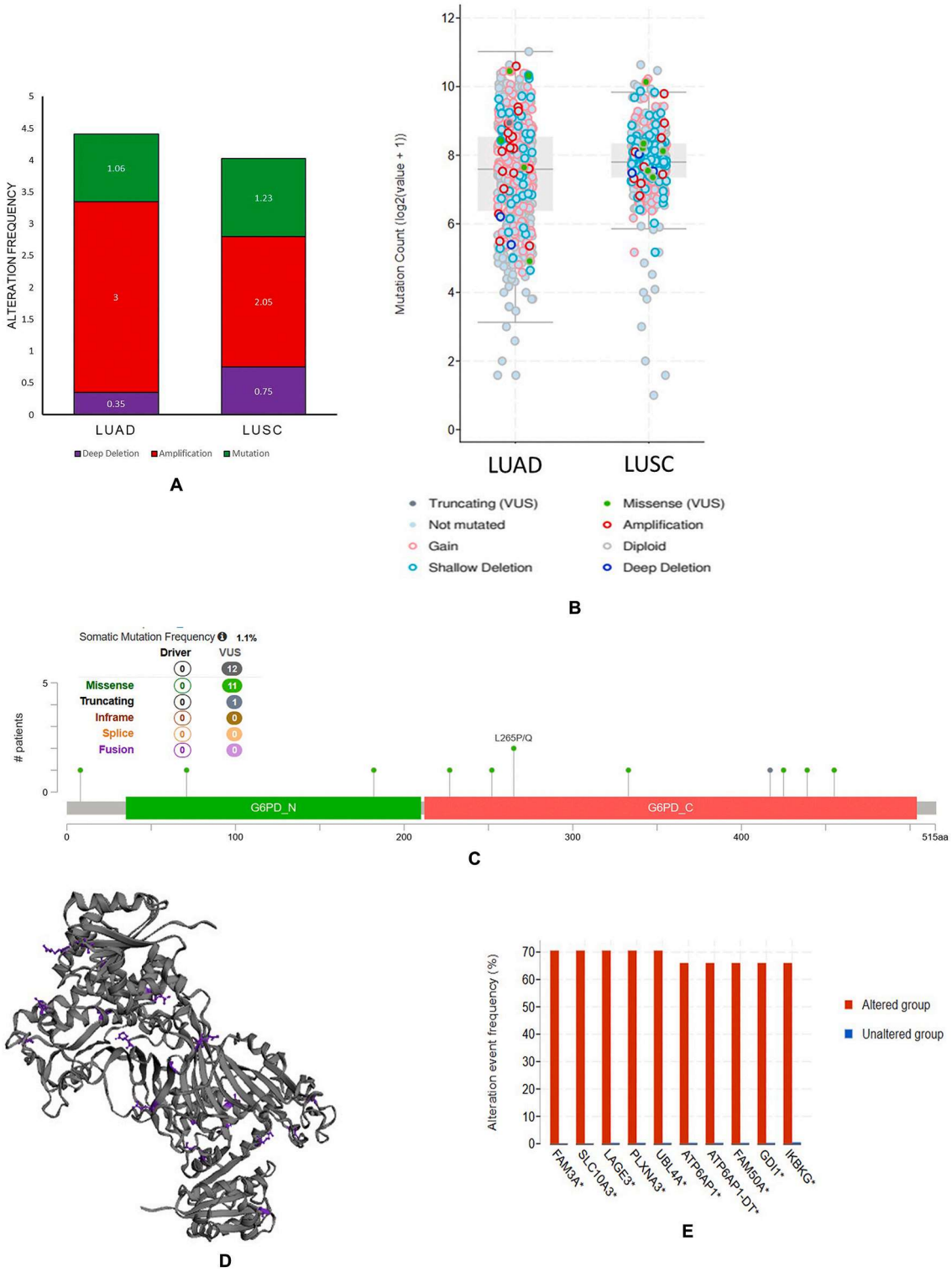

Fig. 6. Mutation leads the changes in the expression level of G6PD (A) The stacked bar graph represents the contribution of the type of mutation in the percentage alteration frequency of G6PD in LUAD and LUSC (B) The graph shows the mutation count along with the types of mutation in G6PD in lung cancer (LUAD and LUSC) (C) The genetic makeup of G6PD, percentage of somatic mutation frequency along with its types represented in the form of lollipop diagram (D) Three-dimensional structure of G6PD with the mutational sites (indicated in purple colour) (E) Copy number variations (including amplification and deletion) in G6PD are associated with altering gene expression (F) Gene expression alteration is due to structural variation or fusion in G6PD (G) Variety of mutations in G6PD affect the alteration of other closely associated genes in lung cancer. The red colour is

the G6PD altered group while blue depicts the unaltered G6PD group in lung cancer (H) Mutation status of TP53 has a close positive concomitant with the expression of G6PD in lung cancer (I) Kaplan-Meier plot represents the overall survival rate of the patients with G6PD alteration and unalteration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

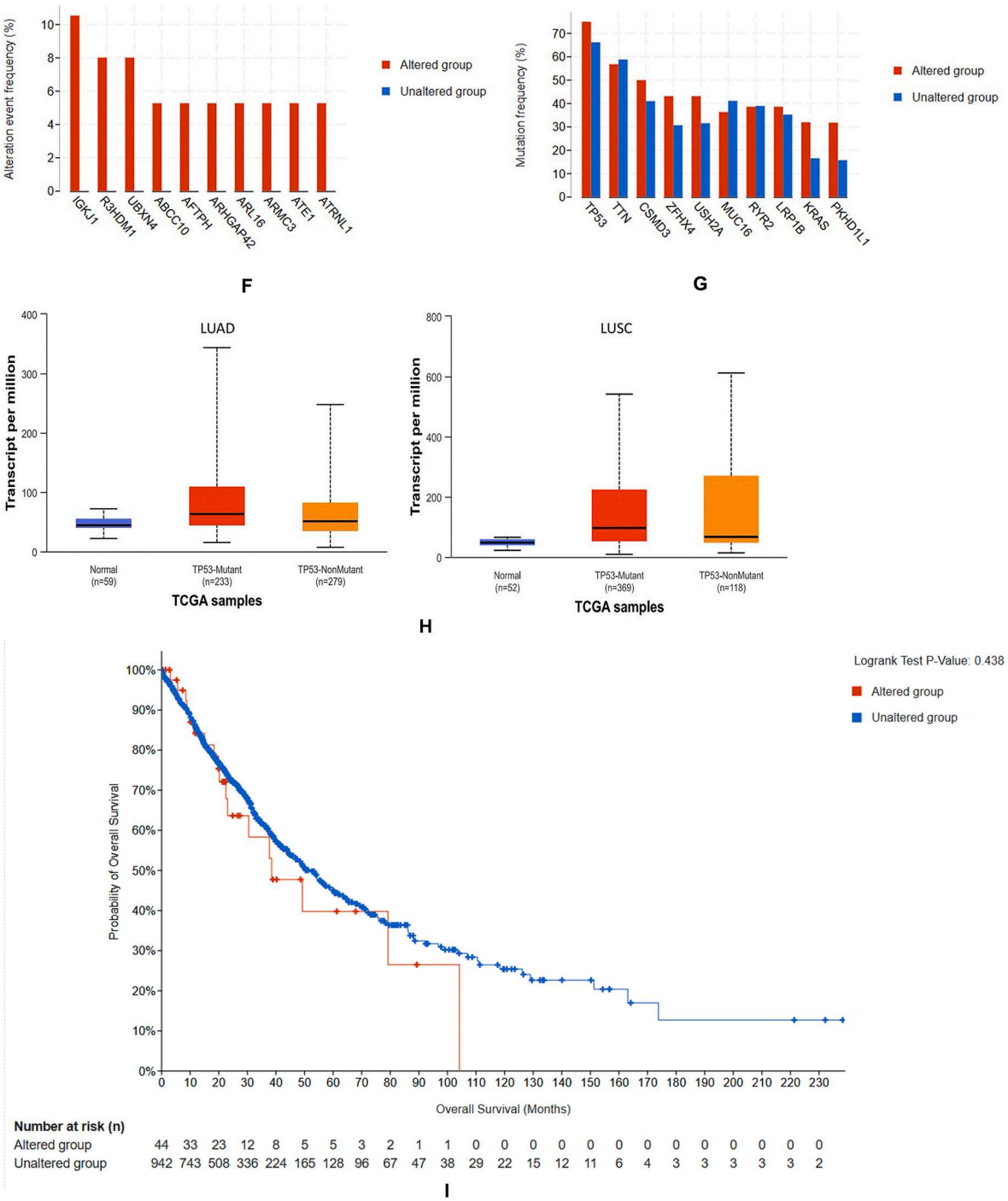

Fig. 6. (*continued*).

log 2 ratio greater than 8.4. The FAM3A gene encodes a cytokine-like protein. Peroxisome proliferator-activated receptor gamma may influence this gene's expression, and the encoded protein may have a role in glucose and lipid metabolism. SLC10A3 is a transporter protein. Compared to normal cells, LAGE3 is ubiquitously expressed in somatic tissues and is related to several forms of tumours, including LUAD and LUSC. PLXNA3 is a plasma protein that regulates cytoskeletal reorganization and apoptosis. In LUAD and LUSC

tumour cells, UBL4A expression was greater than in normal cells, similar to SLC10A3. ATPase H+ transporting accessory protein 1 (ATP6AP1), FAM50A, GDP dissociation inhibitor (GDI1), and IKBKG are the genes that changed more than 65 %. All of these genes are connected with the XQ28 cytoband (Fig. 6E). ATP6AP1 is a predictive marker for kidney cancer, breast cancer, pancreatic cancer, and

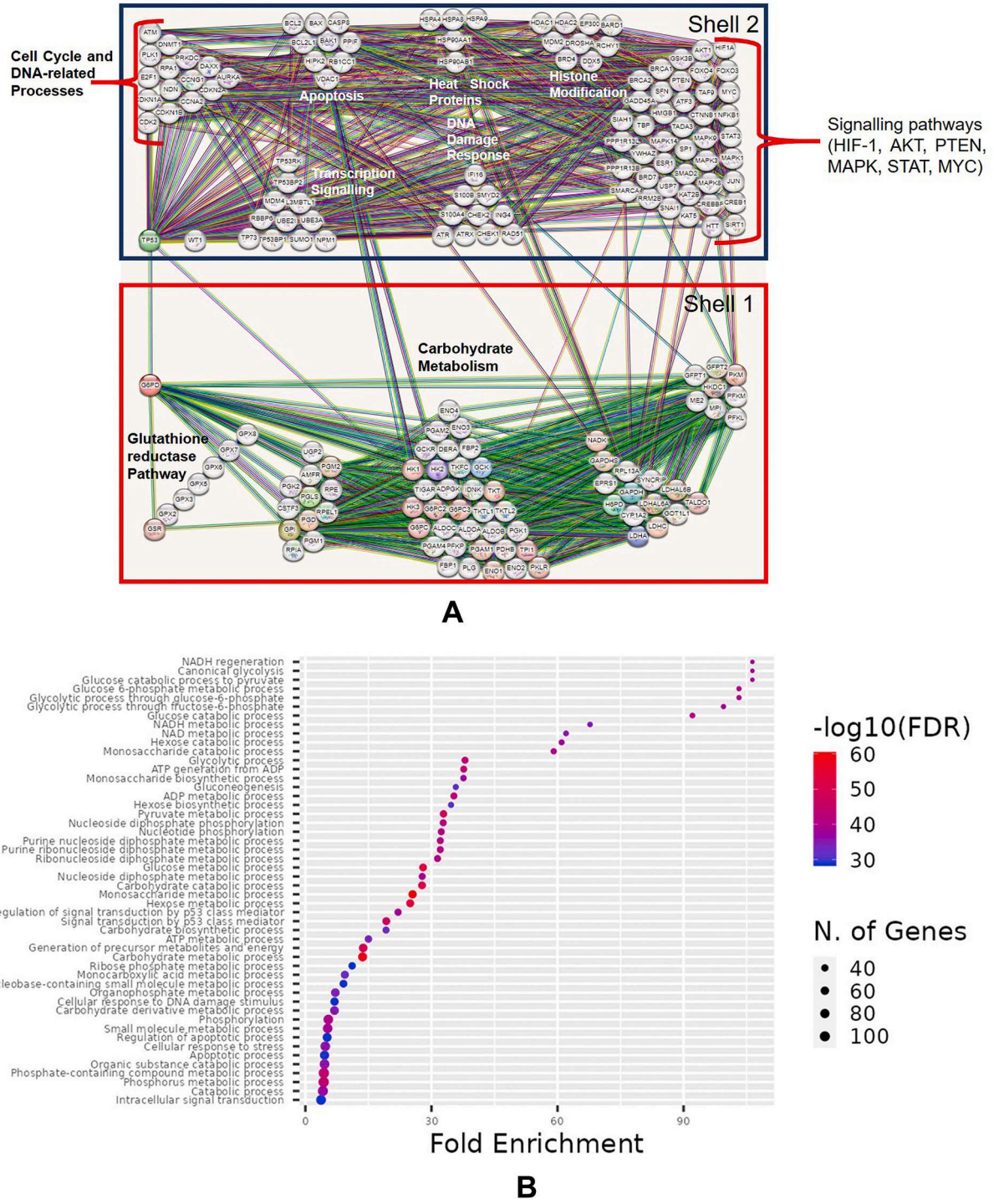

Fig. 7. (A) Protein-Protein Interaction Network of G6PD. Shell 1 represents the proteins involved in carbohydrate metabolism and the glutathione reductase pathway. Shell 2 depicts the proteins involved in various cellular signalling pathways, DNA damage response, cell cycle, and apoptosis-associated events, GO analysis for (B) biological processes (C) cellular compartments (D) molecular functions (E, F) KEGG analysis shows the involvement ofG6PD in the key hallmark events of the cell.

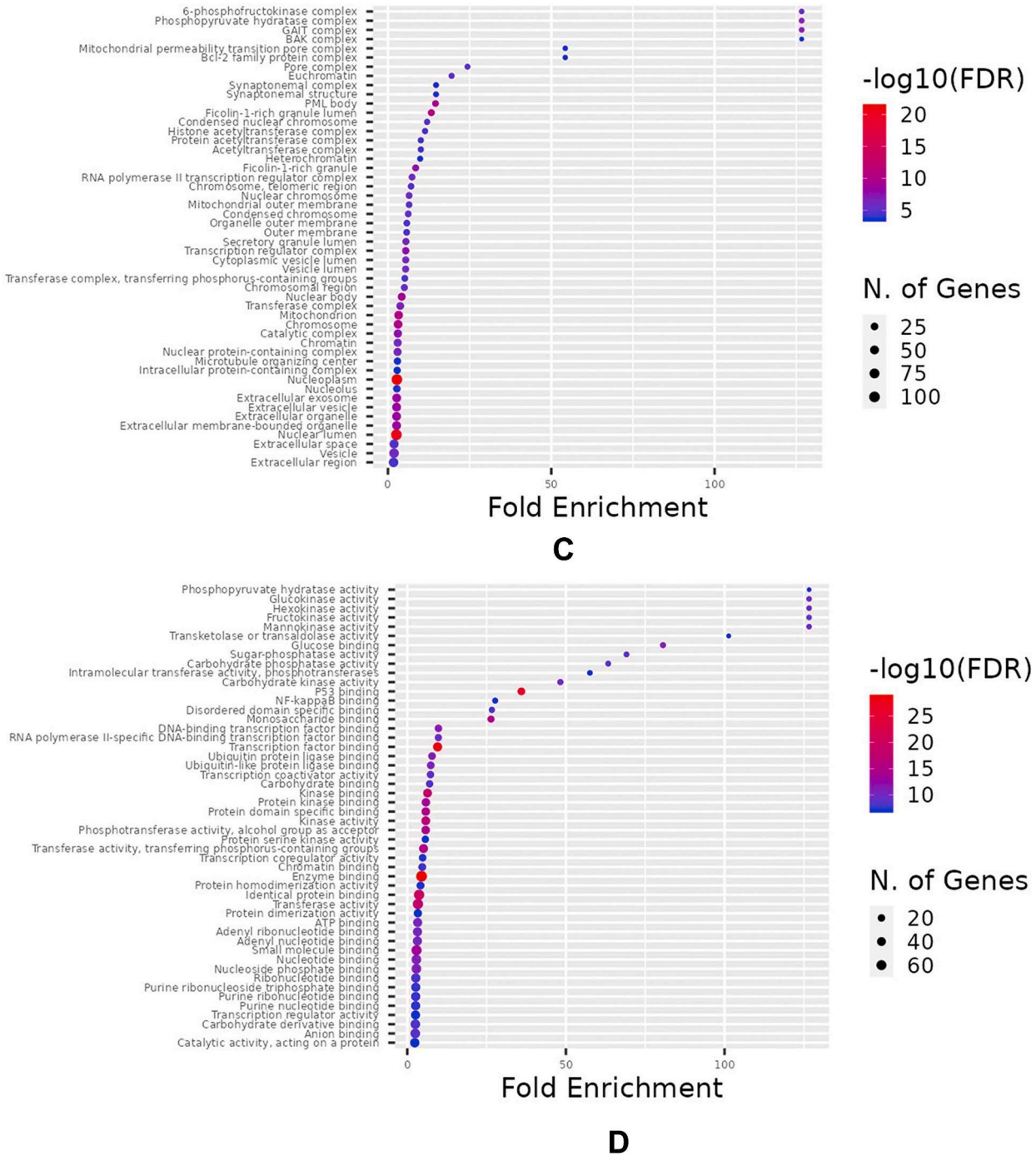

Fig. 7. (*continued*).

glioma. FAM50A, a proto-oncogene, can regulate the immunological microenvironment in several tumours. GDI proteins control the GDP-GTP exchange reaction of Rab family members involved in molecular transport across cellular organelles. IKBKG gene encodes the regulatory subunit of the inhibitor of kappaB kinase (IKK) complex, which activates NF-kappaB resulting in the activation of genes involved in inflammation, immunity, cell survival, and other pathways. Mutations in this gene result in incontinentia pigmenti, hypohidrotic ectodermal dysplasia, and other immunodeficiencies.

Immunoglobulin kappa joining 1 (IGKJ1), R3H Domain Containing 1 (R3HDM1), UBX domain protein 4 (UBXN4), ABCC10, Aftiphilin (AFTPH), ARHGAP42, ARL16, ARMC3, ATE1, and ATRNL1 are the genes that changed more than 5 % (Fig. 6F). IGKJ1 plays an important role in immunity. R3HDM1 enables RNA binding activity and is involved as a prognostic marker in renal and lung cancer.

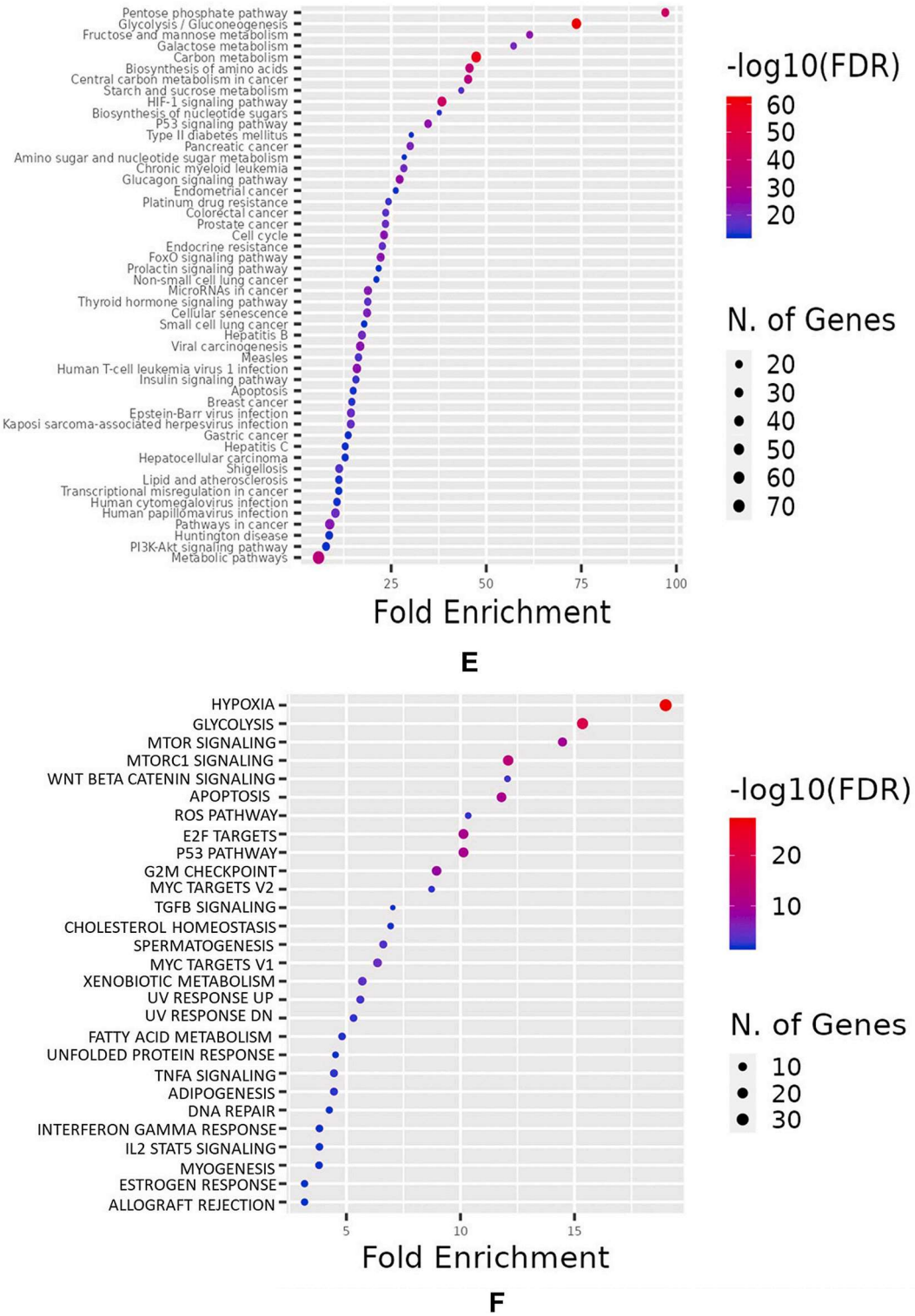

Fig. 7. (*continued*).

UBXN4 served as potential prognostic markers for lung cancer, and M2 predominance and juxtaposition of M2 TAM near tumour cells were associated with poor survival [89]. ABCC10 and AFTPH are the transporter proteins. AFTPH also plays a vital role in LUSC cell proliferation [90].

Fig. 6G depicts the percentage change of the frequency in the G6PD altered and unaltered groups. TP53 alteration frequency is the highest in the altered group (75 %) and unaltered groups (66 %). TTN gene has more than 55 % change frequency in both the groups. With more than 40 % change, CSMD3 also contributed to the mutation. ZFHX4 and USH2A varied by more than 30 % while the RYR2 gene does not show much change in altered and unaltered groups. From Fig. 6G, it is clear that TP53 is the prime candidate gene that mutated with the highest frequency in both groups (altered and unaltered G6PD expression). The correlation between the results indicates the involvement of G6PD and TP53 (p53) in lung cancer. TP53 is a tumour suppressor gene and transcription factor that controls cell cycle arrest, cell survival, and apoptosis via post-translational modification [91]. It also helps to maintain intracellular redox balance and genetic stability [92]. p53 is also a frequently altered gene in various malignancies and controls the PPP by binding directly to G6PD and inhibiting dimerization [93].

Kirsten RAS (KRAS) is considered a proto-oncogene and the most common RAS modifications are identified in lung, pancreatic, and colorectal adenocarcinomas [94]. Up to 30 % of NSCLC patients have KRAS mutations which have been linked with diverse clinicopathological features including ethnicity, sex, histological subtypes, and smoking history [95–97]. In the analysis of the G6PD altered vs unaltered group, it was observed that KRAS mutation has nearly doubled the alteration rate in the altered group (32 % vs. 16.6 % in the unaltered group).

The TTN gene is found on chromosome 2Q31 and has 364 exons [98] which makes it the longest in the entire genome and has many mutation sites [99]. The percentage mutation frequency is around 60, next after TP53 in the G6PD altered and unaltered group. Furthermore, several investigations have found that patients with TTN mutations have an inflammatory tumour immune microenvironment (TIME), which is distinguished by an abundance of activated immune cells with high immunological scores. LUAD patients with TTN mutations include significant levels of immunogenicity, TMB, neoantigen load (NAL), as well as nonsynonymous mutations linked with the DNA damage repair (DDR) pathway [100]. Thus, mutations in the TTN gene are a prognostic factor for patients with lung squamous cell carcinoma [101].

UALCAN results confirmed the TP53 alteration in both LUAD and LUSC. In both forms of lung cancer, there is a significant difference in G6PD expression between the TP53 mutated and non-mutant groups (Fig. 6H).

Mutational variations have an impact on the patient's survival rate and the overall survival rate for lung cancer significantly varied between the G6PD-altered and unaltered groups with a log-rank test p-value of 0.438. The altered group had a survival rate of around 105 months compared to the unaltered group (238 months), indicating that G6PD influences the probability of overall survival (Fig. 6I).

3.6. Enrichment and pathway analysis of G6PD

To investigate the probable mechanisms of G6PD's functions in LUAD and LUSC carcinogenesis and progression, we built a PPI network of 1146 edges and 180 nodes with an average node degree of 12.7 and an average local clustering coefficient of 0.665. Shell 1 represents the proteins involved in carbohydrate metabolism and glutathione reductase pathway, while Shell 2 represents the proteins involved in various signalling pathways, histone modification, cell cycle, apoptosis, and DNA damage response processes (Fig. 7A). Further investigation of the possible biological functions of G6PD, GO and KEGG pathway analysis in ShinyGO v 0.80 with a p-value 0.05 cutoff for False Discovery Rate (FDR) has been performed. GO analysis of G6PD involvement in biological processes suggests that G6PD is primarily involved in NADH regeneration and carbohydrate metabolic processes with high fold enrichment. p53 signal transduction, phosphate-containing compound metabolic processes, ATP generation, nitrogenous base metabolism, and apoptosis, are the other major events associated with more numbers of genes –log10 (FDR) values (Fig. 7B). Fig. 7C depicts the G6PD's involvement in many cellular compartments. Versatile distribution of G6PD observed from nucleoplasm to the mitochondrion. More numbers of genes with high –log10(FDR) were observed in the nucleolus and nuclear lumen. Furthermore, G6PD also engaged in diverse transfer complexes and integrally in the different regions of the chromosome. G6PD is integrally involved in a variety of molecular activities including enzyme binding, transcription factor regulation, p53 signaling, and carbohydrate metabolism (Fig. 7D).

The KEGG pathway analysis revealed that G6PD PPI was predominantly abundant in the cell's core processes, such as glycolysis, carbon metabolism, the pentose phosphate pathway, amino acid biosynthesis, cancer, the cell cycle, HIF-1, p53, and FoxO signalling, and other various metabolic pathways (Fig. 7E). HIF-1, p53, FoxO, and PI3-AKT siganlling play a pivotal role in the metabolic rewiring and shifting metabolic pathways in cancer. G6PD PPI network is also associated with the various hallmark events of the cell including hypoxia, glycolysis, PI3K-AKT, apoptosis, Reactive oxygen species (ROS), p53, Cell cycle checkpoints, MYC, and TGF signalling (Fig. 7F). These characteristic events are interlinked and associated with cancer. The findings suggest that G6PD PPI contributes to cancer development by modulating signalling pathways involved in cell proliferation. Almost all cancers are linked to one of these signature events, either directly or indirectly. It begins with the entry of glucose and its subsequent metabolism in a regulated oxygen environment. Derailing the process alters cell proliferation rates by interfering with HIF-1, p53, PI3K-AKT, ROS, and TGF signalling.

4. Conclusion

In a nutshell, the present study exposed that G6PD expression is higher in all different types of cancer compared to the normal counterparts especially in lung cancer (LUAD and LUSC). Furthermore, there is a substantial positive correlation between the expression of G6PD and different clinical features of lung cancer (disease stage, sex, age, race, and lymph node metastasis), DNA promoter methylation, immune cell infiltration, TMB, MSI, and ICP. Additionally, there is a positive correlation between G6PD expression and mutation, clinical prognosis, and a person's survival. In the meantime, PPI network results and functional analysis revealed the involvement of G6PD in metabolic-related activities, immune responses, cell proliferation, apoptosis, p53, HIF-1, FOXO,

and PI3K-AKT signalling. Interestingly, pathway analysis of G6PD showed the link of hypoxia-induced gene expression via the HIF-1α signaling pathway. These findings help us comprehend G6PD's role in the development of lung cancer. The current study is solely focused on bioinformatics work, which is a constraint, but it does open opportunities for other researchers to do cell and molecular biology research.

Data availability

All datasets are publicly available. TCGA dataset is available via the following link: https://portal.gdc.cancer.gov, http://xena.ucsc.edu. Tumor IMmune Estimation Resource (TIMER) is an online tool available in the public domain via https://cistrome.shinyapps.io/timer/. cBioPortal is available via the link http://cbioportal.org/. All the necessary information is mentioned in the materials and methods section.

CRediT authorship contribution statement

Parth Thakor: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M. Quadir Siddiqui:** Writing – review & editing, Formal analysis. **Trushar R. Patel:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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