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# TTYH3 is a Prognostic Biomarker and Associates with Immune Cell Infiltrations of Lung Adenocarcinoma

## TTYH3 es un Biomarcador Pronóstico y se Asocia con Infiltraciones de Células Inmunitarias del Adenocarcinoma de Pulmón

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**SUMMARY:** We investigated Tweety Family Member 3 (TTYH3) level in lung adenocarcinoma (LUAD) and its relationship with immune infiltration in tumors by bioinformatics. Differential expressions of TTYH3 in lung cancer were analyzed with Oncomine, TIMER, GEO, UALCAN and HPA. Relationship of TTYH3 mRNA/protein levels with clinical parameters was analyzed by UALCAN. Co-expressed genes of TTYH3 in LUAD were analyzed using Cbioportal. Its relationship with LUAD prognosis was analyzed by Kaplan–Meier plotter. GO and KEGG analysis were performed. Correlation between TTYH3 and tumor immune infiltration were tested by TIMER, TISIDB and GEPIA. We found that TTYH3 was significantly increased in LUAD tissues. TTYH3 high expression was closely related to poor overall survival, post progression survival and first progression in LUAD patients. TTYH3 mRNA/protein levels were significantly associated with multiple pathways. Specifically, TTYH3 up-regulation was mostly related to biological regulation, metabolic process, protein blinding, extracellular matrix organization and pathways in cancer. Moreover, TTYH3 was positively associated with immune cell infiltration in LUAD. Finally, TTYH3 was highly expressed in LUAD as revealed by meta-analysis. TTYH3 is closely related to the prognosis of LUAD and immune cell infiltration, and it can be used as a prognostic biomarker for LUAD and immune infiltration.

**KEY WORDS: TTYH3; Immune infiltration; Lymphocytes; Prognosis; Lung adenocarcinoma (LUAD).**

## INTRODUCTION

Lung cancer is one of the most common tumors and the main cause of cancer related death in the world (Sung *et al*., 2021; Chen *et al*., 2021a). It has two subtypes of small cell lung cancer and non-small cell lung cancer (NSCLC). The incidence of NSCLC accounts for about 85 % of all lung cancers (Zhang *et al*., 2021). Lung adenocarcinoma (LUAD) is the most common type of NSCLC, and its proportion in NSCLC has been rising in recent years (Kan *et al*., 2021). Generally, LUAD is malignant with short survival time and high mortality, accounting for about 30 % of cancer-related deaths worldwide (Chen *et al*., 2021b). However, the early symptoms of LUAD are difficult to be identified, and most LUAD patients are already at an advanced stage when they are diagnosed (Wagland *et al*., 2016). Therefore, it is necessary to explore the genes or mechanisms related to LUAD (Wagland *et al*., 2016).

Lung cancer is mainly composed of cancer cells and surrounding matrix, which is a series of mixes of immune cells, endothelial cells, microvascular cells and fibroblasts, as well as soluble signal factors and extracellular matrix. Among them, blood vessels, cancer-associated fibroblasts, extracellular matrix and immune cells constitute the tumor immune microenvironment (Klein-Goldberg *et al*., 2014), which is closely correlated with cancer occurrence and development (Klein-Goldberg *et al*., 2014). Studies have shown that the tumor microenvironment plays an important role in the occurrence and development of NSCLC (Kadota *et al*., 2013; Tang *et al*., 2018; Zeltz *et al*., 2019). Metastasis and invasion are a multi-factor, multi-stage process (Dai *et al*., 2016).

The interaction between tumor cells and the microenvironment causes immune cell infiltration, which

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plays an important role in the evolution of tumors. It has been reported that the tumor microenvironment is related to the prognosis of LUAD patients (Mao *et al*., 2020).

The Tweety gene family (TTYHs) includes three members, namely TTYH1, TTYH2 and TTYH3. They play an important role in cell differentiation, cell division, tumorigenesis and calcium activity regulation (Halleran *et al*., 2015). The main function of TTYH3 is to encode a large conductance transmembrane chloride channel. In addition, TTYH3 is also known as a large-conductivity Ca2+ activated chloride channel. Previous studies have reported that the expression of TTYH2 in colon cancer and renal cell carcinoma was significantly increased (Toiyama *et al*., 2007; Moon *et al*., 2019), which might be involved in the tumor occurrence. Study has also showed that TTYH3 might be a potential prognostic marker and therapeutic target for gastric cancer (Saha *et al*., 2019). However, whether TTYH3 is related to LUAD prognosis needs to be further studied.

In this study, we comprehensively explored the potential role of TTYH3 in LUAD. Using Oncomine, GEO, UALCAN, HPA and other databases, we first analyzed the differential expressions of TTYH3 in LUAD patients. In addition, we further analyzed the relationship between TTYH3 and LUAD survival through the Kaplan–Meier plotter. Meanwhile, co-expressed genes and functions of TTYH3 were analyzed by Cbioportal and Webgestalt, respectively. We also used TIMER and TISIDB databases to evaluate the correlation between the immune status of LUAD and the expression of TTYH3, and used TIMER and GEPIA databases to analyze the correlation between TTYH3 expression and LUAD immune markers, thus further clarifying the immune mechanism of TTYH3 in LUAD.

## MATERIAL AND METHOD

**Oncomine Database Analysis.** Oncomine database (http://www.oncomine.org), a web-based microarray cancer database, was used to analyze the expression difference of TTYH3 in LUAD and normal tissues (Rhodes *et al*., 2007). The fold-change ≥ 1.5, P value = 1e-4, and gene rank ≥ top 10 % were defined as the thresholds.

**GEO Database Analysis.** TTYH3 expression datasets were screened from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). Two human LUAD mRNA expression datasets (GSE31210 and GSE31908) were downloaded from GEO to analyze expression difference of TTYH3 in LUAD (Okayama *et al*., 2012; Yamauchi *et al*., 2012). The GSE31210 dataset, which contains patients with stage I and II only, contains 226 LUAD tissue samples and 20 normal lung samples. The GSE31908 contains 30 LUAD tissue samples and 20 normal lung samples.

**UALCAN Database Analysis.** UALCAN (http://ualcan.path.uab.edu), an open-access interactive web resource for analyzing cancer transcriptome data from The Cancer Genome Atlas (TCGA) and Clinical Proteomic Tumor Analysis Consortium (CPTAC) database (Chandrashekar *et al*., 2017), was used to analyze the relationship between the expression of the TTYH3 (at mRNA and protein levels) and clinical parameters.

**The Human Protein Atlas Database Analysis.** The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/), which include immunohistochemistry staining data of cancer tissues and normal tissues (Pontén *et al*., 2008), was used to compare the expression difference of TTYH3 protein in LUAD tissue and normal tissue.

**Kaplan-Meier Plotter Analysis.** Kaplan-meier Plotter analysis (https://kmplot.com/analysis/) is an open Internet tool to analyze the correlation between the expression of the 540,000 genes and 21 cancer types, including 6234 breast, 2190 ovarian, 3452 lung and 1440 gastric cancer samples (Gyo˝rffy *et al*., 2013). We used Kaplan-Meier plotter to analyze the effects of TTYH3 expression on overall survival (OS), post progression survival (PPS) and first progression (FP) of LUAD patients. The survival curves were plotted. Additionally, we further explored the effect of the relationship between the expression of TTYH3 and clinical parameters on OS of LUAD patients.

**Cbioportal Database Analysis.** Then, the mutated genes (TP53, EGFR, ALK, KRAS, MET, BRAF, ERBB, RET and ROS) and the expression correlated genes of TTYH3 in LUAD were analyzed using Cbioportal database (http://www.cbioportal.org), an open-source containing data retrieved from the TCGA database and a multidimensional cancer genomics data set, which contains many kinds of data, including DNA copy numbers, DNA methylation, gene mutations, gene co-expression, survival analysis and pathways (Cerami *et al*., 2012; Gao *et al*., 2013).

**GO and KEGG analysis.** We conducted GO analysis and KEGG analysis of TTYH3 co-expressed genes through WebGestalt (www.webgestalt.org/option.php), which is a tool for the interpretation of gene lists derived from large scale -omics studies and supports three well-established and complementary methods for enrichment analysis, including Over-Representation Analysis (ORA), Gene Set Enrichment Analysis (GSEA), and Network Topology-based Analysis (NTA) (Liao *et al*., 2019).

**TIMER Database Analysis.** We analyzed the association between the level of TTYH3 gene expression and the abundance of tumor-infiltrating immune cells, including B cells, CD4+ T cells, CD8+ T cells, Neutrophils, Macrophages, and Dendritic cells in LUAD, as well as the tumor purity using TIMER database (https://cistrome.shinyapps.io/timer/) (Li *et al*., 2016, 2017). We also analyze correlation between TTYH3 expression and immune marker in different tumor-infiltrating immune cells.

**TISIDB Database Analysis.** The TISIDB database (http://cis.hku.hk/TISIDB/), an online tool for evaluating tumor and immune System interaction (Ru *et al*., 2019), was used to assess the role of TTYH3 in tumor-immune interplay.

**GEPIA Database Analysis.** We analyzed the correlation between TTYH3 and immune markers in LUAD and normal tissues with GEPIA database (http://gepia.cancer-pku.cn/), which includes 8,587 normal and 9,736 tumor tissue samples from the TCGA and normal projects (Tang *et al*., 2017).

**Meta-Analysis.** We analyzed the expression of TTYH3 in LUAD by Meta-analysis on Lung Cancer Explorer (LCE) database (https://lce.biohpc.swmed.edu/lungcancer/), which is an open web portal to explore gene expression and clinical associations in lung cancer. The standardized mean difference between tumor and normal was summarized by forest plots.

**Statistical analysis.** TTYH3 levels by Oncomine were compared by t-tests and displayed as P-values, fold changes, and ranks. The differential expression of TTYH3 by TIMER was evaluated using the Wilcoxon test. The log rank test was used to calculate the HR and log rank P-value in Kaplan-Meier Plotter. Spearman's correlation and Pearson's correlation were used for correlation analysis. A p value $<0.05$ was considered statistically significant.

## RESULTS

**Expression of TTYH3 mRNA and protein in LUAD.** We first analyzed the difference in TTYH3 mRNA levels between LUAD and normal tissues through Oncomine, GEO and UALCAN (Fig. 1). First, in the Oncomine, we found that studies by Selamat *et al*. (2012) and Hou *et al*. (2010) showed that the expression of TTYH3 was significantly up-regulated in LUAD (Fig. 1A). Second, we analyzed GSE31210 and GSE31908 from the GEO database and found that the expression of TTYH3 mRNA in LUAD was significantly higher than that of the normal control group (Fig. 1B). Third, through the study of the TCGA database in UALCAN, we found that the expression of TTYH3 mRNA in LUAD also increased significantly (Fig. 1C). Finally, we further evaluated the TTYH3 protein levels through the HPA database and CPTAC database in UALCAN, and found that the TTYH3 protein in LUAD patients was significantly higher than that in normal tissues (Fig. 2). In summary, the expression of TTYH3 in LUAD increases significantly at both the mRNA and protein levels.

**Relationship between TTYH3 and clinical characteristics of LUAD patients.** We used UALCAN to analyze the relationship of the TTYH3 mRNA in the TCGA database and the TTYH3 in the CPTAC database with the clinical characteristics of LUAD patients (Fig. 3 and 4). First, in the TCGA database, compared with normal tissues, TTYH3 mRNA was significantly increased with cancer stages (S1, S2, S3, and S4), nodal metastasis status (N0, N1, N2, N3), patient smoking habit (non-smoker, smoker, reformed smoker<15 years, and reformed >15 years), sex (male and female), race (Caucasian, African-American, and Asian), and tumor histological subtypes (Fig. 3A-3G) as well as TP53, EGFR, ALK, KRAS, MET, BRAF, ERBB, RET and ROS mutation status (Mutant, and NonMutant) (Fig. 4A-4I) in LUAD. Interestingly, compared with any other lymph node metastasis status group, the TTYH3 mRNA was the highest in the advanced lymph node metastasis status group (N3) of LUAD patients. In addition, the TTYH3 mRNA only increased in age groups of 41-60, 61-80, and 81-100 years, but not in age group of 21-40 years. In tumor histological subtype, compared with normal groups, there were no differences in Solid Pattern Predominant and Mucinous. The expression of TTYH3 in patients with TP53, EGFR and MET mutation were higher than those with non-mutation.

In the expression level of TTYH3 protein (Fig. 5A-5G), compared with normal tissues, we found that the expression of TTYH3 protein was enhanced in cancer stages (S1, S2, S3), race (Caucasian), sex (male and female), age (21- 40, 41-60, 61-80 years), weight (Normal Weight, Extreme Weight, Obese, and Extreme Obese), tumor grade (Grade1, Grade2, Grade3), and tumor histology (Papillary adenocarcinoma, Adeno- carcinoma, Acinar adenocarcinoma, Other) in LAUD. The above results indicate that the mRNA and protein levels of TTYH3 are related to the clinical parameters of LUAD.

**Relationship between TTHY3 and survival in patients with LUAD.** We used the Kaplan-Meier plotter to analyze the relationship between TTYH3 and LUAD survival and found that compared with LUAD patients with low TTYH3 expression, the OS, FP and PPS in LUAD patients with high TTYH3 expression were significantly shortened (Fig. 6A-6G). We further studied the effects of smoking and sex on OS in LUAD patients expressing TTYH3. The results

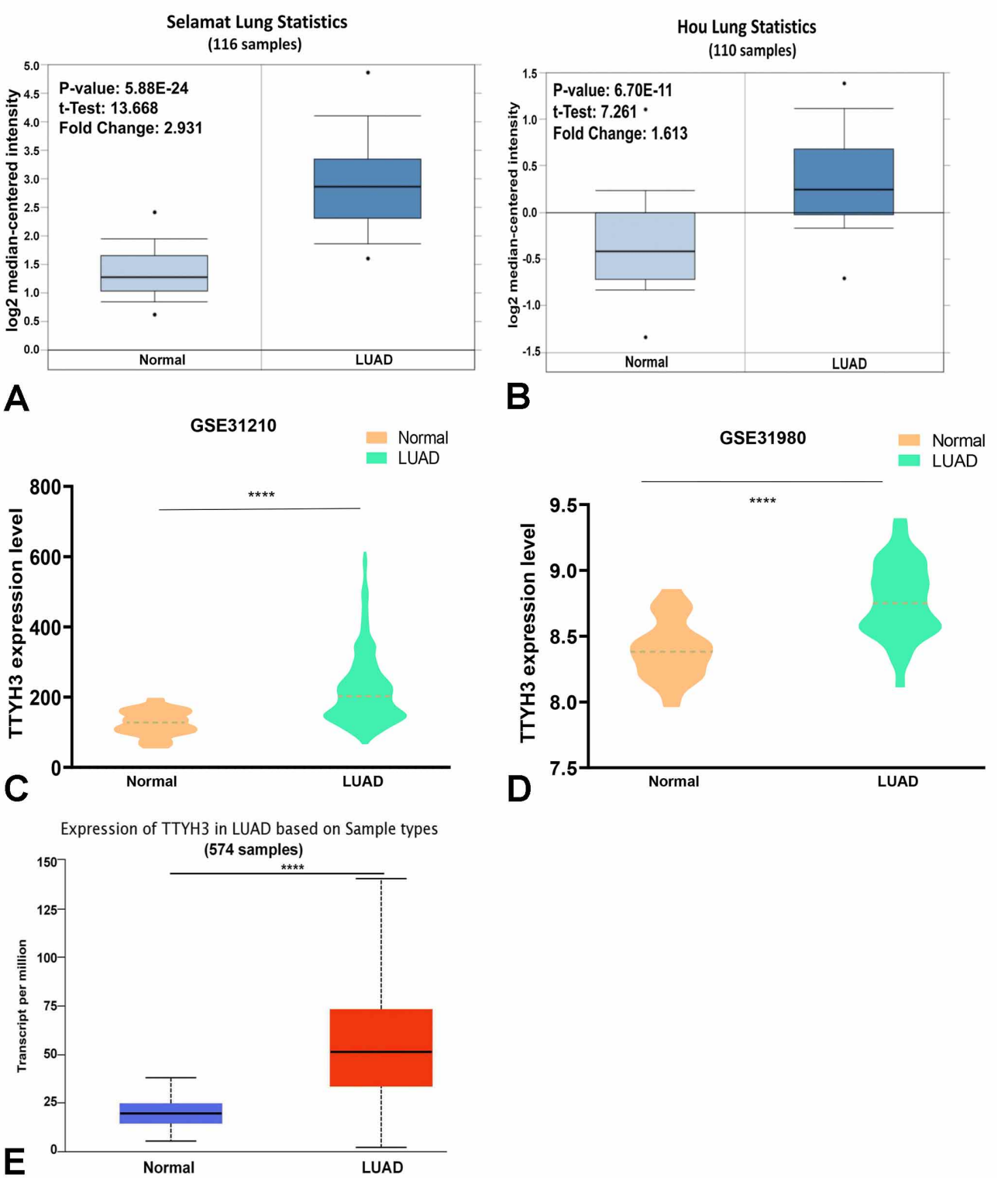


Fig. 1. The mRNA expression levels of TTYH3 in patients with LUAD. The TTYH3 in LUAD from Oncomine database (Selamat *et al*. 2012 (A) and Hou *et al*. 2010 (B)). The TTYH3 in LUAD from GEO database (GSE31210 (C) and GSE31908 (D)). (E) The TTYH3 in LUAD from UALCAN database. **** P<0.0001.

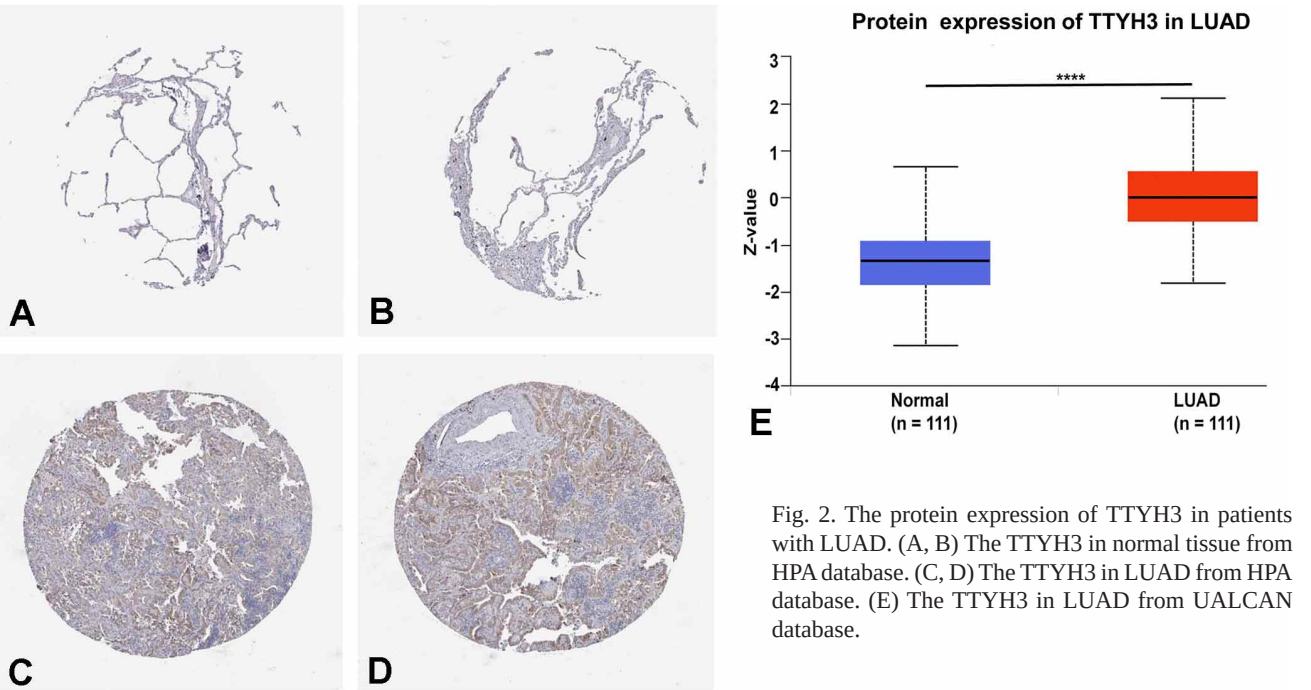


Fig. 2. The protein expression of TTYH3 in patients with LUAD. (A, B) The TTYH3 in normal tissue from HPA database. (C, D) The TTYH3 in LUAD from HPA database. (E) The TTYH3 in LUAD from UALCAN database.

showed that in smoking LUAD patients, the OS of patients with high TTYH3 expression was significantly reduced. In addition, in male patients, high expression of TTYH3 reduced the OS of LUAD. Therefore, TTYH3 is closely related to the survival prognosis of LUAD.

**Analysis of genes correlated with TTYH3 in LUAD.** We analyzed the correlated genes of TTYH3 in LUAD through Cbioportal and found that TTYH3 was most significantly positively correlated with GNA12 (Spearman: r = 0.66; Pearson: r = 0.63) (Fig. 7A). In addition, our data in GEPIA also confirmed that there was a significant positive correlation between TTYH3 and GNA12 (Pearson: r = 0.65, Fig. 7B; Spearman: r = 0.72, Fig. 7C). Finally, we again used the UALCAN and TIMER databases to verify the above results, and the results showed that there was a significant correlation between the expression of TTYH3 and GNA12 in LUAD (Pearson: r = 0.64; Spearman: r = 0.71) (Fig. 7D, E).

**Enrichment analysis of genes co-expressed with TTYH3 in LUAD.** We functionally analyzed the enrichment of TTYH3 and the top 100 co-expressed genes in LUAD using Webgestalt. The top ten biological processes that were significantly enriched included biological regulation, metabolic processes, response to stimuli, positioning, cell component organization, multicellular biological processes, cell communication, developmental processes, cell proliferation, and, multi-biological processes (Fig. 8A). The top ten molecular functions that were also significantly enriched including protein blinding, ion blinding, nucleotide blinding, hydrolase activity, nucleic acid blinding, transferase activity, structural molecule activity, transporter activity, molecular adaptor activity, and molecular transducer activity (Fig. 8B). The cellular components of membrane, protein-containing complex, vesicle, endomembrane system, nucleus, cytosol, membrane-enclosed lumen, extracellular, cell projection, and cytoskeleton, were significantly enriched (Fig. 8C). The top 10 enriched KEGG pathways in TTYH3 high group were extracellular matrix organization, extracellular structure organization, protein heterodimerization, protein trimerization, movement of cell or subcellular component, integrin-mediated signaling pathway, cell junction organization, cell adhesion, biological adhesion, and cell junction assembly (Fig. 8D). Thus, these all indicated the potential function of TTYH3 in LUAD.

**Interaction between TTYH 3 and tumor immune infiltrating cells in LUAD.** We further used TIMER and TISIDB to evaluate the correlation between TTYH3 expression and the level of immune cell infiltration. In the TIMER database, the results showed that TTYH3 was significantly correlated with CD4+ T cells (r = 0.341 p = 1.29e-14), macrophages (r = 0.252, p = 1.77e-08), and neutrophils (r = 0.35, p = 2.27e-15), and dendritic cells (r = 0.353, p = 9.68e-16) (Fig. 9A). However, there was no significant correlation between TTYH3 and CD8+ T cell and B cell infiltration. In addition, we also explored the correlation between TTYH3 and the level of immune cell infiltration through the TISIDB database, and found that TTYH3 was related to infiltration of CD4+ T cells (r = 0.162 p = 0.000221), macrophages (r = 0.123, p = 0.00516),

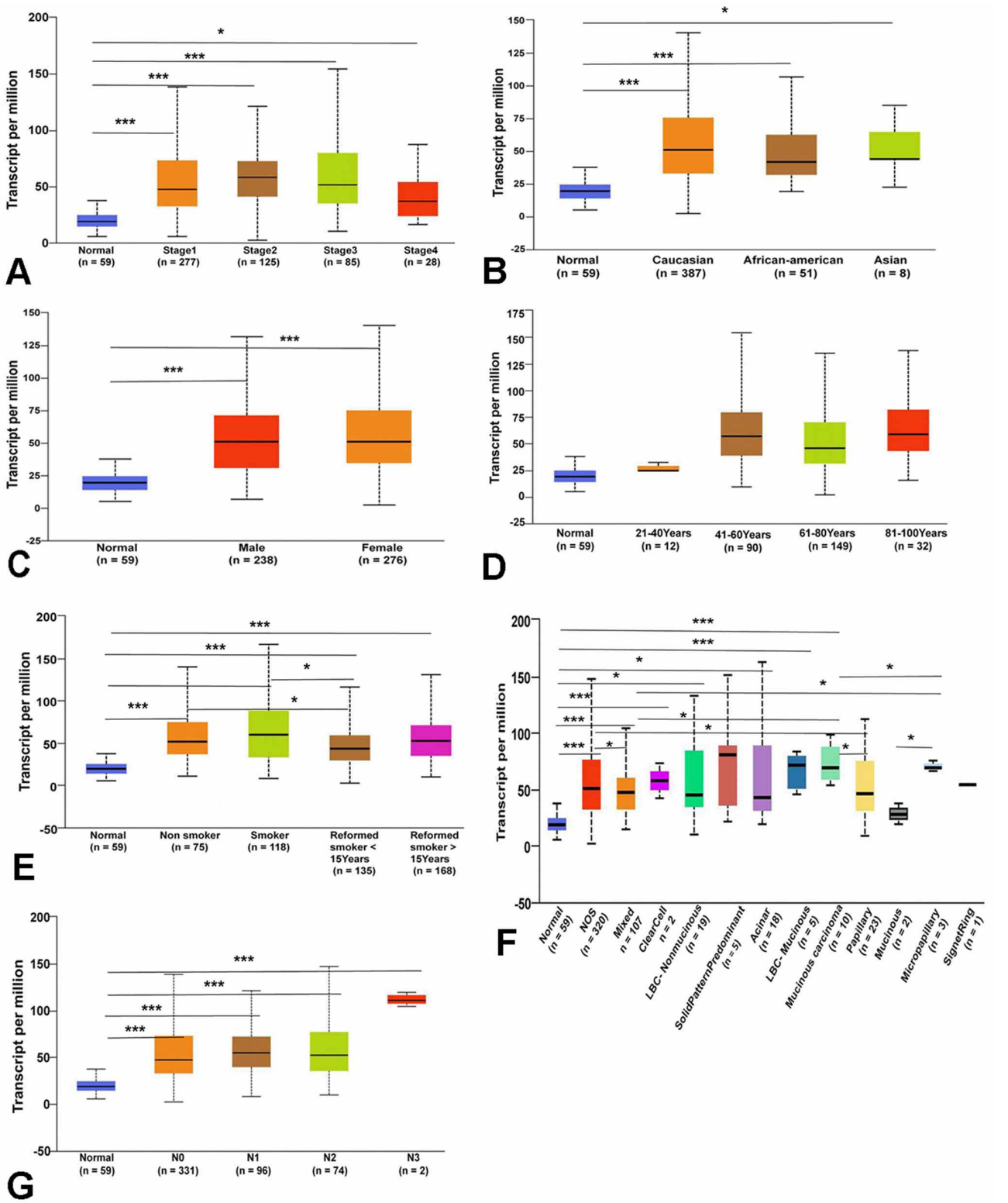


Fig. 3. The associations of the expression of TTYH3 mRNA and clinical features in LUAD. (A) Individual cancer stages. (B) Patient race. (C) Patient gander. (D) Patient age. (E) Patient smoking habits. (F) Histological subtypes. (G) Metastasis status. * P<0.05, ** P<0.001, *** P<0.0001.

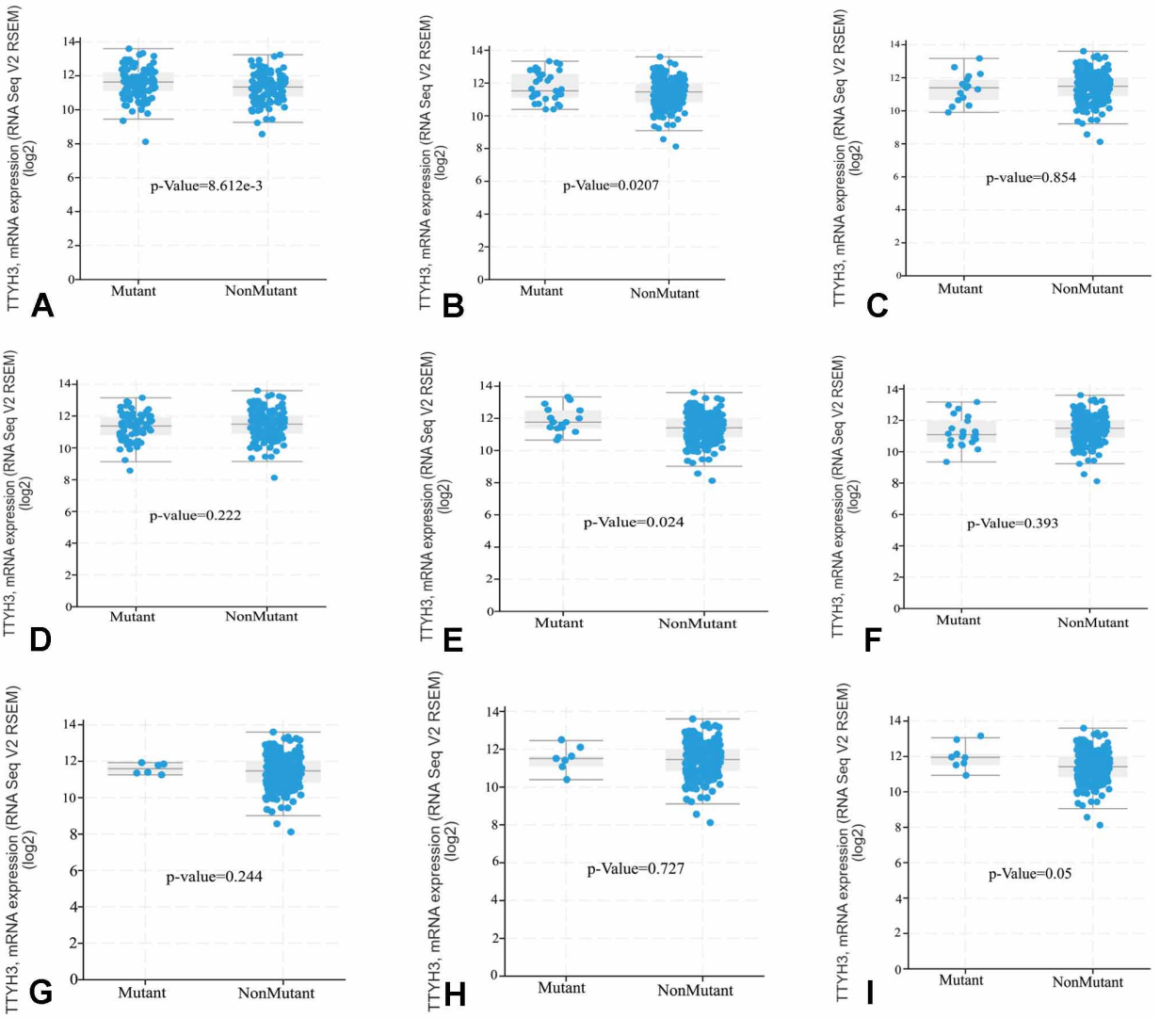


Fig. 4. The associations of the expression of TTYH3 mRNA and gene mutations in LUAD. (A) TP53. (B) EGFR. (C) ALK. (D) KRAS. (E) MET. (F) BRAF. (G) ERBB. (H) RET. (I) ROS.

neutrophils (r = 0.141, p = 0.00134) and dendritic cells (r = 0.137, p = 0.00177) (Fig. 9B). Finally, we compared the relationship between different copy number changes of TTYH3 and the level of tumor invasion. We showed the distribution of each immune subgroup under each copy number status in the LUAD type. The results suggested that there were differences in the level of tumor immune infiltration (B cells, CD8+ T cell, CD4+ T cell, neutrophil, dendritic cell) when TTYH3 was in different copy number variation (Fig. 10). Therefore, there was obvious correlation of TTYH3 expression and immune infiltration in LUAD.

**Correlation analysis between TTYH3 and immune markers.** We used TIMER and GEPIA databases to study the correlation between TTYH3 expression and various immune markers in LUAD. In the TIMER database (Table I), we found that after the correlation adjustment by purity, there was positive correlation between TTYH3 and immune molecular markers (including PTPRC of CD8+T cell; CD14, FUT4 of Monocyte; CCL2, CD68, IL10 of TAM; NOS2, CD80, IRF5, IL6, FCGR1A of M1 Macrophage; CD163, MRC1, VSIG4, MS4A4A of M2 Macrophage; ITGAM, CD15 of Neutrophils; NCAM1 of Natural killer cell; NRP1, IL3RA, ITGAX of Dendritic cell; STAT4, STAT1 of Th1 cell; GATA3 of Th2 cell; BCL6, IL21 of Tfh cell; STAT3, RORC of Th17 cell; FOXP3, IL2RA, CCR8, STAT5B of Treg cell; PDCD1, CTLA4, LAG3, HAVCR2, GZMB of T cell exhaustion). In the GEPIA database, we also confirmed that, the correlation between TTYH3 and markers of immune were similar to those in TIMER (Table I). These findings suggest

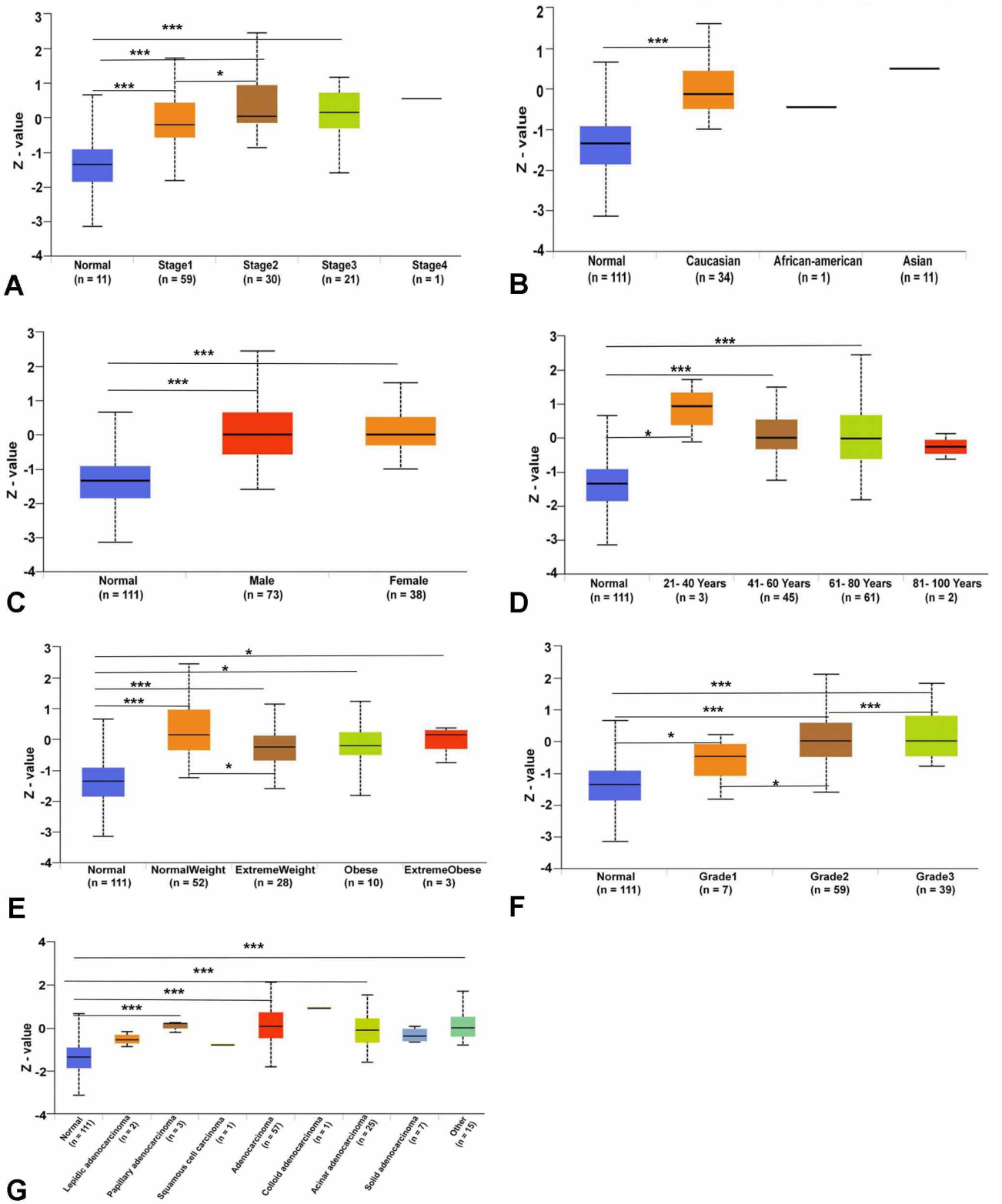


Fig. 5. The associations of the expression of TTYH3 protein and clinical features in LUAD. (A) Cancer stages. (B) Patient race. (C) Patient gander. (D) Patient age. (E) Patient weight. (F) Cancer grade. (G) Histological subtypes. * P<0.05, ** P<0.001, *** P<0.0001.

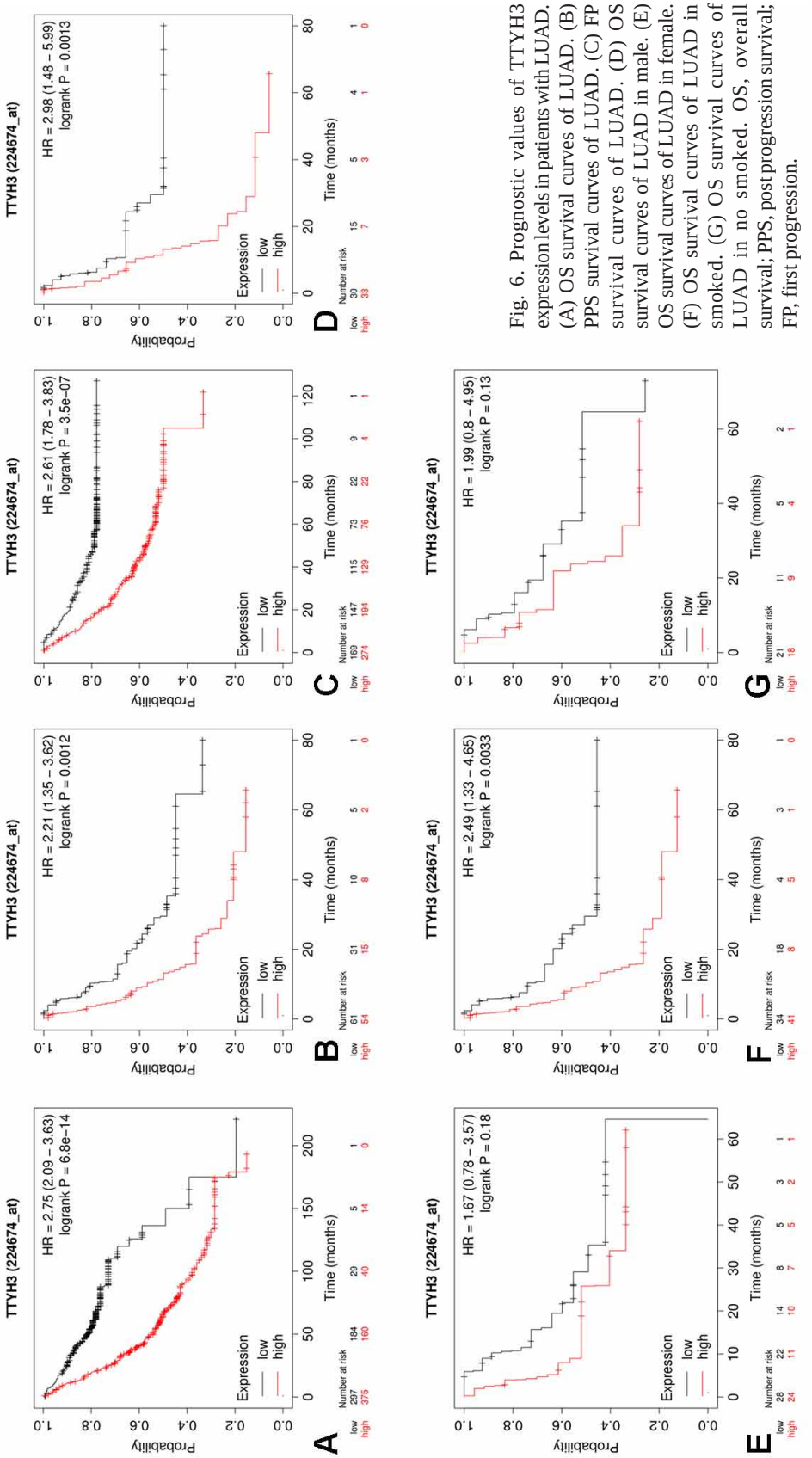


Fig. 6. Prognostic values of TTYH3 expression levels in patients with LUAD. (A) OS survival curves of LUAD. (B) PPS survival curves of LUAD. (C) FP survival curves of LUAD. (D) OS survival curves of LUAD in male. (E) OS survival curves of LUAD in female. (F) OS survival curves of LUAD in smoked. (G) OS survival curves of LUAD in no smoked. OS, overall survival; PPS, post progression survival; FP, first progression.

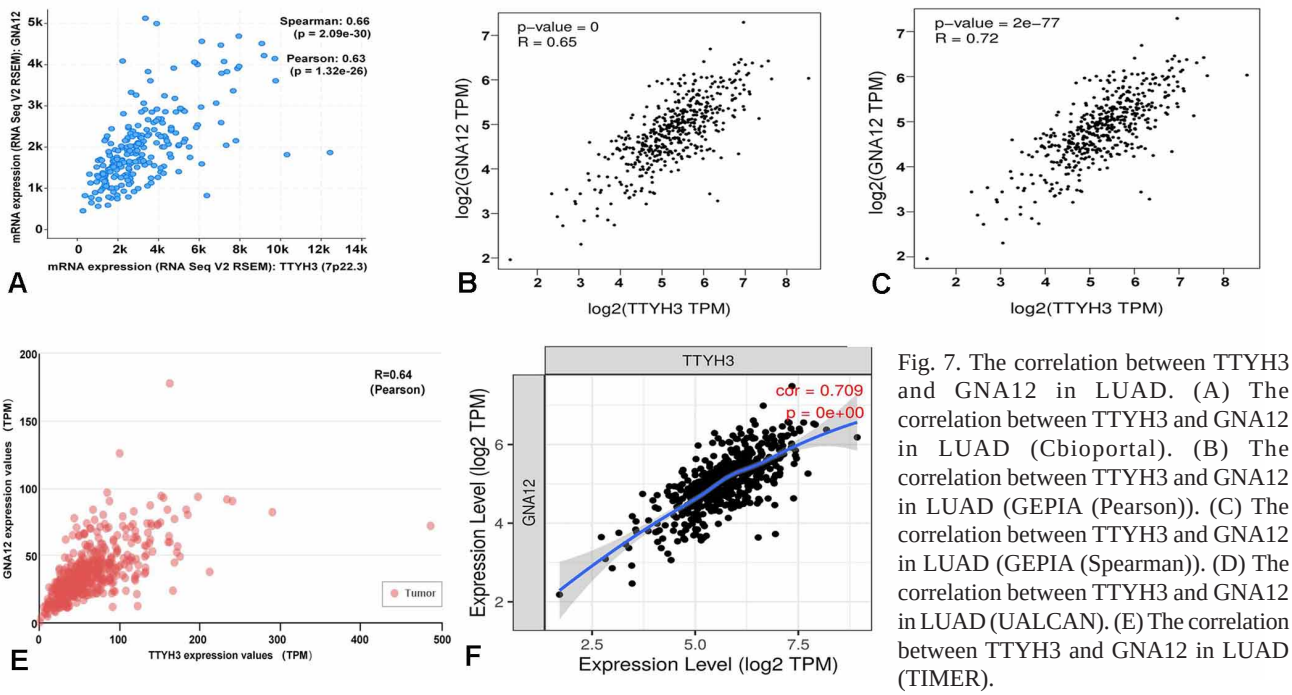


Fig. 7. The correlation between TTYH3 and GNA12 in LUAD. (A) The correlation between TTYH3 and GNA12 in LUAD (Cbioportal). (B) The correlation between TTYH3 and GNA12 in LUAD (GEPIA (Pearson)). (C) The correlation between TTYH3 and GNA12 in LUAD (GEPIA (Spearman)). (D) The correlation between TTYH3 and GNA12 in LUAD (UALCAN). (E) The correlation between TTYH3 and GNA12 in LUAD (TIMER).

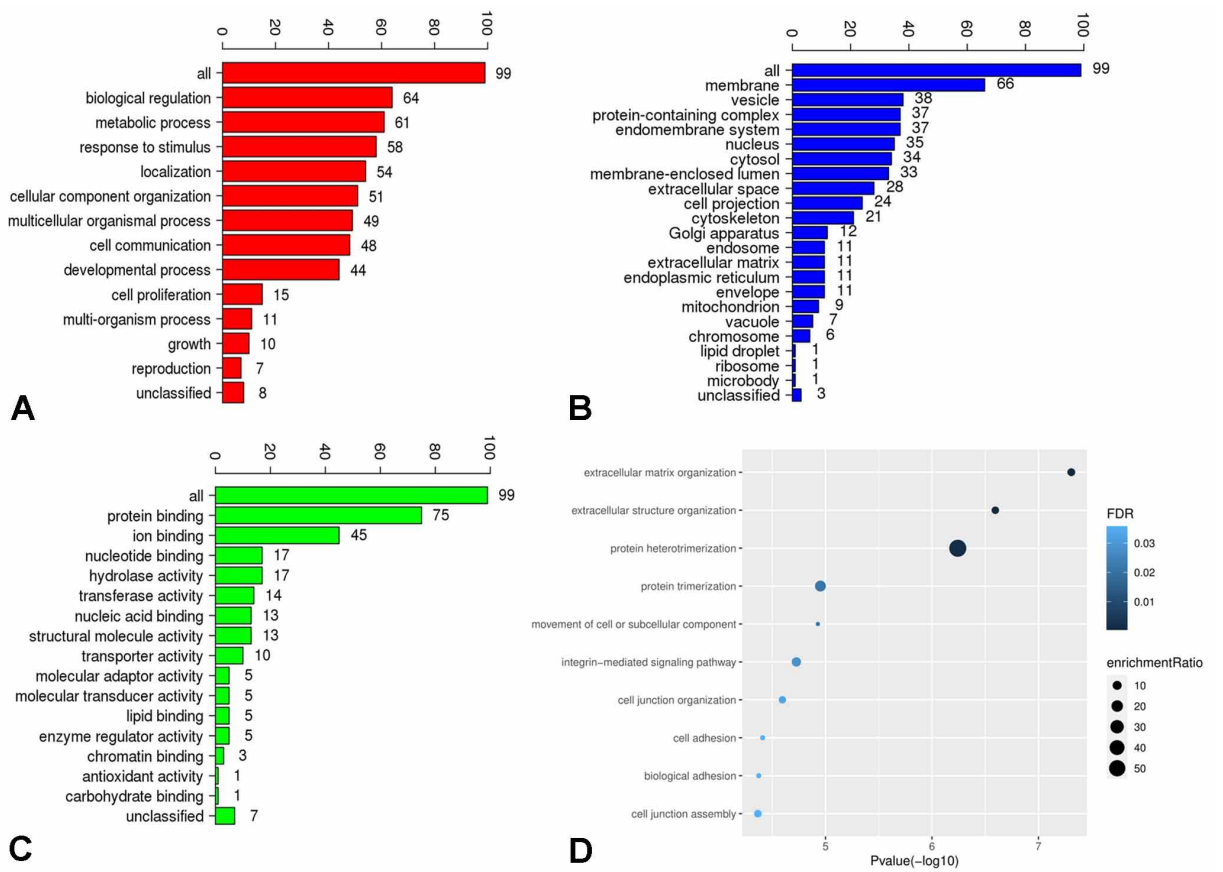


Fig. 8. Top 100 Genes Ontology enrichment function and pathway terms of TTYH3 and co-expression genes in LUAD tissues. Gene Ontology analysis included (A) biological process, (B) cell component, and (C) molecular function. (D) Kyoto Encyclopedia of Genes and Genomes pathway analysis.

that TTYH3 might regulate various immune cell infiltrations in LUAD.

**Meta-analysis of the expression of TTYH3 in LUAD.** We further analyzed the expression of TTYH3 in LUAD by Meta-analysis on LCE. We pooled multiple data sets and found that the expression of TTYH3 in LUAD was higher than that in normal control (Fig. 11).

## DISCUSSION

LUAD is the main manifestation of lung cancer with high morbidity and mortality (Lacroix *et al*., 2008; Testa *et al*., 2022). With the identification of specific molecular and genomic characteristics for LUAD, precision therapy has become a standard for lung cancer treatment. Thus, the molecular characterization of LUAD has become essential for predicting human response to targeted therapy (Selamat *et al*., 2012). However, the 5-year survival rate of LUAD patients who have received multiple comprehensive treatment options including molecular targeted therapy is still not high. Therefore, it is necessary to develop new molecular targets on lung cancer to identify better target molecules.

Chloride ions participate in various physiological functions through trans membrane transport and ion channels, and are the most abundant

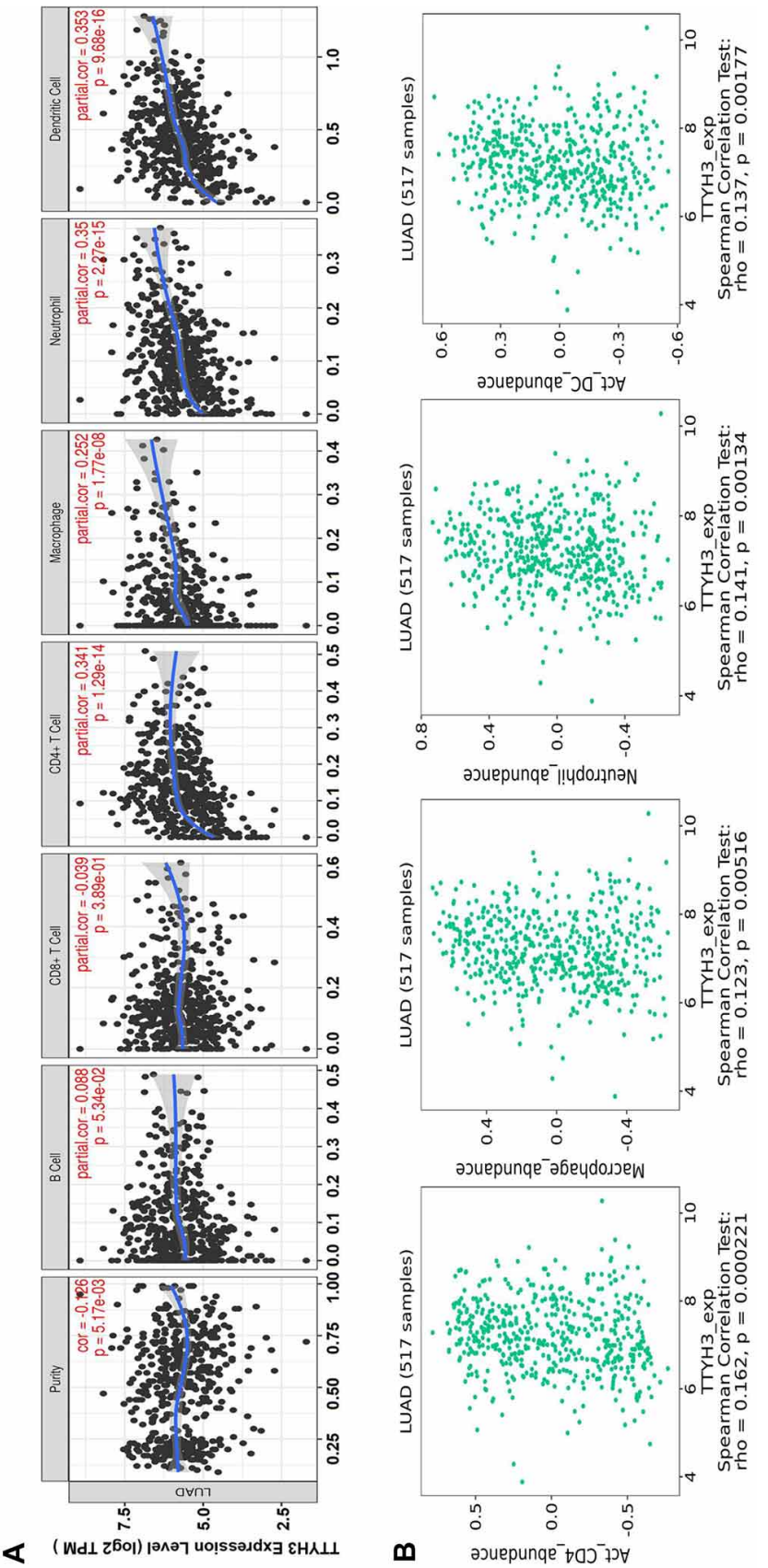


Fig. 9. Correlation of TTYH3 expression with immune infiltration level in LUAD. (A) TIMER database analysis of the correlation between TTYH3 expression and the infiltrating levels of CD4+ T cell, macrophage, neutrophil, and dendritic cell in LUAD. (B) TISIDB database analysis of the correlation between TTYH3 expression and the infiltrating levels of CD4+ T cell, macrophage, neutrophil, and dendritic cell in LUAD.

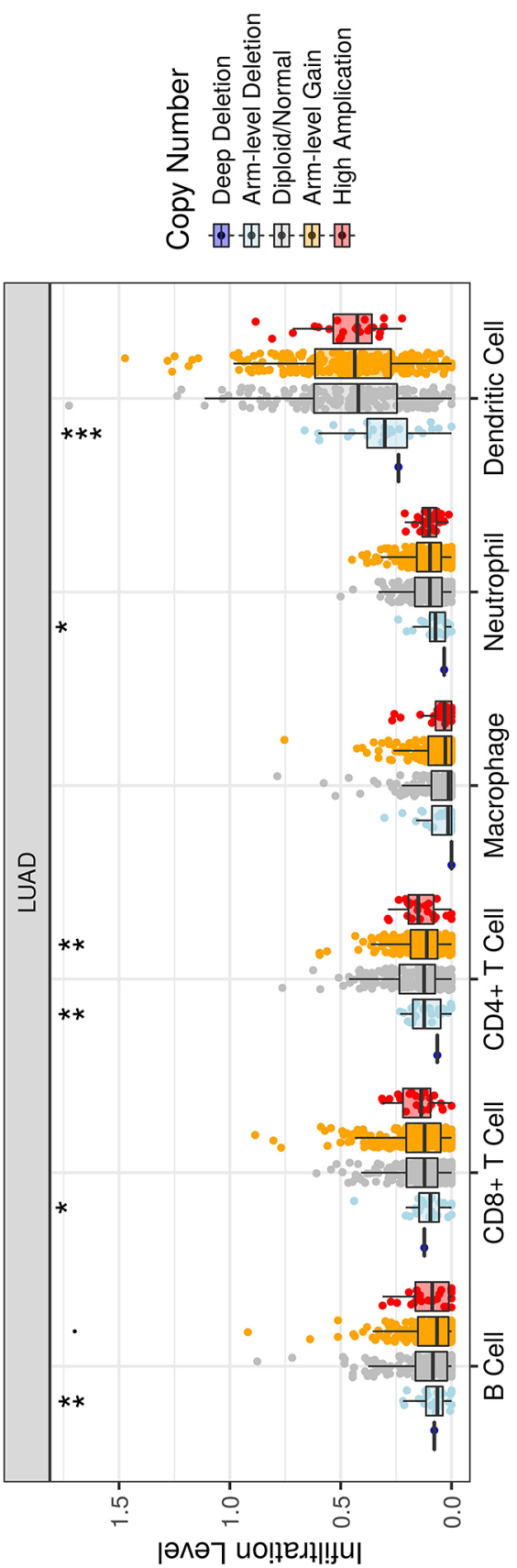


Fig. 10. Correlation of TTYH3 copy number with immune infiltration level in LUAD. TTYH3 copy number had significant positive correlations with infiltrating levels of B cell, CD8+ T cell, CD4+ T cell, neutrophil, and dendritic cell in LUAD (TIMER). * P<0.05, ** P<0.01, *** P<0.001.

anions in organisms. The main function of chloride channels is to regulate epithelial transport, humoral transport, membrane potential and cellular immune response (Shao *et al*., 2016). Abnormal structure or function of chloride channels can lead to a series of diseases. The chloride channel activated by Ca2+ is the main type of chloride ion channel. TTYH3 is also a chloride channel protein activated by Ca2+. Saha *et al*. (2019) found that the expression of TTYH3 in gastric cancer tissue was significantly increased, and it was associated with a decrease in patient survival. However, the expression of TTYH3 in LUAD and its influence on the prognosis of LUAD are still unclear.

In this study, we explored online databases through bioinformatics to comprehensively analyze the expression, prognosis and related mechanisms of TTYH3 in LUAD. To our best knowledge, this is the first study on the expression of TTYH3 in LUAD. First, we used Oncomine and TIMER to evaluate the expression of TTYH3 in different cancers. Compared with the normal control group, TTYH3 was significantly increased in many cancer tissues. These results indicate that TTYH3 might be involved in the occurrence of a variety of tumors and promote tumor growth. Second, we analyzed TTYH3 mRNA in LUAD using Oncomine, GEO and UALCAN, and found TTYH3 mRNA was up-regulated in LUAD. Meta-analysis also showed that TTYH3 expression in LUAD was higher than that in normal control. Subsequently, we showed that there was a significantly increased expression of TTYH3 in LUAD at the protein level using UALCAN and HPA. In

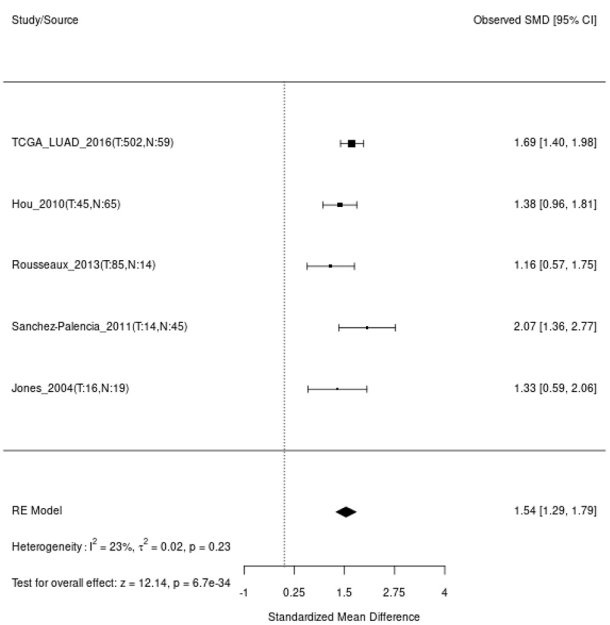


Fig. 11. Meta-analysis of the expression of TTYH3 in LUAD. The high expression of TTYH3 in LUAD by meta-analysis.

Table I. Correlation between TTYH3 and relate markers of immune cells in TIMER and GEPIA.

| Description | Gene markers | TIMER (purity) | | GEPIA | |
|---|---|---|---|---|---|
| | | Cor | P | R | P |
| CD8+T cell | CD8A | 0.054 | 0.227 | 0.06 | 0.19 |
| | CD8B | 0.053 | 0.236 | 0.063 | 0.17 |
| T cell (general) | CD45(PTPRC) | 0.204 | *** | 0.19 | *** |
| | CD3D | 0.023 | 0.607 | 0.042 | 0.35 |
| | CD3E | 0.074 | 0.099 | 0.085 | 0.062 |
| | CD2 | 0.057 | 0.209 | 0.07 | 0.12 |
| B cell | CD19 | -0.001 | 0.981 | 0.023 | 0.61 |
| | CD79A | 0.027 | 0.546 | 0.0034 | 0.94 |
| Monocyte | CD27 | 0.047 | 0.295 | 0.024 | 0.6 |
| | CD20(KRT20) | 0.092 | 0.0402 | 0.17 | ** |
| | CD14 | 0.456 | *** | 0.43 | *** |
| | CD15(FUT4) | 0.374 | *** | 0.41 | *** |
| TAM | CCL2 | 0.337 | *** | 0.35 | *** |
| | CD68 | 0.291 | *** | 0.3 | *** |
| | IL10 | 0.174 | *** | 0.24 | *** |
| M1 Macrophage | INOS(NOS2) | 0.177 | *** | 0.22 | *** |
| | CD80 | 0.230 | *** | 0.25 | *** |
| | IRF5 | 0.369 | *** | 0.39 | *** |
| | IL6 | 0.192 | *** | 0.18 | *** |
| M2 Macrophage | CD64(FCGR1A) | 0.315 | *** | 0.28 | *** |
| | CD163 | 0.364 | *** | 0.34 | *** |
| | CD206(MRC1) | 0.155 | ** | 0.18 | ** |
| | VSIG4 | 0.216 | *** | 0.23 | *** |
| | MS4A4A | 0.222 | *** | 0.22 | *** |
| Neutrophils | CD66b(CEACAM8) | -0.092 | 0.04 | -0.05 | 0.27 |
| | CD11b(ITGAM) | 0.41 | *** | 0.39 | *** |
| | CD15 | 0.374 | *** | 0.41 | *** |
| Natural killer cell | KIR2DL1 | 0.003 | 0.955 | 0.015 | 0.74 |
| | KIR2DL3 | 0.068 | 0.133 | 0.054 | 0.23 |
| | KIR3DL1 | 0.041 | 0.364 | 0.074 | 0.11 |
| | KIR3DL2 | 0.073 | 0.104 | 0.051 | 0.26 |
| | CD56(NCAM1) | 0.124 | * | 0.21 | *** |
| | CD335(NCR1) | 0.089 | 0.0485 | 0.096 | 0.035 |
| Dendritic cell | BDCA1(CD1C) | -0.039 | 0.383 | 0.01 | 0.82 |
| | BDCA3(THBD) | 0.075 | 0.097 | 0.12 | * |
| | BDCA4(NRP1) | 0.238 | *** | 0.27 | *** |
| | CD123(IL3RA) | 0.288 | *** | 0.27 | *** |
| | CD11C_ITGAX_ | 0.425 | *** | 0.39 | *** |
| Th1 | T-BET(TBX21) | 0.108 | 0.0163 | 0.11 | 0.012 |
| | STAT4 | 0.126 | * | 0.17 | ** |
| | STAT1 | 0.273 | *** | 0.29 | *** |
| Th2 | GATA3 | 0.301 | *** | 0.29 | *** |
| | STAT6 | -0.013 | 0.78 | 0.056 | 0.22 |
| | IL13 | -0.024 | 0.599 | 0.00064 | 0.99 |
| Tfh | BCL6 | 0.158 | ** | 0.23 | ** |
| | IL21 | 0.134 | * | 0.130 | * |
| Th17 | STAT3 | 0.152 | ** | 0.2 | ** |
| | IL17A | -0.034 | 0.449 | -0.0086 | 0.85 |
| | RORC | -0.227 | *** | -0.18 | *** |
| Treg | FOXP3 | 0.353 | *** | 0.320 | *** |
| | CD25(IL2RA) | 0.314 | *** | 0.29 | *** |
| | CCR8 | 0.271 | *** | 0.27 | *** |
| | STAT5B | 0.206 | *** | 0.230 | *** |
| T cell exhaustion | PD1(PDCD1) | 0.256 | *** | 0.24 | *** |
| | CTLA4 | 0.172 | ** | 0.17 | ** |
| | LAG3 | 0.198 | *** | 0.18 | *** |
| | TIM3(HAVCR2) | 0.332 | *** | 0.320 | *** |
| | GZMB | 0.171 | ** | 0.13 | * |

Notes: TAM, tumor-associated macrophage; Th, T helper cell; Tfh, Follicular helper T cell; Treg, regulatory T cell; Cor, R value of Spearman's correlation; purity, correlation adjusted by purity. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

conclusion, we confirmed that the expression of TTYH3 in LUAD wassignificantly up-regulated from multiple perspectives.

In order to understand the clinical association between TTYH3 and LUAD, we used UALCAN to analyze the relationship between TTYH3 mRNA / protein levels and the clinical characteristics of LUAD. We found that compared with the normal tissues, TTYH3 mRNA and protein was significantly associated with various clinical indicators (including cancer stage, lymph node metastasis status, tumor grade, tumor histology, race, sex, age, smoking habits, weight). Interestingly, the TTYH3 level in LUAD patients with TP53, EGFR and MET mutation was significantly higher than in those with TP53, EGFR and MET non-mutation. TP53, EGFR and MET are the most common co-mutation genes. Specific TP53 subtype is a biomarker for immune checkpoint inhibitors in LUAD (Sun *et al*., 2020). Molecular targeted therapy for EGFR-mutated LUAD patients can achieve better efficacy and improve the quality of life of patients with advanced LUAD (Zhai *et al*., 2015; Matsubara *et al*., 2020). Additionally, it is believed that MET is an important driver mutation in lung cancer (Lung *et al*., 2019). However, the relationship of TTYH3 expression with TP53, EGFR and MET mutation needs to be further studied. In the prognostic analysis, we used Kaplan-Meier plotter to analyze the clinical relationship between TTYH3 and LUAD survival. We found that LUAD patients with high TTYH3 expression had significantly lower OS, FP and PPS. Further stratification studies found that in smoking and male LUAD patients, high expression

of TTYH3 reduced OS. Therefore, TTYH3 is closely related to the survival prognosis of LUAD. TTYH3 may be an important factor involved in various clinical activities of LUAD, and also an important prognostic indicator of LUAD.

In addition, we studied related co-expressed genes of TTYH3. We found the strongest correlation between TTYH3 and GNA12 genes in the Cbioportal database. The correlation between TTYH3 and GNA12 was confirmed by multiple databases, including GEPIA, UALCAN and TIMER. Udayappan & Casey (2017) reported that GNA12 expression increased in prostate cancer and promoted the tumor progression. Qin *et al*. (2019) found that GNA 12 was a key gene in the "cancer pathway". Therefore, GNA12 may participate in the TTYH3 signaling pathway of LUAD. On the other hand, the GO term and KEGG pathway analysis in this study showed that TTYH3 up-regulation was related to the biological regulation of cancer, metabolic processes, protein binding, and extracellular matrix organization and pathways.

Immune infiltration and tumor microenvironment play a vital role in the occurrence and development of various cancers. The dynamic interaction between tumor molecular characteristics and the immune microenvironment may produce different clinical results (Kadota *et al*., 2013). Tumor immune cell infiltration is an important part of the tumor immune microenvironment (Chen *et al*., 2020). In LUAD, we analyzed the correlation between TTYH3 and tumor immune cell infiltration. We found that TTYH3 was positively correlated with tumor heterogeneity and cell infiltration, including infiltration of CD4 + T cells, macrophages, neutrophils, and dendritic cells. Studies have also shown that the infiltration of T cells, macrophages, neutrophils, and dendritic cells plays an important role in changing the tumor microenvironment, which in turn affects the proliferation of tumor cells (Fei *et al*., 2017; Ge *et al*., 2020). However, the role of TTYH3 in the immune microenvironment has not been reported yet. Our results may provideevidence for the role and mechanism of TTYH3 in the immune microenvironment.

Previous studies have shown that in LUAD, multiple factors are correlated with markers of immune infiltrating cells, which may jointly affect the growth and prognosis of LUAD (Guo *et al*., 2020; Zhong *et al*., 2020). Although TTYH3 is related to the prognosis and immune infiltration of LUAD, the relationship between TTYH3 and the expression of certain immune marker genes is still unknown. Our study found that the immune molecules of various immune cells were positively correlated with the expression of TTYH3. These results suggested that TTYH3 participates in the regulation of immune cell infiltration in the LUAD tumor microenvironment. We observed a significant correlation between TTYH3 and CD8+T cell marker (PTPRC), suggesting that TTYH3 may play an important role in regulating the function of CD8+T cells. We further found that TTYH3 was strongly correlated with relevant markers of M1 Macrophage and M2 Macrophage (NOS2, CD80, IRF5, IL6, FCGR1A, CD163, MRC1, VSIG4, and MS4A4A), suggesting that TTYH3 may play an important role in M1/M2 polarization of macrophages in LUAD. Additionally, TTYH3 and markers of neutrophils (ITGAM and CD15) and dendritic cells (NRP1, IL3RA, and, ITGAX) were also strongly correlated, suggesting that TTYH3 may affect the progression and metastasis of LUAD by regulating neutrophils and dendritic cell. Finally, we found that TTYH3 was positively correlated with markers of multiple T cells (Th1, Th2, Tfh, Th17, Treg, and, T cell exhaustion), suggesting that TTYH3 may gather T cells and play an important role in tumor immune microenvironment. The above results showed that TTYH3 had strong chemotaxis effects on immune-infiltrating cells in LUAD. However, the precise role of TTYH3 in tumor immune microenvironment and tumor progression still needs further study.

However, this study has dome limitations. First of all, although we have verified the role of TTYH3 in LUAD through multiple public databases, the information of public data sets is still limited and there may be selection bias. Secondly, we did not consider the heterogeneity of the immune microenvironment related to the invasion of immune tumor cells. Thirdly, there is no experimental data to verify the results. Therefore, in the future, in vivo and in vitro experimental studies are warranted to confirm these results and the underlying mechanisms.

In summary, our study clarified the expression of TTYH3 in LUAD and its relationship with prognosis from multiple perspectives. Our results indicate that TTYH3 is up-regulated in LUAD and can be used as an early diagnostic marker for LUAD patients. In addition, the high expression of TTYH3 is related to the poor prognosis of LUAD. TTYH3 may induce tumor immune cell activation and immune microenvironment changes, and promote the development of LUAD. These results indicate that TTYH3 plays a crucial role in the immune interaction of LUAD.

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**Data availability statement.** The data utilized in this study is available publicly from [GEO Database] at [http://www.ncbi.nlm.nih.gov/geo/], reference [GSE31210 and GSE31908].

**XU, J.; CHEN, J.; DUAN, H.; ZHANG, D.; LI, Y.; XU, X. & LI, X.** TTYH3 es un biomarcador pronóstico y se asocia con infiltraciones de células inmunitarias del adenocarcinoma de pulmón. *Int. J. Morphol., 41(1)*:118-133, 2023.

**RESUMEN:** Investigamos por bioinformática el nivel de Tweety Family Member 3 (TTYH3) con adenocarcinoma de pulmón (LUAD) y su relación con la infiltración inmune en tumores . Las expresiones diferenciales de TTYH3 en cáncer de pulmón se analizaron con Oncomine, TIMER, GEO, UALCAN y HPA. Con UALCAN se analizó la relación de los niveles de ARNm/proteína de TTYH3 con los parámetros clínicos. Los genes coexpresados de TTYH3 en LUAD se analizaron utilizando Cbioportal. Su relación con el pronóstico LUAD se analizó mediante plotter de Kaplan-Meier. Se realizaron análisis GO y KEGG. TIMER, TISIDB y GEPIA probaron la correlación entre TTYH3 y la infiltración inmune tumoral. Encontramos que TTYH3 aumentó significativamente en los tejidos LUAD. La alta expresión de TTYH3 estuvo estrechamente relacionada con una supervivencia general deficiente, supervivencia posterior a la progresión y primera progresión en pacientes con LUAD. Los niveles de ARNm/proteína de TTYH3 se asociaron significativamente con múltiples vías. Específicamente, la regulación positiva de TTYH3 se relacionó principalmente con la regulación biológica, el proceso metabólico, el cegamiento de proteínas, la organización de la matriz extracelular y las vías en el cáncer. Además, TTYH3 se asoció positivamente con la infiltración de células inmunitarias en LUAD. Finalmente, TTYH3 se expresó altamente en LUAD como lo reveló el metanálisis. TTYH3 está estrechamente relacionado con el pronóstico de LUAD y la infiltración de células inmunitarias, y se puede utilizar como biomarcador pronóstico para LUAD y la infiltración de células inmunitarias.

**PALABRAS CLAVE: TTYH3; Infiltración inmune; Linfocitos; Pronóstico; Adenocarcinoma de pulmón (LUAD).**

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