

Prognostic role and mutational characteristics of N6-methyladenosine-related genes in lung adenocarcinoma

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Received March 20, 2025; Accepted September 1, 2025

DOI: 10.3892/ol.2025.15333

Abstract. Lung adenocarcinoma (LUAD) is an invasive disease that originates from small airway epithelial cells or alveolar type II cells. Abnormal N6-methyladenosine (m6A) RNA methylation serves a key role in the pathogenesis of human diseases, including cancer. RNA sequencing and somatic mutation data in the Genomic Data Commons (GDC) and The Cancer Genome Atlas (TCGA)-LUAD were downloaded from University of California, Santa Cruz (UCSC) Xena database for a comprehensive analysis. m6A-related genes were selected from the content of RNA m6A modification in cancer. m6A genes were further screened by comparing how m6A genes affected survival in normal and tumor groups and analyzing the relationship between m6A genes and LUAD. GDC LUAD data were downloaded from the UCSC Xena public database to analyze the differential expression levels of genes involved with m6A methylation regulators. Next, the mutations of m6A genes were analyzed and a univariate Cox regression analysis and the Kaplan-Meier method were used to determine the relationship between their expression levels and overall survival time shown in TCGA database. Lastly, heterogeneous nuclear ribonucleoprotein C (HNRNPC), insulin-like growth factor 2 mRNA binding protein (IGF2BP1) and IGF2BP3 were selected for subsequent analysis. Enrichment analysis revealed that HNRNPC was mainly enriched in the 'ribonucleoprotein complex biogenesis' pathway, IGF2BP1 in the 'mitotic cell cycle checkpoint' pathway and IGF2BP3 in the 'nuclear division' pathway. The present study identified novel immune-related prognostic markers for LUAD. Furthermore, the potential mechanisms of the prognostic markers in the regulation of LUAD etiology were investigated. The present

study findings may provide novel insights into the treatment of patients with LUAD in the future.

Introduction

Lung cancer (LC) is the leading cause of cancer-related mortality worldwide, with a mortality rate of 18.7% (1) and a 5-year survival rate of ~16.6%. Lung adenocarcinoma (LUAD) is the most frequent histological type, accounting for ~40% of LC cases. Although multiple therapies, such as surgical resection, chemotherapy, radiotherapy and molecular targeted therapy have been developed in the past decades to treat LUAD, the overall survival time of patients with LUAD has not markedly improved, primarily due to the lack of useful molecular biomarkers (2). It remains a matter of debate whether chemoimmunotherapy or immune checkpoint inhibitor monotherapy should be used as first-line treatment for patients with advanced non-small cell LC (NSCLC) and have high expression levels of programmed cell death-ligand 1 (PD-L1) (3). To the best of our knowledge, the lack of prognostic markers for cancer has always limited the choice of first-line therapies (4,5). Similarly, the currently available targeted drugs such as gefitinib, erlotinib, osimertinib, afatinib and crizotinib, have limited clinical efficacy in the treatment of LC due to their off-target effects or drug resistance issues (6). Hu *et al* (7) demonstrated that high Toll-like receptor 7 expression serves as a robust clinical feature predictive of patient prognosis, immunotherapy response and candidate drug efficacy, providing deeper insights for LUAD treatment. In addition, Zhang and Lin (8) identified the TDP-43 co-expressed gene risk score, a prognostic model based on the expression of kinesin family member 20A, WD repeat domain 4, proline rich 11 and glia maturation factor γ , as a reliable biomarker for predicting prognosis and treatment response in LUAD, offering valuable insights for guiding clinical strategies. Therefore, a comprehensive understanding of how LUAD occurs and progresses is essential to improve the diagnosis and prognosis of patients with LUAD in the future.

The methylation of RNA molecules to N6-methyladenosine (m6A) is a universal modification found in all eukaryotes, but its biological relevance remains to be elucidated. Modification of m6A affects mRNA splicing, export, translation and

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Key words: lung adenocarcinoma, N6-methyladenosine-related genes, prognosis, mutational

stability (9). Previous studies have reported that m6A is involved in RNA modulation as ‘writers’ [such as methyltransferase-like (METTL)3/METTL14 complex for methylation] (10), ‘erasers’ [including fat mass and obesity-associated gene (FTO) and AlkB homolog 5 for demethylation] (11), and ‘readers’ [such as YT521-B homology domain-containing family proteins (YTHDFs) that dictate the functional outcomes of m6A modifications] (12), which collectively modulate RNA metabolism (13). Several enzymes, including METTL3 (14) and heterogeneous nuclear ribonucleoprotein A2/B1 (15), participate in the m6A system. Previous studies have reported that modifications to m6A contribute to the progression of a number of diseases, such as obesity (16), cancer (17) and embryonic development (18). However, to the best of our knowledge, comprehensive analysis of the expression of m6A RNA methylation regulators in LC and particularly in LUAD, is largely lacking. The diagnostic and prognostic value of such regulators remains to be explored.

The present study aimed to profile the mRNA expression patterns of m6A-related genes in LUAD using data obtained from The Cancer Genome Atlas (TCGA) and the University of California, Santa Cruz (UCSC) Xena databases. A survival analysis was performed to assess the prognostic value of m6A-related genes in patients with LUAD and correlations between m6A genes and the expression of immunomodulatory factors in LUAD were investigated. The current study aimed to comprehensively analyze the expression and prognostic significance of m6A methylation regulators in LUAD using data from TCGA and UCSC Xena databases.

Materials and methods

Tissue samples. Samples of paracancerous and LUAD tissues used for immunohistochemistry were obtained from 10 patients admitted to the Cancer Center of Guangzhou Twelfth People's Hospital (Guangzhou, China) from February 2019 to May 2021. The cohort comprised 6 men and 4 women, with a median age of 57 years (range, 31–76 years). Clinical data for the patients are shown in Table SI. All patients provided their written informed consent for study participation.

Datasets. Gene expression profiles and clinical information for a cohort of 585 patients with LUAD from The Cancer Genome Atlas (TCGA-LUAD) were downloaded to serve as the training set. Notably, all 585 samples belong to this single TCGA-LUAD cohort, with data obtained from two distinct sources: TCGA database (<http://portal.gdc.cancer.gov/>), which provided 284 tumor and 37 adjacent normal samples, and the UCSC Xena database (<https://xena.ucsc.edu/>), which provided 242 tumor and 22 adjacent normal samples. The dataset used in the analysis is provided in Table SII. In addition, gene expression datasets [accession nos. GSE135222 (19) and GSE126044 (20)] were downloaded from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) series to analyze the expression levels of m6A-related genes.

Identification of differentially expressed immune-related genes and differentially expressed pyroptosis-related genes in LUAD. Based on the method previously described (21), the ‘DESeq2’ R package (v 3.5.1; <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>) was used to identify genes that were differentially expressed between the 526 LUAD and 59 adjacent non-tumor samples, with a threshold of $\log_2 FC > 1$ and $P < 0.05$ was considered to indicate a statistically significant difference.

Construction of a risk model and nomogram based on prognostic markers. The Tumor Immune Estimation Resource (TIMER) database (<http://timer.cistrome.org/>) was used to evaluate the correlation between m6A regulators and the levels of immune cell infiltration [including cancer-associated fibroblasts, bone marrow dendritic cells (BMDCs), CD4⁺ T cells, neutrophils, regulatory T cells (Tregs), CD8⁺ T cells and macrophages]. The Tumor and Immune System Interaction Database (TISIDB) was used to assess the correlation between the expression of m6A regulators and immunoregulators [including immunosuppressant, immunostimulator and major histocompatibility complex (MHC) molecules].

Genetic alteration of m6A regulators in LUAD. cBio Cancer Genomics Portal (cBioPortal; <https://www.cbioportal.org/>), an open-access website that explores, visualizes and analyzes multidimensional cancer genomics data, was used to analyze the genetic alterations of m6A regulators in LUAD.

Mutation analysis of m6A genes in LUAD. Mutation annotation format files were analyzed using the R package ‘Maftools’ (v 2.25.10; Bioconductor; <https://bioconductor.org/packages/devel/bioc/html/maftools.html>) to summarize, visualize and interpret somatic mutations in m6A regulator genes across the LUAD cohort.

Survival analysis. The correlation between m6A regulator aberrations and the survival time of patients with cancer was determined using the cBioPortal database. The correlation between the overall survival time and the expression of m6A regulators was evaluated using Kaplan-Meier curves.

T cell exhaustion correlation analysis. First, the correlation between m6A gene expression and biomarkers of T cell exhaustion [TNF, IL-2, IFN- γ and cytotoxic T lymphocyte (CTL)] was analyzed using the R software package ‘psych’ (version 2.4.3; <https://www.rdocumentation.org/packages/psych/versions/2.4.3>), based on research datasets in a previous study (22).

Next, the expression levels of m6A genes in patients with LUAD treated with anti-programmed cell death protein-1 (PD-1)/PD-L1 were analyzed the GSE135222 and GSE126044 datasets. In these datasets, patients with LUAD were grouped according to whether they had responded to anti-PD-1/PD-L1 treatment [19 non-responders and 8 responders in GSE135222 (23); 11 non-responders and 5 responders in GSE126044 (20)]. Furthermore, analyzing the correlation between the expression levels of m6A-related genes and immune cell marker genes was analyzed using Spearman's rank correlation to clarify the role of m6A-related genes in predicting immune infiltration responses.

Immunohistochemistry (IHC). Samples of LUAD and paracancerous tissue were collected at Cancer Center of

Guangzhou Twelfth People's Hospital. Immunohistochemical procedures were performed on the tissues to assess gene expression according to the methods reported in a previous study (24). In brief, the tissues were fixed in 10% formalin, embedded in paraffin, and cut into 4- μ m sections. Subsequently, the sections were dewaxed using gradient ethanol and subjected to antigen retrieval in citrate buffer (cat. no. G1202; Wuhan Servicebio Technology Co., Ltd.) at 95°C for 20 min. The slides were then treated with 3% H₂O₂ at 25°C for 30 min to block endogenous peroxidase activity, followed by blocking with 3% bovine serum albumin (cat. no. GC305006; Wuhan Servicebio Technology Co., Ltd.) at room temperature for 30 min. Next, the sections were incubated with anti-heterogeneous nuclear ribonucleoprotein C (HNRNPC; cat. no. ab75822; dilution 1:800), anti-insulin-like growth factor 2 mRNA binding protein (IGF2BP1) (cat. no. ab184305; dilution 1:4,000), anti-IGF2BP3 (cat. no. ab179807; dilution 1:1,000), anti-PD-L1 (cat. no. ab205921; dilution 1:1,000), anti-CD4 (cat. no. ab133616; dilution 1:500), anti-CD8 (cat. no. ab217344; dilution 1:2,000) and anti-Forkhead box P3 (FOXP3; cat. no. ab20034; dilution 1:500), respectively, followed by incubation with goat anti-rabbit IgG (HRP; cat. no. ab7090; dilution 1:1,000) (all from Abcam) and DAB chromogen. The tissue sections were then counterstained with hematoxylin, observed and captured under a light microscope.

Statistical analysis. Statistical analyses were performed using SPSS (version 26.0; IBM Corp.) and GraphPad Prism (version 10.1.2; Dotmatics). Differential expression analysis of m6A methylation regulators between tumor and paired adjacent normal tissues was assessed using the paired Student's t-test for parametric data or the Wilcoxon signed-rank test for non-parametric data, with 59 paired tumor and normal tissue samples. Univariate Cox proportional hazards regression analysis was used to evaluate the association between individual m6A regulator expression levels and overall survival time. Kaplan-Meier survival curves were generated and compared using the log-rank test. The correlation between T-cell exhaustion markers and other variables of interest was assessed using Spearman's rank correlation coefficient (ρ), with statistical significance determined by the corresponding P-value. Differences in gene expression across ordinal clinical variables [tumor, lymph node and metastasis (TNM) stages] were assessed using one-way ANOVA with Tukey's post hoc test for multiple comparisons. Mutation analysis of m6A genes was performed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on genes identified through Pearson's correlation analysis ($r > 0.5$, $P < 0.001$) of m6A-related genes from TCGA expression matrix that were co-expressed with HNRNPC, IGF2BP1 and IGF2BP3. $P < 0.05$ was considered to indicate a statistically significant difference for all analyses, unless otherwise indicated.

Results

Tumor mutation load analysis. To evaluate the mutation load in LUAD, the tumor mutation load in 33 TCGA tumor types was compared. The present study data demonstrated that LUAD had a high mutation load, which was lower compared

with the mutation loads of bladder urothelial carcinoma, lung squamous cell carcinoma and skin cutaneous melanoma (Fig. 1).

Differential expression levels of m6A RNA regulators in normal and tumor samples. The differential expression levels of m6A genes in the tumor and control groups and the correlation patterns were analyzed. Excluding YTHDF3, YTHDC protein (YTHDC1, YTHDC2 and IGF2BP2), the expression levels of all m6A genes significantly differed between the tumor and control groups. ($P < 0.01$; Fig. 2A).

A correlation analysis indicated a moderate positive correlation between m6A genes, among which, YTHDF3 had the strongest correlation with vir like m6A methyltransferase associated (KIAA1429) and YTHDC1 a slightly weaker correlation with METTL14 and Zinc finger CCCH domain-containing protein 13 (ZC3H13). Furthermore, FTO was negatively correlated with METTL5 ($r = 0.19$; Fig. 2B).

Mutation analysis of m6A genes in LUAD. To identify somatic mutations in patients with LUAD, mutation data were analyzed using the R software package 'Maftools'. Results indicated that 110 of 561 patients with LUAD had mutated m6A-related genes. As shown in Fig. 3A, the mutated m6A-related genes included KIAA1429 (3%), ZC3H13 (3%), IGF2BP1 (3%), YTHDC1 (2%), YTHDC2 (2%), RNA binding motif protein 15 (RBM15; 2%), IGF2BP2 (1%), IGF2BP3 (1%), HNRNPC (1%), YTHDF1 (1%), METTL14 (1%), YTHDF3 (1%), METTL14 (1%), FTO (1%), Wilms' tumor 1-associating protein (1%), bacterial alkane hydroxylase homolog 5, RNA demethylase (1%) and METTL116 (1%).

Furthermore, the somatic interactions between m6A genes and the status of single nucleotide polymorphisms (SNPs) and highly mutated genomic regions were further explored. C>A and C>T were the two main mutation types. The proportion of each sample is shown in a stacked histogram (Fig. 3B). The rainfall plot demonstrated high mutation genomic regions based on different SNP mutation types (Fig. 3C). As shown in Fig. 3D, IGF2BP1 had significant coexpression frequencies with RBM15 and IGF2BP2, and KIAA1429 had significant coexpression frequencies with IGF2BP2 and YTHDC1 ($P < 0.05$).

Survival analysis for the m6A gene in LUAD. To further evaluate the prognostic value of m6A RNA methylation regulators in LUAD, the relationship between their expression levels and overall patient survival in TCGA database was determined by a univariate Cox regression and Kaplan-Meier analysis. The Cox regression results revealed that five genes were associated with LUAD prognosis. High expression levels of HNRNPC, IGF2BP1, IGF2BP2, IGF2BP3 and METTL5 were with significantly associated with a poor prognosis in LUAD ($P < 0.001$; Fig. 4A-E).

Construction of a nomogram for LUAD. The associations between HNRNPC, IGF2BP1 and IGF2BP3 expression and clinical parameters, including tumor and TNM stages (25), were evaluated. The present study results indicated that there was a significant difference in HNRNPC expression between the normal group and the four stages ($P < 0.001$; Fig. 5A).

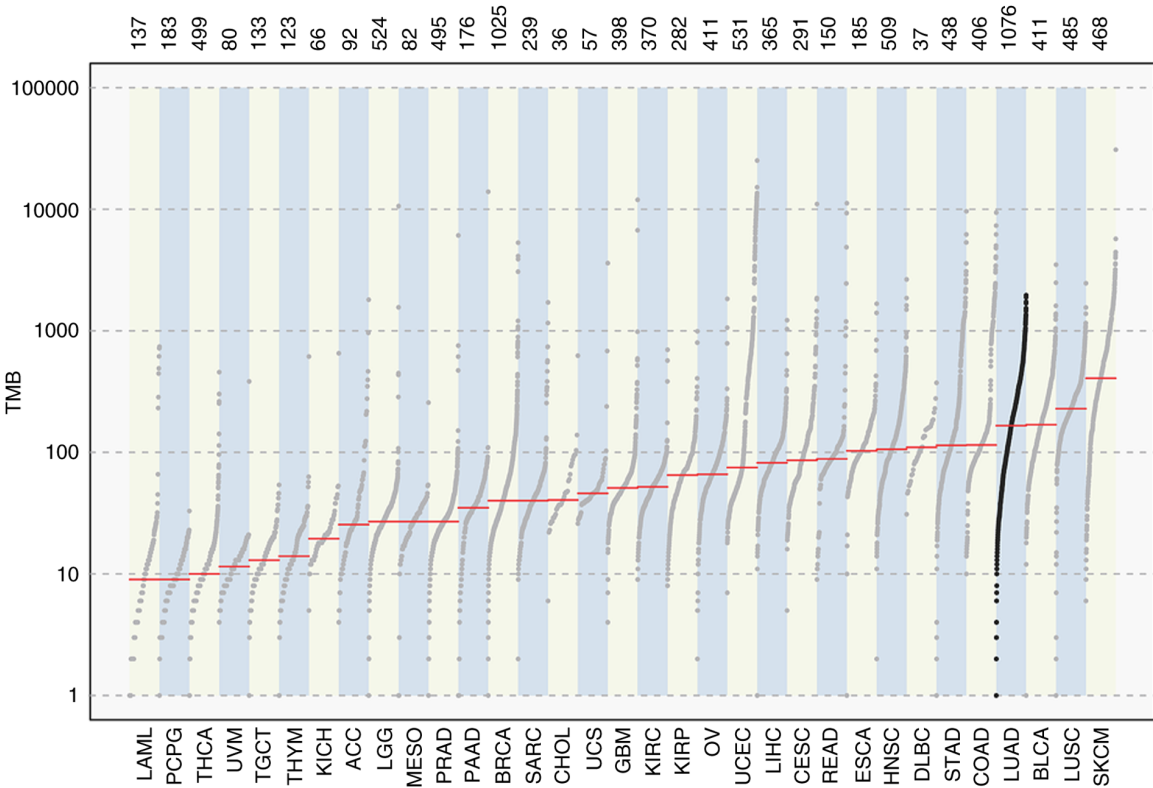


Figure 1. Mutation load in LUAD. TMB, tumor mutation burden; LAML, acute myeloid leukemia; PCPG, pheochromocytoma and paraganglioma; THCA, thyroid carcinoma; UVM, uveal melanoma; TGCT, testicular germ cell tumors; THYM, thymoma; KICH, kidney chromophobe; ACC, adrenocortical carcinoma; LGG, brain lower grade glioma; MESO, mesothelioma; PRAD, prostate adenocarcinoma; PAAD, pancreatic adenocarcinoma; BRCA, breast invasive carcinoma; SARC, sarcoma; CHOL, cholangiocarcinoma; UCS, uterine carcinosarcoma; GBM, glioblastoma; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; OV, ovarian serous cystadenocarcinoma; UCEC, uterine corpus endometrial carcinoma; LIHC, liver hepatocellular carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; READ, rectum adenocarcinoma; ESCA, esophageal carcinoma; HNSC, head and neck squamous cell carcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; STAD, stomach adenocarcinoma; COAD, colon adenocarcinoma; LUAD, lung adenocarcinoma; BLCA, bladder urothelial carcinoma; LUSC, lung squamous cell carcinoma; SKCM, skin cutaneous melanoma.

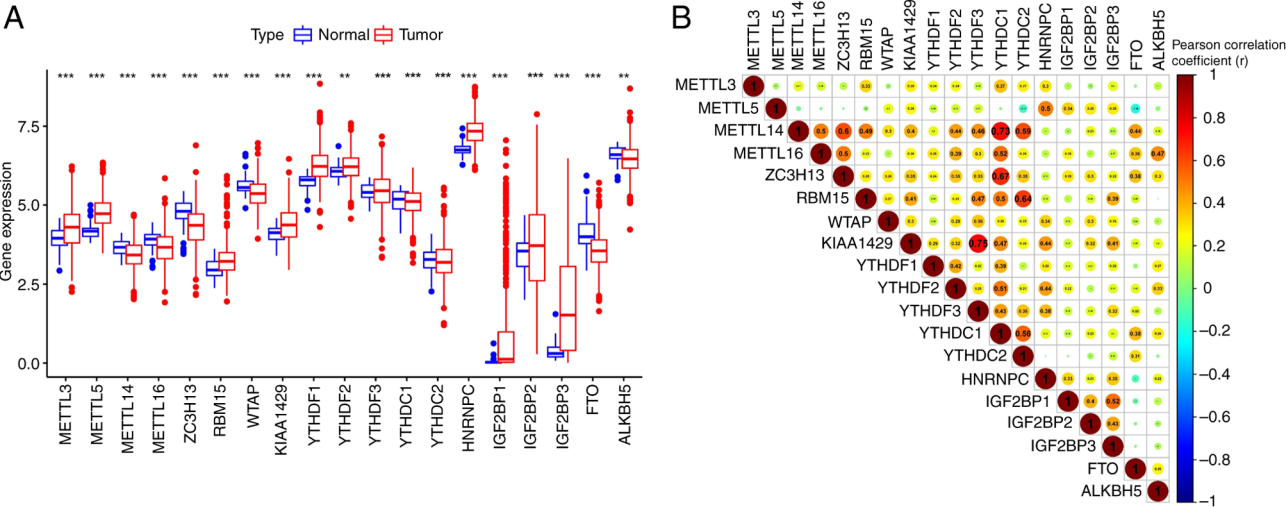


Figure 2. Differential expression levels of m6A genes between the tumor group and control group and the correlation patterns. (A) The expression levels of all m6A genes, excluding YTHDC1, YTHDC2, IGF2BP2 and YTHDF3, were significantly different between the tumor and control groups. (B) Correlation analysis. YTHDF3 had the strongest correlation with KIAA1429. ** $P < 0.01$ and *** $P < 0.001$. m6A, N6-methyladenosine; YTHDC, YT521-B homology domain-containing protein; IGF2BP2, insulin-like growth factor 2 mRNA binding protein 2; YTHDF3, YTHD-containing family protein 3; KIAA1429, vir like m6A methyltransferase associated.

In addition, a statistical difference was observed between stage 1 and 4 ($P=0.031$). There were significant differences between the normal group and the four stages in the T stage (all $P < 0.001$), and there was a significant difference between the

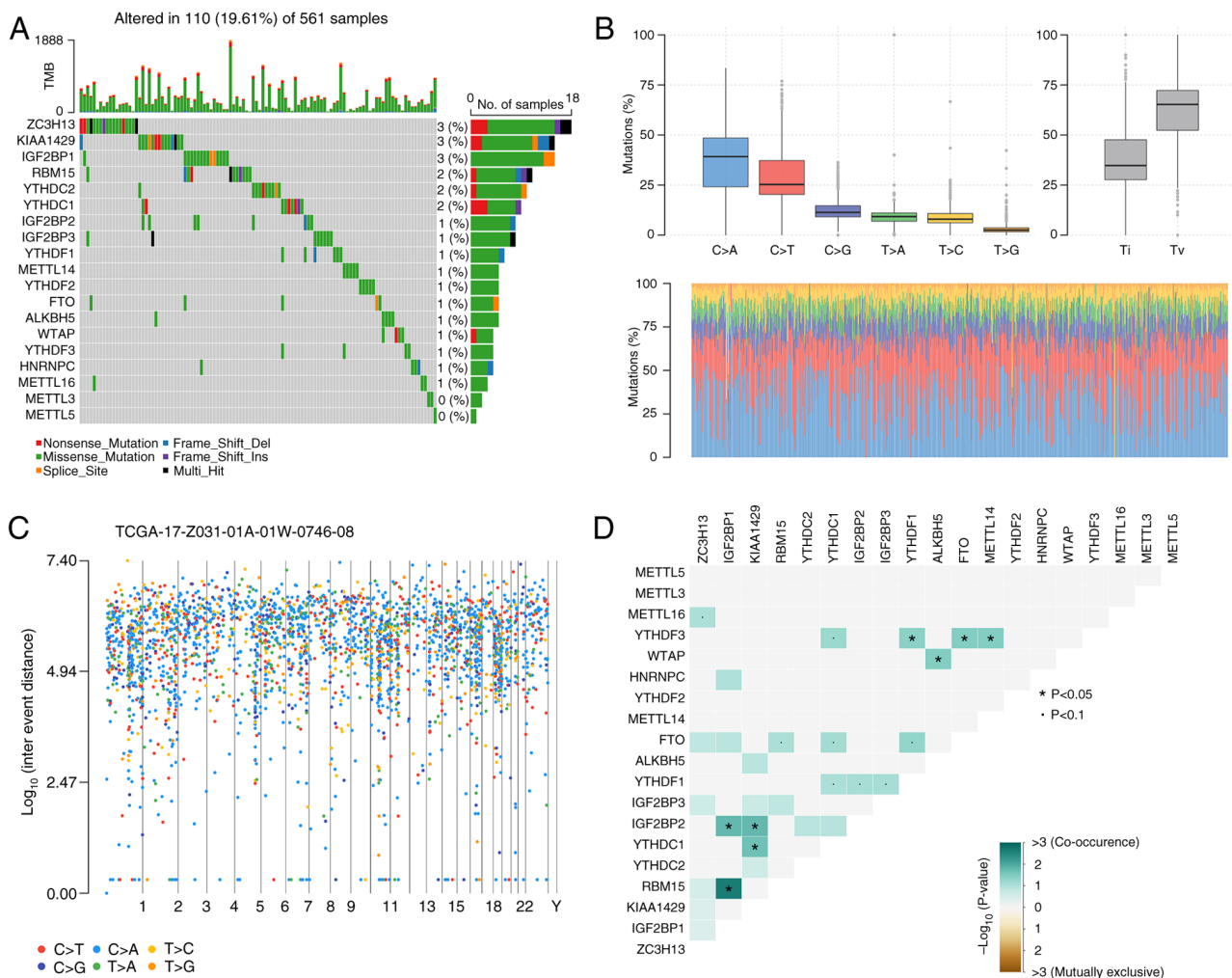


Figure 3. Somatic interaction between m6A genes and the status of SNPs and highly mutated genomic regions. (A) m6A-related mutated genes. (B) Stacked histogram of the proportion of each sample. (C) Rainfall plot indicates highly mutated genomic regions based on different SNP mutation types. (D) IGF2BP1 and RBM15 had significant co-expression frequencies ($P<0.05$). m6A, N⁶-methyladenosine; SNP, single nucleotide polymorphism; TMB, tumor mutation burden; TCGA, The Cancer Genome Atlas; IGF2BP1, insulin-like growth factor 2 mRNA binding protein 1; RBM15, RNA binding motif protein 15.

normal group and the two stages in the M stage (both $P<0.001$). Significant differences were also observed between the normal group and the three stages in the N stage (all $P<0.001$).

For IGF2BP1 expression, results indicated that there was a statistically significant difference between the normal group and the four stages in stage (P<0.001; Fig. 5B). Significant differences were also found between the normal group and the four stages in the T stage ($P<0.01$). Furthermore, a significant difference was observed between T stages 1 and 2 ($P=0.044$). There were significant differences between the normal group and M0 and M1 stages in the M stage ($P<0.001$ and $P<0.01$, respectively). Significant differences were also found between the normal group and the three stages in the N stage ($P<0.001$).

For IGF2BP3 expression, results indicated that there was a statistically significant difference between the normal group and the four stages ($P<0.001$; Fig. 5C). Significant differences were observed between the normal group and the four stages in the T stage (all $P<0.001$). There was also a significant difference between the normal group and M0 and M1 stages in the M stage (both $P<0.001$). Significant differences were also found between the normal group and the three stages in the N stage ($P<0.001$ for all). Next, the expression levels

of HNRNPC, IGF2BP1 and IGF2BP3 were detected in LC tissues by IHC. The results demonstrated that these three genes were all upregulated in LUAD when compared with paracancerous tissues (Fig. 6).

Correlation analysis between m6A gene expression and immune infiltration in LUAD. The TIMER database was used to evaluate the correlation between m6A genes and immune cell infiltration level in LUAD. Results revealed that BMDCs ($\rho=0.494$; $P=8.96\times10^{-32}$) and Tregs ($\rho=-0.181$; $P=5.02\times10^{-5}$) were significantly positively and negatively correlated with HNRNPC, respectively (Fig. 7A). Cancer-associated fibroblasts ($\rho=0.155$; $P=5.02\times10^{-4}$), BMDCs ($\rho=0.374$; $P=6.84\times10^{-18}$) and macrophages ($\rho=0.155$; $P=5.54\times10^{-4}$) were significantly positively correlated with IGF2BP1 (Fig. 7B). IGF2BP3 indicated significant positive correlations with cancer-associated fibroblasts ($\rho=0.208$; $P=3.09\times10^{-6}$), BMDCs ($\rho=0.389$; $P=2.65\times10^{-19}$), neutrophils ($\rho=0.16$; $P=3.56\times10^{-4}$), CD8⁺ T cells ($\rho=0.224$; $P=4.66\times10^{-7}$), and macrophages ($\rho=0.239$; $P=7.39\times10^{-8}$); however, there was a negative correlation with CD4⁺ T cells ($\rho=-0.094$; $P=3.74\times10^{-2}$) (Fig. 7C). Furthermore, IHC staining was performed to assess the expression levels

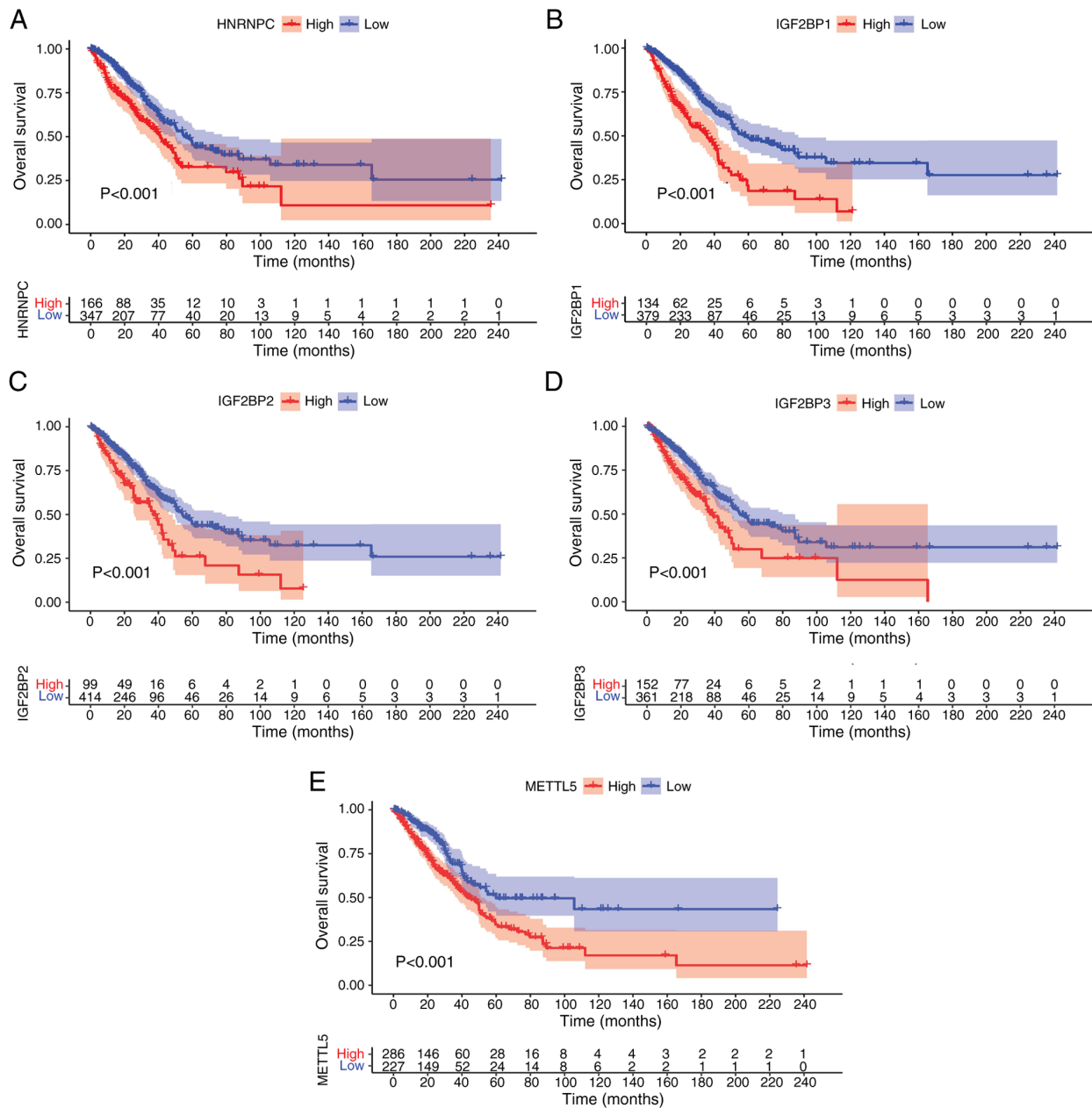


Figure 4. Univariate Cox regression and Kaplan-Meier analysis for the prognostic value of m6A RNA methylation regulators in LUAD. (A) Univariate Cox regression analysis showing that high expression of HNRNPC was associated with poor prognosis in LUAD. (B) Univariate Cox regression analysis showing that high expression of IGF2BP1 was associated with poor prognosis in LUAD. (C) Univariate Cox regression analysis showing that high expression of IGF2BP2 was associated with poor prognosis in LUAD. (D) Univariate Cox regression analysis showing that high expression of IGF2BP3 was associated with poor prognosis in LUAD. (E) Univariate Cox regression analysis showing that high expression of METTL5 was associated with poor prognosis in LUAD. m6A, N6-methyladenosine; LUAD, lung adenocarcinoma; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP, insulin-like growth factor 2 mRNA binding protein; METTL5, methyltransferase-like 5.

of PD-L1, CD4, CD8 and FOXP3 in clinical samples. Results indicated that the expression levels of PD-L1, CD4, CD8 and FOXP3 were elevated in LUAD cancer tissues (Fig. 7D).

Correlation between m6A gene and T cell exhaustion in LUAD. First, the correlation between m6A gene expression and biomarkers of T cell exhaustion (TNF, IL-2, IFN- γ and CTL). The results indicated that HNRNPC was negatively correlated with TNF expression ($P<0.05$), IL-2 expression ($P<0.001$) and CTL expression ($P<0.05$), IGF2BP3 was positively correlated with IFN- γ expression ($P<0.01$), while IGF2BP1 ($P>0.05$) was not correlated with any of the biomarkers (Fig. 8A).

Next, the expression levels of m6A genes in patients treated with anti-PD-1/PD-L1 were analyzed based on GEO datasets (dataset nos. GSE135222 and GSE126044). However, the expression levels of HNRNPC ($P=0.163$; $P=0.583$), IGF2BP1 ($P=0.69$; $P=0.743$) and IGF2BP3 ($P=0.621$; $P=0.583$) were not significantly different between the non-response group and response group (Fig. 8B and C).

Correlation between m6A genes and the expression of immunomodulatory factors in LUAD. The TISIDB database was used to analyze the correlation between m6A genes and the expression of immunomodulators. Furthermore, to

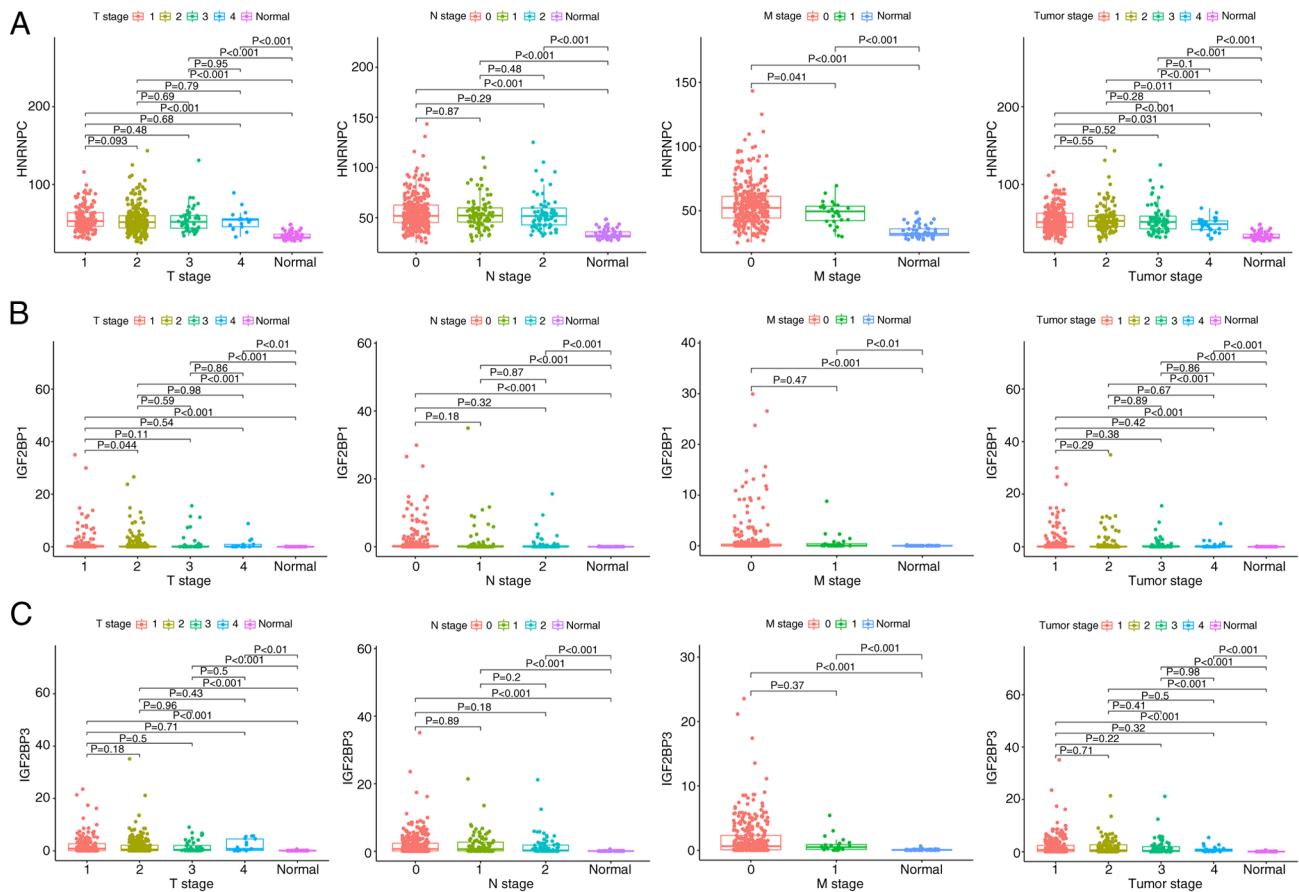


Figure 5. Nomogram construction for LUAD prognosis prediction. Comparisons shown between the normal tissues and stage I-IV groups using one-way ANOVA with Tukey's post hoc test. (A) Differential expression analysis of HNRNPC across TNM stages. (B) Differential expression analysis of IGF2BP1 across TNM stages. (C) Differential expression analysis of IGF2BP3 across TNM stages. LUAD, lung adenocarcinoma; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP, insulin-like growth factor 2 mRNA binding protein; TNM, tumor, lymph node metastasis.

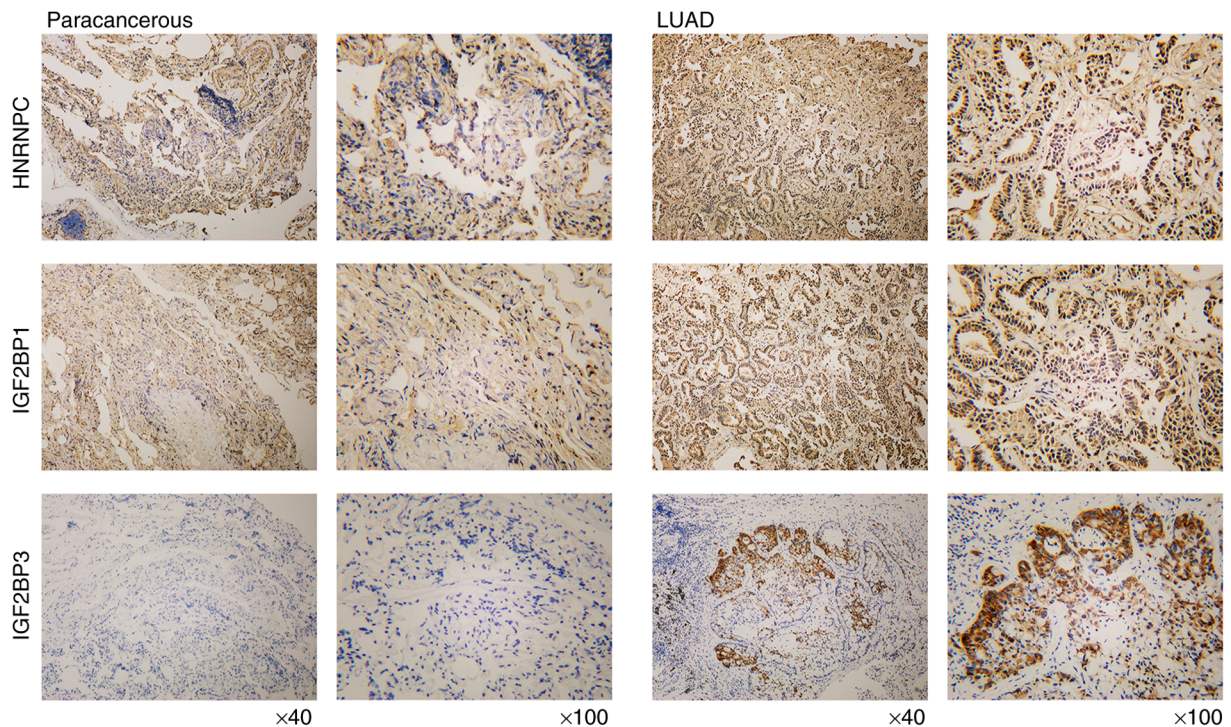


Figure 6. m6A gene expression (HNRNPC, IGF2BP1 and IGF2BP3) in paracancerous and LUAD tissues was analyzed using immunohistochemistry. Magnification, x40 and x100. m6A, N6-methyladenosine; LUAD, lung adenocarcinoma; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP, insulin-like growth factor 2 mRNA binding protein.

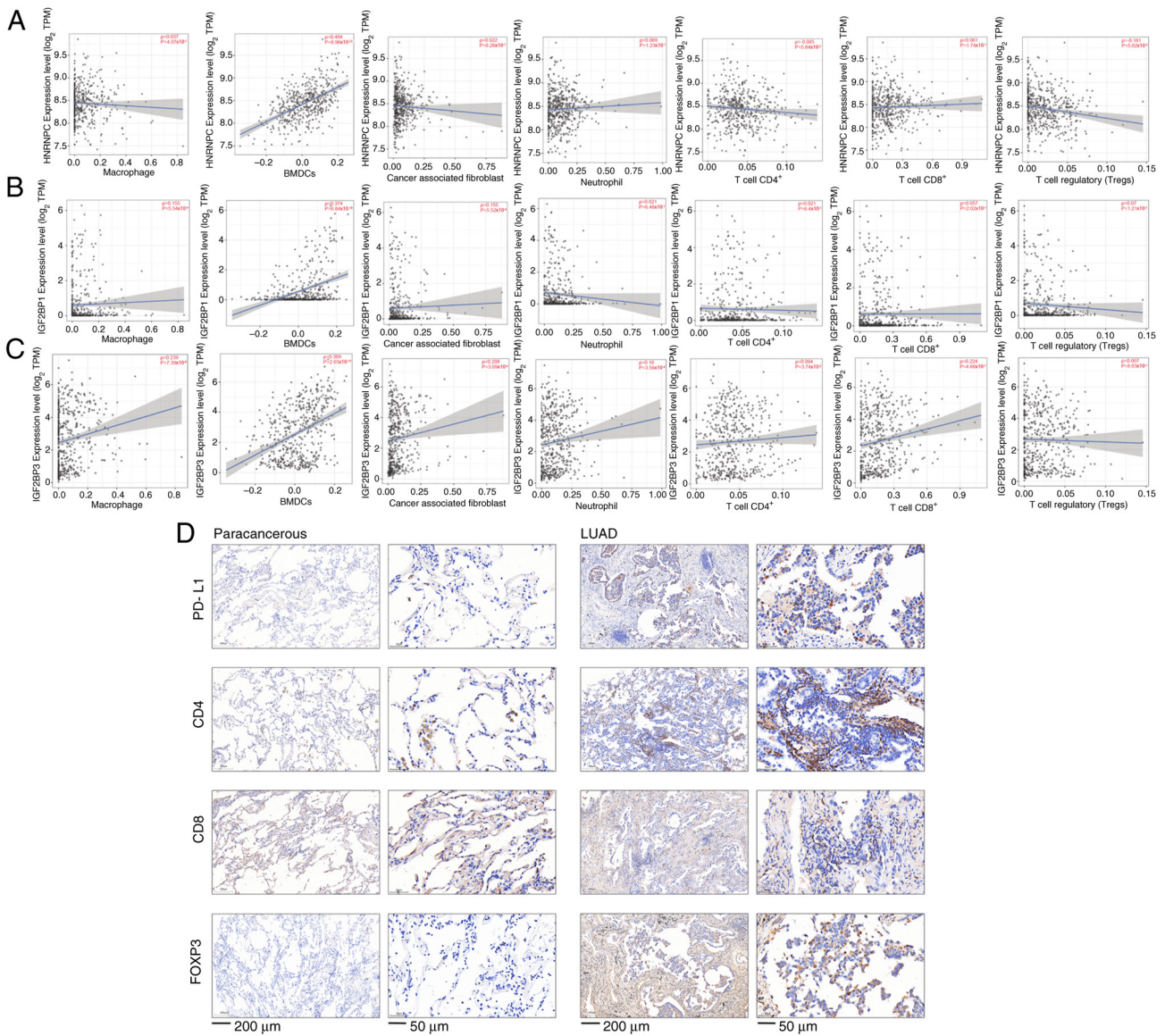


Figure 7. Correlation analysis between m6A gene expression and immune infiltration in LUAD. (A) Bone marrow dendritic cells and regulatory T cells were significantly positively and negatively correlated with HNRNPC, respectively. (B) Cancer-associated fibroblasts, BMDCs and macrophages were significantly positively correlated with IGF2BP1. (C) IGF2BP3 was positively correlated with cancer-associated fibroblasts, BMDCs, neutrophils, CD8⁺ T cells, and macrophages, but negatively correlated with CD4⁺ T cells. (D) Immunohistochemical detection of PD-L1, CD4, CD8 and FOXP3 expression in LUAD (scale bar, 200 and 50 μm) and paracancerous tissues (scale bar, 200 and 50 μm). m6A, N6-methyladenosine; LUAD, lung adenocarcinoma; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP, insulin-like growth factor 2 mRNA binding protein; BMDCs, bone marrow dendritic cells; PD-L1, programmed cell death-ligand 1; FOXP3, Forkhead box P3; TPM, transcripts per million.

explore the effect of m6A regulatory factors on tumor immune response, the correlation between m6A regulatory factors and the expression of immunoregulatory factors was assessed. The present results demonstrated that HNRNPC and IGF2BP1 were negatively correlated with immunosuppressants (Fig. 9A), immunostimulators (Fig. 9B) and MHC molecules (Fig. 9C), whereas IGF2BP3 was negatively correlated with immunostimulators and MHC molecules and positively correlated with immunosuppressants.

Enrichment analysis of the hub gene and its related gene pathways. To identify the genes co-expressed with HNRNPC, IGF2BP1 and IGF2BP3, a Pearson's correlation analysis of m6A genes from TCGA expression matrix was performed. $r > 0.5$ and $P < 0.001$ were used for screening in the subsequent

analysis. A total of 276 genes exhibiting co-expression with HNRNPC were identified, along with 22 for IGF2BP1 and 80 for IGF2BP3. To investigate the downstream pathways of hub m6A regulators in LUAD, GO and KEGG (26–28) analyses were performed using coexpression genes of the three m6A regulators. The present study demonstrated that HNRNPC was mainly enriched in the ‘ribonucleoprotein complex biogenesis’ pathway (Fig. 10A), IGF2BP1 in the ‘mitotic cell cycle checkpoint’ pathway (Fig. 10B) and IGF2BP3 in the ‘nuclear division’ pathway (Fig. 10C).

Discussion

Globally, LUAD is one of the leading causes of cancer-related mortality (29). Numerous studies have confirmed that genomic

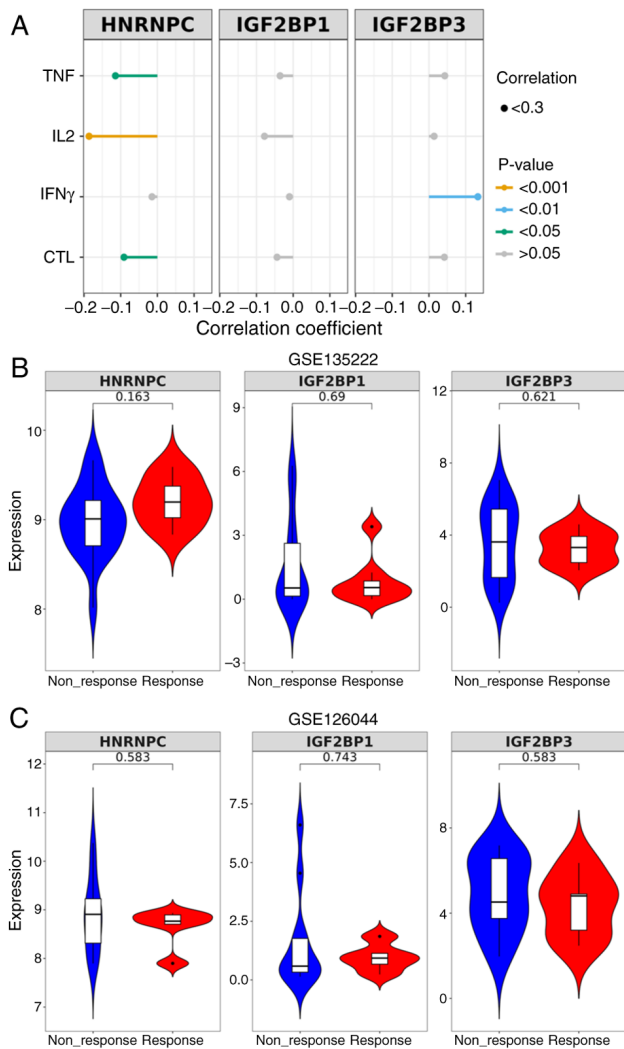


Figure 8. Correlation between m6A gene expression and T cell exhaustion in LUAD. (A) The correlation between m6A gene expression and biomarkers of T cell exhaustion (TNF, IL-2, IFN- γ and CTL) was analyzed based on previous research datasets (PMID, 37284314 and 37284314). (B) Expression levels of m6A genes in patients treated with anti-PD-1/PD-L1 based on a GEO dataset (dataset no. GSE135222). (C) Expression levels of m6A genes in patients treated with anti-PD-1/PD-L1 based on a GEO dataset (dataset no. GSE126044). GEO, Gene Expression Omnibus; m6A, N6-methyladenosine; LUAD, lung adenocarcinoma; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP, insulin-like growth factor 2 mRNA binding protein; PD-L1, programmed cell death-ligand 1; PD-1, programmed cell death protein-1; PMID, PubMed identifier; CTL, cytotoxic T lymphocytes.

and epigenetic changes can facilitate tumor occurrence and progression (30), such as DNA methylation (31). For example, modifications of m6A, one of the most modified mRNAs, are considered to affect tumor proliferation, invasion and metastasis. Zhou *et al* (32) demonstrated that m6A alteration was associated with a pathologic stage in clear cell renal cancer. A previous study by Lin *et al* (33) revealed that METTL3, a modified form of M6A, stimulated the growth and mobility of gastric cancer cells.

The present study assessed whether m6A-related genes could serve as novel biomarkers for LUAD. An examination of TCGA database revealed that the expression levels of certain m6A-associated genes, including YTHDF3, YTHDC1, YTHDC2 and IGF2BP2, was significantly different between

the tumor and control groups ($P < 0.001$). Pearson correlation analysis showed that YTHDF3 had the strongest correlation with KIAA1429 ($r = 0.75$, $P < 0.001$). Luo *et al* (34) reported that YTHDF3 could upregulate the transcription stability of PD-L1 mRNA and accelerate NSCLC immune evasion by targeting CD8 $^{+}$ T lymphocytes. Yuan *et al* (35) suggested YTHDC1 as a tumor progression suppressor in LC and a ferroptosis regulator that functions by modulating ferroptosis suppressor protein 1 mRNA stability. YTHDC2 was found to be suppressed (36). In the present study, further investigation of the somatic interactions between m6A genes and the status of SNPs and highly mutated genomic regions revealed that IGF2BP1, RBM15, IGF2BP2, IGF2BP1 and KIAA1429 had significant co-expression frequencies. Furthermore, the present study results indicated that high expression levels of HNRNPC, IGF2BP1, IGF2BP2, IGF2BP3 and METTL5 were associated with a poor prognosis in LUAD. Similarly, another previous study reported that IGF2BP2 promoted angiogenesis and metastasis in LUAD via exosome-mediated transfer to endothelial cells, where it stabilized FMS-related receptor tyrosine kinase 4 mRNA via m6A modification and activated the PI3K-AKT pathway (37). High expression levels of METTL5 were associated with a poor prognosis in LUAD and the METTL5-associated prognostic signature served as an independent biomarker with immune implications (38).

HNRNPC expression was compared in different clinical parameters, including tumor stage and the TNM stages. The results demonstrated a statistically significant difference between stage 1 and 4 and between the normal group and the four stages in the T, M and N stages. Furthermore, a correlation was observed between m6A genes and immune cell infiltration levels in LUAD. BMDCs and Tregs indicated significant positive and negative correlations with HNRNPC, respectively. The present study indicated that HNRNPC promotes NSCLC progression and metastasis by stabilizing m6A-modified transcription factor activating enhancer binding protein-2 α mRNA (39). A correlation analysis between m6A genes and the expression of immunomodulators also demonstrated that HNRNPC and IGF2BP1 were negatively correlated with immunosuppressants, immunostimulators and MHC molecules. Hu *et al* (40) also reported that IGF2BP1 upregulates budding uninhibited by benzimidazoles 1 mitotic checkpoint serine/threonine kinase B expression via m6A modification to drive malignant progression, stemness and immune resistance in NSCLC stem cells. The GO and KEGG enrichment analyses in the present study demonstrated that HNRNPC was mainly enriched in the 'ribonucleoprotein complex biogenesis' pathway, IGF2BP1 in the 'mitotic cell cycle checkpoint' pathway and IGF2BP3 in the 'nuclear division' pathway. A previous study by Fujiwara *et al* (41) suggested that IGF2BP3 drives malignancy in early-stage LUAD by controlling microRNA structural diversity. The present study identified m6A RNA methylation regulators (HNRNPC, IGF2BP1 and IGF2BP3) as novel prognostic markers and immune modulators in LUAD and therefore provides a bridge between epigenetic regulation and tumor immunology. We hypothesize that small-molecule inhibitors targeting m6A 'writers' (for example, METTL3) or 'readers' (for example, IGF2BPs) might enter clinical trials within the next 5 years.

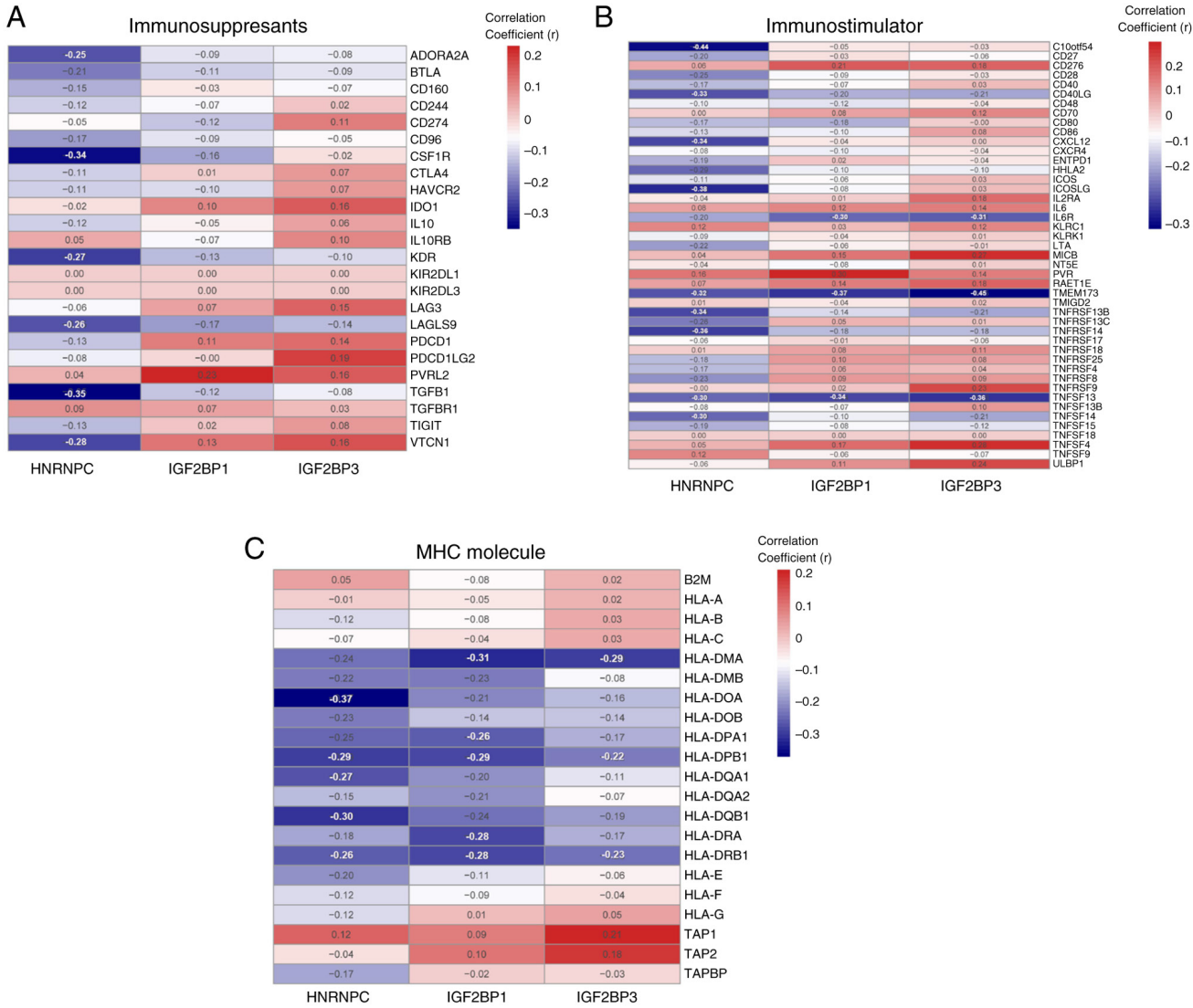


Figure 9. Correlation between m6A gene expression and the expression of immunomodulatory factors in LUAD. HNRNPC and IGF2BP1 were negatively correlated with (A) immunosuppressants, (B) immunostimulators and (C) MHC molecules, whereas IGF2BP3 was negatively correlated with immunostimulators and MHC molecules and positively correlated with immunosuppressants. m6A, N6-methyladenosine; LUAD, lung adenocarcinoma; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP, insulin-like growth factor 2 mRNA binding protein; MHC, major histocompatibility complex.

In the current study, m6A-related genes were found to be partially expressed in LUAD and may serve as potential diagnostic and prognostic indicators. There are three categories of enzymes involved in RNA modification, including ‘writers’, ‘erasers’ and ‘readers’, which modify RNA via the m6A modification mechanism (17,42). Proteins that act as m6A ‘readers’ recognize m6A modifications by binding specific domains and this binding leads to RNA splicing, mRNA decay and translation regulation. (43,44). While it is assumed that m6A-related molecules manipulate the progression and deterioration of human cancer types via those mechanisms, current understanding of the mechanistic relationship between m6A and human cancer remains limited. The findings of the present study suggested an association between m6A-related genes and immune infiltration; however, the correlation lacks experimental validation, a limitation attributable to the retrospective nature and exclusive use of public genomic datasets in the current work, which prevents the confirmation of causal relationships between m6A-related genes and immune infiltration. Therefore, investigating how m6A

contributes to tumor progression is warranted in future research and can potentially provide novel insights into cancer therapy and drug development.

In conclusion, the present study identified HNRNPC, IGF2BP1 and IGF2BP3 as novel immune-related prognostic m6A regulators in LUAD, which holds notable potential in improving patient risk stratification and guiding therapies targeting m6A pathways. However, their precise mechanisms in modulating LUAD immunity and progression remain unclear and it is necessary to focus on functional validation in models in future research. The field may evolve by integrating multi-omics approaches within the tumor microenvironment and potentially advance the development of targeted inhibitors against these specific regulators, paving the way for novel LUAD treatments in the future.

Future studies with larger clinical cohorts are warranted to experimentally validate the predicted correlations between m6A-related genes and immune markers (for example, PD-L1, CD8⁺ T cells) identified in the present bioinformatics analysis.

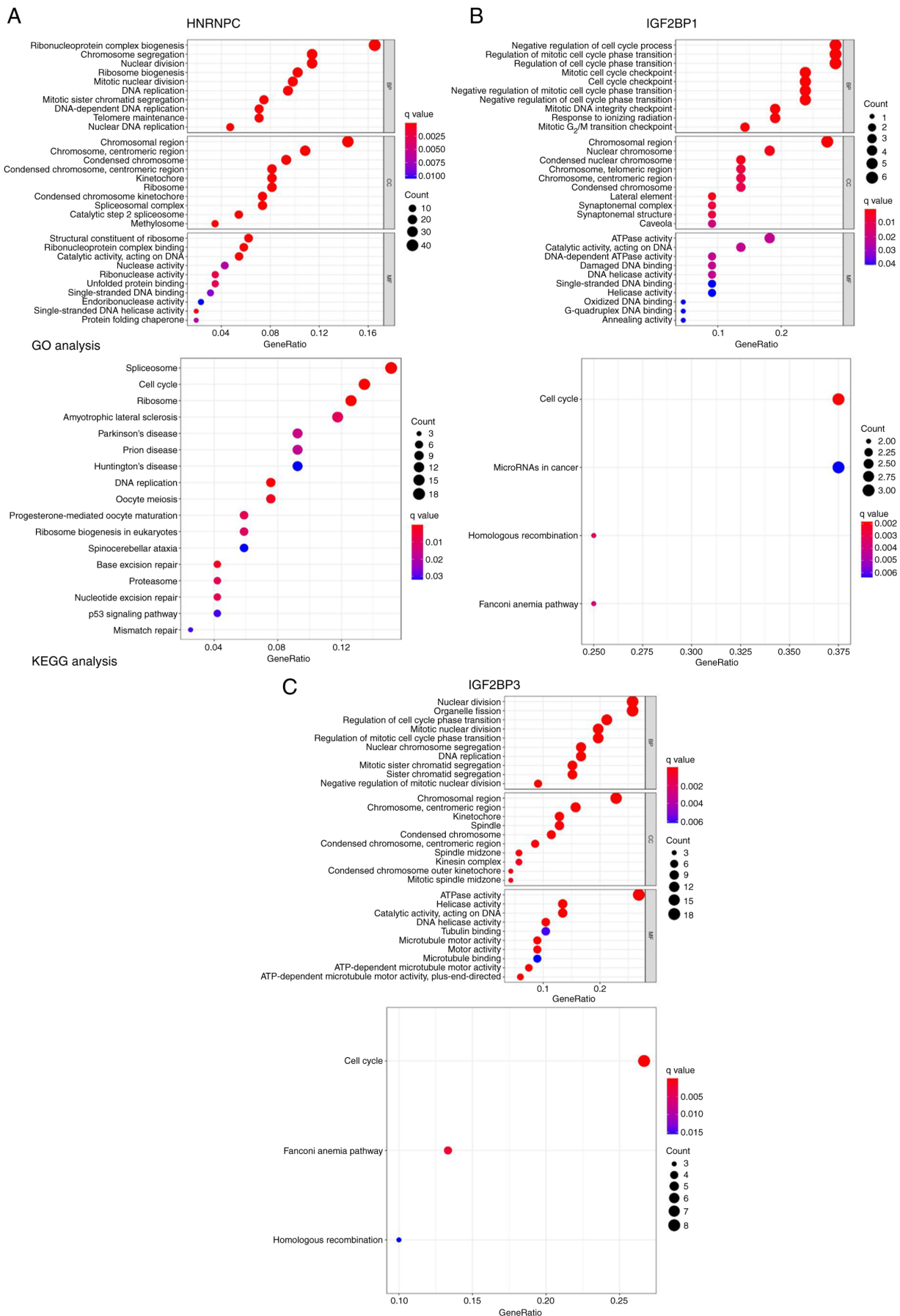


Figure 10. Enrichment analysis of the hub gene and its related gene pathways. (A) HNRNPC was mainly enriched in the 'ribonucleoprotein complex biogenesis' pathway. (B) IGF2BP1 was mainly enriched in the 'mitotic cell cycle checkpoint' pathway. (C) IGF2BP3 was mainly associated with the 'nuclear division' pathway. HNRNPC, heterogeneous nuclear ribonucleoprotein C; IGF2BP, insulin-like growth factor 2 mRNA binding protein; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Acknowledgements

Not applicable.

Funding

The present study was funded by Guangzhou Science and Technology Planning Project (grant no. 2023A03J0491), High-level Hospital Construction Project (grant no. DFJH201801), Guangdong Provincial People's Hospital Young Talent Project (grant no. GDPPHYTP201902), GPDH Scientific Research Funds for Leading Medical Talents and Distinguished Young Scholars in Guangdong Province (grant no. KJ012019449), Guangdong Basic and Applied Basic Research Foundation (grant no. 2019B1515130002), and National Science Foundation of China (grant no. 81872510).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JY and JC conceptualized and devised the methodology for the present study. XC, YL and ZC conducted the formal analysis and investigation. XC and JY prepared the original draft of the manuscript. JC obtained funding and reviewed and edited the manuscript. JY and XC obtained resources. JY and JC confirm the authenticity of the data in the present study. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures performed in the present study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki. The present study was approved by the ethical committee of Guangzhou Twelfth People's Hospital (approval no. 2021065; Guangzhou, China). Informed consent was obtained from all individual participants included in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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