Characterization of m⁶A RNA Methylation Regulators Predicts Survival and Immunotherapy in Lung Adenocarcinoma

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N⁶-methyladenosine (m⁶A) RNA modification is a reversible mechanism that regulates eukaryotic gene expression. Growing evidence has demonstrated an association between m⁶A modification and tumorigenesis and response to immunotherapy. However, the overall influence of m⁶A regulators on the tumor microenvironment and their effect on the response to immunotherapy in lung adenocarcinoma remains to be explored. Here, we comprehensively analyzed the m⁶A modification patterns of 936 lung adenocarcinoma samples based on 24 m⁶A regulators. First, we described the features of genetic variation in these m⁶A regulators. Many m⁶A regulators were aberrantly expressed in tumors and negatively correlated with most tumor-infiltrating immune cell types. Furthermore, we identified three m⁶A modification patterns using a consensus clustering method. m⁶A cluster B was preferentially associated with a favorable prognosis and enriched in metabolism-associated pathways. In contrast, m⁶A cluster A was associated with the worst prognosis and was enriched in the process of DNA repair. m⁶A cluster C was characterized by activation of the immune system and a higher stromal cell score. Surprisingly, patients who received radiotherapy had a better prognosis than patients without radiotherapy only in the m⁶A cluster C group. Subsequently, we constructed an m⁶A score model that qualified the m⁶A modification level of individual samples by using principal component analysis algorithms. Patients with high m⁶A score were characterized by enhanced immune cell infiltration and prolonged survival time and were associated with lower tumor mutation burden and PD-1/CTLA4 expression. The combination of the m⁶A score and tumor mutation burden could accurately predict the prognosis of patients with lung adenocarcinoma. Furthermore, patients with high m⁶A score exhibited greater prognostic benefits from radiotherapy and immunotherapy. This study demonstrates that m⁶A modification is significantly associated with tumor
INTRODUCTION

Lung cancer remains one of the most difficult-to-treat cancers, and its morbidity and mortality are rising rapidly (1). Lung adenocarcinoma (LUAD) accounts for approximately 40% of all lung cancers (2). Driver genes in LUAD include RTKs (aberrantly expressed), EGFR/KRAS (mutations), ALK (rearrangement), and others (3–6). Despite recent advances in surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, the prognosis of patients with LUAD is still unsatisfactory (7). LUAD is a complicated disease with complex pathogenesis and high heterogeneity (8). Therefore, having a good understanding of the molecular mechanisms underlying LUAD is necessary for the selection of optimal therapeutic strategies.

N6-methyladenosine (m6A) RNA modification has recently been identified as a regulatory mechanism for controlling eukaryotic gene expression (9). As a dynamic reversible epigenetic modification, m6A modification exists in mRNAs, microRNAs, circular RNAs, and long noncoding RNAs, accounting for 80% of all RNA methylation modifications in eukaryotic cells (10). m6A modification is mediated by three subtypes of regulatory proteins: methyltransferases (writers), binding proteins (readers), and demethylases (erasers) (11). The modification is mainly regulated by the following components: writers, which catalyze m6A methylation, such as methyltransferase-like 3/14/16 (METTL3/14/16) (12–14), zinc finger CCCH domain-containing protein 13 (ZC3H13) (15), ELAV-like RNA-binding protein 1 (ELAVL1) (16), Cbl proto-oncogene-like 1 (CBLL1) (17), RNA-binding motif protein 15/15B (RBM15/15B) (17, 18), WT1-associated protein (WTAP) (19), and VIR-like m6A methyltransferase associated (KIAA1429) (20). Erasers are proteins involved in maintaining the balance of the m6A content in the transcriptome and include fat mass and obesity-associated protein (FTO) (21) and AlkB homolog H5 (ALKBH5) (22). Readers are proteins that recognize the m6A consensus motif (DRACH) and promote stimulatory and inhibitory effects on translation dynamics, such as YTH domain family 1/2/3 (YTHDF1/2/3) (23, 24), YTH domain containing 1/2 (YTHDC1/2) (25, 26), IGF2 mRNA-binding proteins 1/2/3 (IGF2BP1/2/3) (27, 28), Fragile X mental retardation 1 (FMR1) (29), leucine-rich pentatricopeptide repeat containing (LRPPRC) (29), heterogeneous nuclear ribonucleoprotein C (HNRNPC) (30), and heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1) (31). m6A regulators posttranscriptionally modify RNA molecules and are associated with many biological processes, including carcinogenesis, immune response, cell differentiation, neurodevelopment, and stress responses (9). In addition, m6A mRNA modification may play a significant role in the occurrence and development of human cancers (32), such as lung cancer, hepatic cell carcinoma, and glioblastoma. METTL3 directly promotes YAP translation and increases its activity, which induces resistance to nonsmall cell lung cancer drugs and metastasis (33). Moreover, the upregulation of WTAP contributes to hepatocellular carcinoma tumorigenesis by repressing ETS1 expression (34). Furthermore, the m6A demethylase ALKBH5 maintains the tumorigenicity of stem-like cells by supporting cell proliferation and FOXM1 expression in glioblastoma (35). However, the relationship between m6A modulators and tumors, especially immunotherapy, remains unclear. Therefore, further elucidation of m6A regulatory factors could provide an attractive perspective for cancer therapy (36).

Immune checkpoint therapy has shown unprecedented efficacy for various malignancies by boosting the immune system to fight cancer (37). Immune checkpoints refer to a plethora of inhibitory or stimulatory molecules that maintain self-tolerance, prevent autoimmunity, and control the duration and extent of immune responses, which are hijacked by cancer cells to evade immune eradication (38–40). Immune checkpoint inhibitors (ICIs) target these checkpoints and show remarkable clinical efficacy in a broad spectrum of tumors. Unfortunately, only a considerable proportion of patients receive clinical benefits from ICIs (41). In recent years, many studies have demonstrated the correlation between tumor-infiltrating immune cells and m6A modification patterns, which cannot be explained by RNA degradation mechanisms. Wang et al. reported that the inhibition of m6A modification could enhance the response to anti-PD-1 therapy in pMMR-MSI-L CRC and melanoma (42). ALKBH5 gene expression and mutation status are correlated with response to immunotherapy in melanoma patients, demonstrating that m6A erasers influence the therapeutic effects of immunotherapy (43). However, the overall impact of all m6A regulators on the immune microenvironment and their effect on the response to immunotherapy are still unclear.

In this study, we analyzed genomic information from 936 patients with LUAD to determine their methylation modification patterns. In addition, we constructed an m6A score model to quantify the m6A modification patterns of individual tumors and predict the clinical response to ICI treatment. Our findings

Keywords: m6A, lung adenocarcinoma, tumor microenvironment, tumor-infiltrating immune cells, tumor mutation burden, radiotherapy, immunotherapy
clarify the important role of m^6^A methylation in LUAD and provide clues for improving the efficiency of current immune therapies, which will contribute to the selection of an effective personalized immunotherapy strategy.

**MATERIALS AND METHODS**

**Data Source and Processing**

The expression matrices and corresponding clinical characteristics of LUAD samples were obtained from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. We excluded patients without survival, information, or incomplete clinicopathological characteristics from further assessment. A total of 936 patients were included, encompassing the cohorts GSE68465 (N = 438) and TCGA-LUAD (N = 498). The four validation databases were downloaded from the GEO database, including GSE11969, GSE13213, GSE37745, and GSE50081. The expression matrix data of the TCGA-LUAD cohort (FPKM format) were downloaded from the Genomic Data Commons platform, and FPKM units were converted to transcripts per kilobase million (TPM) units. The “Normalized Between Arrays” function of the R package “limma” was performed for data standardization. Genome mutation data of TCGA-LUAD (including somatic mutation and copy number variation (CNV)) were downloaded from the UCSC Xena platform. Based on previous studies, we collected 24 m^6^A regulators, including 10 writers (CBL11, ELAVL1, METTL3, METTL14, METTL16, KIAA1429, RBM15, RBM15B, WTA, and ZC3H13), two erasers (ALKBH5 and FTO), and 12 readers (YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, FMR1, HNRNPA2B1, HNRNPC, IGF2BP1, IGF2BP2, IGF2BP3, LRPPRC). Clinicopathological information and clinical immunotherapy scores (IPS) were obtained from the TCI database.

**m^6^A Modification Pattern**

Based on mRNA expression levels, 19 m^6^A regulators were extracted from the TCGA-LUAD and GSE68465 cohorts, and the samples were divided into diverse subtypes based on transcriptome data with the R package “Consensus Cluster Plus.” A thousand repetitions were performed to guarantee the stability of the classification (44).

**Pathway Enrichment Analysis**

To investigate the difference between m^6^A modification patterns in the biological process, we explored the variation in signaling pathways between each of the two subtypes of m^6^A regulators by using “Gene set variation analysis (GSVA)” R packages (45). We downloaded the gene set file “c2.cp.kegg.v7.4.symbols” from the MSigDB database for the GSVA analysis. An adjusted p-value of less than 0.05 was considered statistically significant.

**Analysis of Immune Cell Infiltration**

To estimate the relative abundance of 23 immune cell types in the tumor microenvironment of LUAD, single-sample gene set enrichment analysis (ssGSEA) was used to calculate the enrichment scores and represent the relative abundance of each tumor-infiltrating immune cell type in each sample. The set of genes used to label each tumor-infiltrating immune cell type was obtained from the study by Charoentong (46).

**Identification of Differentially Expressed Genes Among Subtypes of m^6^A Regulators**

The patients were divided into three subtypes according to the expression level of 19 m^6^A regulators. We identified differentially expressed genes (DEGs) among different subtypes using the empirical Bayesian method in the R package “limma,” and selected p-values less than 0.001 as DEG candidates for further analysis (47).

**Construction of the m^6^A Score Model**

To quantify the level of m^6^A modification in a single tumor, we established an m^6^A score model by performing principal component analysis (PCA). The procedure for the established m^6^A score model was as follows: first, overlapping DEGs were identified from different m^6^A clusters, and significant prognosis-related genes were identified by univariate Cox regression analysis; second, PCA was performed on the gene expression profile, and the principal components 1 and 2 were extracted as feature scores. This method minimized the deprivation of information contained in the original index and reduced the indicators to be analyzed, thereby allowing a comprehensive analysis of the collected data; lastly, the m^6^A score was defined by performing a formula similar to that used in previous studies (48, 49). m^6^A score = Σ (PC1i + PC2i), where i is the expression of m^6^A phenotype-related genes.

**Statistical Analysis**

All statistical analyses were performed with R software (version 4.0.3). The R package “limma” was used for differential gene expression analysis. Spearman’s correlation analysis was used to calculate the correlation coefficients between the levels of different tumor-infiltrating immune cell types and the expression of m^6^A regulators. Kruskal-Wallis and one-way analysis of variance were used to compare differences between more than two groups. The Kaplan-Meier (KM) method was used to draw the survival curve (5-year survival rate), and univariate Cox regression analysis was used to calculate the hazard ratios (HRs) for m^6^A regulators and m^6^A phenotype-related genes. LUAD samples were divided into high and low m^6^A score subgroups using the “surv-cutpoint” function in the R package “survival.”

**RESULTS**

**Genetic m^6^A Regulator Variation in LUAD**

Twenty-four m^6^A RNA methylation regulators were selected for LUAD according to previous studies, including 10 writers, 12 readers, and two erasers (Table S1). We summarized the
dynamic reversible epigenetic modification behavior of these m^6^A regulators and their biological functions in RNA, including mRNA export, mRNA translation, mRNA decay, and mRNA degradation/stability (Figure 1A). Somatic mutations in m^6^A regulators were found in 151 (26.63%) of 567 samples. ZC3H13 had the highest mutation frequency, followed by KIAA1429 and IGF2BP1, while no CBL1 mutations were found in the samples (Figure 1B). In addition, YTHDC1 was significantly positively correlated with ZC3H13, YTHDC2, FMR1, and HNRNPA2B1 (Supplementary Figure S1B). There was widespread CNV in the 24 regulatory factors; METTL16, RBM15B, METTL14, ELAVL1, and RBM15 had copy number losses, while YTHDF1, KIAA1429, FMR1, IGF2BP2, and METTL3 showed numerous copy number gains (gene amplification) (Figure 1C). The locations of the CNVs in the 24 m^6^A regulators were labeled on the chromosomes (Figure 1D). Tumor samples were distinguished from normal samples by three-dimensional PCA (3D-PCA) of the 24 m^6^A regulators. The results showed that the two groups were completely separated from each other (Figure 1E). We then compared mRNA expression in normal and tumor samples to explore whether the expression levels of the m^6^A regulators were affected by the above gene variation. Seventeen of the 24 m^6^A regulators showed significant overexpression or downregulation in LUAD samples. YTHDF1, KIAA1429, HNRNPC, and METTL3 were most significantly upregulated in tumor samples, and METTL16 and METTL14 were markedly downregulated (Figure 1F). These results showed that the CNV was an important factor in controlling the expression of m^6^A regulators. Most m^6^A regulators underwent remarkable expression changes in LUAD, suggesting that the abnormal status of m^6^A regulators is involved in the development of LUAD.

Identification of m^6^A Modification Patterns in LUAD

Meta-analysis was performed using TCGA-LUAD and GEO (GSE68465) datasets to further elucidate the modification characteristics of the m^6^A regulators in LUAD. This algorithm finally identified 19 m^6^A regulators through data consolidation. To examine whether the 19 m^6^A regulators could be used as prognostic markers for LUAD, we used univariate Cox regression analysis (Supplementary Figures S1C and S2A–S). Eight of the 19 genes were significantly correlated with prognosis, including HNRNPA2B1, HNRNPC, IGF2BP2, IGF2BP3, LRPPRC, RBM15, WTAP, and ZC3H13 (HR >1). The prognostic significance of 19 m^6^A regulators in LUAD, the connection to the regulator, and the interactions are shown in the m^6^A regulator network (Figure 2A). The results showed a remarkable correlation among writers, readers, and erasers, and this crosstalk was essential for the generation of different m^6^A modification modes. The landscape also suggested that the occurrence and progression of LUAD are related to m^6^A regulators. Effective immune infiltration in tumors is considered a key factor in carcinogenesis and prognosis (50, 51). The correlations between individual regulators and each tumor-infiltrating immune cell type were analyzed using Spearman’s correlation analysis (Figure 2B). All m^6^A regulators were significantly correlated with some types of tumor-infiltrating immune cells, suggesting that m^6^A regulators are critical for immune infiltration in tumors. Most m^6^A regulators were significantly positively correlated with immune infiltration, while WTAP, an m^6^A methyltransferase, was negatively correlated.

Based on the expression of the 19 m^6^A regulators, the R package “Consensus Cluster Plus” classified patients into various m^6^A modification patterns. Consequently, the unsupervised consensus clustering algorithm revealed that patients were well defined when k = 3 (Supplementary Figure S3A). Thus, three distinct patient clusters were identified based on m^6^A modification patterns, including 253 cases of cluster A, 324 cases of cluster B, and 359 cases of cluster C. The Kaplan-Meier analysis of the three clusters revealed that cluster A had the worst prognosis, while cluster B had a significant survival advantage (Figure 2C). Next, the expression levels of the m^6^A regulators in the three clusters were analyzed; the expression profiles of most m^6^A regulators significantly differed among the three clusters. Cluster A was upregulated compared with the other two clusters, including CBL11, ELAVL1, FMR1, HNRNPA2B1, HNRNPC, IGF2BP2, IGF2BP3, LRPPRC, METTL3, RBM15, RBM15B, WTAP, YTHDF1, and ZC3H13 (Supplementary Figure S3B). The expression levels of IGF2BP2 and IGF2BP3 were significantly downregulated in cluster B but upregulated in cluster A (Figure 2D). These results indicate that IGF2BP2 and IGF2BP3 are the dominant risk factors for malignant progression. Based on the KEGG gene set, GSVA enrichment analysis was used to explore the biological processes of the two m^6^A modification patterns (Figure 2E; Supplementary Figures S3C, D). We found that cluster A was clearly enriched in the process of DNA repair, such as nucleotide excision repair, mismatch repair, DNA replication, and base excision repair; cluster B was prominently enriched in metabolism-associated pathways, such as fatty acid metabolism, amino acid metabolism, arachidonic metabolism, drug metabolism cytochrome P450, and primary bile acid biosynthesis. Cluster C was enriched in pathways associated with immune activity, including intestinal immune network production, antigen processing and presentation, and cytokine receptor interaction.

Immune Landscapes With Distinct m^6^A Modification Patterns

Subsequently, the correlation between 23 tumor-infiltrating immune cell types and three m^6^A cluster subsets was examined by ssGSEA analysis. The results showed that cluster C was associated with more adaptive and innate immune cell infiltration, including CD8 cells, NK cells, Tregs, MDSCs, macrophages, and B cells (Figure 3A). Consistently, cluster C exhibited a comprehensively elevated expression of MHC molecules (Figure 3B). Immune cell infiltration in the three m^6^A cluster subsets was assessed using the ESTIMATE algorithm. The results showed that cluster C exhibited higher immune and stromal scores, indicating that cluster C had
significantly increased immune cell infiltration (Figures 3C, D), which was mainly due to increased immune infiltration and high MHC expression.

**m6A Phenotype-Related DEGs in LUAD**

To further elucidate the underlying biological processes and functional annotation of a single m6A modification pattern, seventy-three m6A phenotype-related DEGs were identified using the R package “Limma” and represented on a Venn diagram (Figure 4A). GO enrichment analysis for DEGs was performed using the R package “ClusterProfiler.” Surprisingly, these genes were remarkably related to nuclear division and organelle fission (Supplementary Figure S3E). To further validate this regulatory mechanism, we obtained 68
**FIGURE 2** | m^6^A methylation patterns and related biological processes. (A) The interplay among the m^6^A regulators in LUAD. The m^6^A regulators in three RNA modification types were indicated by the different colors in the circle left. Favorable factors for patients' survival were indicated by grass green in the circle right and risk factors indicated by blue in the circle right. The circle size indicates the influence of each regulator on prognosis, and the range of values calculated by Log-rank test was represented by the size of each circle. The lines connecting the regulators show their interplay, and the thickness indicates the strength of the association between the regulators. Negative correlation was marked with blue and positive correlation with red. (B) Analysis of the relationship between each tumor-infiltrating immune cell type and each m^6^A regulator in LUAD using Spearman's analysis. Red indicates positive correlation; blue indicates negative correlation. *p < 0.05, **p < 0.01. (C) Survival analyses for the three m^6^A modification patterns in from TCGA-LUAD and GSE68645, including 208 cases of m^6^A cluster A, 240 cases of m^6^A cluster B, and 282 cases of m^6^A cluster C. Log-rank test, p < 0.0001. (D) Heatmap showing the correlation between the three m^6^A clusters and the clinicopathological characteristics. Clinicopathological information including age, gender, fustat, and tumor stage, as well as the m^6^A cluster, is shown in annotations above. Red represents high expression, and blue represents low expression. (E) Heatmap showing the biological processes in different m^6^A modification patterns obtained by GSVA enrichment analysis. Red shows activated pathways and blue shows inhibited pathways. m^6^A cluster A vs. m^6^A cluster B.
prognosis-related DEGs using univariate Cox regression analysis. Unsupervised cluster analysis was performed on the 68 prognosis-related DEGs, which were divided into three subgroups: gene clusters a, b, and c. We performed PCA on the expression of the 68 DEGs, showing that the three groups were completely separated from each other (Figure 4B). The results showed 226, 193, and 311 patients in gene clusters a, b, and c, respectively. Patients in gene cluster a were associated with a worse prognosis, patients in gene cluster b had a better prognosis, and patients in gene cluster c had a moderate prognosis (Figure 4C). m6A regulator expression was significantly different between the three gene clusters, which was the same as that of the m6A modification patterns (Figure 4D).

**Construction of an m6A Score Model**

To predict the immune status and prognosis in a single patient, we sought to develop an m6A score based on the 68 DEGs identified. Therefore, patients were divided into high- or low-m6A score groups based on the cutoff value. The high m6A score group had a better clinical survival profile (Figure 5A). In addition, we externally verified m6A score model to predict the prognosis of patients with lung adenocarcinoma from GSE11969, GSE13213, GSE37745, and GSE50081 datasets (Supplementary Figures S4A–D). An alluvial diagram was used to illustrate the workflow of the m6A score construction and to visualize the attribute changes in individual patients (Figure 5B). The results indicated that gene cluster b was associated with a high m6A score, whereas gene cluster c was associated with a lower m6A score. Notably, most patients who were still alive were included in the high m6A score group. Most patients with m6A cluster A were defined as low m6A, while patients with m6A cluster B had a high m6A score. The m6A cluster C was similarly distributed (Figure 5C). The group with a statistically low m6A score had more advanced patients (Figure 5D). We also examined the relationship between the above subtypes and the m6A score. Kruskal–Wallis analysis revealed that m6A cluster B and gene cluster b exhibited the highest m6A score, while m6A cluster A and gene cluster a showed the lowest score (Figures 5E, F). Multimodel crossvalidation suggested that the m6A score could serve as a prediction model for LUAD. Based on Spearman’s analysis, a heat map demonstrated a positive correlation between the m6A score and tumor-infiltrating immune cells (Figure 5G). Furthermore, the high m6A score group also exhibited a comprehensively elevated expression of MHC molecules (Figure 5H). These results indicated that the m6A score may be used as a model to predict the immune status in LUAD.
Characteristics of Tumor Somatic Mutations in Patients From the High and Low m6A Score Groups

Several studies have confirmed correlations between tumor somatic mutations, genomic alterations, and immunotherapeutic effects. Therefore, we evaluated the distribution of the tumor mutation burden (TBM) in different m6A score groups. A box and scatter diagram showed that the high m6A score group had a lower TMB and that the m6A score was negatively correlated with TMB (Figures 6A, B). K-M survival analysis showed that TMB alone was not sufficient to accurately predict the prognosis of LUAD (Figure 6C). To further understand the relationship between TMB, m6A score, and survival outcomes, a K-M survival analysis based on the combination of m6A score and TMB was performed. The results revealed that the low TMB and low m6A score subgroups were associated with poor prognosis. The combination of the m6A score and TMB could accurately predict the quality of life of patients with LUAD (Figure 6D). These data emphasize the impact of the m6A score and TMB on cancer development. A list of the somatic mutations in the high- and low-m6A score groups is shown (Figures 6E, F). The low m6A score group presented more extensive TMB than the high m6A score group, except for KRAS (22% vs. 31%).

Strong Association of the m6A Score With Clinicopathological Characteristics in LUAD

We examined the relationship between the m6A score and clinicopathological characteristics, and observed that the m6A score significantly differed between patients by stage, node (N), but not tumor (T) and metastasis (M) (Figures 7A–D). In this study, the prognostic value of the m6A score was determined for different clinicopathological characteristics, and it was found that patients with high m6A score had significantly longer overall survival than patients with low m6A score in stages I/II, T1/2, N0, and M0 (Figures 7E–L). Therefore, we considered the m6A score to be a possible prognostic factor for LUAD.

The Role of the m6A Score in Predicting Benefits From Radiotherapy and Immunotherapy

The prognostic value of the m6A score was investigated for patients with LUAD who accepted radiotherapy. Surprisingly, only in the m6A cluster C group did patients with radiotherapy had a better quality of life than those without radiotherapy (Figures 8A–C). Interestingly, patients with radiotherapy had a
better quality of life than those without radiotherapy in the high m6A score group. The survival advantage from radiotherapy in the low m6A score group was virtually zero (Figures 8D, E).

Furthermore, the expression levels of PD-1 and CTLA4 were examined, and a remarkable elevation was observed in the low m6A score group (Figures 8F, G). The levels of other immune checkpoints were then compared in the high and low m6A score groups. The high m6A score group exhibited higher expression of CD27, CD28, LGALS9, TNFSF14, and TNFSF18, while the low m6A score group had higher expression of CD70, IDO1, LAG3, PDCD1LG2, and PVR (Figure 8H). In addition to the well-known TMB and checkpoint, IPS is one of the newly discovered predictive factors that is widely used and is strongly suggested to assess patients’ reaction to immunotherapy (52, 53). Compared with patients with low m6A score, patients with high m6A score exhibited significant clinical benefits from anti-PD-1/CTLA4
immunotherapy (Figures 8I–L). These findings confirmed that the levels of tumor m\(^6\)A modification modes play a significant role in the regulation of the expression of immune molecules.

**DISCUSSION**

m\(^6\)A methylation, the most common form of mRNA modification, plays an indispensable role in posttranscriptional regulation. In recent years, increasing studies have demonstrated the importance of m\(^6\)A modification in congenital immunity and inflammation, and its antitumor effects through cooperation with unequal m\(^6\)A regulators (54–56). Many studies have focused on single m\(^6\)A regulators or tumor-infiltrating immune cell types; however, the association between overall tumor microenvironment characteristics and integrated m\(^6\)A regulators remains poorly understood. Therefore, the distinction of inverse m\(^6\)A modification modes in tumors will contribute to understanding the relationship between m\(^6\)A regulators and the antitumor immune response. Here, we constructed a prognostic model for effective therapeutic strategies.

Based on the presentation level of 21 m\(^6\)A regulators, this study revealed three m\(^6\)A modification modes with different characteristics. The m\(^6\)A cluster A was characterized by a poor prognosis and enrichment in the process of DNA repair. The m\(^6\)A cluster B was characterized by a favorable prognosis and enrichment in metabolism-associated pathways. The m\(^6\)A cluster C was characterized by activation of the immune system and a higher stromal cell score. Previous studies have reported that the immune microenvironment plays a key role in tumor evolution and immunotherapy (57). The characteristics of tumor immune infiltration, including the activity of CD4 and CD8 T cells,
macrophages, and natural killer cells, are associated with immunotherapeutic efficacy (58–60). Here, we verified that the m^6^A cluster C was significantly associated with elevated immune cell permeation and high stromal cell infiltration. Previous studies demonstrated that tumors with immune-excluded phenotype showed the presence of abundant immune cells, whereas these immune cells do not penetrate the parenchyma but instead are retained in the stroma that surrounds nests of tumor cells (61). Moreover, stromal cells affect the killing effect of IL-12 delivery, empowering CAR-T immune infiltrating cells (62). Thus, it was not surprising to find that m^6^A cluster C aroused congenital immunity but a poor prognosis.

DEGs that discerned disparate m^6^A modification patterns were deemed to be m^6^A phenotype-related gene signatures. Parallel to the m^6^A clustering construction, three gene cluster types were constructed according to the m^6^A-related DEGs, which were significantly related to distinct clinical outcomes and landscapes of immune infiltration. These findings suggest that m^6^A modification is involved in tumorigenesis, tumor development, and immune cell infiltration. To qualify the m^6^A

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**FIGURE 7** Relationship between the m^6^A score and different clinical characteristics. (A–D) Box plot showing differences in m^6^A score among patients with different clinical characteristics. (A) Stages I–II vs. stages III–V; (B) T 1–2 vs. T 3–4; (C) N 1–3 vs. N 0; (D) M 0 vs. M 1+X. (E–I) Kaplan-Meier curves showing the differences in survival depending on the m^6^A score and different clinical characteristics. (E) Stages I–II; (F) Stages III–V; (G) T 1–2; (H) T 3–4; (I) N 0; (J) N 1–3; (K) M 0; (L) M 1+X. The asterisks represented the statistical p-value (ns p > 0.05; * p < 0.05; ** p < 0.01).
FIGURE 8 | The m6A score model predicts the benefits of radiotherapy and immunotherapy. (A–C) Kaplan-Meier curves of overall survival for patients in m6A cluster A (A), m6A cluster B (B), and m6A cluster C (C) based on acceptance/rejection of radiotherapy. Log-rank test. (D, E) Kaplan-Meier curves of overall survival for patients with a high (D) low (E) m6A score based on acceptance/rejection of radiotherapy. Log-rank test. (F, G) Comparison of the PD-1 (F)/CTLA4 (G) expression levels between the high and low m6A score groups. Log-rank test. *p > 0.05; **p < 0.05; ***p < 0.01. (H) Differences in the expression of other immune checkpoints between the high and low m6A score groups. Log-rank test. *p < 0.05; **p < 0.01; ***p < 0.001. (I–L) Box plot representing the relative distribution of the immunophenoscore between the low and high m6A score groups in LUAD patients based on TCGA database, (I) CTLA4+ PD1−; (J) CTLA4+ PD1+; (K) CTLA4− PD1−; and (L) CTLA4− PD1+.

The asterisks represented the statistical p-value (*p < 0.05; **p < 0.01; ***p < 0.001).
advantages in a fraction of patients with high m6A score, more addition, since immunotherapy showed strong clinical

collection; therefore, a future cohort of patients with LUAD to the small number of samples and the clinical heterogeneity of

promoting the clinical success of immunotherapy. However, due provides a basis for improving current immune therapies and

understanding of the tumor microenvironment and the

expression of PD-1 and CTLA4. These findings provide new insights into the relationship between tumor-infiltrating immune cells and cancer immunotherapy, and increase our capacity to select clinical immunotherapy strategies.

We compiled a list of 24 identified m6A regulators; however, newly recognized regulators must be integrated into the model to achieve the highest precision with the m6A modification patterns. Furthermore, the m6A modification patterns and m6A score were distinguished by employing retrospective data collection; therefore, a future cohort of patients with LUAD accepting immunotherapy is required to confirm our findings. In addition, since immunotherapy showed strong clinical advantages in a fraction of patients with high m6A score, more clinical cases and tumor types should be introduced into the predicted models to increase precision.

CONCLUSION

In conclusion, we systematically analyzed m6A modification patterns among 936 LUAD samples considering 24 m6A regulators, and comprehensively evaluated their prognostic value and correlation with tumor-infiltration immune cell characteristics. The comprehensive analysis of individual tumor m6A modification patterns will greatly enhance our understanding of the tumor microenvironment and the characterization of immune cell infiltration. This study provides a basis for improving current immune therapies and promoting the clinical success of immunotherapy. However, due to the small number of samples and the clinical heterogeneity of the study cohort, large-scale cohort studies and prospective studies are necessary to verify the predictive value of the m6A score in the clinical treatment and prognosis of LUAD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Guangzhou Medical University Tumor Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Medical Ethics Committee of Guangzhou Medical University Tumor Hospital. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MZ and YC have contributed equally to this work. MZ, YL, XZ, and QY designed the experiments. MZ, YL, and QY wrote the paper. MZ, YC, QM, JZ, TZ, YX, ZW, and DS analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.782551/full#supplementary-material

Supplementary Figure S1 | The correlation and prognostic analysis of 19 m6A regulators. (A) Overview of this analysis. (B) Correlation analyses for 19 m6A regulators. Red indicates positive correlation; blue indicates negative correlation. (*P < 0.05; **P < 0.01; ***P < 0.001). (C) Prognostic analyses for 19 m6A regulators using univariate Cox regression analysis. Hazard ratio >1 indicated risk factors for survival and represent by red, hazard ratio <1 indicated risk factors for survival and represented by blue.

Supplementary Figure S2 | Prognostic analysis of 19 m6A regulators. (A–S). Kaplan-Meier survival curves are used to analyze the survival difference between high and low gene expression groups of 19 m6A regulators. High gene expression groups indicated by red. Low gene expression groups indicated by blue.
Supplementary Figure S3 | Consensus clustering of 24 m6A regulators in lung adenocarcinoma. (A) Consensus clustering matrix for k = 3. (B) Expression of the 24 m6A regulators in the three m6A clusters. The upper and lower ends of the boxes represented interquartile range of values. The lines in the boxes represented median value, and black dots showed outliers. The asterisks represented the statistical p value (*P< 0.05; **P< 0.01; ***P< 0.001). (C, D) Heatmap showing the biological processes in different m6A modification patterns obtained by GSEA enrichment analysis. Red shows activated pathways and blue shows inhibited pathways. B m6A cluster A vs m6A cluster C; C m6A cluster B vs m6A cluster C. (E) GO enrichment analysis of the 73 m6A phenotype-related DEGs.

Supplementary Figure S4 | External validation of the m6A score model in lung adenocarcinoma patients from GSE11969, GSE13213, GSE37745, and GSE50081 datasets. (A) Kaplan-Meier curves showing the differences in survival of the high and low m6A score groups in GSE11969 (P = 0.002, Log-rank test). (B) Kaplan-Meier curves showing the differences in survival of the high and low m6A score groups in GSE13213 (P = 0.002, Log-rank test). (C) Kaplan-Meier curves showing the differences in survival of the high and low m6A score groups in GSE37745 (P = 0.032, Log-rank test). (D) Kaplan-Meier curves showing the differences in survival of the high and low m6A score groups in GSE50081 (P < 0.001, Log-rank test).

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### GLOSSARY

| Term                | Definition                                           |
|---------------------|------------------------------------------------------|
| N6mA                | N6-methyladenosine                                   |
| LUAD                | lung adenocarcinoma                                  |
| METTL3              | methyltransferase-like 3                             |
| METTL14             | methyltransferase-like 14                            |
| METTL16             | methyltransferase-like 16                            |
| ZC3H13              | zinc finger CCCH domain-containing protein 13        |
| ELAVL1              | ELAV-like RNA binding protein 1                      |
| CBLL1               | Cbl proto-oncogene-like 1                            |
| RBM15               | RNA-binding motif protein 15                         |
| RBM15SB             | RNA-binding motif protein 15B                        |
| WTAP                | WT1-associated protein                               |
| KIAA1429            | VIR-like m6A methyltransferase associated            |
| FTO                 | fat mass and obesity-associated protein              |
| ALKBH5              | AlkB homolog H5                                     |
| YTHDF1              | YTH domain family 1                                  |
| YTHDF2              | YTH domain family 2                                  |
| YTHDF3              | YTH domain family 3                                  |
| YTHDC1              | YTH domain containing 1                              |
| YTHDC2              | YTH domain containing 2                              |
| IGF2BP1             | IGF2 mRNA-binding proteins 1                         |
| IGF2BP2             | IGF2 mRNA-binding proteins 2                         |
| IGF2BP3             | IGF2 mRNA-binding proteins 3                         |
| RMR1                | Fragile X mental retardation 1                       |
| LRPPRC              | leucine-rich pentatricopeptide repeat containing     |
| HNRNPC              | heterogeneous nuclear ribonucleoprotein C            |
| HNRNPA2B1           | heterogeneous nuclear ribonucleoprotein A2/B1        |
| NMF                 | nonnegative matrix factorization                     |
| ssGESA              | single sample GSEA                                   |
| PCA                 | principal component analysis                         |
| TME                 | tumor microenvironment                               |
| TMB                 | tumor mutation burden                                |
| ICIs                | immune checkpoint inhibitors                         |
| TOGA                | The Cancer Genome Atlas                              |
| GEO                 | Gene Expression Omnibus                              |
| GSVA                | gene set variation analysis                          |
| DEGs                | differentially expressed genes                       |
| HR                  | hazard ratios                                         |
| CNV                 | copy number variation                                |
| 3D-PCA              | three-dimensional principal component analysis       |
| KEGG                | Kyoto Encyclopedia of Genes and Genomes             |
| MHC                 | major histocompatibility complex                     |
| MSI                 | microsatellite instability                           |
| IPS                 | immunoscore                                          |
| B cell              | activated B cell                                     |
| CD4 cell            | activated CD4 T cell                                 |
| CD8 cell            | activated CD8 T cell                                 |
| DC                  | activated dendritic cell                             |
| CD56+ NK cell       | CD56bright natural killer cell                       |
| CD56− NK cell       | CD56dim natural killer cell                          |
| eosinophil          | eosinophil                                           |
| γδ T cells          | gamma delta T cell                                   |
| iB cell             | immature B cell                                      |
| IDC                 | immature dendritic cell                              |
| NK cells            | natural killer cell                                  |
| pDC                 | plasmacytoid dendritic cell                          |
| Treg                | regulatory T cell                                    |
| Thf                 | T follicular helper cell                             |
| Th1 cell            | T helper 1 cell                                      |
| Th2 cell            | T helper 2 cell                                      |
| Th17 cell           | T helper 17 cell                                     |