m⁶A regulator expression profile predicts the prognosis, benefit of adjuvant chemotherapy, and response to anti-PD-1 immunotherapy in patients with small-cell lung cancer

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Abstract

Background: Small cell lung cancer (SCLC) is lethal and possesses limited therapeutic options. Platinum-based chemotherapy—with or without immune checkpoint inhibitors (anti-PDs)—is the current first-line therapy for SCLCs; however, its associated outcomes are heterogeneous. N⁶-methyladenosine (m⁶A) is a novel and decisive factor in tumour progression, chemotherapy resistance, and immunotherapy response. However, m⁶A modification in SCLC remains poorly understood.

Methods: We systematically explored the molecular features and clinical significance of m⁶A regulators in SCLC. We then constructed an m⁶A regulator-based prognostic signature (m⁶A score) based on our examination of 256 cases with limited-stage SCLC (LS-SCLC) from three different cohorts—including an independent cohort that contained 150 cases with qPCR data. We additionally evaluated the relationships between the m⁶A score and adjuvant chemotherapy (ACT) benefits and the patients’ responses to anti-PD-1 treatment. Immunohistochemical (IHC) staining and the HALO digital pathological platform were used to calculate CD8+ T cell density.
Background
Small cell lung cancer (SCLC) is the most lethal high-grade neuroendocrine malignancy and features fast growth, early metastasis, and drug resistance. SCLC accounts for about 15% of all lung cancers; however, it has the highest mortality and worst outcomes—with a 5-year survival of < 7% [1, 2]. Regrettably, therapeutic strategies for SCLC have not significantly improved over recent decades. Conventional platinum-based chemotherapy remains the first-line treatment for patients with SCLC. Meanwhile, there have been few improvements in our ability to combat chemotherapy resistance for patients with SCLC [3]. Given the favourable achievements of immune checkpoint blockade (ICB) therapy for various tumours, this type of immunotherapy may be useful for SCLC treatment [4, 5]. Notably, a significant proportion of patients with ICB therapy resistance cannot benefit from such novel treatment [6-8]. Because of this, accurate and timely screening for patients who are more likely to benefit from immunotherapy is important.

PD-L1 expression is a classical biomarker for immunotherapy in various tumours, which is usually low or absent in SCLC. Consequently, it may fail to function as an immunotherapeutic biomarker [9, 10]. Therefore, there is an urgent and unmet need for a reliable predictive biomarker to guide the clinical application of chemotherapy and immunotherapy in patients with SCLC.

Dysregulation of epigenetic modifications relates to progression and treatment resistance in SCLC [11]. N6-methyladenosine (m6A) is the most prevalent type of RNA modification in eukaryotic cells [12], is responsible for various RNA-related biological processes—including RNA decay, stabilization, translation, splicing, and exportation—and ultimately regulates target gene expression [13]. Modification of m6A is a dynamic, multi-layered, and reversible process regulated by m6A methyltransferases, demethylases, and binding proteins [14]. Aberrant expression of m6A regulators appears closely related to carcinogenesis, tumour development, and immunological abnormalities [15, 16]. Multiple studies have revealed that m6A dysregulation dramatically enhances chemotherapy resistance in various tumours [17, 18]. Moreover, some m6A regulators can affect the response to immunotherapy [19, 20]. Increasing evidence suggests that m6A regulators are promising prognostic biomarkers which help determine chemotherapy and immunotherapy resistance. As the relevant research continues, these regulators’ relevance to a variety of tumours has been gradually confirmed [21, 22]. However, to the best of our knowledge, almost nothing is known about the roles of these m6A regulators in SCLC.

We examined the expression profiles, molecular characteristics, and prognostic significance of m6A regulators in SCLC. As early screening for lung cancer continues, the proportion of patients with limited-stage SCLC (LS-SCLC) is expected to similarly increase. We examined 265 cases with LS-SCLC from three independent cohorts and constructed an m6A regulator-based prognostic risk stratification score (m6A score) for patients with LS-SCLC. We additionally investigated the relationship between m6A score and adjuvant chemotherapy (ACT) benefit and response to anti-PD-1 treatment. Our findings may advance our ability to create individualized therapy regimens and guide SCLC prognostication.

Methods
Clinical samples and SCLC tissue specimens
We downloaded the training cohort somatic mutations and expression profiles (the international cohort) from...
Cbioportal (https://www.cbioportal.org/study/summary?id=sclc_uccologne_2015). During data processing, all RNA-seq data was subjected to log2 transformation. The mean expression values were selected when targeting genes that had more than one probe. We chose the GSE40275 database to explore the expression profile of m^6A regulators, both in normal and LS-SCLC tissues. This dataset was downloaded from the Gene Expression Omnibus (GEO) dataset (https://www.ncbi.nlm.nih.gov/geo/) via the GPL15974 platform. Two validation cohorts—including the Shanghai cohort (GSE60052) downloaded from the GEO dataset and the National Cancer Centre (NCC) cohort, collected from the National Cancer Centre of China from January 2009 to November 2017—were collected. The NCC cohort included 150 LS-SCLC samples with formalin-fixed paraffin-embedded (FFPE) archived tissues were collected during surgeries. All enrolled patients from the NCC cohort were pathologically reconfirmed, had no other tumours, and carried clinically confirmed diagnoses of LS-SCLC. The relapse-free survival (RFS) and overall survival (OS) were defined as the day of surgical removal to recurrence, metastasis, or latest follow-up and the day of surgical removal to the date of death or latest follow-up. The study was approved by the Ethics Committee Board of our institute. The demographic and clinicopathologic parameters of the 150 LS-SCLC samples are displayed in Table 1.

Collection of samples with anti-PD-1 treatment
We included 14 patients with SCLC who received sequential chemotherapy and anti-PD-1 treatment in our hospital from April 2019 to January 2021. Their baseline biopsy FFPE specimens before immunotherapy were collected. The RECIST V1.1 Criteria were used to evaluate the response to therapy.

Immuochemistry and quantification of CD8+ T cells
The 4-μm FFPE slides were subjected to immunohistochemical staining. After deparaffinization and rehydration with high-concentration ethanol and pure water, the slides were incubated in 3% H_2O_2 or 15 min to block endogenous peroxidase activity. Then, the slides were subjected to heat antigen retrieval and non-specific site blocking using an EDTA buffer (PD 9.0) and 10% standard serum, respectively. Next, the slides were incubated overnight at 4 °C. The final counterstaining was performed using secondary antibodies (CD8, Abcam, ab17147, 1:100) and the 3, 3′-diaminobenzidine (DAB, Dako, Glostrup, Denmark) and haematoxylin.

The digital pathological system (HALO) was utilized to quantify the density of CD8+ T cells on the whole slides. We scan the slides images at high resolution (×400) using the Pannoramic MIDI II slide scanner (3DHISTECH). The tumour regions were identified by a trained pathologist (LYX). The “Membrane IHC

### Table 1 Clinical characteristics of the patients from multiple institutions

| Characteristics | International cohort (N = 68) | Shanghai cohort (N = 47) | NCC cohort (N = 150) |
|-----------------|-----------------------------|------------------------|---------------------|
| Age, years      |                             |                        |                     |
| < 60            | 16 (23.53%)                 | 26 (55.32%)            | 81 (54.00%)         |
| ≥ 60            | 52 (76.47%)                 | 21 (44.68%)            | 69 (46.00%)         |
| Sex             |                             |                        |                     |
| Male            | 48 (70.59%)                 | 42 (89.36%)            | 119 (79.33%)        |
| Female          | 20 (29.41%)                 | 5 (10.64%)             | 31 (20.67%)         |
| Smoking history |                             |                        |                     |
| Yes             | 64 (94.12%)                 | 32 (68.09%)            | 94 (62.67%)         |
| No              | 3 (4.41%)                   | 15 (31.91%)            | 56 (37.33%)         |
| NA              | 1 (1.47%)                   | 0 (0.00%)              | 0 (0.00%)           |
| SCLC staging    |                             |                        |                     |
| I               | 33 (48.53%)                 | 8 (17.02%)             | 49 (32.67%)         |
| II              | 14 (20.59%)                 | 39 (17.02%)            | 50 (33.33%)         |
| III             | 21 (30.88%)                 | 31 (65.96%)            | 51 (34.00%)         |
| OS state        |                             |                        |                     |
| Alive           | 28 (41.18%)                 | 24 (51.06%)            | 69 (46.00%)         |
| Death           | 40 (58.82%)                 | 23 (48.94%)            | 81 (54.00%)         |

Data are n (%).

NCC, National Cancer Center; NA, not available; SCLC, small cell lung cancer; OS, overall survival
Quantification module was selected for absolute counting of CD8+ T cells on the CaseViewer 2.3.

RNA isolation and quantitative RT-PCR

Only biopsies with at least 70% tumour cells were collected, and ~30-μm sections were cut from the FFPE samples. The Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher, Waltham, MA, USA) was used to isolate the FFPE tissue total RNA. The NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to quantify the extracted RNA. Meanwhile, the extracted RNA with an A260/A280 ratio between 1.8 and 2.2 were chosen for the quantitative RT-PCR analysis. We used 200 ng RNA of a 20-μL reaction to reverse transcription through the FastKing Reverse Transcription Kit (Tiangen Biotech, Beijing, China). Next, we used 1 μL cDNA for PCR reaction with quantiNova PCR Kits (Qiagen, Düsseldorf, Germany) using 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA; Indianapolis, IN). The qRT-PCR analysis was performed on all validation and independent cohort samples. The 2^ΔΔCt method was used to calculate the expressions of interested m^6A regulators. The details of the target m^6A regulators primer sequences for qRT-PCR are shown in Additional file 1: Table S1.

Construction of m^6A regulator-based signature and statistical analysis

Firstly, we screened out the m^6A regulators with prognostic significance based on the optimal cut-off point in the international cohort. We used the least shrinkage and selection operator (LASSO) Cox model to determine the most prognostic m^6A regulators; the minimum criteria were chosen during the analysis process. Lastly, the final m^6A score equation was accomplished based on the expression of the five chosen m^6A regulators and corresponding Cox regression coefficients.

R version 3.5.1 (https://www.r-project.org) was used for all data analysis and image generation. The 30 m^6A regulator protein-protein interaction analysis was conducted using the STRING interaction database and visualized using the Cytoscape software. The optimum cut-off survival analysis was completed using the "surv_cutpoint" function of the "survminer" R package. The Kaplan-Meier curve model was used to determine the prognostic value of the m^6A regulator-based signature in the training and validation sets. The R package "survival" was employed to determine if the m^6A score was an independent prognostic predictor for SCLC. The time-dependent receiver operating characteristic (ROC) curve analysis was created with the "survivalROC" R package. The Mann–Whitney U test was chosen to calculate the CD8+ T cell density between high- and low-score patients. A P-value less than 0.05 was considered statistically significant.

Results

Molecular characteristics of m^6A regulators in SCLC

After reviewing the latest relevant literature [23–26], we finally identified 30 m^6A regulators, including 11 methyltransferases (METTL3, METTL14, METTL16, METTL5, WTAP, VIRMA, RBM15, RBM15B, ZC3H13, CBL11, and ZCCHC4), 2 demethylases (ALKBH5 and FTO), and 17 binding proteins (YTHDF1, YTHDF2, YTHDF3, YTHDC1, YTHDC2, HNRNPA2B1, HNRNPC, FMR1, EIF3A, IGF2BP1, IGF2BP2, IGF2BP3, ELAVL1, G3BP1, G3BP2, PRRC2A, and RBMX) (Fig. 1A; Additional file 1: Table S1). Firstly, we explored the incidence of somatic mutations for m^6A regulators in LS-SCLC. Mutations were present in 20 of 88 samples (22.73%; Fig. 1B). FMR1 displayed the highest mutation frequency, while approximately 14 m^6A regulators exhibited no mutations within the LS-SCLC samples, including demethylases ALKBH5 and FTO (Fig. 1B). We identified co-occurrent mutations between METT3 and YTHDC2 and between HNRNPC and IGF2BP3 (Additional file 2: Fig. S1).

To determine if this genetic alteration affected the expression of m^6A regulators in LS-SCLC, we explored the mRNA expression of regulators between LS-SCLC and normal lung specimens. Principal component analysis revealed markedly different expression patterns of m^6A regulators in LS-SCLC and normal samples (Fig. 1C). We also generated a heatmap for different expression profiles of these m^6A regulators in LS-SCLC and normal tissues (Fig. 1D). The regulators’ expression details—between LS-SCLC and normal groups—are shown in Fig. 1E. Notably, almost all methyltransferases and binding proteins were upregulated in LS-SCLC; however, the two demethylases tended to exhibit lower expression in LS-SCLC than their normal counterparts. These results suggested that there may be abundant m^6A modifications in LS-SCLC and significant heterogeneity in the m^6A regulator genetic profile expression between LS-SCLC and normal lung tissues. Disordered m^6A regulator expression may be involved in tumorigenesis and SCLC development.

Association of various m^6A regulators in SCLC

Various m^6A regulators cooperatively promote tumour development. Therefore, we also tried to explore the expression relationships for the 30 m^6A regulators in SCLC. Notably, we detected remarkable correlations among the expressions of methyltransferases, demethylase, and binding proteins (Additional file 2: Fig. S2). Several significant positive correlations were also identified, including a correlation coefficient between
Fig. 1 Molecular characteristics of m⁶A regulators in LS-SCLC. A Summary of the m⁶A regulators incorporated in this study. B The mutation frequency of m⁶A regulators in LS-SCLC. C Principal component analysis of SCLC and normal lung tissues based on the expression profile of 30 m⁶A regulators from GSE40275. D The heatmap of 30 m⁶A regulator expression from GSE40275. E The expression detail of m⁶A regulators in SCLC and normal lung tissues from GSE40275.
KIAA1429 and YTHDF3 as high as 0.820 (Fig. 2A). Our protein-protein interaction network analysis determined that these 30 m6A regulators were in frequent communication (Fig. 2B). Importantly, the methyltransferases exhibited the most frequent interactions. Various methyltransferases formed a protein complex to perform biological functions. Therefore, the crosstalk among multiple m6A regulators may be actively involved in the SCLC development and progression.

Clinical significance of m6A regulators in SCLC
Next, we investigated the clinical significance of m6A regulators in patients with LS-SCLC based on the optimal cut-off point derived from the international cohort. Most regulators were significantly associated with survival (Fig. 2C). Some regulators exhibited procarcinogenic properties, such as RBM15, RBM15B, ALKBH5, IGF2BP3, and PRRC2A. Some regulators functioned as tumour suppressors, including METTL5, YTHDC2, and G3BP1. Higher expression levels of these regulators often conveyed a favourable clinical prognosis. Given that most regulators exhibited clinical significance, we felt that an m6A regulator-based prognostic signature (m6A score) may be a useful molecular model for LS-SCLC. Therefore, using the LASSO Cox model, we included the above 22 regulators and determined five significant candidates—G3BP1, METTL5, ALKBH5, IGF2BP3, and RBM15B—for the subsequent m6A score creation (Fig. 2D, E).

Establishment of the m6A score for LS-SCLC
Based on the expression levels of these five regulators and corresponding coefficients, we constructed the m6A score system for patients with LS-SCLC. The formula is as follows: m6A score = (0.0906 × G3BP1 expression) + (0.4096 × METTL5 expression) − (0.6365 × ALKBH5 expression) − (0.0912 × IGF2BP3 expression) − (0.0660 × RBM15B expression). We calculated the m6A scores for each patient and classified them into high- (m6A score ≥ 1.271) and low-score (m6A score < 1.271) groups according to the optimal cut-off point (Fig. 3A). The principal component analysis revealed high heterogeneity between the high- and low-score groups (Fig. 3B). The Kaplan-Meier survival curve results indicated that high-score patients suffered significantly worse OS (Fig. 3C, hazard ratios (HR) 5.19, 95% confidence interval (CI) 2.75–9.77, P < 0.001). To evaluate the prognostic performance of the m6A score, a time-dependent ROC analysis was conducted. The m6A score achieved area under the curve (AUC) values of 0.672, 0.812, and 0.793 for predicting OS within the international cohort at 1, 3, and 5 years, respectively (Fig. 3D). Further ROC analysis revealed that the m6A score performed better than clinicopathological characteristics for predicting OS (Fig. 3E). The C-index of the m6A score reached 0.881. This indicated that the prognostic accuracy of the m6A score was also higher than other clinicopathological parameters (Fig. 3F).

Validation of the m6A score in multiple cohorts
To further assess the reliability and robustness of the classifier, we incorporated another two independent cohorts of 197 samples for validation, including the Shanghai cohort (N = 47) and the NCC cohort (N = 150). The cohorts’ clinicopathological features are presented in Table 1. Using the same formula, the two cohorts were divided into low- and high-score groups. Firstly, we tested the m6A score in the Shanghai cohort. As expected, the signature showed excellent repeatability and stability during validation (Fig. 4A). The high-score patients in the Shanghai cohort suffered shorter OS than low-score patients (Fig. 4B, P = 0.006). The AUCs were 0.652, 0.733, and 0.731 for predicting 1-, 3-, and 5-year OS in the Shanghai cohort, respectively (Fig. 4C). In the Shanghai cohort, both the m6A score (C-index = 0.862) and SCLC staging (C-index = 0.880) accurately predicted OS for patients with LS-SCLC (Fig. 4D).

The clinical expressability of the m6A score was further evaluated in the FFPE specimens from the NCC cohort. Here, the low-score patients tended to have a significant better clinical outcomes in terms of OS (Fig. 4E, F, P < 0.001), and the m6A score achieved AUCs of 0.794, 0.691, 0.686 at 1-, 3-, and 5-year OS, respectively (Fig. 4G). Also, the C-index of the m6A score for OS was up to 0.769 and higher than other factors in the NCC cohort (Fig. 4H).

We evaluated the predictive performance of the m6A score for RFS in patients with SCLC (Fig. 4I). The high m6A score was also predictive of poorer RFS in the NCC cohort (Fig. 4I, P < 0.001). The AUCs of m6A score for 1-, 3-, and 5-year RFS predictions were 0.713, 0.682, and 0.695, respectively, and the C-index was 0.748 in the NCC cohort (Fig. 4K, L). Thus, the m6A score was superior to the TNM system and sufficiently reliable to predict prognosis in patients with SCLC—both for OS and RFS.

We additionally explored the prognostic significance of the m6A score in relationship to various clinicopathological features—including sex, age, and smoking history. Because the sample size of the Shanghai cohort was small, we only performed clinical subgroup analyses on the international and NCC cohorts. As illustrated in Additional file 2: Fig. S3-S4, in the international cohort, low-score cases achieved longer OS and RFS across all clinical subtypes, including male, female, smoker, older (age ≥ 60), and younger (age < 60) (P < 0.05). The same results were obtained during the NCC cohort validation (Additional file 2: Fig. S3-S4, P < 0.05).
Fig. 2 The clinical significance of m^6A regulators in LS-SCLC. A Correlation between the expression of each m^6A regulator in LS-SCLC. Negative correlations are marked with blue, and positive correlations are marked with red. The scatter plot indicates the highest correlation coefficient group (YTHDF3 and KIAA1429, Pearson R = 0.820). B Protein-protein interactions among the m^6A regulators. C Forest plot of the association between m^6A regulators and prognosis in SCLC. D 100-fold cross-validation for tuning parameter selection in the LASSO model. E LASSO coefficient profiles of the most useful prognostic regulators.
Fig. 3 (See legend on next page.)
The m^6A score was an independent prognostic predictor in LS-SCLC

To confirm whether the m^6A score is an independent predictor of prognosis in SCLC, we carried out univariate and multivariate Cox regression analyses on three independent cohorts. Sex and the m^6A score were significantly related to OS in the international cohort; staging and the m^6A score were also correlated with the prognosis in the Shanghai cohort. Age and the m^6A score were significantly associated with survival in the NCC cohort (Fig. 5A, P < 0.05). Moreover, after integrating these clinical parameters into the multivariate Cox regression analyses, the m^6A score was the only stable, independent prognostic indicator for patients with SCLC across all three cohorts (Fig. 5B, P < 0.05). Additionally, after multivariable adjustment by clinicopathological features, the m^6A score remained a significant independent prognostic factor for RFS in the NCC cohort (Additional file 1: Table S3).

The m^6A score predicts the benefits of ACT

Considering the decisive role of m^6A regulators in chemotherapy resistance, we further explored whether the m^6A score could predict ACT treatment benefit. In the international and NCC cohorts, 42 and 129 cases underwent ACT, respectively. The m^6A score divided 23 and 19 of 42 patients into high- and low-score groups in the international cohort (Fig. 6A), respectively, and divided 75 cases into the high-score group and 54 cases into the low-score group NCC cohort (Fig. 6D). Those low-score patients benefited considerably from ACT and achieved much longer OS than the high score cases in either cohort (Fig. 6A, D, both P < 0.001). Additionally, ROC curves showed that the AUCs of m^6A score for predicting ACT OS benefit were 0.768, 0.901, and 0.82, and 0.807, 0.68, and 0.67 in the international cohort and NCC cohort for 1-, 3-, and 5-year, respectively (Fig. 6B, E). Meanwhile, the C-index of the m^6A score for OS was also higher than other clinicopathological characteristics and as high as 0.956 and 0.750 in the two cohorts, respectively (Fig. 6C, F). In the NCC cohort, high-score cases suffered shorter RFS than the low-score ones (Fig. 6F, P < 0.001). The m^6A score also achieved a reliable predictive ability to stratify different RFS statuses for patients with ACT. For AUCs of 0.708, 0.683, 0.66 at 1-, 3-, and 5-year RFS, the corresponding C-index was up to 0.734 (Fig. 6G, H). Collectively, the m^6A score was able to identify those patients with SCLC most likely to benefit from ACT.

Relationship between the m^6A score and the anti-PD-1 immunotherapy response

Previous studies have demonstrated that m^6A regulators relate to anti-tumour immune effects and tumour immune microenvironment (TIME) characterizations [27]. Since the m^6A score is based on various m^6A regulators, we decided to probe the relationship between the m^6A signature and TIME features. Considering the centrality of CD8+ T cells in TIME, we explored the relationship between CD8+ T cell infiltration and the signature in SCLC. Using strict quality controls, we finally incorporated 117 FFPE samples in the NCC cohort. The density of CD8+ T cells in the tumour regions of SCLC was detected and calculated using the HALO digital pathological platform. Each patient’s m^6A score was also matched. Representative pictures of CD8+ T cell distribution from high- and low-score groups are displayed in Fig. 7A. Low-score patients tended to have more CD8+ T cell infiltration than high-score patients (Fig. 7B). In addition, the m^6A score was negatively correlated with CD8+ T cell density in SCLCs (Fig. 7C, R = −0.34, P < 0.001).

We collected the pre-treatment samples from 14 patients with SCLC who received anti-PD-1 treatment to investigate the relationship between the m^6A score and responses to immunotherapy. The overall response rate was 35.71%. Interestingly, patients with low scores seemed to benefit more from immunotherapy, while those with high scores tended to be resistant to immunotherapy (Fig. 7D, E). Meanwhile, ROC analyses indicated that the m^6A score could predict non-responders with an AUC of 0.8. The m^6A score showed superior performance than age or sex for identifying non-responders to immunotherapy (Fig. 7F).

Discussion

Recent studies have indicated that m^6A modification and multiple regulators play pivotal roles in
Fig. 4 (See legend on next page.)
tumorigenesis, tumour progression, and the anti-tumour immune response [11]. We also know that m6A regulators actively participate in mediating responses to chemotherapy and immunotherapy. Some proof-of-concept preclinical data have found that various m6A regulators inhibitors exhibit significant antitumor therapeutic potential, especially enabling dramatic increases in immunotherapy efficacy [19, 20, 28]. Therefore, the relevant mechanisms and clinical significance of m6A regulators are extremely important.

Although the functions of m6A modification and regulators in various tumours have been elucidated [21, 22], their roles and clinical values in SCLC were unknown. As our ability to detect and diagnose early-stage lung cancer increases, the proportion of LS-SCLC cases has similarly increased. We constructed an m6A regulator-based signature to predict prognosis for patients with LS-SCLC. We also explored the signature's predictive role for chemotherapy and immunotherapy in SCLC. Our findings should enhance our understanding of tumorigenesis and help inform the clinical management of this disease.

Various epigenetic abnormalities are closely associated with the malignant phenotype, aggressiveness, metastasis, and therapeutic resistance of SCLC [11]. The m6A modification is the most essential RNA modification in eukaryotic cells; however, the m6A modification is poorly explored in SCLC. In the present study, we comprehensively revealed the m6A modification patterns in SCLC and identified that aberrant expression of m6A regulators was closely involved in SCLC tumorigenesis. We also found that most m6A methyltransferases and binding proteins were remarkably upregulated, while m6A demethylases were downregulated. Thus, abundant m6A modification may play a dominant role in SCLC progression.

We additionally excluded over 22 m6A regulators closely associated with SCLC prognosis and then established a five-regulator-based m6A score to effectively divide patients with SCLC into low- and high-score groups. During this process, the LASSO model was chosen because the collinearity relationships were found among the regulators. The low-score patients exhibited a more favourable prognosis than their high-score counterparts for OS and RFS. The signature was well-validated in various validation cohorts and was identified as an independent prognostic indicator for patients with SCLC. Moreover, we have also confirmed that our signature possesses significantly superior stratification ability for multiple clinical parameters among the three multi-centre cohorts.

The m6A regulator-based signature included protective (ALKBH5, IGF2BP3, and RBM15B) and risk-enhancing (G3BP1 and METTL5) factors. ALKBH5, one of the classical m6A demethylases, decreases m6A modification in the target RNA. ALKBH5 is involved in the progression of multiple cancers, playing an oncogenic role in glioblastoma while suppressing the tumour proliferation and development in pancreatic cancer and NSCLC [29–31]. Meanwhile, a higher expression of ALKBH5 was also positively correlated with a favourable prognosis in gastric cancer; however, it was associated with worse clinical outcomes in colorectal cancer and NSCLC [32–34]. IGF2BP3 is a member of the IGF2 mRNA binding protein family—also known as the m6A binding protein—which exerts its biological functions in various human cancers [35]. IGF2BP3 functions as an oncofoetal factor in multiple tumour types, facilitating tumorigenesis by regulating the cell cycle, proliferation, and angiogenesis [36, 37]. In the previous studies, IGF2BP3 was considered a poor prognostic factor for NSCLC, prostate cancer, and endocrine system tumours [38–40]. RBM15B was classified into the m6A methyltransferase type, responsible for confirming that the m6A classical methyltransferase complex can function in specific regions. Higher expression levels of RBM15B tend to confer better clinical outcomes for patients with kidney renal cell carcinoma [41].

Among the risky candidates, G3BP1 was a novel m6A-binding protein that affects mRNA stability via an m6A modification manner. This further regulated some essential oncogenic signal pathways to promote tumour
metastasis and aggressiveness [42]. The elevated expression of G3BP1 confers a worse prognosis for patients with lung cancer after surgery [43]. METTL5 is a novel m^6^A methyltransferase, mainly adding m^6^A modification for ribosomal RNA [44]. Our earlier work found that METTL5 was significantly associated with a worse prognosis in NSCLC [45]. One small-scale study sought to determine the function of METTL5 in carcinogenesis; however, additional studies are needed.

We could also use the m^6^A score to identify patients with SCLC who were more likely to benefit from ACT. Our novel m^6^A score possessed a better predictive capacity of ACT efficacy than TNM staging. This may be useful for the individualized application of ACT in patients with SCLC. Additionally, some m^6^A regulators in the signature appeared closely associated with chemotherapy resistance. ALKBH5 can induce cisplatin resistance by decreasing the m^6^A modification on the

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**Fig. 5** Cox regression analyses of the m^6^A score across multiple centres. A Univariate Cox regression analyses of m^6^A score and clinicopathological in three independent cohorts. B Multivariate Cox regression analyses of m^6^A score and clinicopathological in three independent cohorts.
FOXM1 and NANOG transcripts and increasing their expression [46]. Also, upregulating ALKBH5 expression sensitizes pancreatic ductal adenocarcinoma cells to chemotherapy treatment, indicating that ALKBH5 may play the same role in SCLC [30]. Chen et al. reported that IGF2BP3 sustained the pluripotency in hepatocellular carcinoma (HCC) cells and triggered chemoresistance in HCC [47]. Lower expression of G3BP1 increases the chemotherapy sensitivity in gastric cancer cells and predicts favourable benefits of chemotherapy.

Fig. 6 The predictive value of the m^6A score for the benefit of adjuvant chemotherapy in different cohorts. A Kaplan-Meier curves of OS in patients with adjuvant chemotherapy in the international cohort. B ROC analysis of m^6A score for the prediction of OS at 1, 3, and 5 years in patients with adjuvant chemotherapy in the international cohort. C C-index values of m^6A score and clinicopathological parameters for OS in patients with adjuvant chemotherapy in the NCC cohort. D Kaplan-Meier curves of OS in patients with adjuvant chemotherapy in the NCC cohort. E ROC analysis of m^6A score for predicting survival at 1, 3, and 5 years in patients with adjuvant chemotherapy in the NCC cohort. F C-index values of m^6A score and clinicopathological parameters for OS in patients with adjuvant chemotherapy in the NCC cohort. G Kaplan-Meier curves of RFS in patients with adjuvant chemotherapy in the NCC cohort. H ROC analysis of m^6A score for the prediction of RFS at 1, 3, and 5 years in patients with adjuvant chemotherapy in the NCC cohort. I C-index values of m^6A score and clinicopathological parameters for RFS in patients with adjuvant chemotherapy in the NCC cohort.
Fig. 7 (See legend on next page.)
We established a comprehensive m\textsuperscript{6}A regulator program to further personalize immunotherapy application in patients with SCLC. To understand the nature of SCLC and provide some clues for developing effective anti-tumour agents, we investigated the relationship between these regulators and chemotherapy resistance in SCLC.

We discovered a relationship between the m\textsuperscript{6}A score and immunotherapy responses in SCLC. PD-L1 expression and CD8+ T cells are closely associated with the efficacy of immunotherapy on various malignancies. Notably, PD-L1 expression is typically low or absent in SCLC. Given the obvious subjectivity and uncertainty in interpreting PD-L1 expression, we finally explored the relationship between CD8+ T cells and m\textsuperscript{6}A score in SCLC [49]. As expected, the m\textsuperscript{6}A score was closely correlated with CD8+ T cells in SCLC, and patients with low scores exhibited higher CD8+ T infiltration.

Then, we investigated the potential role of the m\textsuperscript{6}A score in predicting the immunotherapy response in patients with SCLC. Consistent with the above observations, low-score patients were more likely to benefit from immunotherapy. We also noted that some signature members appeared to relate to immunotherapy efficacy, especially demethylase ALKBH5. ALKBH5 regulates the immunotherapy responses by manipulating the accumulation of suppressive immune cells in the TIME, actively modulating the infiltration of Tregs and myeloid-derived suppressor cells [50]. ALKBH5 may participate in the composition and function of CD8+ T cells in the TIME, ultimately affecting the response to immunotherapy in SCLC, while other regulators may also function in the same way. Further exploring the functions of these five m\textsuperscript{6}A regulators may help us understand the nature of SCLC and provide some clues to further personalize immunotherapy application in patients with SCLC.

To our best knowledge, this is the first systematic examination of m\textsuperscript{6}A modification patterns in LS-SCLC. We established a comprehensive m\textsuperscript{6}A regulator prognostic signature based on data obtained from over 265 patients with LS-SCLC from three centres. Large-scale retrospective SCLC analyses are exceptionally rare due to challenges in obtaining available tumour specimens within the context of standardized treatment regimens.

Our innovative signature has certain advantages. Firstly, the large size of our study cohort increases the reliability and robustness of our model. Additionally, our signature is the first molecular model to predict chemotherapy and immunotherapy efficacy for patients with SCLC based on tissue samples. This signature may therefore be useful in treating and clinically managing patients with SCLC.

In addition to these advantages, our study also possesses some limitations which warrant consideration. Firstly, we validated the NCC cohort using retrospective FFPE specimens. Future, studies should collect and examine fresh specimens in a prospective manner. Secondly, despite we did our best efforts to collect the immunotherapy samples for validation, we only included 14 patients with SCLC who received immunotherapy. This is likely insufficient for conducting a comprehensive analysis. Thirdly, given that this was a retrospective study, there is likely to be unavoidable bias and error in the analysis. Prospective, well-powered studies are needed to further validate the reliability of the signature.

**Conclusions**

In conclusion, we demonstrated the significance of m\textsuperscript{6}A modification in SCLC and developed the first and most comprehensive multicentre m\textsuperscript{6}A regulator-based prognostic signature for patients with LS-SCLC. This m\textsuperscript{6}A signature can accurately predict OS, RFS, chemotherapy benefit, and immunotherapy response in patients with SCLC. The m\textsuperscript{6}A signature can therefore serve as both a prognostic and predictive tool for SCLC. Further prospective validation of the predictive ability of the m\textsuperscript{6}A score will aid our ability to effectively treat patients with SCLC.

**Abbreviations**

ACT: Adjuvant chemotherapy; AUC: Area under the curve; CI: Confidence interval; FFPE: Formalin-fixed paraffin-embedded; GEO: Gene Expression Omnibus; HCC: Hepatocellular carcinomas; HR: Hazard ratios; ICB: Immune checkpoint blockade; LASSO: Least shrinkage and selection operator; LS-SCLC: limited-stage SCLC; m\textsuperscript{6}A: N\textsuperscript{6}-methyladenosine; NCC: National Cancer Centre; OS: Overall survival; RFS: Relapse-free survival; ROC: Receiver operating characteristic; SCLC: Small cell lung cancer; TIME: Tumour immune microenvironment

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12916-021-02148-5.
Additional file 1: Table S1. Primer sequences of the samples from the NCC cohort for qPCR. Table S2. The descriptions of the 30 mA regulators collected in this study. Table S3. Univariable and multivariable Cox regression of mA score and clinicopathological characteristics and relapse free survival in SCLC.

Additional file 2: Fig. S1. Co-occurrence of genetic alterations of the mA regulators in SCLC. Fig. S2. Correlation between the expression of mA regulators in SCLC. Fig. S3. Validation of the OS predictive performance of the mA score across clinical subgroups. Fig. S4. Validation of the RFS predictive performance of the mA score across clinical subgroups.

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Authors’ contributions
NS and JH supervised the project, designed, edited, and led the experiments. ZHZ, PW, and YJL conducted the experiments and data analysis. CQZ and ZHZ prepared all the figures and tables. ZHZ, CQZ, and YJL drafted the manuscript. CQZ, GCZ, QPZ, LDW, ZYY, LYX, BZ, HZ, FWT, QX, and SGG collected the clinical samples and provided material support. All the authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
The protocol of this study was approved by the Ethics Committee and Institutional Review Boards of Cancer Hospital, Chinese Academy of Medical Sciences, and Peking Union Medical College.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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References
1. van Meerbeeck JP, Fennell DA, De Ryuysscher DK. Small-cell lung cancer. Lancet (London, England). 2011;378(9784):1741–55.
2. Gazdar AF, Minna JD. Developing new, rational therapies for recalcitrant small cell lung cancer. J Natl Cancer Inst. 2016;108(10):dwv119. https://doi.org/10.1093/jnci/diwv119.
3. Alvarado-Luna G, Morales-Espina D. Treatment for small cell lung cancer, where are we now?-a review. Transl Lung Cancer Res. 2016;5(1):26–38. https://doi.org/10.3978/j.issn.2218-6751.2016.01.13.
4. Welsh JW, Heymach JV, Guo C, Menon H, Klein K, Cushman TR, et al. Phase I/II trial of pembrolizumab and concurrent chemoradiation therapy for limited-stage SCLC. J Thorac Oncol. 2020;15(12):1919–27.
5. Gong J, Chehrazi-Raffle A, Reddi S, Salgia R. Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. J Immunother Cancer. 2018; 6(1):8. https://doi.org/10.1186/s40425-018-0316-z.
6. Roviello G, Corona SP, Nesi G, Mini E. Results from a meta-analysis of immune checkpoint inhibitors in first-line renal cancer patients: does PD-L1 matter? Ther Adv Med Oncol. 2019;11:1758859119861905. https://doi.org/10.1177/1758859119861905.
7. Lei Q, Wang D, Sun K, Wang L, Zhang Y. Resistance mechanisms of anti-PD-1/PD-L1 therapy in solid tumors. Front Cell Dev Biol. 2020;8:672. https://doi.org/10.3389/fcell.2020.00672.
8. Zhang C, Zhang Z, Sun N, Zhang Z, Zhang G, Wang F, et al. Identification of a costimulatory molecule-based signature for predicting prognosis risk and immunotherapy response in patients with lung adenocarcinoma. Oncoimmunology. 2020;9(1):1824641. https://doi.org/10.1080/2162402X.2020.1824641.
9. Lu S, Stein JE, Rimm DL, Wang DW, Bell JM, Johnson DB, et al. Comparison of biomarker modalities for predicting response to PD-1/PD-L1 checkpoint blockade: a systematic review and meta-analysis. JAMA Oncol. 2015;5(8):1195–204. https://doi.org/10.1001/jamaoncol.2015.1549.
10. Thommen DS, Koelzer VH, Herzig P, Roller A, Trefny M, Dimeloe S, et al. A single-cell RNA sequencing atlas of the human immune system. Nature. 2017;548(7667):338–44. https://doi.org/10.1038/nature23450.
11. Khan P, Siddiquia JA, Mauya SK, Lakshmanan I, Jain M, Ganti AK, et al. Epigenetic landscape of small cell lung cancer: small image of a giant recalcitrant disease. Semin Cancer Biol. 2020. https://doi.org/10.1016/j.semcancer.2020.11.006.
12. Roundtree IA, Evans ME, Pan T, He C. DNA modification in gene expression regulation. Cell. 2017;169(7):1187–200. https://doi.org/10.1016/j.cell.2017.05.045.
13. Wang T, Kong S, Tao M, Ju S. The potential role of RNA N6-methyladenosine in Cancer progression. Mol. Cancer. 2020;19(1):88. https://doi.org/10.1186/s12949-020-01204-7.
14. Yang Y, Hsu PJ, Chen YS, Yang YC. Dynamic transcriptomic m6A decoration: writers, erasers, readers and functions in RNA metabolism. Cell Research. 2018;28(8):616–24. https://doi.org/10.1038/s41422-018-0040-8.
15. Pinello N, Sun S, Wong JJ. Aberrant expression of enzymes regulating m6A mRNA methylation: implication in cancer. Cancer Biol Med. 2018;15(4):323–34. https://doi.org/10.20892/jissn.2095-3941.2018.0365.
16. Tong J, Cao G, Zhang T, Sekif E, Ameczua Vesely MC, Broughton JP, et al. m6A mRNA methylation sustains Treg suppressive functions. Cell Res. 2018;28(6):253–6. https://doi.org/10.1038/s41422-018-0137-7.
17. Jin D, Guo J, Wu Y, Du J, Yang L, Wang X, et al. m6A mRNA methylation initiated by METTL3 directly promotes YAP translation and increases YAP activity by regulating the MALAT1-miR-191-3p-YAP axis to induce NSCLC drug resistance and metastasis. J Hematol Oncol. 2019;12(1):135. https://doi.org/10.3978/j.issn.1678-0661.2019.01.13.
18. Zhou S, Bai ZL, Xia D, Zhao ZJ, Zhao R, Wang YY, et al. FTO regulates the chemo-radiotherapy resistance of cervical squamous cell carcinoma (CSCC) by targeting β-catenin through mRNA demethylation. Mol Carcinog. 2018; 57(5):590–5. https://doi.org/10.1002/mc2.2782.
19. Li HB, Tong J, Zhu S, Bastia PJ, Duffy EE, Zhao J, et al. m6A mRNA methylation controls T cell homeostasis by targeting the IL-7/STAT5/SCDS pathways. Nature. 2017;548(7667):338–42. https://doi.org/10.1038/nature23450.
Han D, Liu J, Chen C, Dong L, Liu Y, Chang R, et al. Anti-tumour immunity controlled through mRNA m^6A methylation and YTHDF1 in dendritic cells. Nature. 2019;566(7743):270–7. https://doi.org/10.1038/s41586-019-0916-x.

Zhao X, Cui L. Development and validation of a m^6A RNA methylation regulators-based signature for predicting the prognosis of head and neck squamous cell carcinoma. Am J Cancer Res. 2019;9(10):2156–69.

Zhou J, Wang J, Hong B, Ma K, Xie H, Li L, et al. Gene signatures and prognostic values of m^6A regulators in clear cell renal cell carcinoma - a retrospective study using TCGA database. Aging. 2019;11(6):1633–47. https://doi.org/10.18632/aging.101856.

Li Y, Xiao J, Bai J, Tian Y, Yu B, Chen X, et al. Molecular characterization and clinical relevance of m^6A regulators across 33 cancer types. Mol Cancer. 2019;18(1):137. https://doi.org/10.1186/s12943-019-1066-3.

Liu J, Harada BT, He C. Regulation of gene expression by N^6-methyladenosine in cancer. Trends Cell Biol. 2019;29(6):487–99. https://doi.org/10.1016/j.tcb.2019.02.008.

Huang H, Weng H, Chen J. m^6A modification in coding and non-coding RNAs: roles and therapeutic implications in cancer. Cancer Cell. 2020;37(3):270–88. https://doi.org/10.1016/j.ccell.2020.02.004.

Nombela P, Miguel-Lopez B, Blanco S. The role of m^6A, m^6C and W^4 RNA modifications in cancer: novel therapeutic opportunities. Mol Cancer. 2021; 20(1):18. https://doi.org/10.1186/s12943-020-01263-w.

Wang L, Hui H, Agrawal K, Kang Y, Li N, Tang R, et al. m^6A RNA methyltransferases METTL3/14 regulate immune responses to anti-PD-1 therapy. EMBO J. 2020;39(20):e104514. https://doi.org/10.15252/embj.2020104514.

Wang H, Hu X, Huang M, Liu J, Gu Y, Ma L, et al. METTL3-mediated mRNA m^6A methylation promotes dendritic cell activation. Nat Commun. 2019;10(1):1898. https://doi.org/10.1038/s41467-019-09093-6.

Zhang S, Zhao BS, Zhou A, Lin K, Zheng S, Lu Z, et al. m^6A demethylase ALKBH5 maintains tumorigenesis of glioblastoma stem-like cells by sustaining FoxM1 expression and cell proliferation program. Cancer Cell. 2017;31(4):491–506.e506.

Tang B, Yang Y, Kang M, Wang Y, Wang Y, Bi Y, et al. m^6A demethylase ALKBH5 inhibits pancreatic cancer tumor growth by decreasing IF-1 RNA methylation and mediating Wnt signaling. Mol Cancer. 2020;19(1):3. https://doi.org/10.1186/s12943-019-1128-6.

Jin D, Guo J, Wu Y, Yang L, Wang X, Du J, et al. m^6A demethylase ALKBH5 inhibits tumor growth and metastasis by reducing YTHD5-mediated YAP expression and inhibiting miR-107/LATS2-mediated YAP activity in NSCLC. Mol Cancer. 2020;19(1):1. https://doi.org/10.1186/s12943-020-01161-1.

Su Y, Huang J, Hu J. m^6A RNA methylation regulators contribute to malignant progression and have clinical prognostic impact in gastric cancer. Front Oncol. 2019;9:1038. https://doi.org/10.3389/fonc.2019.01038.

Liu X, Liu L, Dong Z, Li J, Yu Y, Chen X, et al. Expression patterns and prognostic value of m^6A-related genes in colorectal cancer. Am J Transl Res. 2019;11(7):397–91.

Zhuang Z, Chen L, Mao Y, Zheng Q, Li H, Huang Y, et al. Diagnostic, progressive and prognostic performance of m^6A methylation RNA regulators in lung adenocarcinoma. Int J Biol Sci. 2020;16(11):1785–97. https://doi.org/10.7150/ijbs.39046.

Lederer M, Bley N, Schliefer H, Hüttemüller S. The role of the oncofetal IGF2 mRNA-binding protein 3 (IGF2BP3) in cancer. Semin Cancer Biol. 2014;29:3–12. https://doi.org/10.1016/j.semcancer.2014.07.006.

Yang Z, Wang T, Wu D, Min Z, Tan J, Yu B. RNA N^6-methyladenosine reader IGF2BP3 regulates cell cycle and angiogenesis in colon cancer. J Exp Clin Cancer Res. 2020;39(1):203.

Huang W, Li Y, Zhang C, Zha H, Zhou X, Fu B, et al. IGF2BP3 facilitates cell proliferation and tumorigenesis via modulation of JAK/STAT signalling pathway in human bladder cancer. J Cell Mol Med. 2020;24(2):13940–60. https://doi.org/10.1111/jcmm.16003.

Gil G, Cao L, He S, Gong Y, Song G, Li X, et al. Comprehensive analysis of m^6A regulators prognostic value in prostate cancer. Aging. 2020;12(14):14863–84. https://doi.org/10.18632/aging.105349.

Li W, Li N, Gao L, You C. Integrated analysis of the roles and prognostic value of RNA binding proteins in lung adenocarcinoma. PeerJ. 2020;8:e8509. https://doi.org/10.7717/peerj.8509.

Li K, Luo H, Luo H, Zhu X. Clinical and prognostic pan-cancer analysis of m^6A RNA methylation regulators in four types of endocrine system tumours. Aging. 2020;12(23):23931–44. https://doi.org/10.18632/aging.104064.