Evaluating the utility of an immune checkpoint-related lncRNA signature for identifying the prognosis and immunotherapy response of lung adenocarcinoma

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Lung adenocarcinoma (LUAD) is the most frequent subtype of lung cancer globally. However, the survival rate of lung adenocarcinoma patients remains low. Immune checkpoints and long noncoding RNAs are emerging as vital tools for predicting the immunotherapeutic response and outcomes of patients with lung adenocarcinoma. It is critical to identify lncRNAs associated with immune checkpoints in lung adenocarcinoma patients. In this study, immune checkpoint-related lncRNAs (IClncRNAs) were analysed and identified by coexpression. Based on the immune checkpoint-related lncRNAs, we divided patients with lung adenocarcinoma into two clusters and constructed a risk model. Kaplan–Meier analysis, Gene Set Enrichment Analysis, and nomogram analysis of the 2 clusters and the risk model were performed. Finally, the potential immunotherapeutic prediction value of this model was discussed. The risk model consisting of 6 immune checkpoint-related lncRNAs was an independent predictor of survival. Through regrouping the patients with this model, we can distinguish between them more effectively in terms of their immunotherapeutic response, tumour microenvironment, and chemotherapy response. This risk model based on immune checkpoint-based lncRNAs may have an excellent clinical value for predicting the immunotherapeutic response and outcomes of patients with LUAD.

Lung adenocarcinoma (LUAD) is one of the most common subtypes of lung cancer and it ranks first in cancer-related death1. Despite the reported efficacy of surgical techniques, radiotherapy, and chemotherapy as treatments for LUAD, the survival of patients with LUAD is still unfavourable2. In the past decade, immune checkpoints, mainly including PD-1, PD-L1, and CTLA-4, have become a promising and effective treatment strategy capable of significantly prolonging the survival of LUAD patients3. However, few biomarkers can predict the efficacy of anti-PD-1/PD-L1/CTLA-4 immunotherapy and stratify the LUAD population by its benefits. Therefore, identifying immune checkpoint-related biomarkers is pivotal for improving the therapy and prognosis of LUAD.

Programmed cell death protein 1 (PD-1), which can bind to its ligand, programmed cell death ligand 1 (PD-L1), is expressed by activated T cells4,5. The interactions of PD-L1 on tumour cells with PD-1 signalling have proven to be a potent mechanism to counter the activation of T cells during their escape from host immune responses, which elicits a vitally crucial role in an antitumor immune response6–9. Therefore, PD-1/PD-L1 inhibitors (nivolumab and pembrolizumab) were first approved by the US Food and Drug Administration (FDA) for treating melanoma10 and renal cell carcinoma11 and have also been confirmed to be a significant clinical advance for patients with lung cancer12–14. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) belongs to the CD28 receptor family, which was identified to be activated on the exterior of conventional T cells and attenuate tumour
cell proliferation by inhibiting T-cell proliferation and IL-2 secretion. The FDA also approved anti-CTLA-4 inhibitors (ipilimumab) for melanoma, non-small cell lung cancer, and other incurable tumours.

Long noncoding RNAs (lncRNAs) that exceed two hundred nucleotides in length regulate diverse biological processes and cellular functions. Accumulating studies have revealed that lncRNAs regulate tumour aggression, metastasis, treatment sensitivity, and prognosis by affecting immune cell lineages. Although immune checkpoint therapies have been developed as effective therapeutic strategies in tumour immune evasion, no research has yet been carried out on analysing the application values of lncRNAs that act as immune regulators for LUAD clinical immunotherapy.

Our study is the first to develop and validate the IClncRNA-related signature of LUAD by identifying IClncRNAs based on Pearson correlation analysis of data from TCGA-LUAD. Then, we revealed the interaction with tumour-infiltrating immune cells and tumour microenvironment scores and examined the treatment response of LUAD patients to immunotherapy and chemotherapy. Overall, our work developed a new signature that can contribute to immunotherapeutic strategies for treating patients with LUAD.

**Materials and methods**

**Data processing.** We downloaded the RNA-seq data (FPKM) and the corresponding clinicopathological characteristics from the TCGA-LUAD database. After screening for data quality, 34 healthy lung and 464 LUAD tissues were selected from individuals with an OS longer than 1 month.

**Selection of immune checkpoint genes and IClncRNAs.** The “limma” package extracted the expression data of the lncRNAs and PD1, PD-L1, and CTLA4 from the LUAD expression profile. Next, we identified IClncRNAs by Pearson correlation analysis, and a total of 75 IClncRNAs were found. The screening criteria were as follows: | Pearson R| > 0.4 and p values less than 0.001.

**Identification of LUAD subtypes.** Univariate Cox regression models were used to develop the most relevant IClncRNAs for OS of LUAD patients under the R package “survival.” We performed nonnegative matrix
factorization (NMF) clustering to analyse IClncRNAs associated with significant prognostic value. Using the R package "NMF" on the gene expression matrix, unsupervised NMF clustering procedures were executed, and the optimal cluster number was calculated based on a coexistence correlation coefficient $K = 2$.

**Construction and verification of the IClncRNA-related model.** All LUAD patients were randomly stratified into a training ($n = 232$) or testing ($n = 232$) cohort. The training group was used to identify prognostic IClncRNA-related signatures, while the testing and the entire group were used to validate its prognostic value. First, based on the IClncRNAs, we screened IClncRNAs in the training set and further screened IncRNAs distinctly related to overall survival (OS) using the R package "glmnet" impinged on a least absolute shrinkage and selection operator (LASSO)-penalized Cox regression analysis. Eventually, IClncRNAs with OS values were incorporated into the established model. The following formula was computed: risk score ($RS$) = $\sum_{i=1}^{N} (\text{IncRNA Exp} \times \text{coeft})$, where coeft means the coefficients, and $N$ is the number of IncRNAs. Next, the corresponding risk scores were used to validate the patients in the test set and the entire cohort.

**Assessment of the IClncRNA-related model.** Independent prognostic factor analysis of the contributions of each clinical variable and the IClncRNA-related signature was conducted through univariate and multivariate Cox regression analyses. Statistical significance was defined as a $P$ value $< 0.05$. The nomograms of three clinical features and risk scores were analysed with the "rms" R package to show the predicted survival probabilities for the 1-, 3- and 5-year survival rates of LUAD patients with IClncRNA-related signatures. A calibration plot was then carried out to determine the nomogram accuracy.

**Analysis of tumour immune infiltration.** To estimate the relationship between the immune infiltration landscape and the risk score, we utilized the "CIBERSORT" algorithm to estimate the fraction of 22 immune cell types among the LUAD samples, and Spearman correlation was used to assess the relevance between signature-related IncRNAs and immune cells. A $P$ value less than 0.05 was considered significant.

**Gene set enrichment analysis.** By using gene set enrichment analysis (GSEA) enrichment analyses were conducted to explore the potential mechanisms and functions between Cluster 1 and Cluster 2, with the following parameters: nPerm = 1000, minGSSize = 10, maxGSSize = 1000, and nominal $P$ value $< 0.05$.

**Immunotherapy and chemotherapy.** We implemented the Tumour Immune Dysfunction and Exclusion (TIDE) score, which has proven to be an effective predictor of the ICI therapeutic response. In addition, the "pRRophetic" R package has been utilized in studies evaluating drug sensitivity in cancers by calculating each LUAD sample's IC50 value based on the GDSC website.

**Statistical analyses.** All statistical analyses were carried out with R software (version 3.6.1). Patients were randomly grouped using the "caret" R package. Univariate and multivariate Cox regression models were used to evaluate the prognostic significance. Kaplan–Meier curves were plotted to analyse the OS between different groups by the log-rank test. The prediction accuracy of the IClncRNA-related risk model was determined by ROC curve analysis. The above statistical analysis was regarded as significantly different at $P$ value $< 0.05$.

**Ethics statement.** We obtained RNA sequence transcriptome data and relevant clinical information of the LUAD patients from the TCGA (https://cancergenome.nih.gov) database. Their use did not require ethical approval.
indicated that there were 13 immune infiltrating cell differences between clusters (Fig. 3A), with memory B cells, plasma cells, CD8 T cells, activated memory CD4 T cells, regulatory T cells (Tregs), and M1 macrophages having higher infiltration in Cluster 2, while native B cells, gamma delta T cells, activated NK cells, M2 macrophages and activated mast cells had more infiltration in Cluster 1 (P < 0.05). The TME scores in Cluster 1 were considerably higher than those in Cluster 2 (Fig. 3C–E; p < 0.05). However, the response rate to ICIs predicted by the TIDE score showed no difference between Clusters 1 and 2 (Fig. 3F). In addition, to predict the functions or pathways involved in IClncRNAs from LUAD, GSEA was selected for comparison between the clusters. The results were highly enriched in Cluster 2, including the non-small cell lung cancer pathway, T-cell receptor signalling pathway, B-cell receptor signalling pathway, NK-cell-mediated cytotoxicity, and VEGF signalling pathway (P < 0.05; Fig. 3B), and the unwarping IClncRNAs proved to be remarkably associated with the immune status of patients in the TCGA-LUAD cohort.

Construction and validation of the IClncRNA-associated risk model in LUAD patients. The results of the univariate Cox regression model showed that 18 out of the 75 IClncRNAs were significantly associated with the overall survival of patients with LUAD. Based on the Lasso Cox regression model, these 18 IncRNAs were screened out to avoid overfitting, improve the accuracy and obtain the best penalty parameters. Hence, 6 IClncRNAs (AC022613.1, LINC00892, TSP0AP1-AS1, HIF1A-AS1, ADPGK-AS1, and LINC02390, Table 2) were eventually used to construct the IClncRNA-associated signature (Fig. 4A,B). K-M survival analysis showed worse OS in the high-risk group (Fig. 4C). The ROC curves showed that our signature had a robust predictive ability, with AUCs predicting 1-year, 3-year, and 5-year overall survival of 0.710, 0.703, and 0.659, respectively (Fig. 4D). Next, we ranked the training cohort by the risk score from low to high; the follow-up time and genetic heatmap of the population are also shown by this standard (Fig. 4E). The heatmaps showed that LINC00892,

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**Figure 1.** Identification of IClncRNAs of prognostic value in patients with LUAD. (A) Network diagram for PD1, PD-L1, CTLA4 and 75 IClncRNAs. (B) Sankey relational diagram for three immune checkpoint genes and the IClncRNAs. (C) Univariate Cox regression analysis revealed that the 18 selected lncRNAs were significantly correlated with the clinical prognosis. (D) Heatmap for the expression difference of IClncRNAs between tumour and normal tissue. (E) Boxplot for the expression difference of IClncRNAs between tumour and normal tissue (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).
Figure 2. Identification of LUAD subtypes using NMF consensus clustering in LUAD patients. (A, B) NMF clustering using 18 IClncRNAs. The patients were divided into two clusters (Cluster 1 and Cluster 2). (C) Survival analysis of patients in Clusters 1 and 2 in the LUAD cohort. (D) Heatmap of two clusters defined by IClncRNA expression. (E–G) Differences in PD1, PD-L1, and CTLA4 expression between lung cancer tissue and normal tissue. (H–J) Differences in PD1, PD-L1, and CTLA4 expression between Cluster 1 and Cluster 2.

Figure 3. Identification of the immune cell infiltration landscape, tumour microenvironment score, KEGG pathway, and TIDE score in the two clusters. (A) The difference in immune cell infiltration between Cluster 1 and Cluster 2. (B) KEGG pathway analysis between Cluster 1 and Cluster 2. (C–E) ImmuneScore, StromalScore and ESTIMATEScore between Cluster 1 and Cluster 2. (F) The difference in TIDE_score between the two clusters.
TSPOAP1-AS1, ADPGK-AS1, and LINC02390 expression in the low-risk group was significantly greater than that in the high-risk group, while the other lncRNAs were downregulated (Fig. 4E).

To further examine the predictive efficacy of the model, the IClncRNA-associated risk model was verified in the testing and entire groups. We calculated the optimal cut-off point and randomly separated patients into low-risk and high-risk groups. The survival analysis showed significantly longer OS in the high-risk group than in the low-risk group (P < 0.05; Fig. 5A,D). As shown in Fig. 5B, all of the time-dependent ROC curve results obtained superior AUC values for the 1-year, 3-year, and 5-year OS of LUAD patients (AUC = 0.693, 0.595, and 0.617). The AUCs in the entire set for predicting patient OS at 1, 3 and 5 years were 0.700, 0.652 and 0.640 (Fig. 5E), respectively. The six-lncRNA expression heatmap sorted by the risk score is also shown in Figs. 5C,F.

Stratified survival analysis by the universal clinicopathologic characteristics, gender, age, stage, or tumour stage subgroups in low-risk group patients was significantly unfavourable to the low-risk group (P < 0.05; Fig. 6).

The IClncRNA-related risk score as an independent risk factor. To evaluate whether this risk model of IClncRNAs had independent prognostic characteristics, stage and risk scores were strongly associated with prognosis via univariate and multivariate analyses, for which the HRs of the risk score were 2.449 and 1.990 (p < 0.001; Fig. 7A,B), indicating that the signature was an independent prognostic factor for LUAD.

| Gene         | Coef   |
|--------------|--------|
| AC022613.1   | 0.101634 |
| LINC00892    | -0.42989 |
| TSPOAP1-AS1  | -0.18157 |
| HIF1A-AS1    | 0.1437  |
| ADPGK-AS1    | -1.05709 |
| LINC02390    | -1.35457 |

Table 2. The model information of lung adenocarcinoma.
Construction and evaluation of the nomogram. We then constructed a nomogram with the IClnRNA risk score and other clinicopathological variables to predict the 1-, 3-, and 5-year survival of LUAD patients (Fig. 7C). The concordance index of the risk grade was always higher than that of the other clinical factors, indicating a promising prognostic ability (Fig. 7D). Moreover, the calibration plot was identified as a sensitive prediction strategy for the prognosis of LUAD patients (Fig. 7E–G).

Clinical correlation analysis between the tumour immune microenvironment and risk model. We further explored the relationship between risk scores and clinical characteristics. The outcomes
Figure 7. Assessment of the prognostic risk model and clinical characteristics in the entire cohort; construction and evaluation of a prognostic nomogram. (A,B) Univariate and multivariate analyses of the clinical characteristics and risk level with OS. (C) The nomogram predicts the probability of 1-, 3-, and 5-year OS. (D) Concordance indices of the risk score and clinical characteristics. (E–G) The nomogram's calibration plot predicts the probability of 1-, 3-, and 5-year OS.
demonstrated that the expression levels of the 4 IClncRNAs were high in the low-risk group (Fig. 8A). We also analysed the risk score in different LUAD subgroups (Fig. 8A) to explore their correlations with clinico-pathological features in LUAD, among which age and metastasis were not significantly different between the risk groups. At the same time, there was a significant difference in sex and stage (Fig. 8B–G). Compared to the low-risk group, the results of the stage and three types of TME scores were greater for patients in the high-risk group (Fig. 8H–K), which implies a potential correlation between the model and the LUAD microenvironment.

Therapeutic response assessment. We then explored the differences in PD1, PD-L1, and CTLA4 expression between the IClncRNA model and ICI biomarkers. The response to immunotherapy was better in patients in the low-risk group (P < 0.05; Fig. 9A–C). Intriguingly, TIDE, which has emerged as a vital predictive immunotherapeutic biomarker36, had significantly lower scores in the low-risk group than in the high-risk group, indicating that our signature could better predict the response of LUAD to immunotherapy (p < 0.05; Fig. 9D).

The IPS has promising potential for cancer patients treated with CTLA-4 and PD-1 blockers37. In our research, the patients with high-risk scores/PD-1 negative, high-risk scores/PD-1 positive were found to have worse survival than those with low-risk scores/PD-1 positive and low-risk scores/PD-1 negative (p < 0.05; Fig. 9E). Similarly, PD-L1 or CTLA-4 stratification in patients with a risk score showed the same survival pattern following the PD-1 trend (p < 0.05; Fig. 9F,G), which indicated that the high-risk group patients showed a better opportunity for ICI application. After discussing the immunotherapy possibility of the signature, we investigated the risk score’s links to immune infiltration. As shown in Fig. 9H–T, it was inversely correlated with the abundance of memory B cells, resting dendritic cells, M1 macrophages, resting mast cells, plasma cells, CD8 T cells,
and regulatory T cells (Tregs) (p < 0.05), whereas it had the same trend as activated dendritic cells and activated M0 macrophages (p < 0.05). These observations indicate that the IClncRNA-related signature could predict the efficacy of immune checkpoint inhibitors for LUAD.

Apart from the above analysis, we explored the resistance to chemotherapy changes to estimate the IC50 between the two risk groups to investigate whether this signature also had a chemotherapeutic value. The results revealed significant differences in 36 targeting drugs between the groups, and patients in the high-risk group had strong drug sensitivity (P < 0.05; Fig. 10), suggesting it also has utility in predicting the response to chemotherapy.

Discussion

LUAD is the most common subtype of lung cancer that threatens human health\(^1\). Despite advances in therapeutic strategies such as surgical techniques, radiotherapy, and chemotherapy, the survival of patients with LUAD is still unfavourable. Therapies targeting immune checkpoints that mainly involve PD1, PD-L1, and CTLA4 have been widely applied in the treatment of advanced cancers, including melanoma\(^38\) and non-small cell lung cancer\(^39–41\). However, there are some limitations regarding immune checkpoints: their expression level does not directly reflect the tumour's sensitivity to immunotherapy or the OS\(^42\). LncRNAs, a large class of noncoding RNAs > 200 nucleotides (nt) in length, have recently received increasing attention. They have been reported to function as essential regulators in tumour-infiltrating immune cells\(^24,43–45\). However, little is known about the roles of IClncRNAs in immunity assessment and immunotherapeutic responses in LUAD.

Using data from the TCGA-LUAD dataset, we first identified 18 of 75 IClncRNAs that had confirmed prognostic value. Moreover, we categorized the patients into two clusters by consensus clustering analysis to explore the immune checkpoint-related subtypes of LUAD. The results showed that tumour stage, OS, immune...
checkpoint expression, immune cell infiltration, and tumour microenvironment score exhibited significant differences between the clusters. However, there was no significant difference in TIDE, which is employed to evaluate the immunotherapy response.

In our risk model, we identified a signature of six lncRNAs associated with OS that was constructed by multivariate regression analysis. Researchers previously found that LINC00892 is associated with the tumour microenvironment and immunotherapy response in bladder cancer\(^46\). These results are consistent with ours. The long noncoding RNA TSPOAP1-AS1 is a potential diagnostic biomarker for paediatric septic shock\(^47\). Other
studies have shown that the long noncoding RNA TSPOAP1-AS1 is associated with obesity and influenza A virus replication. Consistently, researchers previously reported that TSPOAP1-AS1 is a prognostic biomarker for pancreatic cancer. HIF1a-AS1 is involved in many types of malignant tumours and it plays a vital role in liver fibrosis. Inhibition of HIF1a-AS1 could promote apoptosis of hepatoma cells induced by starvation. These results are consistent with ours. Long noncoding RNA ADPGK-AS1 was associated with a poor prognosis of osteosarcoma, breast cancer, pancreatic cancer, and gastric cancer, and was also related to molecular subtypes of prostate cancer. These results indicate that ADPGK-AS1 could play an essential role in regulating the occurrence and development of many cancers. Its mechanism is worthy of further study. Reports on the long noncoding RNAs LINC02390 and AC022613.1 are rare and they are worthy of further study.

Based on the above lncRNAs, the lncRNA-related signature that was constructed in the training population was validated successfully in the testing and entire set. The risk scoring model had good prediction effectiveness and was an independent risk factor in multivariate Cox regression analysis. The associated nomogram showed perfect consistency for 1-year, 3-year, and 5-year OS. Patients with a high-risk score in all three risk cohorts had significantly worse OS rates than the low-risk group. Moreover, we found that not only clinical stages but also tumour-infiltrating immune cells, immune checkpoint gene expression, and the tumour microenvironment score had significantly different distributions between the two risk groups. Above all, our results revealed that patients at high risk have higher TIDE scores, which has been confirmed to predict the efficacy of anti-PD1 and anti-CTLA4 therapy. Therefore, we hypothesize that the prediction model might have massive potential for selecting LUAD patients likely to benefit from immunotherapy.

Some limitations of the current study need to be highlighted. First, due to the limited sample sizes, more large-scale data are warranted for external verification. Second, to sufficiently understand their potential mechanisms, it is necessary to conduct in vitro and in vivo experiments on the identified lncRNAs. In summary, we successfully established and verified an lncRNA-associated signature for predicting the survival of patients with LUAD. This signature based on 6 lncRNAs was better than two molecular subtype methods in predicting the immunotherapeutic response of LUAD patients, especially the TIDE score prediction. Consequently, this signature in LUAD could offer novel insights into a theoretical foundation for future studies on immune treatment and be helpful for personalized management of LUAD in the clinical environment.

Data availability
The data are available in a public, open access repository. The datasets analysed during the current study are available in The Cancer Genome Atlas (TCGA) network (https://cancergenome.nih.gov/).

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Author contributions
H.Z. designed the experiments and wrote the paper; M.L., G.D. and B.Y. collected data and modified the language of the article. Z.Y. revised the paper. Y.G. and L.C. collected data; X.L. and B.T. conducted the experiments and provided financial support.

Competing interests
The authors declare no competing interests.

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