Model based on five tumour immune microenvironment-related genes for predicting hepatocellular carcinoma immunotherapy outcomes

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Abstract

Background: Although the tumour immune microenvironment is known to significantly influence immunotherapy outcomes, its association with changes in gene expression patterns in hepatocellular carcinoma (HCC) during immunotherapy and its effect on prognosis have not been clarified.

Methods: A total of 365 HCC samples from The Cancer Genome Atlas liver hepatocellular carcinoma (TCGA-LIHC) dataset were stratified into training datasets and verification datasets. In the training datasets, immune-related genes were analysed through univariate Cox regression analyses and least absolute shrinkage and selection operator (LASSO)-Cox analyses to build a prognostic model. The TCGA-LIHC, GSE14520, and Imvigor210 cohorts were subjected to time-dependent receiver operating characteristic (ROC) and Kaplan–Meier survival curve analyses to verify the reliability of the developed model. Finally, single-sample gene set enrichment analysis (ssGSEA) was used to study the underlying molecular mechanisms.

Results: Five immune-related genes (LDHA, PPAT, BFSP1, NR0B1, and PFKFB4) were identified and used to establish the prognostic model for patient response to HCC treatment. ROC curve analysis of the TCGA (training and validation sets) and GSE14520 cohorts confirmed the predictive ability of the five-gene-based model (AUC > 0.6). In addition, ROC and Kaplan–Meier analyses indicated that the model could stratify patients into a low-risk and a high-risk group, wherein the high-risk group exhibited worse prognosis and was less sensitive to immunotherapy than the low-risk group. Functional enrichment analysis predicted potential associations of the five genes with several metabolic processes and oncological signatures.

Conclusions: We established a novel five-gene-based prognostic model based on the tumour immune microenvironment that can predict immunotherapy efficacy in HCC patients.

Keywords: HCC, Risk model, Immune environment, Prognosis, Immunotherapy

Background

Hepatocellular carcinoma (HCC) is a highly malignant cancer and ranks as the third leading cause of cancer-related deaths worldwide [1]. The 5-year survival and overall survival (OS) rates are below 12%. Precursors of most HCC cases include liver cirrhosis, chronic...
hepatitis viral infections, alcohol-related liver disease, non-alcoholic fatty liver disease, and drug-induced hepatitis. As HCC is usually diagnosed at advanced stages [2], it can be difficult to treat. Thus, it is important to elucidate the molecular mechanisms underlying HCC progression and develop novel therapeutic targets to improve patient survival outcomes.

The immune microenvironment plays a critical role in tumorigenesis and is correlated with tumour progression and treatment efficacy [3, 4]. Systemic immune therapeutics have shown efficacy against HCC, especially for patients without an opportunity to undergo resection or liver transplantation [2, 5]. Common immunotherapy strategies include chimeric antigen receptor-engineered T cells (CAR-T cells), cancer vaccines, cytokine therapy, and immune checkpoint inhibitors (ICIs). Currently, ICIs are the most successful class of immune therapeutics, both as monotherapy and combination therapy [6]. For example, the efficacy of anti-programmed cell death 1 (anti-PD-1) and anti-programmed death ligand 1 (anti-PD-L1) in HCC has been investigated. PD-L1 expressed by T cells regulates immune responses at the initiation phase in lymph nodes and at the effector phase in tumour cells [7]. The restoration of function in “exhausted” T cells and the depletion of immunosuppressive regulatory T lymphocytes using monoclonal antibodies targeting these receptors have opened up new avenues for the treatment of several malignancies [8]. However, only approximately 25% of HCC patients with high infiltration of PD-1 expressing T cells respond to ICIs [9], and identification of patients who will respond well to ICIs is challenging.

Conventional strategies using tumour-node-metastasis (TNM) classification to predict HCC prognosis can help guide decisions in HCC clinical therapy [10]. However, their predictive efficacies are less than satisfactory. Notably, the use of genome-sequencing technologies coupled with bioinformatics analyses has improved tumour diagnosis and prognosis capabilities. Gene-based prognostic models have been established to identify differential mRNA expression patterns between cancer and normal tissues. Datasets reporting the expression profiles of long noncoding RNAs (lncRNAs) [11], genes that regulate epigenetic modifications [12], and immune-related genes [13] from The Cancer Genome Atlas (TCGA) and NCBI Gene Expression Omnibus (GEO) databases have been increasingly explored to study disease prognosis. However, there is no mature model that can stably predict patient response to HCC immunotherapy and treatment outcome. Therefore, we established a novel five-gene-based model pertaining to the immune microenvironment and conducted bioinformatics analyses to assess the ability of this model to predict HCC immunotherapy outcomes.

In the present study, we conducted univariate Cox regression analyses and least absolute shrinkage and selection operator (LASSO)-Cox regression analyses to build a risk model. Risk score, time-dependent receiver operating characteristic (ROC) AUC, and Kaplan–Meier survival analyses were used to assess the prognostic ability of the model. The results indicate that our model can effectively predict the efficacy of immunotherapy and that the five genes can serve as potential independent biomarkers in clinical applications. Functional enrichment analysis predicted the potential associations of the upregulated and downregulated genes identified in our model with relevant biological mechanisms. Conclusively, we established a five-gene-based model based on the influence of the tumour immune microenvironment that could potentially be applied to predict patient response to HCC immunotherapy.

Methods

Data collection

Data from a total of 365 HCC patient samples from the TCGA-liver hepatocellular carcinoma (LIHC) dataset were retrieved and used for the analysis of prognostic gene expression signatures and the development of a prognostic model. Genes were excluded if corresponding patient sample data were lacking. Random sampling with arrangement was performed, wherein the 365 samples (including the training set \( n = 219 \) and validation set \( n = 146 \)) were randomly sampled 100 times with replacement. There was no significant difference in TNM stage, grade, OS, sex, or age between the training set and validation set \( p > 0.05 \). The clinical data and mRNA expression data of the GSE14520 dataset \( n = 221 \) were retrieved from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/), and an immunotherapy dataset (Imvigor210) was obtained from published study [14]. All patient data that were used in the present study had complete clinical information, including TNM stage, grade, survival time, sex, age, and immune-related gene expression.

Establishment of the five-gene model based on immune-related genes

First, we screened for immune-related genes associated with HCC prognosis. The immune-related genes involved in HCC pathogenesis were collected from previous studies [15–17]. In the TCGA-LIHC training set, immune-related gene and survival data were analysed by univariate Cox regression analysis using the R package "coxph". The criterion of \( p < 0.001 \) was selected as the filtering threshold. We screened the immune-related
genes associated with HCC prognosis. Second, a LASSO-Cox regression analysis was used to further filter the prognostic genes[18]. This method enables the simultaneous selection of variables and parameter estimation and can better solve the multicollinearity problem in regression analyses [18]. A prognostic gene signature was established based on the LASSO-Cox regression model coefficients (β values) multiplied by the mRNA expression level. The risk score = \[0.307 \times \text{mRNA expression level of } LDHA\] + \[0.268 \times \text{protein expression level of } PPAT\] + \[0.455 \times \text{mRNA expression level of } BFSP1\] + \[0.234 \times \text{mRNA expression level of } NR0B1\] + \[0.109 \times \text{mRNA expression level of } PFKFB4\].

Assessment of the five-gene-based model by tissue microarray (TMA) analysis
To assess the prognostic ability of the five-gene-based model, we constructed a TMA comprised of 90 carcinoma tissues from HCC patients (Shanghai Outdo Biotech Co. Ltd., Shanghai, China) according to a reference method that was described previously [19]. Subsequently, immunohistochemistry (IHC) and integrated optical density (IOD) analyses were performed as described previously [20]. The primary antibodies used are shown in Additional file 1: Table S1. The IHC scores were obtained from independent assessments by three senior pathologists without any prior knowledge of patient characteristics. The IHC score of each patient was calculated using the following formula: 

\[
\text{IHC score} = 0.307 \times \text{mRNA expression level of } LDHA + 0.268 \times \text{protein expression level of } PPAT + 0.455 \times \text{protein expression level of } BFSP1 + 0.234 \times \text{protein expression level of } NR0B1 + 0.109 \times \text{protein expression level of } PFKFB4
\]

A Kaplan–Meier log-rank analysis was used to evaluate the difference in survival rates between the groups with a high IHC score and low IHC score.

Validation of the performance and prognostic ability of the five-gene-based model
Time-dependent ROC analyses and Kaplan–Meier log-rank tests were used to evaluate the performance and prognostic ability of the model using verification datasets from the TCGA-LIHC dataset, the entire TCGA-LIHC dataset, the GSE14520 cohort, and the immunotherapy dataset (Imvigor210).

Functional enrichment analyses
To explore the underlying molecular mechanisms of the five-gene-based model, single-sample gene set enrichment analysis (ssGSEA) of the gene expression profiles was used to identify the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways predicted to be correlated with the risk score [21, 22]. Clustering analyses were performed by gene set variation analysis (GSVA). A \(p < 0.05\) and false discovery rate (FDR) < 0.05 were considered statistically significant.

Statistical analysis
Statistical analyses were performed using SPSS v25 (IBM, Chicago, IL, USA), GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA), and R software (version 3.5.1). Student’s \(t\)-test was used for statistical comparisons, the Kaplan–Meier method was used to estimate OS, and \(p < 0.05\) was considered statistically significant.

Results
Identification of HCC prognostic gene expression signatures to construct the HCC prognostic model
A flowchart of the analysis workflow is illustrated in Fig. 1a. Using the TCGA-LIHC training set, univariate Cox regression analysis of the screening results, including 4,227 immune-related genes, led to the identification of 245 immune-related genes as potential prognostic indicators of HCC OS.

Construction of the five-gene-based HCC prognostic model
After primary filtering, a LASSO-penalized Cox analysis was performed to further narrow down the mRNA expression profiles (Fig. 1b and c). Five genes were identified: lactate dehydrogenase A (\(LDHA\)), phosphoribosyl pyrophosphate amidotransferase (\(PPAT\)), beaded filament structural protein 1 (\(BFSP1\)), nuclear receptor subfamily 0 group B member 1 (\(NR0B1\)), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (\(PFKFB4\)). A stepwise multivariate Cox regression analysis was then performed to establish the prognostic model. The risk score was calculated by summing the weighted gene expression level of each of the five genes multiplied by their respective LASSO coefficients: 

\[
\text{risk score} = 0.307 \times \text{mRNA expression level of } LDHA + 0.268 \times \text{mRNA expression level of } PPAT + 0.455 \times \text{mRNA expression level of } BFSP1 + 0.234 \times \text{mRNA expression level of } NR0B1 + 0.109 \times \text{mRNA expression level of } PFKFB4
\]

All five genes showed positive LASSO coefficients in the multivariate Cox regression analysis. Next, the five-gene-based model was further evaluated for stability and reliability.

Evaluation of the predictive efficacy of the five-gene-based model using the TCGA-LIHC training set
To determine the association between the gene expression signatures of these five genes and HCC patient survival outcome, risk scores (AUC values) were calculated with the five-gene-based model for each sample
separately, and the optimal cut-off for the risk score was defined using the TCGA-LIHC training set (Fig. 2a). Higher AUC values indicated better classification performance of the five-gene-based HCC prognostic model. AUC values of 0.80, 0.77 and 0.73 were obtained for the 1-year, 3-year and 5-year survival rates, respectively. Kaplan–Meier survival analysis revealed that patients in the high-risk group showed worse prognosis than patients in the low-risk group \((p < 0.0001; \text{Fig. 2a})\). Taken together, these results indicate good performance of the established five-gene-based model for predicting HCC survival outcomes.

Validation of the five-gene-based HCC prognostic model using the GSE14520 dataset, TCGA-LIHC testing set, and whole TCGA-LIHC dataset

To validate the stability of the five-gene-based model, a similar workflow was employed for the training set, wherein three datasets (the GSE14520 dataset, TCGA-LIHC testing set, and whole TCGA-LIHC dataset) were analysed. Risk scores for the five-gene-based model were obtained by calculating the AUC values of the respective ROC curves. AUC values of 0.75, 0.73 and 0.67 were obtained for 1-year, 3-year and 5-year survival rates, respectively, for the GSE14520 validation dataset. The results indicated that patients in the high-risk group showed significantly worse survival rates than patients in the low-risk group \((p < 0.001; \text{Fig. 2b})\). Consistent with the results of the GSE14520 dataset, patients in the high-risk group showed poorer OS than patients in the low-risk group \((all \ p < 0.05)\) and the AUC values were above 0.6 for the whole TCGA-LIHC validation dataset and the TCGA-LIHC dataset (Additional file 2: Figure S1a and b). The results collectively showed that the five-gene-based model could predict patient survival duration based on gene expression levels.

Upregulated genes (LDHA, PPAT, BFSP1, NR0B1, and PFKFB4) identified from the IHC TMA analysis predict poor prognosis

Next, the protein expression levels of the five genes LDHA, PPAT, BFSP1, NR0B1 and PFKFB4 were determined with
The five-gene-based model can predict different clinical characteristics

After confirming the efficacy of the five-gene-based model in predicting patient response to immunotherapy, whether the five-gene-based model could be applied to determine the survival outcomes in subgroups with different clinical characteristics was investigated. The results indicated that the five-gene-based model could be used to predict different clinical characteristics ($p < 0.05$; Fig. 5).

Risk model and prognostic analyses of different gene mutation subtypes

To determine the clinical efficacy of the model in predicting treatment response in HCC patients with somatic mutations, Kaplan–Meier survival analyses were performed. First, 11 genes that are commonly mutated in HCC (tumour protein p53 [TP53], phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha [PIK3CA], retinoblastoma protein [RB1], cyclin-dependent kinase inhibitor 2A [CDKN2A], tuberous sclerosis-2 [TSC2], β-catenin [CTNNB1], AT-rich interactive domain-containing protein 2 [ARID2], axin 1 [AXIN1], ribosomal protein S6 kinase A3 [RPS6KA3], AT-rich interactive domain-containing protein 1A [ARID1A], and lysine methyltransferase 2D [KMT2D]) were selected. TP53, CTNNB1, AXIN1, and ARID1A showed the highest mutation frequencies (28%, 24%, 8% and 7%, respectively) and were therefore used for the Kaplan–Meier analysis (Additional file 3: Figure S2). There was a significant difference in OS between patients in the high-risk and low-risk groups (Additional file 3: Figure S2a with TP53 mutation; Figure S2b without TP53 mutation; Figure S2c with CTNNB1 mutation; and Figure S2d without CTNNB1 mutation $p < 0.05$; Additional file 4: Figure S3). Patients with AXIN1 or ARID1A mutations showed no significant difference in OS between the high-risk and low-risk groups, but a significant difference was observed for patients without AXIN1 or ARID1A mutations between the high-risk and low-risk groups. The results suggest that the five-gene-based model can also be used to predict the survival outcomes of HCC patients with genetic mutations.
The five-gene-based model can serve as an independent biomarker in clinical applications

Next, we evaluated whether the five-gene-based model could serve as an independent biomarker with clinical implications. Univariate and multivariate Cox regression analyses of the clinicopathological parameters (including age, sex, T stage, stage, and tumour grade) of 365 patients (Table 2) revealed that the HR of the risk model was approximately 1.7 \((p<0.001)\), and the HR of the T stage model was approximately 1.3 \((p<0.001; \text{Fig. 6a})\). Multivariate Cox regression analysis showed that T stage and the prognostic model were independent risk factors associated with OS.

Comparison of the five-gene-based model and other models

Next, the ability of our established model to determine HCC prognosis was compared with those of three other prognostic models: the four-gene signature \([23]\), the HCC prognostic evaluation model \([24]\) and the six-gene signature \([25]\). We calculated the risk score of the corresponding genes in these three models for the TCGA-LIHC dataset using a method similar to that used for the establishment of our five-gene-based model (described above).

The four-gene-based model had lower AUC values for 1-, 3- and 5-year survival rates than our model; the HCC prognostic evaluation model and the six-gene-based model had slightly higher AUC values for the 1-year survival rate than our model but had lower AUC values for 3- and 5-year survival rates (Fig. 6b-d). These results indicated that our model performed better at predicting the long-term survival (3-year and 5-year survival) than the short-term survival (1-year) of HCC patients. Similar to our model, these three models could also predict the OS of the high- and low-risk groups (log-rank \(p<0.001; \text{Fig. 6e–g})\).

Relationship between risk score and KEGG pathways

We performed ssGSEA to identify potential KEGG pathways (with a correlation coefficient > 0.45) associated with the risk score. A total of 21 KEGG pathways were identified (Fig. 7a). Analysis of the relationship between gene sets and the risk score revealed that the KEGG pathways positively correlated with the risk score included DNA replication, mismatch repair, cell cycle, homologous recombination, spliceosome, oocyte meiosis, progesterone-mediated oocyte maturation, and pathogenic *Escherichia coli* infection. There were also downregulated pathway

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**Table 1** Univariate and multivariate analyses of factors correlated with overall survival

| Variables                | Univariate analysis |          |          | Multivariate analysis |          |          |
|--------------------------|---------------------|----------|----------|-----------------------|----------|----------|
|                          | HR                  | 95% CI   | p value  | HR                    | 95% CI   | p value  |
| Five-gene-based model    | 2.054               | 1.087–3.882 | 0.027   | 2.19                  | 1.152–4.163 | 0.017   |
| Sex                      | 1.499               | 0.463–4.847 | 0.499   |                       |          |          |
| Grade                    | 1.936               | 1.062–3.529 | 0.031   | 1.878                 | 1.024–3.443 | 0.042   |
| Age                      | 1.442               | 0.779–2.668 | 0.244   |                       |          |          |
| TNM stage                | 1.792               | 1.054–3.046 | 0.031   | 1.79                  | 1.033–3.103 | 0.038   |

*HR* hazard ratio, *CI* confidence interval, *TNM* tumour-node-metastasis
terms that negatively correlated with the risk score in HCC, including butanoate metabolism, peroxisome, fatty acid, linoleic acid, tryptophan and tyrosine metabolism (Fig. 7b). The enrichment analysis revealed potential critical pathways implicated in HCC pathogenesis.

Discussion

Immune subsets were found to be significant effectors of immune defence [26–28]. The use of gene expression signatures to determine the outcomes of treatments has been reported by several groups. We showed that the correlation between gene signatures and the tumour immune microenvironment can be used to predict HCC immunotherapy outcome. Similarly, He et al. [13] established a model based on the expression of immune-related genes to predict treatment outcome, Shen et al. [29] identified a clinical-immune signature to estimate OS in ovarian cancer, and Tekpli et al. [30] discovered a novel independent prognostic factor based on the tumour immune microenvironment in breast cancer. Recently, integrative analysis of multi-omics data revealed epidermal growth factor receptor (EGFR) as a critical node in the gene regulatory network that is related to immune phenotype, and the inclusion of therapeutic EGFR inhibition enhanced head and neck squamous cell carcinoma patient response to ICIs [31]. This finding suggests that critical genes associated with the tumour immune microenvironment may serve as prognostic signatures and be useful for clinical immunotherapy.

HCC is a highly heterogeneous tumour at the molecular level and is pathological [32]. The most frequent mutations were identified in telomerase reverse transcriptase (TERT), ARID1A, TP53, and CTNNB1 [33–37]. These gene mutations were also associated with cell differentiation, proliferation, and clinical features [32, 38]. In our study, the five identified genes had a low frequency of mutations, and they were conducive to constructing a stable prognostic model. In addition, the five-gene-based model established in the present study could predict HCC treatment outcomes for patients with or without
tumour-specific mutations in the \textit{TP53}, \textit{CTNNB1}, \textit{AXIN1} or \textit{ARID1A} genes. Thus, the five-gene-based prognostic model was a useful classification tool for patients with various genetic mutation backgrounds.

TMB is an emerging biomarker of sensitivity to immunotherapy [39, 40]. Cai et al. [41] showed that high TMB in liver cancer patients with radical resection was significantly correlated with poor prognosis. Stenzinger et al. [42] reported that high TMB correlated with increased

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\hline
Variables & Univariate analysis & & & Multivariate analysis & & & & & & \\
& & HR & 95\% CI of HR & & & HR & 95\% CI of HR & & \\
& & & lower & upper & & & lower & upper & & \\
\hline
Age & 1.013 & 0.999 & 1.027 & 0.073 & 1.013 & 0.999 & 1.027 & 0.069 \\
Sex & 0.815 & 0.572 & 1.161 & 0.257 & 0.904 & 0.626 & 1.305 & 0.589 \\
T stage & 1.577 & 1.337 & 1.860 & 6.4E-08 & 1.317 & 1.037 & 1.672 & 0.024 \\
Stage & 1.382 & 1.218 & 1.569 & 5.3E-07 & 1.096 & 0.896 & 1.340 & 0.372 \\
Grade & 1.164 & 0.949 & 1.427 & 0.145 & 1.038 & 0.841 & 1.283 & 0.727 \\
Risk score & 1.834 & 1.564 & 2.151 & 8.6E-14 & 1.730 & 1.463 & 2.046 & 1.5E-10 \\
\hline
\end{tabular}
\caption{Univariate and multivariate analyses of all TCGA datasets}

\textit{HR} hazard ratio, \textit{CI} confidence interval, TCGA The Cancer Genome Atlas
\end{table}
patient response rates and survival benefits from immune checkpoint inhibitors. Tumour-specific NEO that form as a consequence of mutations are thought to be another important biomarker for the therapeutic efficacy of cancer immunotherapies [43, 44]. In our study, ROC curve analysis showed that the combined consideration of NEO burden, TMB and risk score output a higher AUC value than NEO burden, TMB, or risk score alone. Combining the five-gene-based model with NEO burden and TMB could enhance the assessment of immunotherapy efficacy and identify patients who will respond to immunotherapy. In the present study, we identified five genes (LDHA, PPAT, BFSP1, NR0B1, and PFKFB4) associated with the tumour immune microenvironment in HCC patient
cohorts that may be applied as potential biomarkers of HCC immunotherapy outcome. The correlation between the risk score and HCC prognosis was determined by analysing the mRNA expression and protein expression of the identified genes with an HCC TMA by qPCR and IHC, respectively. We showed for the first time that BFSP1 is correlated with HCC outcomes. PPAT catalyses the first committed step of de novo purine nucleotide biosynthesis [45, 46], suggesting that targeting PPAT might be a promising cancer strategy [47]. PPAT was also identified as a prognostic biomarker in HCC [48]. Many studies have reported that upregulated LDHA promotes tumour metastasis and is correlated with poor prognosis in several cancers, including lung adenocarcinoma [49], breast cancer [50], HCC [51], gallbladder carcinoma [52], and renal cell carcinoma [53]. Since high LDHA expression can reduce the oxygen dependency of tumour cells by promoting efficient anaerobic/glycolytic metabolism, targeting LDHA is a potential anti-tumour strategy. Epigenetic modification of NR0B1 leads to its ectopic activation in Ewing's sarcoma and lung cancer, enabling it to promote cancer cell proliferation [54–57]. The overexpression of PFKFB4 was found to be associated with a poor prognosis in gastric cancer [58], bladder cancer [59], colon cancer [60], acute monocytic leukaemia [61], glioblastoma [62], thyroid cancer [63], and breast cancers [64–66]. A better understanding of the molecular mechanisms underlying the ability of the five-gene signature to predict HCC pathogenesis as well as prospective studies to validate its utility in clinical applications are needed for the further development of new therapeutic and prognostic strategies (Additional file 5: Table S2).

In this work, we established a prognostic signature for HCC OS prediction that also effectively predicts the efficacy of immunotherapy through combined analysis of gene expression datasets from GEO and TCGA. The model was based on gene mRNA expression but not gene mutations or epigenetic modifications of these five genes. Therefore, it has good clinical feasibility, as it does not require whole-genome sequencing for all patients. Moreover, the methods used in this study might also be suitable for other types of malignancies. In further studies, we plan to detect the expression of these five genes in circulating tumour cells. However, there are several limitations in our study. First, the prognostic role of the five genes at the protein level warrants further research. Second, the model was established with tumour tissues, so it can only predict the prognosis of HCC patients after surgery and cannot detect and diagnose tumours at the early stage. Third, further functional experiments are needed, and the underlying mechanism of the five genes needs to be clarified (Additional file 6).

Conclusions

In this study, we established a novel five-gene-based prognostic model based on the tumour immune microenvironment. Importantly, our model could effectively predict the efficacy of immunotherapy and might serve as a potential independent biomarker in clinical applications.

Supplementary Information

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reviewed the manuscript and made critical corrections. All authors read and approved the final manuscript.

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Availability of data and materials
All data in our study are available upon request.

Ethics approval and consent to participate
The study was approved by the institutional ethical committee, and all patients signed informed consent forms.

Consent for publication
Consent for publication was obtained from all authors.

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

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