A Novel Differentially Expressed Cuproptosis-Related IncRNAs Signature to predict the prognosis and immune characteristics of hepatocellular carcinoma

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Research Article

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Abstract

Objective: According to a growing body of research, long non-coding RNAs (lncRNAs) participate in the progress of hepatocellular carcinoma (HCC). Cuproptosis is a distinct kind of programmed cell death, separating it from several other forms of programmed cell death that may be caused by genetic programming. Consequently, our aim was to investigate the relationship between Differentially Expressed Cuproptosis-Related lncRNAs (DECRLs) and clinical outcome and immune characteristics of HCC.

Method: The Cancer Genome Atlas (TCGA) database was used to retrieve related data. The GSE101728 dataset was downloaded from the Gene Expression Omnibus (GEO) database. A list of cuproptosis-related genes (CRGs) was obtained from a recently published article in Science. Combined analysis of TCGA dataset and the GSE101728 dataset identified differentially expressed CRGs (DECRGs). We can obtain DECRLs via co-expression. Then, using DECRLs, we developed a risk prediction model using Cox regression analysis and the least absolute shrinkage selection operator (LASSO) regression analysis. To evaluate the diagnostic accuracy of this model, a Kaplan-Meier (K-M) survival analysis and a receiver operating characteristic (ROC) curve analysis were used. Next, principal component analysis (PCA) was carried out. Moreover, the relationships between the risk model and immune characteristics, somatic mutation, and drug sensitivity were also investigated. Finally, Real-Time quantitative PCR (RT-qPCR) and Western Blot confirmed the expression of DECRGs or DECRLs in HCC.

Results: Three high-risk DECRLs (AL031985.3, AC107959.3, MKLN1-AS) that can guide HCC prognosis and immune microenvironment were obtained through cox regression analysis. Immune functions such as APC co-inhibition, Type-II-IFN-Response, Paraflammmation, MHC-class-I, and Tumor Immune Dysfunction and Exclusion (TIDE) score, and Tumor Mutation Burden (TMB) were significantly different in high-risk and low-risk groups. Moreover, this research also found that the IC50 values for 87 chemotherapeutic drugs varied widely across patients within high and low-risk groups. The expression of GLS at both mRNA and protein levels was significantly raised in HCC, and that of CDKN2A was dropped in HCC. The mRNA expression level of AL031985.3, AC107959.3 and MKLN1-AS was upregulated in HCC.

Conclusion: The proposed 3-DECRLs that can predict clinical prognosis or guide the immune characteristics and drugs that may have a potential curative effect on HCC received in our research may play a major role in patient management and immunotherapy.

Introduction

Liver cancer is one of the most common malignant tumors of digestive system in the world. According to the global cancer data released in 2020, liver cancer accounts for 4.6% of all new cancers in the world, and its deaths account for 8.3% of all cancer deaths, which is the sixth largest cause of cancer incidence and the third largest cause of cancer death in the world[1]. Because of the large population base, 50% of the world’s new liver cancer cases and deaths are all in China. The main risk factors of liver cancer include chronic infection caused by hepatitis B virus (HBV) or hepatitis C virus (HCV), eating food contaminated
with aflatoxin and drinking heavily etc[2–3]. 85% – 90% of liver cancer are hepatocellular carcinoma (HCC) [4]. The prognosis of HCC is extremely poor due to its insidious onset and lack of specific early markers. Most patients are already in an advanced stage at the time of diagnosis, usually with an average survival time of only 6 months, and a 5-year average survival rate of less than 10%[5]. Therefore, it is urgent to find new therapeutic targets and prognostic factors to improve the therapeutic efficiency and prognosis.

Cuproptosis is a novel kind of cell death that occurs when copper is directly coupled with lipoylated components of the tricarboxylic acid (TCA) cycle [6]. The molecular weight of long non-coding RNAs (lncRNAs) is more than 200 nucleotides [7]. Although they are not involved in protein translation, they play a crucial function in gene regulation. Many recent studies have shown that lncRNAs significantly regulate tumor proliferation, metastasis, invasion, and programmed death [8–10]. Nevertheless, the role of cuproptosis in cancer progression is unknown, and the role of differentially expressed cuproptosis-related lncRNAs (DECRLs) in the prognosis of HCC is also rarely reported. In this study, we explored the role of DECRLs in HCC through the TCGA database (http://portal.gdc.cancer.gov/). At the same time, the correlation between the expression of DECRLs and clinical prognosis and immune characteristics of HCC was also been investigated.

Results

Identification of Differentially Expressed Cuproptosis-Related IncRNAs in HCC Patients

Difference analysis of the GSE101728 dataset obtained 2612 DEGs: 1095 upregulated DEGs and 1517 downregulated DEGs and the TCGA dataset obtained 2910 DEGs: 2463 upregulated DEGs and 447 downregulated DEGs. R was used to visualize volcano plots (figure 1 a,b). Finally, we identified 2 differentially expressed HCC-related CRGs (figure 1d). Subsequently, the expression of 2 DECRGs and 16,876 LncRNAs were downloaded from the TCGA database. According to the filtering conditions (|R|>0.4, P-value <0.05), a total of 453 DECRLs were screened by Pearson correlation analysis.

Development of Risk Model Based on DECRLs

Firstly, through univariate Cox regression analysis, 89 DECRLs with significant correlation with OS in HCC patients were screened via P-value <0.05 as the threshold. Subsequently, according to the optimal penalty parameter (λ) value, the LASSO regression analysis determined that the optimal number of DECRLs participating in the model construction was 4 (Figure 1c,e). Finally, a stepwise multivariate Cox proportional hazards regression analysis was performed, leading to identifying 3 DECRLs AL031985.3, AC107959.3, MKLN1-AS independently related to OS. Following this, a risk model was developed to predict the prognosis of HCC patients.

Evaluation and Validation of the DECRLs Prognostic Signature
At first, the risk score for each sample was obtained using the risk model formula. Then, we classified the patients in the training group, test group, and total sample as high-risk or low-risk based on the median value of the risk score. Patients in high-risk group had shorter OS than patients in low-risk group in the training set (p<0.001), test set (p=0.001), and total sample set (p<0.001) (Figures 2a, 3a, 4a). The survival chart of the training, test, and total sample set shows that the mortality of patients from low- to high-risk group increases gradually (Figures 2b, 3b, 4b). The risk heat map of the training, test, and total sample group shows that the expression level of AL031985.3, AC107959.3, and MKLN1-AS gradually increases from the low- to the high-risk group, indicating that these DECRLs are high-risk LncRNAs (Figures 2c, 3c, 4c).

The ROC curve of the training group had a higher AUC at 1 year (AUC=0.800), 3 years (AUC =0.767), and 5 years (AUC= 0.685) (figure 2d). The ROC curve of the test group had a higher AUC at 1 year (AUC=0.715), 3 years (AUC=0.607), and 5 years (AUC=0.648) (figure 3d). The ROC curve of the total sample group had a higher area under curve (AUC) at 1 year (AUC =0.753), 3 years (AUC =0.678), and 5 years (AUC =0.665) (figure 4d). In addition, the ROC curve of the training, test, and total sample group was significantly higher than other clinical features such as age, gender, stage (Figures 2e, 3e, 4e).

Univariate independent prognostic analysis of the training set showed that the risk score (p<0.001, HR=1.652) and stage (p=0.002, HR =1.598) can be used as independent prognostic factors, which are high-risk factors (figure 2f). However, multivariate independent prognostic analysis of the training set showed that only the risk score (p<0.001, HR=1.623) could be used as an independent prognostic factor, which was a high risk factor (figure 2g).

Univariate independent prognostic analysis of the test set showed that the risk score (p<0.001, HR=1.290) and stage (p<0.001, HR =1.780) can be used as independent prognostic factors, which are high-risk factors (figure 3f). Multivariate independent prognostic analysis of the test set showed that the risk score (p=0.007, HR=1.205) and stage (p<0.001, HR=1.679) can be used as independent prognostic factors, which are high-risk factors (figure 3g). Univariate independent prognostic analysis of the total sample group showed that the risk score (p<0.001, HR=1.393) and stage (p<0.001, HR =1.680) can be used as independent prognostic factors, which are high-risk factors (figure 4f). Multivariate independent prognostic analysis of the training set showed that the risk score (p<0.001, HR=1.306) and stage (p<0.001, HR=1.530) can be used as independent prognostic factors, which are high-risk factors (figure 4g). These results suggest that risk score may be an independent high risk prognostic factor for HCC patients.

In addition, the risk score of risk model can better predict the clinical prognosis of patients in different age, gender and stage (figure 5a-h). At the same time, in order to provide a quantitative tool for clinical application, we also established a nomogram, which contain age, gender, grade, stage and risk score, to predict the survival rate (figure 5i). The calibration chart shows the consistency between the actual versus predicted rates of the 1, 3, and 5-year OS (figure 5j). At last, the PCA analysis showed that the expression level of DECRLs which were used to construct the risk model could significantly distinguish patients in
high- and low-risk group, indicating the accuracy of the model (figure 6a-c). Therefore, all the above results indicate that our risk prediction model can well predict the survival rate of HCC patients.

Cancer-Related Gene Mutation between two Groups

With the use of "maftool" package, a comparison was made between the distribution differences of somatic mutations found in groups with high and low-risk scores. The waterfall diagram shows that the high-risk group has a greater gene mutation rate than the low-risk group (Figures 6i-j). Besides, the low-risk group's tumor mutation burden (TMB) was significantly lower than the high-risk group in the TMB quantification analysis (Figure 6d).

Based on the TMB cutoff value provided by the 'survminer' package, all of the HCC patients in our research were classified as either having a low or high TMB level. The K-M method and log-rank tests illustrate that patients in the TMB\textsubscript{low} group and risk\textsubscript{low}+TMB\textsubscript{low} group had better OS than other groups(Figure 6e-f).

Immunity-Related Analyses and Drug Sensitivity between two Groups

The immunity-related difference analysis showed that the TIDE score of low-risk group is significantly higher than that of high-risk group, which indicates that high-risk HCC patients may be more sensitive to immunotherapy (figure 6h). Differential analysis of immune function showed that Type_II_IFN_Reponse, APC\_co\_stimulation, chemokine receptor (CCR), Parainflammation, Cytolytic\_activity, and MHC\_class\_I were significantly different between high- and low-risk group (figure 6g). A Spearman correlation analysis was conducted to investigate how the risk score impacts medication response to evaluate the relationship between the risk score and the IC50 for various pharmaceuticals. Eighty-seven drugs were connected with risk scores (p<0.05). The sensitivity of low-risk patients to Erlotinib is higher than that of high-risk patients. The sensitivity of patients in high-risk group to Sunitinib is higher than that of patients in low-risk group (figure 6k-n).

Validation of the expression of DECRGs and DECRLs in vitro

To further evaluate the expression of DECRGs and DECRLs, we selected three HCC cell lines (HepG2, SMCC7721 and Huh-7 ), normal liver cell (L-02 ), HCC tumor tissues and adjacent normal tissues to detect their mRNA and protein expression levels. Compared with adjacent normal tissue, the mRNA expression level of AL031985.3, AC107959.3 and MKLN1-AS were significantly upregulated in HCC tissue (figure 7a-c). At the same time, the mRNA expression level of AL031985.3, AC107959.3 and MKLN1-AS were significantly increased in three HCC cell lines HepG2, SMCC7721 and Huh-7 compared to that in normal liver cell L-02 (figure 7d-f). The mRNA expression level of CDKN2A in the HepG2, SMCC7721 and Huh-7 cell line or HCC tissue was decreased compared with the normal liver cell L-02 or adjacent normal tissue, and GLS was increased. (figure 7g-j).
Western blotting results showed that compared with adjacent normal tissue, the protein expression level of GLS was significantly highly expressed and CDKN2A was significantly downregulated in HCC tissue (figure 7k). Besides, the protein expression level of GLS was significantly elevated and CDKN2A was significantly low expressed in three HCC cell lines HepG2, SMCC7721 and Huh-7 (figure 7l).

On the whole, it was experimentally confirmed that the expression of AL031985.3, AC107959.3 and MKLN1-AS at mRNA level was significantly elevated in HCC cell lines or tissue compared with normal group. This is consistent with the results of our bioinformatics analysis. AL031985.3, AC107959.3 and MKLN1-AS may be potential targets for the diagnosis and treatment of HCC. Besides, both mRNA and protein expression level of CDKN2A was decreased in HCC, and that of GLS was upregulated in HCC.

Discussion

HCC is the sixth cancer in the world in 2020 and the third leading cause of cancer related death. At present, surgical resection, ablation or liver transplantation are the main treatment methods for liver cancer in clinical practice, which has a good effect on the treatment of early liver cancer [11–12]. However, less than 20% of patients are diagnosed in the early stages of the disease. Postoperative recurrence rate of patients with advanced or metastatic liver cancer is as high as 70%, and 5-year survival rate is less than 10%. Recent research conducted by Tsvetkov P et al. conclusively showed that copper-dependent death is caused by the direct connection of copper with the lipoylated component of the TCA cycle. It leads to the aggregation of lipoylated proteins and the subsequent loss of iron-sulfur cluster proteins, which leads to proteotoxic stress and, ultimately, cell death. This copper-dependent cell death was defined as coproptosis. Previous studies have shown that TCA cycle is also involved in the occurrence and development of HCC [13–14]. However, whether DECRLs can effectively predict the clinical prognosis of HCC has not been reported yet. In this study, we identified DECRLs and constructed a prognosis model, which is related to mutation landscape, immune characteristics and immunotherapy of HCC patients.

Firstly, we obtained two DECRGs related to HCC via GEO and TCGA databases, and then obtained 453 DECRLs through co-expression analysis. Finally, AL031985.3, AC107959.3, MKLN1-AS were conformed and developed DECRL signature for prognostic prediction. Different kinds of predictive cuproptosis signatures for HCC patients have been reported in previous studies. Yang et al [15] reported that cuproptosis-related gene signature with AUC of 0.734, 0.659, and 0.646 at 1-, 3–5-year. Chen et al [16] also reported that cuproptosis-related LncRNA signature with AUC of 0.719, 0.695, and 0.638 at 1-, 3–5-year. While the highest AUC was 0.741 in the study by Huang [17]. In our study, the highest AUC is 0.800, which indicates this DECRLs signature has strong predictive power.

Additionally, we integrated risk scores and predictive clinical features (including age, sex, pathological stage, grade, and T stage) to construct a nomogram for predicting the prognosis of patients. The results displayed that the higher the calculated Risk Score, the worse the predicted prognosis, which indicates that the nomogram provides a personalized and accurate survival prediction. As a biomarker, TMB is significantly associated with the clinical prognosis of cancer patients [18]. The waterfall diagram shows
that TP53, as a tumor suppressor gene, has a significantly higher mutation rate in the high-risk group than in the low-risk group, so we speculate that the prognosis of patients in the high-risk group is worse. Then we found through K-M analysis and log-rank test that the OS of IMB$_{\text{low}}$ group and risk$_{\text{low}}$+TMB$_{\text{low}}$ group was significantly better than that of other groups, as shown in Fig. 6.

In order to further evaluate the role of DECRLsig risk model in HCC, we also discussed the relationship between risk model and immune characteristics. As shown in Fig. 6, immune functions such as APC$_{\text{co}}$stimulation, CCR, Parainflammation, and MHC$_{\text{class}}$I were significantly up-regulated in high-risk group, while Type$_{\text{IL}}$IFN$_{\text{Response}}$ and Cytolytic$_{\text{activity}}$ were significantly down regulated. TIDE, as a new computing architecture, represents tumor immune dysfunction and rejection, and is used to evaluate the possibility of tumor immune escape[19–20]. As illustrated in Fig. 6, the TIDE score of patients in the low-risk group is significantly higher than that in the high-risk group, indicating that this group of patients has a poor effect on immune checkpoint blocking therapy (ICB) and a shorter survival time after receiving ICB treatment[21]. Currently, chemotherapy is still an important method for treating HCC[22–23], and drug resistance is the main cause of treatment failure[24]. Therefore, we analyzed the resistance and sensitivity of chemotherapeutic drugs to validate the predictive ability of DECRLsig in determining treatment effectiveness. In our research, we also identified eighty-seven drugs with significant sensitivity in the prognosis model, such as Vinorelbine, Sunitinib, Erlotinib, which may improve the clinical efficacy of OS. All the above findings indicate that risk score-based classification can not only effectively predict the clinical outcome of HCC patients, but also may has the potential to guide individualized chemotherapy and immunotherapy treatment strategies for individual tumors, hence improving the prognosis of patients with HCC.

At last, we also experimentally verified that the expression of GLS at both mRNA and protein levels was significantly elevated in HCC, and that of CDKN2A was decreased in HCC. Besides, the mRNA expression level of AL031985.3, AC107959.3 and MKLN1-AS was upregulated in HCC. It had been reported that abnormal expression of MKLN1-AS contributes to tumorigenesis. Silence of MKLN1-AS suppressed HCC cell growth, angiogenesis, migration, and invasion[25]. As displayed in the study of Bian et al[26] the level of MKLN1-AS in the urine of bladder cancer patients was higher than that of the control group, which showed good efficacy in the diagnosis of bladder cancer. Meanwhile, we also found that AL031985.3 and AC107959.3 were significantly related to the prognosis of HCC[27–29], which also reflected to some extent the reliability of the results of this study.

There are a few limits to our study despite our best efforts. First, cuproptosis, a newly identified kind of cell death, has an unknown role in tumor growth. Second, although we have verified DECRGS and DECRLsig by RT-qPCR and western blot, however, functional biological experiments should be carried out to further validate the results.

To summarize, in our study, a new OS prognosis model was established. Three DECRLs were involved in the construction of the risk model, including three high-risk DECRLsig AL031985.3, AC107959.3 and MKLN1-AS. As the expression of high-risk DECRLs in HCC increases, the patient risk increases. Our
findings could provide new insights into the molecular mechanisms involved in the genesis and progression of HCC.

**Methods And Materials**

**Flow Chart**

**Data Capture and Specimen collection**

We acquired the liver hepatocellular carcinoma (LIHC) data from the TCGA database, including RNA sequencing data, pertinent clinical information, and somatic mutation data. The TCGA database contains transcriptome data from 424 HCC patients, including 374 LIHC tissues and 50 normal tissues. In the interim, clinical data were also obtained. This data comprised gender, age, clinical stage and grade, and TNM stage. The GSE101728 dataset, including 7 HCC tissue samples and 7 adjacent tissue samples, was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). In addition, 19 cuproptosis-related genes (CRGs) were gathered from the previous study [6], and GENCODE v36 was applied for the annotation of the genes (https://www.gencodegenes.org/human/release 36. html).

HCC tissues and adjacent normal tissues were collected from 30 pathologically confirmed HCC patients who had received surgical treatment in the ** from January 2022 to September 2022. Those tissues were immediately frozen in liquid nitrogen after surgery and stored at -80°C. This study was approved by the Ethics Committee of the **.

**Identification of Differentially Expressed Cuproptosis-Related LncRNAs**

The limma package of R software performed differential analysis on the TCGA transcriptome data and GSE101728 dataset to obtain differentially expressed genes (DEGs). The screening criteria were \( p<0.05 \) and \( |\log FC|>1 \). The list of CRGs were compiled from the prior literature and intersected with DEGs to identify the differentially expressed HCC-related CRGs (DECRGs). Then, we use the limma package again to perform co-expression analysis of DECRGs and lncRNAs in HCC transcriptome data to obtain differentially expressed HCC-related LncRNAs (DECRLs), and the screening criteria were \( |R|>0.4 \) and \( p<0.05 \).

**Development of Risk prognostic Model**

The candidate DECRLs were first screened using univariate Cox regression analysis \( (p<0.05) \) and the least absolute shrinkage selection operator (LASSO). For the generation and validation of risk models, 370 GC patients were randomly assigned to either a training cohort or a test cohort in a ratio of 1:1. Developing the prognostic risk signature of DECRLs included using a linear combination of the expression values of all prognostic DECRLs. This signature was then applied to the training set. The multivariable Cox proportional hazard regression analysis was used to assign weights to the predicted regression coefficients, as can be seen in the table below: Risk score=(Expression of
IncRNA_{AL031985.3} \times 0.67162) + (Expression of IncRNA_{AC107959.3} \times 0.323451) + (Expression of IncRNA_{MKLN1-AS} \times 0.608972). Therefore, the median risk score was used to place each group of HCC patients into either a high-risk or low-risk category.

Validation of Risk Prognostic Model

We evaluated the predictive effectiveness of the risk model for overall survival (OS) using the Kaplan-Meier (K-M) survival analysis and receiver operating characteristic (ROC) analysis. Utilizing principal component analysis (PCA), the expression differential of CRLs in HCC patients was determined. Using univariate and multivariate Cox proportional hazard regression, the independent prognostic determinants of OS were evaluated. Additionally, using the "rms" package of R software, nomograms were produced utilizing all independent prognostic markers and other clinical characteristics to analyze the 1-, 3-, and 5-year survival rates of HCC patients.

Tumor Mutational Burden (TMB)

Somatic mutations were analyzed by “maftools” R package and illustrated in waterfall plots. TMB of each sample was calculated according to the definition of the total number of variations per million bases via Perl script (version: 5.32.1) (https://www.perl.org/). Two-sample Wilcoxon test was used to detect the difference in TMB between the high-risk and low-risk groups. Then, the K-M survival curve was used to evaluate the predictive ability of TMB for OS.

Immunity-Related Analysis and Drug Sensitivity Prediction

Two-sample Wilcoxon test was used to detect the difference in Tumor Immune Dysfunction and Exclusion (TIDE) http://tide.dfci.harvard.edu/ score between the high-risk and low-risk groups. Immune function analysis using the "GSVA" package of R. The half-maximal inhibitory concentration (IC50) indicates the effectiveness of the substance in inhibiting specific biological or biochemical processes. The "pRRophetic" package of R is applied to the IC50 of chemotherapy drugs.

Cell Culture

The normal liver cell line L-02 and the human HCC cell lines HepG2, SMCC7721 and Huh-7 were all purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, Gaithersburg, Maryland, USA) supplied with 10% fetal bovine serum (FBS) and maintained in a CO2 incubator at 37°C. When cell confluence reached 80%-90%, the cells were treated with 0.25 g/dL trypsin (Shanghai Rugi Biotechnology) for subculture.

Real-Time quantitative PCR (RT-qPCR)

Total RNA was extracted from the HCC samples and cells using TRIzol reagent (15596018, Life Technologies, Carlsbad, California, USA). RNA was reverse transcribed into cDNA following the
instructions of the reverse transcription kit and amplified using a RT-qPCR machine (PIKOREAL 96, Thermo Scientific, Waltham, MA, USA). Each reaction mixture consisted of 1μL of cDNA, 5μL of SYBR Green Master Mix, 1μL of the probe, 2μL of forward and reverse primers, and 2μL of RNase-free water. The reaction conditions were as follows: 95°C for 1 min, 95°C for 20 s, and 60°C for 60 s, for 40 cycles. The relative LncRNA and mRNA level of the target gene was determined using the 2-△△Ct method, \( \Delta Ct = Ct(\text{target gene}) - Ct(\text{Reference gene-\(\beta\)-actin}) \), \( \Delta \Delta Ct = \Delta Ct(\text{HCC group}) - \Delta Ct(\text{control group}) \). \(\beta\)-actin was used as internal references and the primers were listed in Table 1.

Table 1  Primer sequences for RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’→3’</th>
<th>Reverse primer 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-actin</td>
<td>CCCTGGAGAAGAGCTACGAG</td>
<td>GGAAGGAAGGCTGGAAGAGT</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>GGGAGGGGTTCCTGGAC</td>
<td>ATCTATGCGGGCATGTTAC</td>
</tr>
<tr>
<td>GLS</td>
<td>CTTAGGCGGAGCGAAGAG</td>
<td>ATGACGAAGAGGAAAGGGC</td>
</tr>
<tr>
<td>AL031985.3</td>
<td>TTTGACGTGACACTCTCCAC</td>
<td>TAGTTGGTCCGCCATGTATC</td>
</tr>
<tr>
<td>AC107959.3</td>
<td>TCAATCTCTGTTCCCCCTA</td>
<td>CTCACTGCCTGAATTITGG</td>
</tr>
<tr>
<td>MKLN1-AS</td>
<td>GTTTCTCTCTGAAAGCAGCG</td>
<td>GGCATGTTTTGGACTGTCTC</td>
</tr>
</tbody>
</table>

Western blot

The cells were lysed with protein lysate radio immunoprecipitation assay lysis buffer (RIPA) (P0013B, Beyotime, Shanghai, China), homogenized, and centrifuged (12,000 rpm for 15 min). The supernatant containing total protein was taken, and the protein concentration was determined using a protein assay (BCA) kit. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the cells were transferred to a polyvinylidene fluoride (PVDF) membrane and sealed with 5% skimmed milk at room temperature 2h. The samples were incubated with the primary antibody (CDKN2A,bs-23797R,Bioss,China;GLS,bs-10341R,Bioss,China;GAPDH,TA-08,Zsbio,China) and then the secondary antibody overnight at room temperature for 1.2h and developed with an ECL kit (340958, Thermo, Waltham, MA, USA). ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used to analyze the film strip, and the ratio of gray value to GAPDH was determined as the relative expression level of the target protein.

Statistical Analysis

Statistical analysis and visualization were performed using R.v.4.1.2, GraphPad Prism v9 and SPSS 22.0, and the t-test or one-way ANOVA was used for differences analysis. The "survival" and "survminer" packages were used to conduct a Kaplan-Meier survival curve analysis of the OS rate. The "survivalROC" package is then used for ROC analysis. The AUC values were obtained to assess the prediction accuracy of the DECRLs-based prognostic model. p <0.05 was considered statistically significant. Each experiment was performed at least three times independently.
Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Ethics Committee of the Second Affiliated Hospital of Anhui Medical University. Written informed consent was obtained from individual or guardian participants.

Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

CLY and XXZ conceived and designed the experiments; MG and YSS interpreted the data; CLY and HL were the major contributor in writing the manuscript; XXZ has substantively revised manuscript; All authors read and approved the final manuscript.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Funding Statement

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References


Figures
Figure 1

(a) Volcano plot of DEGs in GSE 101728, red for high expression and green for low expression;(b) Volcano plot of DEGs in TCGA, red for high expression and green for low expression;(c) The intersection of DEGs in GSE101728, TCGA, and Science obtained differentially HCC-related CRGs;(d) LASSO regression analysis;(e) Selection of the optimal penalty parameter for LASSO regression.
Figure 2

Training group.(a)Survival curve;(b)Risk score;(c)Risk heatmap;(d)ROC curve at 1-year, 3-years and 5-years;(e)ROC curve of the risk score and clinical variables;(f)Univariate COX regression analysis;(g)Multivariate COX regression analysis;
Figure 3

Test group. (a) Survival curve; (b) Risk score; (c) Risk heatmap; (d) ROC curve at 1-year, 3-years and 5-years; (e) ROC curve of the risk score and clinical variables; (f) Univariate COX regression analysis; (g) Multivariate COX regression analysis;
Figure 4

Total sample group. (a) Survival curve; (b) Risk score; (c) Risk heatmap; (d) ROC curve at 1-year, 3-years and 5-years; (e) ROC curve of the risk score and clinical variables; (f) Univariate COX regression analysis; (g) Multivariate COX regression analysis;
Figure 5

(a-h) Subgroup survival analysis between two groups, (a) Female; (b) Male; (c) Age < 60; (d) Age ≥ 60; (e) Stage -; (f) Stage -; (g) Grand 1-2; (h) Grand 3-4; (i) A nomogram to predicts 1, 3, and 5 years OS of HCC patients; (j) Calibration plots for 1-, 3-, and 5-years survival predictions.
Figure 6

(a) PCA base on DECRLsig; (b) PCA base on DECRLs; (c) PCA base on all genes; (d) Differential analysis of TMB; (e) K-M survival analysis of the TMB; (f) K-M survival analysis of combined TMB and risk scores; (g) Immune function differential analysis; (h) Differential analysis of TIDE scores; (i) Waterfall diagram in low-risk groups; (j) Waterfall diagram in high-risk groups; (k-n) Drug sensitivity analysis.
Figure 7

Experimental verification of mRNA or protein expression level of DECRGs or DECRLs in HCC tissue and cell lines. (a-c) The mRNA expression level of DECRLs in HCC tissue. (d-f) The mRNA expression level of DECRLs in HCC cell lines. (g-h) The mRNA expression level of DECRGs in HCC tissue. (i-j) The mRNA expression level of DECREs in HCC cell lines. (k) The protein expression level of DECRGs in HCC tissue. (l) The protein expression level of DECREs in HCC cell lines. **p < 0.0001 each experiment was repeated three times.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- CDKN2AinCell.tif
- CDKN2AinTissue.tif
- GAPDHinCell.tif
- GAPDHinTissue.tif
- GLSinCell.tif
- GLSinTissue.tif
- flowchart.png