Anoikis-Related LncRNA Signature Predicts Prognosis and Is Associated With Immune Infiltration in Hepatocellular Carcinoma

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Abstract

Anoikis is a term used to describe the programmed cell death that takes place when cells become disconnected from the extracellular matrix[1]. Numerous long non-coding RNAs (lncRNAs) have been found to be linked to anoikis resistance in various tumors, including glioma[2], breast cancer[3], and bladder cancer[4]. There is a lack of studies examining the relationship between ARLs and HCC prognosis. Further research is needed to explore this potential link and understand the role of ARLs in HCC progression.

Patients and methods:

In this study, we acquired 36 genes associated with anoikis through the GO and GSEA databases, and identified 22 differentially expressed Incrnas that were correlated with these genes based on data from The Cancer Genome Atlas (TCGA). Using Cox regression analyses, we created an anoikis-related IncRNA signature (ARIncSig) in a training cohort, which we then validated in both a testing cohort and a combined cohort composed of data from both cohorts. Then, we collected 8 pairs of liver cancer tissues and adjacent tissues from Affiliated Tumor Hospital of Nantong University.

Results:

Five ARLs were developed as a risk stratification system to categorize patients into low- and high-risk groups. The high-risk group exhibited a notably lower overall survival (OS) rate in comparison to the low-risk group. The model's predictive performance is supported by the receiver operating characteristic curve, calibration of nomogram, clinical correlation analysis, and clinical decision curve. Furthermore, differential analysis of immune function, immune checkpoint, response to chemotherapy, and immune cell subpopulations indicated significant discrepancies between the high and low-risk groups. IC50 analysis determined that commonly prescribed drugs were more effective for patients in the high-risk group. Lastly, quantitative real-time PCR validated the expression levels of the five IncRNAs in specific drug-resistant cell lines. In conclusion, our ARIncSig model possesses critical predictive value in the prognosis of HCC patients, and it may provide clinical guidance for personalized immunotherapy.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for the sixth most common type of cancer and the second leading cause of death among all cancers. Its 5-year survival rate is only 18%[5]. Despite significant advances in clinical and experimental cancer treatments, the overall prognosis for HCC patients remains poor due to high rates of tumor recurrence and metastasis after surgery. Previous studies have suggested that HCC progression is a multi-step process involving various factors and genetic changes. Therefore, understanding the molecular mechanisms underlying HCC recurrence and metastasis is crucial for the prognosis and treatment of HCC[6].
Anoikis is a type of apoptotic cell death that occurs when cells lose their interaction with the neighboring extracellular matrix (ECM)[7]. Overcoming this process can promote the metastasis of cancer cells. Anchored growth is a crucial step in tumorigenesis, especially in the process of cancer cell metastasis and diffusion[8]. The potential mechanism of tumor cells' resistance to Anoikis is not completely clear. Current research suggests that factors such as epidermal mesenchymal transformation (EMT), Integrins, intracellular cell adhesion complexes, intercellular cadherin and IgCAMs, growth factor signaling, and cell cycle progression may all play a role in resistance formation[9]. Anoikis resistance is also related to drug resistance and immune cell infiltration in hepatocellular carcinoma, such as HRP-3 promoting anchor-dependent growth and drug resistance of HCC cells[10]. Chen's research has identified anovulatory apoptosis-related subgroups showing that BAK1, SPP1, BSG, PBK, and DAP3 are highly correlated with immune infiltration[11].

Long-chain noncoding RNA (lncRNA) is a non-protein coding RNA with a length of more than 200 nucleotides. Increasing literature has reported that long-chain noncoding RNA (lncRNA) plays a significant role in the occurrence and development of liver cancer, such as cell proliferation, apoptosis, metastasis, differentiation, and chemical resistance in cancer. LncRNA can exert its biological activity through various biological mechanisms such as genomic imprinting, chromatin remodeling, miRNA-lncRNA, and lncRNA-protein interaction[12]. Some lncRNA has been shown to be involved in the regulation of Anoikis or anchored independent growth processes. For example, Liu et al. demonstrated that the LncRNA-FOXD2-AS1 /miR7/TERT axis enhanced the dryness, cell survival, and anchored independent growth of thyroid cancer cells[13]. Sun et al. found that lncRNA-LEF1-AS1/miR-30-5p/SOX9 can promote anoikis-related drug resistance and enhance the dryness of rectal cancer cells[14]. Nevertheless, there have been few studies on the role of lncRNA in the formation of Anoikis resistance in hepatocellular carcinoma, and the application of Anoikis-related research and clarification.

Results

Identification of Differentially Expressed Anoikis-Related Genes and Functional Enrichment Analysis

The flow chart of this study is shown in Figure 1A. We obtained a directory of 36 genes related to anoikis from the GO database and the MSigDB database (Supplementary File S3). Expression levels of these 36 anoikis-associated genes were compared using gene expression data from 365 HCC tumor tissues versus 50 adjacent tissues. Based on the criteria of |fold-change| >1 and P <0.05, we identified 21 differentially expressed genes (Figure 2A,B), all of which were up-regulated in hepatocellular carcinoma tissues. Functional enrichment analysis was used to identify gene ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) terms associated with the 21 DEGs. The following GO projects were significantly enriched: anoikis (P=4.94E-57), regulation of anoikis (P=1.67E-34), negative regulation of anoikis (P=1.68E-23), intrinsic apoptotic signaling pathway (P=2.17E-08), and negative regulation of Wnt signaling pathway (P=1.13E-06) (Figure 2C). KEGG results showed that DEGs were mainly enriched in the infection-related biological pathway including hepatitis B (Figure 2D).
Construction of anoikis-related ArlncSig

To identify IncRNA associated with anoikis, Pearson correlation analysis was conducted on 1202 IncRNA (|Cor|>0.4, P < 0.001) (Supplementary File S4), resulting in 880 differentially expressed IncRNA (logFC > |1|) (Figure 3A, Supplementary File S5). From this pool, 104 at-risk IncRNA were selected based on results from univariate Cox regression analysis (HR > 1, p <0.001) (Supplementary File S6). The HCC samples were randomly divided into training (70%, n=256) and test (30%, n=119) cohorts to construct a risk model. LASSO Cox regression analysis was performed to obtain a risk model, which included six variables when the partial likelihood deviation reached the minimum(Figure 3B,C), and stepwise multivariate Cox regression analyses were used to confirm the prognostic significance of five genes- AL031985.3, AC026412.3, DDX11-AS1, MKLN1-AS, and TMCC1-AS1 (table2). The expression levels of these IncRNA were found to be significantly associated with poor prognosis in Kaplan-Meier survival analysis (P < 0.05) (Figure 3D), and a risk score formula was calculated. Co-expression networks were established using the five independent IncRNA and anoikis-related genes, revealing the risk association of all five IncRNA (Figure 3E,3F). Patients were divided into high-risk and low-risk groups according to the median score in the training, test and the entire sets (Supplementary File S7-9).

Evaluation of ARlncSig

The model performance was assessed by using the ROC curve over time. The AUCs for 1-, 3-, and 5-year survival in the overall cohort were 0.771, 0.685, and 0.692, respectively. Consistent with these results, the AUCs for 1-, 3-, and 5-year survival in the training cohort were 0.795, 0.694, and 0.714, respectively. Similarly, the AUCs for the 1-, 3-, and 5-year survival in the test cohort were 0.706, 0.677, and 0.710, respectively (Figure 4A).In the training cohort, patients with higher expression levels of the five PRlncRNAs were classified as high-risk, and their overall survival time decreased as their risk scores increased. The high-risk group had a significantly shorter overall survival time than the low-risk group(Figure 4B). The same risk score formula was applied to the entire cohort testing cohort (Figure 4C) and the training cohort (Figure 4D), and the results were consistent with those obtained in the testing cohort(Figure 4E).

Correlation between ARlncSig and clinical pathological features of hepatocellular carcinoma

We conducted univariate and multivariate Cox regression analyses to investigate whether ARlncSig was an independent prognostic factor in HCC, considering variables such as age, gender, grade, stage, and TNM staging in the entire cohort. Results of the univariate Cox regression analysis revealed that ARlncSig and tumor stage were significantly associated with overall survival of HCC patients (Figure 5A). Furthermore, multivariate Cox regression analysis demonstrated that ARlncSig and tumor stage were independent prognostic factors for overall survival of HCC patients (Figure 5B).We also compared the ROC curve, AUC at 1 year, and the predictive ability of risk scores with Age, Gender, Grade, Stage, T, N, and M. The results showed that the risk scores had better prediction ability than other factors, with an AUC of 0.765 (Figure 5C).We investigated the association of ARlncSig with clinical pathology across the cohort by stratifying cohorts according to age, gender, grade, stage, T, N, and M. Heat map results showed high
expression of ARlncSig in the high-risk patient group, and the prognostic significance of risk score in HCC patients was related to the clinical pathological characteristics of grade (P < 0.001) and stage (P < 0.05) (Figure 5D).

Construction and Evaluation of the Prognostic Nomogram

To further investigate the role of ARlncSig in predicting 1-, 3-, and 5-year survival in HCC patients, we constructed nomograms incorporating risk scoring models and other clinical pathology features using data from TCGA-HCC cohort. The results indicated that the nomogram was a better predictor of overall survival than single factors (Figure 6A). The calibration curves of survival probability were also found to be close to the standard curves (Figure 6B), suggesting that the nomograms can accurately predict the prognosis of patients.

In addition, the cumulative risk curve demonstrated that the risk values of patients in the high-risk group were higher than those of patients in the low-risk group at the same survival time (Figure 6C). To further validate the clinical utility of ARlncSig, we used decision curve analysis (DCA) to compare the net benefits of the combined predictive models and the predictive value of any single variable. The results indicated that the combined predictive models showed better clinical utility than the predictive value of any single variable (Figure 6D). Overall, these findings suggest that ARlncSig may serve as an effective prognostic tool for the management of HCC patients.

Gene Set Enrichment Analysis

To investigate the potential molecular mechanisms underlying the association between PRlncSig and HCC, we used GSEA to perform biological functional annotation. Our analysis revealed that the high-risk group was primarily enriched in ECM receptor interaction, cell cycle, and neuroactive ligand receptor interaction pathways, while the low-risk group was mainly enriched in metabolic pathways such as fatty acid metabolism, primary bile acid biosynthesis, glycine, serine, and threonine metabolism, tryptophan metabolism, beta-alanine metabolism, and butanoate metabolism (Figure 7A).

The involvement of cell cycle and extracellular mechanisms in tumor cell anoikis resistance has been widely established[15]. Recent studies have also suggested that neural receptors such as the nutritional receptor TrkB play an important role in the resistance to anoikis. Furthermore, abnormal metabolism of glucose, amino acids, fatty acids, nucleotides, and other substances may also be related to anoikis resistance[16]. Our gene ontology analysis showed that high-risk groups were enriched in immune-related pathways related to the biological activities of immunoglobulin, including immunoglobulin complex, immunoglobulin receptor binding, immunoglobulin complex circulating, humoral immune response mediated by circulating immunoglobulin, B cell receptor signaling pathway, antigen binding, and phagocytosis recognition (Figure 7B).

Transforming cells continuously reprogram their signaling characteristics to sense and modulate stimuli from cell surface molecules such as integrins, cadherins, and immunoglobulin family of cell adhesion
molecules at adhesion complexes, which enables them to resist anoikis and metastasize to different organs[9]. Our GSEA results support the biological significance of the PRLncSig marker for immune regulation and suggest the potential role of our risk score in guiding immunotherapy for HCC patients.

Immune analysis of risk scoring

Anoikis is a type of programmed cell death triggered by the loss of ECM anchoring, and cancer-derived ECM can regulate immune cells, resulting in ineffective immunotherapy and tumor immunosuppression[17]. To investigate the correlation between PRLncSig and tumor immune cell infiltration, we analyzed the correlation between risk score and immune cells (Figure 8A). Furthermore, we found that the immunologic infiltration levels of multiple factors, including APC co-stimulation, CCR, checkpoint, cytotoxic activity, MHC class I, parainflammation, type I IFN response, and type II IFN response, were different between the high and low subgroups (Figure 8B).

The importance of immune checkpoints in clinical treatment strategies for HCC led us to study the association between risk scores and multiple immune checkpoints. We found that BTNL2, CD40, and TNFRSF25 were differentially expressed between the high and low-risk groups (Figure 8C). Additionally, N6-methyladenosine (m6A) is the most common RNA modification and regulates immune cells through various mechanisms in hcc[18], m6A methylation helps regulate the immune infiltration of long-chain non-coding RNA (lncRNA). Our findings indicated that m6A regulators METTL3, METTL14, WTAP, KIAA1429, RBM15, ZC3H13, YTHDC1, YTHDC2, YTHDF1, YTHDF2, HNRNPC, FTO, and ALKBH5 had significant differences between the high and low-risk groups (Figure 8D).

Drug reaction characteristics of ArLncSig

Extracellular matrix (ECM) plays a role in inducing resistance to cell death known as anoikis and cell adhesion-mediated resistance (CAM-DR). TACE treatment is widely used in advanced liver cancer patients, with a notable objective tumor response rate of 60% to 70%. Long-term benefits in survival also reach 20% to 25% compared to best supportive care. In this study, we assessed the relationship between the efficacy of chemotherapeutic agents, including immunotherapy, and the risk score we developed. Our findings revealed that the high-risk group demonstrated higher sensitivity to cisplatin, oxaliplatin, and sorafenib than the low-risk group, whereas the high-risk group showed lower sensitivity to 5-fluorouracil (9A-D). We collected 8 pairs of liver cancer tissues and adjacent tissues from Affiliated Tumor Hospital of Nantong University to validate the expression of seven ARLs in our signature. After extracting total RNA from the tissues and performing RT-PCR, we found that three out of the five high-risk ARLs (AL031985.3, AC026412.3, DDX11-AS1, MKLN1-AS, TMCC1-AS1) had significantly higher expression levels in liver cancer tissues than in adjacent tissues (Figure 9E).

Discussion

Liver cancer is a prevalent malignancy and ranks third in cancer-related deaths. The survival rate of patients greatly depends on distant metastasis after treatment[19], with the majority of cancer-related
deaths occurring due to it[20]. The complex mechanism of distant metastasis involves various factors, one of which is resistance to anoikis[21]. Thus, it is crucial to identify relevant biomarkers that predict patient prognosis. Previous studies already developed anoikis-related gene prognosis models for different cancers, yet none have explored IncRNA prognosis models for liver cancer[22–24]. In this study, we constructed a predictive signature consisting of five anoikis-related Incrnas based on various downstream targets and validated its specificity and sensitivity in three cohorts.

Several studies have developed candidate IncRNA models for apoptosis-related pathways of hepatocellular carcinoma, with DDX11-AS1 used in prognosis models related to ferroptosis, cuproptosis, and cancer cell stemness[25–27]. AL031985.3 is associated with liver cancer ferroptosis, cuproptosis, pyroptosis, and autophagy-related prognosis models[28–30], while AC026412.3 is included in the model predicting copper mortality and focal mortality in hepatocellular carcinoma[31, 32]. MKLN1-AS and TMCC1-AS1 are also part of liver cancer-related models such as pyroptosis, ferroptosis, autophagy, necroptosis, and autophagy, respectively[29, 31, 33–35]. Collectively, the identified IncRNAs may be closely related to liver cancer apoptosis and may offer new avenues for targeted therapy.

Anoikis is a crucial apoptotic mechanism that inhibits tumor metastasis, preventing cancer cells from escaping from the natural extracellular matrix and metastasizing at a distance[16]. Tumor cells that evade anoikis include those that break away from the extracellular matrix by adapting to the transcription signaling pathway, oxidative stress, crosstalk with cell cycle, growth protein, and EMT-related mechanisms. Recent studies suggest that the material metabolism of glucose, fatty acids, amino acids, and nucleotides may also play key roles in or resistant to anoikis[36]. Neurotrophic factor family members and their receptors, such as BDNF and TrkB, are related to anoikis resistance in various cancers like endometrial carcinoma, cervical carcinoma, and renal cell carcinoma[37–39]. Furthermore, GSEA analysis suggests that obtaining anoikis resistance allows malignant cells to survive and anchor themselves, improving their lifespan in the biological cycle. Anoikis resistance becomes a prerequisite for intrahepatic and extrahepatic metastasis of hepatocellular carcinoma[40].

Autophagy, ferroptosis, pyroptosis, and necroptosis are other apoptotic methods that are related to tumor immunity, but the connection between immunity and anoikis remains unclear[41, 42]. Results from our study show that the risk score we constructed correlates with immune cells such as CD4 + T cells, CD8 + T cells, NK cells, monocytes, and macrophages, which promote hepatitis to liver cancer[43]. Immunologic functions like Cytolytic_activity, MHC_class_I, Type_I_IFN_Reponse, and Type_II_IFN_Reponse also differ between the high and low-risk groups. Effector T lymphocytes improve the cytolytic activity of hepatocellular carcinoma and offer a promising new treatment option[44]. Low expression of MHC class I is associated with high postoperative recurrence in liver cancer[45]. Moreover, immune checkpoints such as PD-1, PD-L1, TME, CD27, CD28, CD40, and CD48 are highly expressed in patients with HCC, and our results show high expression of immune checkpoints such as CD27, CD28, CD40, and CD48[46]. Therefore, our risk model can provide a reference for clinicians to develop tailored immunotherapy for their diseases.
Despite our promising results, our study has limitations. We primarily based our analysis on bioinformatics results and lack systematic experimental verification of lncRNA functions. We also require further research to predict and explain the relationship between immune infiltration results and risk scoring models for specific clinical treatment. In addition, the number of samples included in our experiment is too small.

Conclusion: The prognostic signature established in this study has the potential to be utilized for evaluating both the prognosis and microenvironment of patients with HCC. Additionally, it can function as a novel standard for choosing optimal treatment strategies for HCC.

Materials and methods

Data Collection

We downloaded RNA sequencing (RNA-seq) data and clinical information on hepatocellular carcinoma (LIHC) from the Cancer Genome Atlas (TCGA) website (https://gdc.cancer.gov/). To correct for batch effects from non-biological bias, we used the "combat" algorithm from the SVA R package. We obtained GTF files from Ensembl to annotate and identify lncRNAs (Supplementary File S1) and mRNAs (Supplementary File S2). After removing duplicate patients, those without complete follow-up information, and those with zero survival time, we included 50 normal samples and 365 tumor samples in our analysis.

Acquisition of anoikis-related genes (ARGs)

To identify anoikis-related genes (ARGs), we retrieved a total of 36 protein-encoding genes from the MSigDB (http://www.gsea-msigdb.org/gsea/msigdb) and GO databases (http://geneontology.org/) using the keyword "anoikis". We used the limma R package[47] to analyze the differential expression of ARGs between normal and tumor samples. We set \( p < 0.05 \) and \(|\log|FC|> 1\) as screening criteria and identified 22 differentially expressed genes related to anoikis.

Acquisition of anoikis-related LncRNA

We analyzed 1203 differentially expressed lncRNAs that were found to be co-expressed with 36 ARGs. Using Pearson correlation analysis with the criteria of \(|\text{Cor}|> 0.4 \) and \( P < 0.001 \), we evaluated all lncRNAs. Next, we used the limma R package to analyze the differential expression of these lncRNAs between normal and tumor samples, with the screening criteria of \( p < 0.05 \) and \( \log|\text{FC}|> 1\). As a result, we obtained 881 differentially expressed anoikis-associated lncRNAs.

Random grouping of data

The "Caret" package in R (version 6.0.88) was used to randomly assign 365 patients with HCC into a training cohort (70%) and a test cohort (30%), each with complete prognostic information and a non-zero survival time. Univariate Cox proportional hazards regression was performed on the batch-adjusted
cohort (P < 0.05) to identify lncRNAs associated with survival. The training cohort was subject to the minimum absolute contraction and selection operator (LASSO) algorithm and multi-factor Cox regression model, which underwent 10-fold cross-validation and 1000 cycles of 1000 random stimuli to avoid overfitting[48, 49]. The R-package "glmnet" identified genetic markers containing biomarkers helpful for prognosis and calculated the risk score for each sample in the entire cohort based on the model, using the formula: Risk score = (lncRNA1 coefficient × lncRNA1 expression) + (lncRNA2 coefficient × lncRNA2 expression) + ... + (lncRNA_n coefficient × lncRNA_n expression). Patients in TCGA were divided into high-risk and low-risk groups according to median risk score in the training and test sets, respectively. Kaplan-Meier (K-M) curves were plotted to compare OS between the risk groups, and the patient's risk curve was drawn. The ROC package was used to draw the receiver's work characteristic (ROC) curve and calculate the area under the curve (AUC) to evaluate model accuracy. Univariate and multivariate Cox regression analyses were conducted on clinical information of all HCC patients to evaluate whether the risk scoring model showed good predictive ability independent of other clinicopathological characteristics such as age, gender, grade, and stage. Nomograms that included relevant clinical parameters (Sex, Grade, Age, Stage), tumor-lymph node metastasis (TNM) stage, and risk score were constructed using the "rms" and "regplot packages" to evaluate patient survival at 1, 3, and 5 years. The model's superiority was further assessed through correction curves, cumulative risk curves, and clinical decision curves.

Functional enrichment analysis

Gene set enrichment analysis (GSEA) was performed using the "clusterProfiler" software R package to identify potential enrichment pathways or potential functional pathways of anoikis marker-related lncRNA markers between samples from high-risk group and low-risk group[50]. TCGA samples were divided into high-risk group and low-risk group according to the optimal threshold. Set pvalue cut off = 1, minGSSIZE = 15, maxGSSIZE = 300, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) dataset c2.cp.kegg.symbols.gmt and the Gene Ontology(GO) dataset c5.go.symbols.gm for GSEA enrichment analysis. P < 0.05 was considered statistically significant.

Cell lines and culture conditions

Four HCC cell lines (SNU-387, Huh7, HepG2, and Hep3B) and normal hepatic epithelial cell line (L02) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Huh7 and HepG2 cells were cultured in modified medium (DMEM)(Gibco-BRL, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100µg/ml streptomycin. Hep3B cells were cultured in MEM medium (HyClone), supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% non-essential amino acids (Gibco, #11140050). SNU-387 and L-02 cells were cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were incubated at 37 in a cell incubator containing 5%CO2.

RNA extraction and quantitative real-time fluorescent quantitative PCR(qRT-PCR)
RNA Extraction Total RNA was extracted from HCC cell lines or clinical samples using TRizol's reagent (Takara BioInc, Shiga Prefecture, Japan) according to the manufacturer's instructions. The cDNA was extracted using a reverse transcription system reagent (Vazyme, #R233-01). Quantitative real-time PCR (qRT-PCR) was conducted on the SteponePlus (Applied Biosystems) using SYBR qPCR Master Mix (Vazyme, #Q511-02) and 10 µM primers. Relative expression values were normalized to the control gene (GADPH). The primer pairs used in this study are shown in Table 1.

Tumor immune cell infiltration

The immune infiltration levels of high-risk and low-risk groups were estimated by TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPCOUNTER, XCELL and EPIC algorithm[51]. GSVA, GSEABase, limma software packages, and single-sample gene set enrichment analysis (ssGSEA) algorithms were used to evaluate immune function between high-risk and low-risk ARlncSig-based populations. Subsequently, differences between the two groups in gene expression levels of 47 immune checkpoints and 12 m6A factors were analyzed.

Drug sensitivity analysis

Semi-maximal inhibitory concentration (IC50) values for chemotherapeutic agents were obtained from the cancer susceptibility genomics (GDSC) database (https://www.cancerrxgene.org/)[52]. Based on ArlncSig, 198 drugs were tested for susceptibility between high and low subgroups by oncoPredict and limma software packages.

**Statistical analysis**

Statistical analysis was performed using RStudio and the corresponding software packages. Overall survival was analyzed using the Kaplan-Meier method and the log-rank test. The prognostic significance of IncRNA in patients with HCC was calculated using univariate and multivariate Cox regression analyses. Person is used in correlation analysis. P < 0.05 was considered to be statistically significant.

**Declarations**

Acknowledgments

None.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors. We thank the contributor of TCGA (https://cancergenome.nih.gov/) for sharing the LIHC dataset on open access. We are grateful to all the participants who have enabled this research.
Ethics Statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Nantong Tumor Hospital. The patients/participants provided their written informed consent to participate in this study.

Author Contributions

JH and WJ made equal contributions to this study. JK and CL carried out the data analysis; JH drafted the manuscript; JH and SM reviewed and revised the manuscript; WJ and YL were responsible for funding acquisition. All authors have read and approved the final version of the manuscript for publication.

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Conflict of interest

The authors state that the study was conducted without any commercial or financial relationships that could be considered a potential conflict of interest.

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Tables

Table 1: Multivariate cox regression analysis of five prognostic IncRNAs.

<table>
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<th>LncRNAs</th>
<th>Coefficient</th>
<th>HR</th>
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<td>AL031985.3</td>
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<td>DDX11-AS1</td>
<td>0.4675</td>
<td>1.5960</td>
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<td>AC026412.3</td>
<td>0.5321</td>
<td>1.7024</td>
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<td>MKLN1-AS</td>
<td>0.3445</td>
<td>1.4113</td>
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<td>TMCC1-AS1</td>
<td>0.2528</td>
<td>1.2877</td>
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Table 2: PCR primer sequences.

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<th>Gene</th>
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<tr>
<td>AL831985.3</td>
<td>F: TCTCActATGTGTTGCTGGACTGG</td>
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<tr>
<td></td>
<td>R: CcAcAgATCActAACACGcC</td>
</tr>
<tr>
<td>AC026412.3</td>
<td>F: TGTGAGGTGAGGGAGCGAT</td>
</tr>
<tr>
<td></td>
<td>R: TGAGCCAAAGGGATCTACGC</td>
</tr>
<tr>
<td>MKLN1-AS</td>
<td>F: GTGTTTCTCTCTGAAAGCAGCG</td>
</tr>
<tr>
<td></td>
<td>R: TTCAAAAGTGACCAAAGCCAGG6</td>
</tr>
<tr>
<td>TMCC1-AS1</td>
<td>F: GGTAGGGTAGCAGGTGACATATC</td>
</tr>
<tr>
<td></td>
<td>R: TTGTCACAGGCCAGACTACCAG</td>
</tr>
<tr>
<td>DDX11-AS1</td>
<td>F: CCTCTGCTACAATACAAAAGTCA</td>
</tr>
<tr>
<td></td>
<td>R: CAGGGTAAATGTACTTCAGCCAC</td>
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Figures
Figure 1

Flow chart of the study.
Figure 2

Functional enrichment analysis of differentially expressed anoikis-related genes.

(A) The volcano plot of 21 differentially expressed genes.

(B) Heatmap of differentially expressed anoikis-related genes in HCC and adjacent tissues (blue: low expression level; red: high expression level).

(C) Enriched GO terms and for differentially expressed anoikis-related genes.

(D) Enriched KEGG pathways for differentially expressed anoikis-related genes.
Figure 3

Construction of prognostic risk signature for patients with HCC based on anoikis-related IncRNAs in the training set. (A) The volcano plot of anoikis-related differentially expressed IncRNAs. (B) Distribution plot of the partial likelihood deviation of the LASSO regression. Six variables were retained when the partial likelihood deviation reached the minimum (log lambda = –2.4). (C) Distribution plot of the LASSO coefficient. (D) The expression of the five IncRNAs and poor prognosis for the high expression group. (E) Sankey diagram of the relationship between IncRNAs and mRNAs. *P < 0.05. (F) The study established a prognostic co-expression network to depict the correlation between the most important five pyroptosis-associated IncRNAs and mRNAs.
Evaluation and validation of anoikis-related IncRNA signature for overall survival in patients with HCC in three datasets. Risk scores and expression profiles of five-IncRNA signature in the high- and low-risk groups showed in the training cohort. (A,B) ROC analyses and Kaplan–Meier survival in the entire cohort (left), training cohort (middle), and testing cohort (right), respectively. Risk scores and expression profiles of five-IncRNA signature in the high- and low-risk groups showed in the entire cohort (C), training cohort (D), and testing cohort (E).
Figure 5

Evaluation of the prognostic anoikis-related IncRNAs signature (A) Forest plot for univariate Cox regression analysis. (B) Forest plot for multivariate Cox regression analysis. (C) The ROC curve of the risk score and clinicopathological variables. Heatmap to show the connection between clinical pathology, clusters, and risk scores. (D) The results indicated that the expression of the five IncRNAs in the high-risk group was significantly higher than that in the low-risk group, and this trend was more obvious in the high-risk group. N, lymph node; M, metastasis; T, tumor.
Figure 6

Construction and evaluation of the nomogram for clinicopathological characteristics and risk signature. (A) Nomogram combining ARlnCSig and clinicopathological characteristics for predicting prognosis of HCC patients in the entire cohort. (B) Calibration curve analysis of the nomogram for the probability of overall survival at 1, 3, and 5 years. (C) Cumulative risk curve. (D) Decision curve analysis (DCA) of the predictions. N, lymph node; M, metastasis; T, tumor. ***P < 0.001.
Figure 7

Functional GSEA analysis of PrlncSig. (A) Immune-related GO terms significantly enriched in high-risk patients. (B) Representative KEGG pathways significantly enriched in high-risk patients.
Figure 8

Immune microenvironment analysis in different risk groups. (A) Heatmap of all significantly differential immune responses between high- and low-risk groups based on TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPCOUNTER, XCELL, and EPIC algorithms. Immune cell scores. Immune function scores (B), immune checkpoints (C) and m6A methylation (D) in high- and low-risk groups based on ssGSEA algorithm.
Figure 9

Drug effectiveness of different risk groups and expression of ARLs in HCC patients. (A) 5-Fluorouracil. (B) Cisplatin. (C) Oxaliplatin. (D) Sorafenib. (E) Relative expression of five ARLs in HCC patients.

Supplementary Files

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