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Bioinformatics analysis to identify effect on silencing LIX1L gene in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a prevalent and aggressive liver cancer with high morbidity and mortality rates, often associated with hepatitis B and C virus infections. In this study, RNA sequencing data from HCCLM3 cells transfected with siLIX1L or siControl were analyzed to identify differentially expressed genes (DEGs) and their functional implications. Using the HISAT2 pipeline, we identified a total of 746 DEGs, including 312 upregulated and 434 downregulated genes. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses revealed significant enrichment in biological processes such as angiogenesis, cell proliferation, cell migration, and apoptosis. Protein–protein interaction (PPI) network and module analyses identified 20 hub genes and 4 clusters, which were predominantly associated with cancer-related pathways. Of these hub genes three (TP53, STAT3, VEGFA) were upregulated, while 17 (HSP90AA1,HIF1A,NOTCH1, PLK1, CD44, TGB1, TOP2A, FGF2, RAD51, MKI67, PXN, TCP1, NPM1, RRM2,ANLN,CDC20,TPN11) were downregulated in siLIX1L-transfected samples. These results showed that HCCLM3 cells transfected with siLIX1L strongly reduced HCC cell proliferation compared to control cells. The results suggest that targeting LIX1L could be an effective strategy for mitigating the progression of Hepatocellular Carcinoma. However, further research, including in vitro and in vivo studies, is necessary to fully validate LIX1L as a therapeutic target and to understand the underlying mechanisms of its role in HCC.

Keywords: Hepatocellular carcinoma (HCC), RNA sequencing (RNA-Seq), Differentially expressed genes (DEGs), Gene Ontology, KEGG pathway enrichment, Protein–protein interaction network, LIX1L silencing.

Introduction

Hepatocellular carcinoma (HCC), which accounts for over 80% of liver cancer cases globally, is one of the biggest risks to human health (Burton et al., 2024). There are several types of liver cancer that spread from other organs to the liver. Hepatocellular carcinoma, on the other hand, is a cancer that begins in the liver (Kinsey et al., 2024). Due to the disease's complex pathogeny, the heterogeneity of cancer cells, and the low rates of early diagnosis, individuals with this illness have a terrible prognosis (Leowattana et al., 2023). HCC is being treated with non-surgical procedures such radiofrequency ablation, trans-arterial embolization and percutaneous ethanol injection in addition to potentially curative surgical resection (Stefani-

ni et al., 2024). Estimating the overall survival (OS) of patients with HCC remains a tough task for the purpose of treatment plan selection (Ninomiya et al., 2023). Tumor categorization systems such as TNM, Okuda, Cancer of the Liver Italian Programme, and Barcelona Clinic Liver have been widely used in prognostic evaluation (He et al., 2023). Nevertheless, there is presently disagreement about the best staging strategy for determining an HCC patient's prognosis due to the variety of cancers. A-fetoprotein (AFP) and carbohydrate antigen (CA19-9), two commonly used biomarkers, perform relatively poorly as predictors of long-term prognosis.

Many kinds of cancer have high expression levels of the putative RNA-binding protein (RBP) limb expression 1-like (LIX1L), which contains a double-stranded RNA binding motif. It has been determined that LIX1L is an important regulator of cell proliferation. limb expression 1 (LIX1), the Vertebrate Ortholog of Lowfat (Lft) gene, and LIX1L were originally found to be genes expressed in chicken limbs. Because human cancer cells preferentially produce the LIX1L protein, it may be possible to target it for therapeutic effects in a range of cancer types. By controlling the glucose metabolism through the down-regulation of FBP1 (fructose-1,6-bisphosphatase), LIX1L encouraged the development of HCC (Li et al., 2020). The evident up-regulation of LIX1L in HCC tissues is associated with a poor prognosis for HCC patients (Pessino et al., 2024). LIX1L enhances cancer cell proliferation. Functional investigations revealed that LIX1L knockdown increased gluconeogenesis, upregulated FBP1, and decreased the growth and spread of HCC (Zou et al., 2021).

RNA Seq technology can be used to find novel cancer biomarkers, possible therapeutic targets, and guide targeted therapy during early treatment decisions by detecting differentially expressed genes (DEGs) as well as high molecular risk mutations (Hong et al., 2020). The current study used differential expression analysis, enrichment analysis, and network analysis to determine whether silencing the LIX1L gene is an effective technique for inhibiting the proliferation of hepatocellular carcinoma.

Materials and methods

Data source

RNA Seq data of HCC was retrieved from ENA (https://www.ebi.ac.uk/ena/) database with the accession number SRP269155. A total of six samples (SRR12105763, SRR12105764, SRR12105765, SRR12105766, SRR12105767 and SRR12105768), three of which were transfected with siLIX1L and three of which were transfected with siControl were retrieved from the database.

DEG analysis

Following the download of the FASTQ file containing the paired-end raw reads of the RNA-Seq data [SRP269155] from ENA, the quality of the raw reads was assessed using FastQC . Using the HISAT2 tool version 2.2.1 , reads were mapped to the human reference genome GRCh38 (Kim et al., 2015). StringTie was used to assemble transcripts (Pertea et al., 2015). Differential expression was analyzed using Ballgown (Frazee et al., 2015). Genes that satisfied the criteria of (logFC) > 1 and logFC < − 1 and a p value threshold of 0.05 were chosen as significant differentially expressed genes (Udhaya Kumar et al., 2020).

Go and pathway functional enrichment analyses

The DAVID tool (Database for Annotation, Visualisation and Integrated Discovery) was used to perform GO enrichment and KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway analysis of specific DEGs. The three categories in the Gene Ontology analysis were cellular component, biological processes, and molecular function. A t-test (ANOVA) was conducted as the default setting for statistical analysis (Krušič et al., 2024). Genes were mapped to KEGG pathways for the pathway functional analysis, and the cutoffs were set at p-value 0.05 and count > 2 (Li et al., 2018).

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PPI network construction and module functional analysis

The protein-protein interaction network was constructed with the STRING (Search Tool for the Retrieval of Interacting Genes) (version 11.5) on-line database. With a confidence score > 0.4, the PPI network of upregulated and downregulated DEGs created using STRING was deemed statistically significant. The protein interaction network was then visualised by importing the data into Cytoscape (version 3.9.1) . Module analysis was used to find subsets of genes that have comparable expression patterns across multiple conditions. To explore the interaction linkages between the DEG and the PPI network, significant modules were chosen. MCODE in Cytoscape was used, with default thresholds set to 2 degrees, 0.2 node scores, 2 k-cores, and 100 maximum depths.

Identification of hub genes

Genes with significant interconnections are known as hub genes. The hub genes were found using the hybrid centrality metric (HCM) (Arya et al., 2021). To calculate the HCM, the closeness, betweenness, and mean degree of extra interacting genes were considered. Hub genes were defined as the genes that produced the greatest HCM scores, as shown below:

Hybrid centrality measure of one gene = (degree of node + closeness + betweenness) + (∑ Degree of connected nodes)/ No. of connected nodes)

Results and Discussion

Identification of DEGs

The RNA sequencing analysis identified 6,631 genes. Among these, 746 genes were found to be differentially expressed (DEGs) based on an adjusted p-value of less than 0.05, with a log fold change logFC > 1 for upregulation and logFC < -1 for downregulation. Out of the 746 DEGs, 312 genes were upregulated, and 434 genes were downregulated.

GO Function and KEGG Pathway Enrichment Analysis

These DEGs' possible functions were investigated through the use of DAVID and GO analysis. The overexpressed DEGs were extremely evident in biological processes such as angiogenesis, apoptosis, and cell division. (Figure 1). There were eight genes involved in the biological process small GTPase mediated signal transduction.

Five genes related to biological processes including the Notch signalling system and positive control of angiogenesis were among the six genes involved in cell cycle arrest. Among the molecular functions that were enriched in these were transcription factor binding, transcriptional activator activity, and RNA polymerase II core promoter proximal region sequence-specific binding.

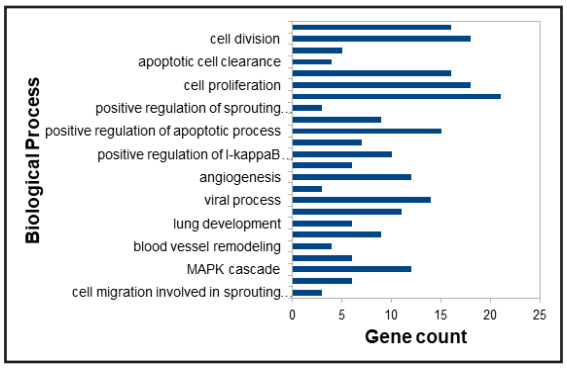


Figure 1. Common biological processes enriched in upregulated DEGs

Significantly more abundant in the downregulated DEGs were the biological processes of angiogenesis, cell division, proliferation, and the cellular components cytoplasm, nucleus, and nucleoplasm as well as molecular functions such as protein, enzyme, and calcium ion binding, receptor activity, kinase activity, and Poly (A) RNA binding. (Figure 2). There were eighteen genes involved in the cell proliferation and cell division. Fifteen genes involved in positive regulation of apoptotic process, twelve genes involved in the biological process such as angiogenesis etc. These findings are consistent with the effects

seen in gene silencing, where the reduction or complete inhibition of gene expression often leads to decreased cellular activities and altered molecular interactions, demonstrating the similar impact of downregulation and silencing on cellular functions.

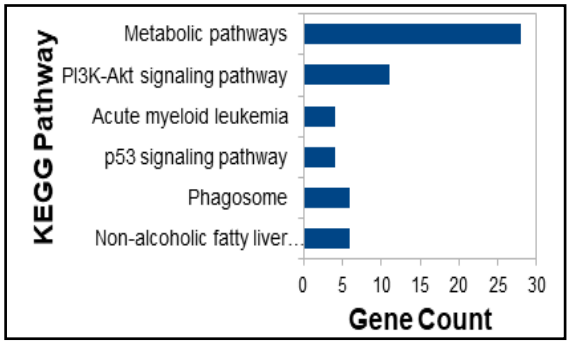


Figure 2. Common biological processes enrichment in downregulated DEGs

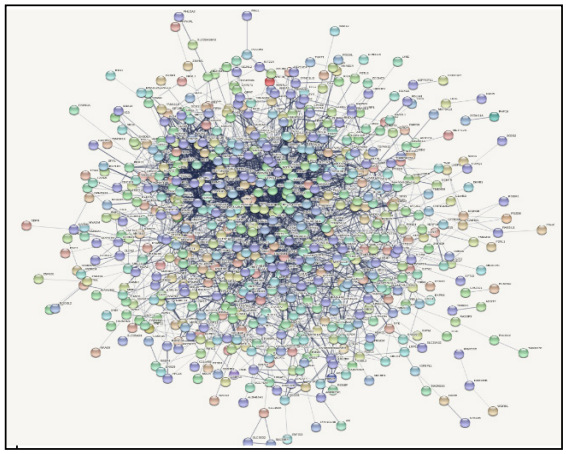
The most significantly enriched KEGG pathways of the upregulated differentially expressed genes were notably enriched in several pathways, including renal cell carcinoma, pancreatic cancer. In particular, twenty-eight genes were involved in metabolic pathways, while six genes were associated with biological processes such as non-alcoholic fatty liver disease (NAFLD) and phagosome formation.

The downregulated genes were enriched in the pathways such as cancer, Proteoglycans in cancer, Pancreatic cancer, PI3K-Akt signalling pathway. There were twenty-two genes involved in pathways in cancer. Seventeen genes involved in PI3K-Akt signaling pathway, fourteen genes involved in Proteoglycans in cancer etc. This analysis suggests that the downregulation of these genes may disrupt crucial oncogenic pathways, potentially reducing cancer cell viability. These findings align with Zou et al. (2021), who demonstrated that LIX1L promotes cancer progression through multiple pathways. This comparison indicates that the silencing of LIX1L impacts key pathways involved in cancer, which is consistent with its role in enhancing cancer progression.

PPI Network Construction, Module Analysis, and Identification of Hub Genes

Protein function and cellular regulation mechanisms were explored through a genome-wide analysis of protein-protein interactions (PPIs) using data from the STRING database, resulting in the construction of a PPI network with 708 nodes and 2,774 edges (Figure 3). To identify significant modules within this network, the Cytoscape plugin MCODE was utilized, focusing on modules with an MCODE score >5. Four notable modules were identified (Figure 4). Of these, three modules (Modules 1, 3, and 4) were enriched in pathways related to tumor progression and angiogenesis, indicating their functional relevance. Specifically, Module 3 was closely associated with the PI3K-Akt signaling pathway (involving ten genes) and pathways in cancer (seven genes). Module 4 was primarily linked to pathways in cancer (six downregulated genes) and proteoglycans in cancer (five downregulated genes), highlighting the impact of these downregulated genes on critical cancer-related processes.

Figure 3. Analysis of the protein-protein interaction network of DEGs. The Search tool for the Retrieval of Interacting Genes (STRING) created the PPI network of DEGs and analyzed using Cytoscape.



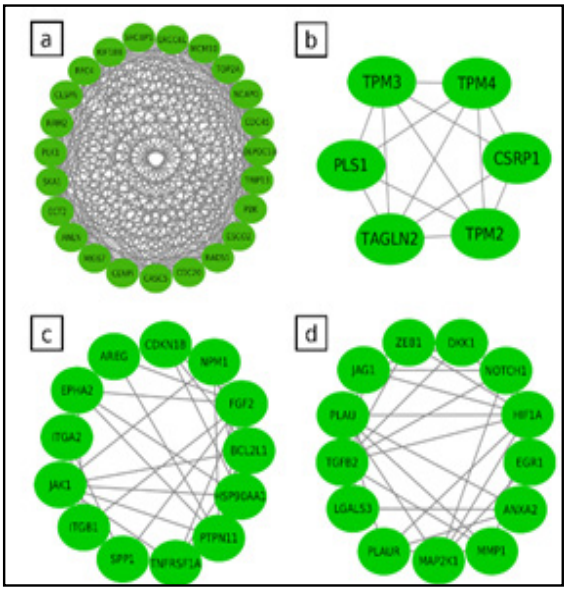


Figure 4. Modules from the PPI were extracted using Cytoscape's MCODE plugin, with the default thresholds of degree cut-off: 2, node score cut-off: 0.2, k-core: 2, and max depth: 100. The MCODE score >5 was used to determine which modules were the most important. There were 23 nodes and 224 edges in Module 1 (a), 6 nodes and 14 edges in Module 2 (b), 13 nodes and 31 edges in Module 3 (c), and 13 nodes and 30 edges in Module 4 (d).

Hub genes were identified from the protein-protein interaction (PPI) network using a hybrid centrality measure, with a score threshold set above 12. A total of 20 hub genes were classified based on their log fold change (logFC) values into upregulated and downregulated categories. Upregulated hub genes included TP53, STAT3, and VEGFA, all with positive logFC, indicating increased expression and roles in tumor suppression, cell growth, and angiogenesis. Downregulated hub genes, with negative logFC values, included HSP90AA1, HIF1A, NOTCH1, PLK1, CD44, ITGB1, TOP2A, FGF2, RAD51, MKI67, PXN, TCP1, NPM1, RRM2, ANLN, CDC20, PTPN11 and several others, reflecting decreased expression and involvement in processes like cell cycle regulation, hypoxia response, and cancer progression. These findings, visualized in Figure 5, highlight the critical regulatory roles of these genes and their potential implications in disease mechanisms.

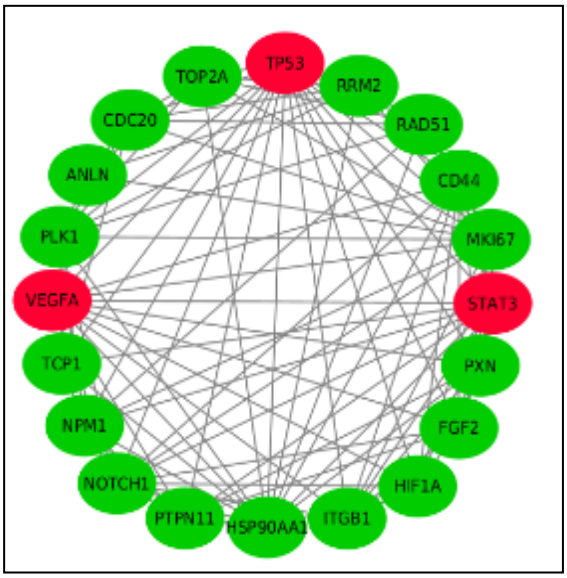


Figure 5. Analysis of the network of interactions between the 20 hub genes' proteins. Using STRING, a PPI network of 20 hub genes was constructed, and it was deemed statistically significant with a confidence value higher than 0.4. Red is used to indicate upregulated genes and green is used to indicate downregulated genes. The hub genes are represented by circles, and their interactions are shown by connecting lines.

The upregulated hub genes' KEGG pathway analysis revealed a strong enrichment in networks like viral carcinogenesis, Ras signaling pathway. Nonetheless, Table 1 shows that the downregulated hub genes were significantly concentrated in pathways related to prostate cancer, the PI3K-Akt signalling pathway, cancer pathways, and proteoglycans in cancer. This indicates that gene silencing could disrupt these critical pathways, reducing their activity and potentially impacting cancer progression and cell lar signaling processes.

Table 1. KEGG pathway analysis of hub genes

KEGG Pathway	Hub Genes
Ras signaling pathway	PTPN11, FGF2, VEGFA
Renal cell carcinoma	PTPN11, HIF1A, VEGFA
Viral carcinogenesis	CDC20, PXN, STAT3, TP53
PI3K-Akt signaling pathway	ITGB1, HSP90AA1, TP53, FGF2, VEGFA
MicroRNAs in cancer	NOTCH1, STAT3, TP53, CD44, VEGFA
Pancreatic cancer	RAD51, STAT3, TP53, VEGFA
Pathways in cancer	ITGB1, HSP90AA1, RAD51, STAT3, HIF1A, TP53, FGF2, VEGFA
Proteoglycans in cancer	ITGB1, PXN, STAT3, PTPN11, HIF1A, TP53, FGF2, CD44, VEGFA

The enrichment analysis was conducted using DAVID, using a significant enrichment cut-off of p 0.05 and a set count >2. A list of hub genes with several pathway enrichments exists.

Discussion

In the present study, we identified that the HCC cells transfected with siLIX1L strongly reduced HCC cell proliferation as compared to HCC control cells. A series of bioinformatics analysis was performed to screen key genes and pathways closely related to HCC. Six ENA datasets were analysed using HISAT pipeline which shared 746 common DEGs. The genes with logFC (fold change) > 1 and logFC (fold change) < -1 were selected as DEGs . For a further in-depth knowledge related the functional DEGs, GO function and KEGG pathway analysis of these DEGs were carried out. PPI network complex was constructed using these DEGs and four modules with the most significant degree were filtered from the PPI network complex by the MCODE plug-in. There were a total of 20 hub genes predicted, 3 of which were upregulated and 17 of which were downregulated. The upregulated hub genes (TP53,STAT3,VEGFA) were enriched in the KEGG pathways such as Pancreatic cancer, Proteoglycans in cancer, Ras signaling pathway. The downregulated hub genes (HSP90AA1,HIF1A,NOTCH1, PLK1, CD44, TGB1, TOP2A, FGF2, RAD51, MKI67, PXN, TCP1, NPM1, RRM2, ANLN, CDC20, TPN11) were associated with Proteoglycans in cancer, cell cycle, pancreatic cancer, PI3K-Akt signaling pathway etc. CDC20 is involved in the activation of the anaphase-promoting complex (APC), a key regulator of cell division. PLK1 (Polo-like kinase 1) plays a pivotal role in various stages of mitosis. MKI67 is a well-known marker for cell proliferation. ANLN is involved in cytokinesis, the final step of cell division. RRM2 is essential for DNA synthesis and repair, supporting cell proliferation. TOP2A is crucial for DNA replication and chromosome segregation. The downregulation of these genes following LIX1L silencing suggests a disruption in the normal progression of the cell cycle. This disruption likely leads to a reduced proliferation rate of HCC cells, which aligns with the observed decrease in cell growth in this study. Essentially, LIX1L may support cell cycle progression, and its silencing could induce cell cycle arrest or slow down cell division, thus inhibiting tumor growth. The observed downregulation in metabolic pathways, which were also highlighted in the study by Zou et al., suggests that LIX1L might influence metabolic reprogramming in cancer cells. The reduction in these pathways upon LIX1L silencing could impair the metabolic flexibility of HCC cells, leading to reduced proliferation. The downregulation of genes involved in cancer-specific pathways following LIX1L knockdown supports the hypoth-

esis that LIX1L contributes to the activation of oncogenic signaling in HCC. The suppression of these pathways might be directly responsible for the observed anti-proliferative effects.

TP53 is accepted as the 'guardian of the genome'. During cell division, it serves as a master checkpoint. When it detects DNA damage, it causes cell arrest, and if the damage is not repaired, it promotes apoptosis. In HCC, STAT3 is typically considered as an oncogene. Several STAT3 target genes have been linked to the development of aggressive carcinomas, angiogenesis, and hepatocellular tumors. Depending on the genetic context and the etiology of the HCC, STAT3 has the potential to function as a tumor suppressor . Vascular endothelial growth factor (VEGF), which is overexpressed in HCC, is thought to be the driving force behind both healthy and pathological angiogenesis. The enhanced VEGF expression suggested a possible role of angiogenesis in HCC carcinogenesis (ElGhandour et al., 2021).

The higher expression of HSP90AA1 in cancer tissues are reported to be connected with poor overall survival (Liu et al., 2021). In lung cancer patient tissues, greater levels of HSP90AA1 transcription, expression, and AKT1/ERK pathway activation were confirmed (Niu et al., 2022).HSP90AA1 transcript levels were considerably greater in HCC patients than in cirrhotic patients, with late HCC patients having stronger up-regulation of these transcripts. umerous cancers, including breast, endometrial, ovarian, colon, lung, and prostate cancers, have high levels of HSP90AA1 expression. It aids in controlling mitochondrial apoptosis and signal transduction brought on by growth factors, stress signals, and death receptors during the development of malignant tumors.

High levels of accumulated HIF-1 under hypoxia increase the expression of several angiogenic factors, including VEGF, and improve the stability of VEGF mRNA, ultimately driving tumor angiogenesis. Many cell processes, including as cell specification, proliferation, and apoptosis, which affect the development and control of multiple organs, are influenced by notch signalling. The proliferation of different prostate cancer cells was inhibited by the overexpression of active Notch1, indicating that Notch activation can also cause growth arrest and, it appears, reduce the neoplastic potential of tumors.

The siRNA-mediated suppression of PLK1 can considerably slow the course of the hepatocellular cell cycle and increase cell death. Even while the correlation between PLK1 and metastasis was not as strong, upregulated PLK1 expression could be a precursor to HCC and the cancerization of cirrhosis. A considerable reduction in cell proliferation and an increase in cell death can result from inhibiting PLK1 expression in HCC cell lines. In HCC, CD44 was overexpressed to encourage HCC cell migration and proliferative growth via oncogenic YAP, the primary downstream regulator in the Hippo pathway. Given its possible significance in the pathogenesis of HCC, the CD44-YAP axis offers potential targets for HCC management as well as an understanding of the pathophysiology of the disease (Zhang et al., 2021).

FGF plays a crucial role in the development and progression of human HCC due to the fact that FGF controls a number of biological functions, including metastasis, drug resistance, cell migration, invasion, and proliferation (Seitz et al., 2022). The serum FGF2 levels were higher in individuals with chronic hepatitis (CH), liver cirrhosis (LC) or HCC compared with healthy volunteers. It is interesting to note that the progression of chronic liver disease was strongly correlated with serum FGF2 levels. In terms of the underlying mechanisms, FGF18 was down-regulated by its siRNA, which prevented cell proliferation. In contrast, FGF8, FGF17, and FGF18 caused cell proliferation and tube formation. However, siRNA-mediated downregulation of FGF19 expression greatly reduced HCC cell growth and triggered apoptosis.

Topoisomerase II alpha (TOP2A) was shown to be strongly upregulated in HCC cancer

tissues (Meng et al., 2022). Significant cirrhosis, a bigger tumor diameter, and poor tumor differentiation have all been associated with high TOP2A expression (Cai et al., 2020). A poor prognosis and invasiveness of HCC tumors are associated with high expression of TOP2A, which can also act as a prognostic indicator for radical HCC resection (Hasan et al., 2023). In 2020, Cai et al. In cases of hepatocellular carcinoma, TOP2A promotes metastasis and growth. In HCC tissues, TOP2A was upregulated and connected with both the T and M phases and a dismal prognosis for the illness. (Wang et al., 2022).

The expression of MKI67 was upregulated in liver cancer tissues (Song et al 2024). The kind and stage of liver cancer tissue affected the expression levels of TGFB1 and MKI67. Moreover, MKI67 expression was linked to TGFB1 expression in HepG2 cells and liver cancer tissues. Patients with increased MKI67 expression levels and HBV-related HCC had a worse prognosis. In HCC tissues and cell lines, ANLN was upregulated. A high ANLN level was linked to a worse outcome in patients with HCC. ANLN expression may be a valuable predictive biomarker and contributed to the onset and development of human HCC. In HCC, anillin stimulates cell division and tumor formation. Take-down Anillin suppresses Hep3B.

The small subunit of ribonucleotide reductase, RRM2, is frequently expressed highly in malignancies (Zuo et al., 2024). RRM2 is known to promote tumor growth and is a target for cancer treatment. When used alone or in conjunction with current treatments, SiRNA, RRM2 inhibitors, kinase inhibitors, and other compounds successfully lower RRM2 expression levels or activity in cell lines and animal models, and they show potential as a novel anticancer therapy. (Zhan et al., 2021).

In this study, it was observed that these 17 downregulated hub genes are associated with cell proliferation in HCC, were downregulated when the HCCLM3 cells were transfected with siLIX1L. Cell proliferation was significantly decreased compared with siLIX1L untreated cells. Based on the literature reports, the above-mentioned genes were associated with cell proliferation. But under this condition when LIX1L got silenced, we could notice that LIX1L has crucial role in silencing the HCC associated genes. Our study provides initial insights into how LIX1L silencing impacts HCC by disrupting key pathways involved in cell proliferation and survival. However, we acknowledge that further experimental validation is required to fully elucidate the mechanistic underpinnings of these observations. Future work will involve detailed in vitro and in vivo studies to confirm the roles of these pathways and the potential of LIX1L as a therapeutic target in HCC.

Conclusion

In this study, we demonstrated that silencing the LIX1L gene in HCCLM3 cells led to a significant reduction in hepatocellular carcinoma (HCC) cell proliferation compared to control cells. LIX1L appears to play a key role in cell proliferation, as its silencing resulted in the downregulation of 17 genes, many of which are implicated in cancer progression. These findings suggest that targeting LIX1L could be an effective strategy for mitigating the progression of Hepatocellular Carcinoma.

However, several limitations should be acknowledged. The study is based on a relatively small number of RNA sequencing datasets, which may limit the generalizability of the findings. The lack of experimental validation in vitro and in vivo means that the observed effects of LIX1L silencing on gene expression and cell proliferation need further confirmation.

Future research should address these limitations by incorporating larger sample sizes and experimental validation to confirm the functional relevance of LIX1L and to explore its mechanisms in HCC. This will provide a more comprehensive understanding of LIX1L's role and its potential as a therapeutic target in HCC.

Declarations

Availability of data and materials

Supplementary information SI contains all of the data created or examined throughout this investigation.

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Conflict of interest

Regarding this work, the authors declared that they have no conflicts of interest.

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