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Proteomic profiling identifies miR-423-5p as a modulator of oncogenic metabolism in HCC

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Abstract

Background Hepatocellular carcinoma (HCC) remains a significant clinical challenge due to limited diagnostic and therapeutic options. Non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), play key roles in cancer biology. Our previous findings showed that miR-423-5p enhances anti-cancer effects on HCC patients treated with sorafenib by promoting autophagy. Here, we investigated the molecular mechanisms underlying miR-423-5p function through a comprehensive proteomic approach.

Methods We generated an HCC cell line stably overexpressing miR-423-5p via lentiviral transduction. Total proteins were extracted from SNU-387 cells, enzymatically digested into peptides, and subsequently analysed by liquid chromatography-tandem mass spectrometry (LC-MS/M). Raw spectral data were processed and quantified using MaxQuant. Differentially expressed proteins (DEPs) were defined based on fold-change ($|log2FC| \geq 1$) and false discovery rate (FDR < 0.05). The full proteomic dataset is available via the ProteomeXchange repository (identifier: PXD064869). Functional enrichment analysis of DEPs were performed using DAVID and Reactome. To assess clinical relevance, predicted and validated miR-423-5p targets were integrated with The Cancer Genome Atlas (TCGA) Liver Hepatocellular Carcinoma (LIHC) dataset using GEPIA platform. Survival analyses were performed using the Kaplan–Meier method.

Results Proteomic profiling identified 698 DEPs in miR-423-5p-overexpressing cells compared to controls with significant enrichment in metabolic pathways, related to purine/pyrimidine metabolism and gluconeogenesis. Integration with bioinformatic predictions and miRTarBase validation identified 43 DEPs as potential direct targets of miR-423-5p. Among these, seven proteins (ACACA, ANKRD52, DVL3, MCM5, MCM7, RRM2, SPNS1, and SRM) were significantly associated with patient prognosis in the TCGA-LIHC cohort. These targets were downregulated in miR-423-5p-overexpressing cells but upregulated in advanced-stage HCC tissues, suggesting a potential role for miR-

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423-5p in the regulation of HCC pathogenesis. Stage-specific expression analysis showed increased levels from stage I to III, followed by a decline at stage IV. Notably, we experimentally confirmed miR-423-5p-mediated suppression of MCM7, DVL3, IMPDH1, and SRM (SPEE), supporting their functional involvement in HCC progression.

Conclusion Overall, our findings support a tumour-suppressive role for miR-423-5p in HCC, mediated by modulation of metabolic pathways and suppression of oncogenic proteins. These results suggest that miR-423-5p and its downstream effectors may serve as promising biomarkers and potential therapeutic targets in HCC.

Highlights miR-423-5p acts as a tumor suppressor in HCC by targeting key nodes of pro-tumorigenic signalling.

miR-423-5p significantly altered metabolic pathways, including purine/pyrimidine metabolism and gluconeogenesis.

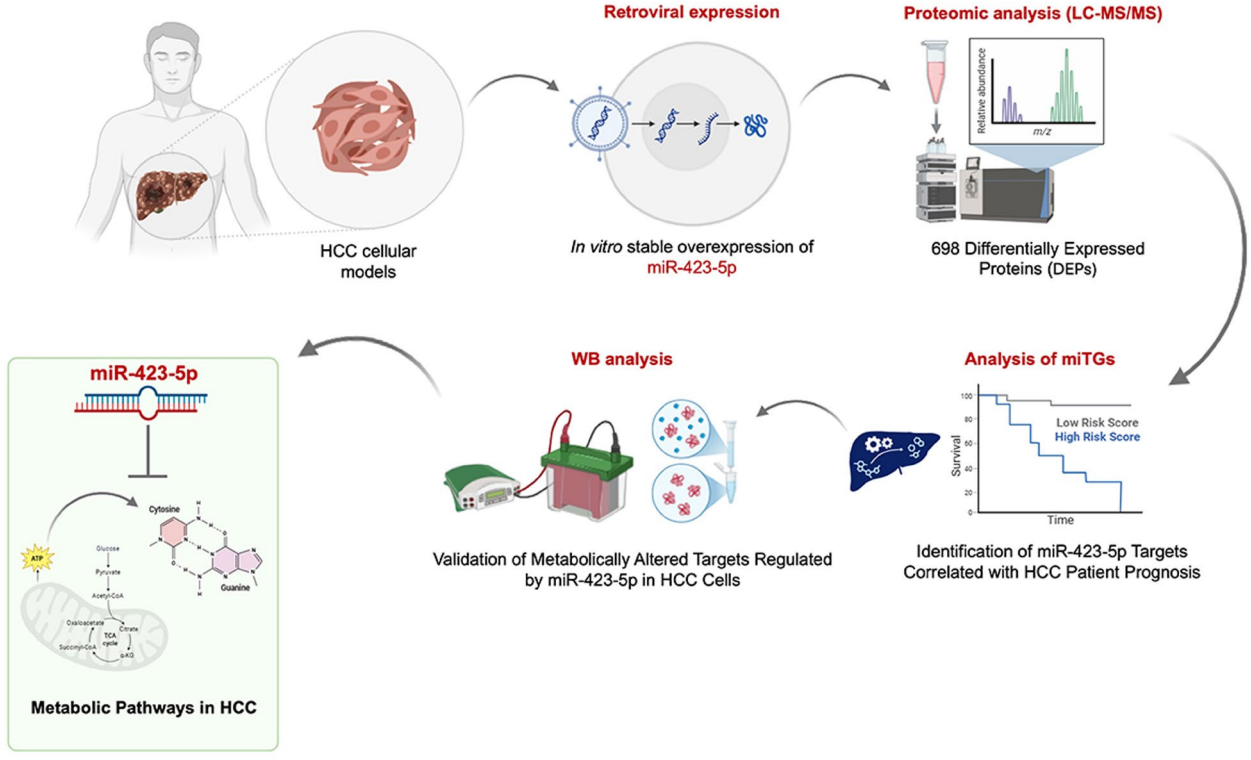
Seven miR-423-5p targets correlate with poor prognosis in TCGA-LIHC patients and are downregulated in miR-423-5p overexpressing HCC cells.

miR-423-5p over-expression induces a significant downregulation of MCM7, DVL3, IMPDH1, SPEE in HCC cell models.

miR-423-5p limits tumor metabolic plasticity, suggesting therapeutic potential.

Keywords Hepatocellular carcinoma, miR-423-5p, Proteomics, Spermidine synthase, Nucleotide metabolism, Gluconeogenesis, Overall survival, Stage plot

Graphical abstract



Background

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the fourth leading cause of cancer-related mortality, with a relative 5-year survival rate of approximately 18% [1–3]. HCC accounts for about 90% of liver cancer cases worldwide [1, 4] and it is considered a complex multistep process inflammation-associated cancer, being connected to liver injury, prolonged and chronic inflammation for viral hepatitis (hepatitis B virus, HBV or hepatitis C virus, HCV), alcohol abuse or alcoholic steatohepatitis (ASH), nonalcoholic fatty liver disease (NAFLD or nonalcoholic steatohepatitis (NASH). Although the liver can adapt to changes in environmental, dietary, and viral conditions; the accumulation of these epigenetic modifications may also trigger or contribute to HCC progression through by disrupting the expression of tumour suppressor genes and oncogenes [5–8]. Over the past decades, therapeutic strategies for

both early and advanced stages of HCC have significantly evolved. In recent years, many tissue biomarkers have been evaluated for selecting appropriate treatments and predicting responses to immunotherapy with checkpoint inhibitors in HCC [9, 10]. Substantial evidence has been accumulated highlighting the dysregulation of multiple cellular processes involved in HCC, including cell cycle progression, DNA methylation, chromosomal stability, immunomodulation, epithelial-to-mesenchymal transition, cancer stem cells and microRNAs (miRNAs) regulation [11, 12]. MiRNAs are small nucleotide sequences able to regulate gene expression at both post-transcriptional and translational level, acting either as oncogenes or tumour suppressor genes. In this context, we have demonstrated that miR-423-5p is significantly upregulated in HCC serum of patients responsive to sorafenib treatment, suggesting its potential role as a surrogate prediction marker of therapeutic response. We have also shown that miR-423-5p inhibits HCC cell proliferation by promoting autophagy [13]. In a separate study using a prostate cancer (PCa) model, we demonstrated that miR-423-5p interacts with the long non-coding RNA (lncRNA) MALAT1, leading to its downregulation. This interaction results in reduced cell proliferation and invasion in vitro, as well as decreased metastatic potential in vivo in a murine model [14]. Moreover, stable transfection of miR-423-5p in PCa cells was found to induce changes in glucose and amino acid metabolism, together with the modulation of several tumor-associated processes, as revealed by a proteomic analysis. It was found that mir-423-5p could induce a metabolic change by inhibiting essential energy processes such as glycolysis and interfering with amino acid biosynthetic pathways in PCa cells. Furthermore, the effects of miR423-5p overexpression are accompanied by interference with angiogenic processes in PCa cells and are consistent with the activation of the miR-423-5p-mediated autophagic pathway in other cancer models [15]. Multiple studies have highlighted the involvement of various microRNAs in the regulation of metabolic pathways and resistance to sorafenib. For instance, miR-22 has been shown to modulate the HIF-1α signaling pathway, enhancing cell survival under stress, promoting a glycolytic shift, and increasing cellular plasticity and resistance to sorafenib in HCC cells by targeting GLUT1 [16]. Elevated serum levels of miR-22 have been linked to sorafenib resistance in both HCC patients and rat models [16]. In a similar manner, miR-494 promotes a metabolic shift in HCC cells toward a glycolytic phenotype by targeting the glucose-6-phosphatase catalytic subunit (G6pc) and activating the HIF-1α pathway. The miR-494/G6pc axis plays a key role in supporting the metabolic plasticity of cancer cells, contributing to the accumulation of glycogen and lipid droplets, which enhances cell survival under adverse environmental conditions. Consistently, high serum levels of miR-494 have been associated with sorafenib resistance in preclinical models and in a preliminary cohort of HCC patients [17]. Metabolic reprogramming is a well-established hallmark of HCC and contributes to its molecular and genetic classification of HCC [18] often involving miRNA-mediated regulatory mechanisms. In fact, it was also reported that KLF4 reduces ATP synthesis in HCC by suppressing the expression of RICTOR, a core component of mTORC2. This impairment promotes glutaminolysis to replenish the TCA cycle and increase ATP levels, facilitated by the promotion of miR-206 transcription [19]. MiR-10b-5p has been shown to inhibit cell growth by targeting the amino acid transporter SLC38A2 (Solute Carrier Family 38 Member 2). Similarly, miR-148a-3p suppresses glycolysis and proliferation in HCC cells by regulating transmembrane protein 54 [20, 21]. Two additional miRNAs, mitomiR-181a-5p and miR-885-5p, play opposite effects on glucose metabolism in HCC cells, thereby differently influencing cell proliferation. MitomiR-181a-5p promotes glycolysis and cell growth by targeting PTEN and activating the AKT signaling pathway [22]. In contrast, miR-885-5p impairs glucose metabolism by downregulating key glycolytic enzymes, such as HK2 and PFKP, leading to inhibited cell proliferation [23]. Chen Z., et al. reported that miR-3662 inhibits HCC cell growth by reducing ERK and JNK signalling pathways and regulates HCC progression under hypoxic conditions, inhibiting the Warburg effect and decreasing the expression of its target HIF-1α [24]. Despite significant advances in therapeutic approaches and biomarker discovery for early diagnosis, HCC remains hindered by the lack of highly sensitive and specific predictive markers. Emerging evidence highlights the pivotal role of miRNAs that serve not only as promising biomarkers for patient classification and therapy monitoring, but also as novel therapeutic targets capable of modulating the tumor microenvironment and overcoming resistance to treatments. In this context, our project aims to investigate the biological pathways underlying the overexpression of miR-423-5p in the inhibition of the development and progression of HCC. This integrative approach will also contribute to a better understanding of the metabolic alterations that characterize HCC, reinforcing the potential of miR-423-5p as a novel biomarker and therapeutic target.

Experimental procedures

Cell lines and growth conditions

Hepatocellular carcinoma cell lines, SNU-387 (ATCC® CRL-2237) and Hep3B (DSMZ no. ACC 93) were purchased from ATTC and DSMZ, respectively. SNU-387 cells were cultured in RPMI1640 (11530586, Gibco Life Technologies, Carlsbad, CA, USA) instead, Hep3B cells

were grown in EMEM. The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), 100 µg/mL penicillin, 100 µg/mL streptomycin, 1% (w/v) L-glutamine (Lonza, Basel, Switzerland). Cells were incubated at 37 °C in 5% (v/v) CO_2 and 100% (v/v) humidity.

Retroviral expression of empty vector and miR-423-5p-mimic in HCC cell models

SNU-387 and Hep3B hepatocellular cell lines were seeded in a 96-well, 25,000 cells/ well, and treated with Sh MIMIC Lenti miR-423-5p lentiviral particles (GE Healthcare Dharmacon V1SMHS_000254) at 2.5, 5, 10 and 20 MOI. Control empty backbone cells were obtained using lentiviral particles generated from the pLKO.1 Empty plasmid (MISSION® pLKO.1. Empty Vector Control Plasmid DNA Sigma Aldrich SHC001). Hexadimethrine Bromide (SIGMA H9268-5G) was used in complete growth media for each cell line to facilitate the infection. After 16 h of incubation, the infection medium was removed and replaced with complete growth medium for each cell line. Puromycin (INVIVOGEN) was then added to each cell complete growth medium at a final concentration of 1.5 µg/ml for the selection. The lentiviral particles used to infect SNU-387 and Hep3B empty backbone clones were produced according to the manufacturer's recommendations. The lentiviral packaging mix was also purchased from Sigma (SHP001).

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis

Samples (~ 50 µg protein) were reduced/alkylated and digested as described previously [25]. Then, samples were dried and resuspended in 5% (v/v) acetonitrile + 0.1% (v/v) formic acid and transferred to an HPLC vial for MS analysis in both SWATH (Sequential Window Acquisition of All Theoretical Mass Spectra) and IDA (information-dependent acquisition) modes. Each sample was analyzed on a SCIEX TripleTof 6600 mass spectrometer coupled in line with an Eksigent ekspert nano LC 425 system running in microflow as described previously with minor modifications [26]. For IDA (Information-Dependent Analysis) to generate a spectral library, 8 µL of pooled sample in triplicate were injected by autosampler (Eksigent nanoLC 425 LC system, Dublin, OH, USA) in microflow at 5 µL/min directly onto a YMC Triart-C18 column (15 cm, 3 µm, 300 µm i.d.) using gradient elution (2–40% Mobile phase B, followed by washing at 80% B and re-equilibration) over 87 min. For SWATH/DIA (Data-Independent Analysis), 3 µL was injected on the same gradient elution profile over 57 min. Mobile phases consisted of A: 0.1% formic acid; B: acetonitrile containing 0.1% (v/v) formic acid. IDA analysis was performed in positive ion mode with a 250 ms survey scan, m/z range 400–1250; The top 30 peaks were selected for fragmentation, with an accumulation time of 50 ms per experiment and a cycle time of 1.8 s. SWATH analysis used 100 variable windows, 25 ms per window, 100–1500 m/z and was performed using the SCIEX Duospray source with a 50 µm electrode at 5500 V.

Library generation, MS data processing and fold change analysis

Raw data files were processed using DIA-NN [27] (version 1.8.2), with a Human SwissProt FASTA database (Sept 2023), which enables library-free search and deep learning-based spectra prediction for library generation. Analysis against the predicted peptide library was carried out with a match between runs on. Labelfree quantification was performed at a precursor FDR of 1.0%, with default DIA-NN parameters unless otherwise specified. Output from the DIA-NN pg_matrix file was further processed using LIMMA via the AMICA [28] 3.0.1 R-based software platform for analysis of proteomics data to generate protein differential expression for downstream analysis, with a min-based imputation, Log2FC >0.6, <-0.6 and adjusted p-value of 0.05.

Identification of differentially expressed proteins (DEPs) and functional enrichment analysis in SNU-387 transduced models

Quantitative proteomics approach was used to identify differentially expressed proteins (DEPs) in hepatocellular carcinoma (HCC) SNU-387 cells overexpressing miR-423-5p compared to control (empty plasmid, pLKO.1). Data table of all the differentially expressed abundant proteins from MS analysis processed using AMICA previously was filtered to identify significant DEPs using adjusted p-value ≤ 0.05 and log2FC |0.6|. Volcano plot of DEPs was created using VolcaNoseR web app https://huygens.science.uva.nl/VolcaNoseR/] and a heatmap of the top 40 upregulated and downregulated DEPs was created using online Morpheus software [https://software.broadinstitute.org/morpheus/]. Functional enrichment analysis including pathways and Gene Ontology (GO) terms (biological process, molecular function and cellular component) regulated by DEPs was analyzed using the clusterProfiler R package.

Identification of experimental and predicted targets of hsa-miR-423-5p in HCC cells

Targets of hsa-miR-423-5p were predicted using four different online prediction tools including DIANA-MicroT v22 (https://dianalab.e-ce.uth.gr/html/dianauniverse/index.php?r=microT_CDS), TargetScan Human v8.0, mirDIP_v5.3 (https://ophid.utoronto.ca/mirDIP/) and mirDB (https://mirdb.org/) Only miR-423-5p target genes (miTGs) predicted by at least three of the above four prediction tools were considered for further

analysis. Common predicted targets of miR-423-5p were identified by creating Venn diagram using online "Calculate and draw custom Venn diagrams" web application [https://bioinformatics.psb.ugent.be/webtools/Venn/]. Furthermore, miR-423-5p experimental targets were identified using mirTarBase release v9.0 (https://dianalab.e-ce.uth.gr/html/diana/web/index.php?r=tarbasev8/index) [29–32]. These experimental and predicted targets of miR-423-5p were combined with list of DEPs experimentally modulated by miR-423-5p in HCC cells. Identified miTGs were further considered for downstream analysis by Gene Expression Profiling Interactive Analysis 2 (GEPIA2, http://gepia2.cancer--pku.cn/).

Comprehensive expression and survival analysis of miRNA target genes (miTGs) in HCC using TCGA-LAST LIHC and GTEx datasets

Gene expression pattern of miTGs was assessed in Liver Hepato Cellular Carcinoma (TCGA-LIHC) dataset from The Cancer Genome Atlas (TCGA) project in comparison with the TCGA and GTEx (Genotype-Tissue Expression) normal tissue (control) datasets by using the web server GEPIA2 [33]. Comparative expression analysis was performed using "Expression DIY" module of GEPIA2 with the TCGA-LIHC tumour dataset. Expression of the TCGA-LIHC tumor dataset was compared with the TCGA and GTEx normaldataset by deriving a box plot with a p-value and log2FC threshold 0.05 and |1|, respectively. In addition, to comprehend the expression pattern of miTG in relation to TCGA-LIHC patient pathological stages, pathological stage plots were obtained with GEPIA2. A stage-wise differential expression plot was derived by using a one-way ANOVA approach and assessing F and Pr(>F) values. Pr(>F) cut-off less than < 0.05 was used to describe the significant expression of a miTGs within different pathological stages including I, II, III and IV. Furthermore, overall (OS) and disease-free survival (DFS) plots were analysed to study the association between miTGs expression level and patient survival in the TCGA-LIHC dataset.

Protein extraction and Western blot analysis

HCC cell proteins were collected in 30 µL of cold lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM EDTA, 1% Triton X-100) supplemented with 1 bmM DTT, 1 mM PMSF, and Protease and Phosphatase Inhibitor (Halt Protease and Phosphatase Inhibitor Single-Use Cocktail - Thermo Fisher Scientific - Cat# 78442) and electro-transferred to nitrocellulose membranes (Trans-Blot Turbo – Mini format – BioRad, Hercules, CA). Membranes were washed in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and blocked for 1 h with 1x Tris Buffered Saline (TBS) with 1% Casein (Bio-rad- #1610782). Membranes with transferred proteins were incubated overnight with primary antibodies in 1x Tris Buffered Saline (TBS) with 1% Casein (Bioad-#1610782). Membranes were then washed and incubated with secondary antibodies for 1 h at room temperature (Table S1). Blots were developed using enhanced chemiluminescence detection reagents ECL (Immobilion ECL Ultra Western HRP Substrate - Cat# WBULS0500 Millipore) and acquired using ChemiDoc Imaging System (BioRad). Primary and secondary antibodies were shown in Table S1. Statistical analyses were conducted using GRAPHPAD PRISM version 8.0 (GraphPad Software, San Diego, CA). Data were expressed as the mean ± SD. In detail, the two-tailed Student's t test was used to calculate two-group comparisons; one-way ANOVA was carried out to analyze multiple-group comparisons.

Results

Identification of differentially expressed proteins (DEPs) and functional enrichment analysis reveals miR-423-5p–mediated modulation of key oncogenic and tumor-suppressive pathways in SNU-387 model

Having previously transduced two HCC cell models, SNU-387 and Hep-3B, with miR-423-5p, we chose to focus our investigations on the SNU-387 characterized by enhanced proliferative, invasive, and metastatic potential [34]. SNU-387 is characterized by enhanced proliferative and invasive capabilities, which makes it a suitable model to better understand the molecular mechanisms through which miR-423-5p may influence tumor progression and aggressiveness in HCC. The SNU-387 are derived from an HBV-positive, stage IV/V pleomorphic HCC. While the Hep3B are HBV-positive epithelial HCC line, p53-null, widely used in drug-response and gene-function studies, with lower baseline invasiveness and higher transfection efficiency. Using these two different models allowed us to understand the effects of miR-423-5p overexpression in HCC cells with different basal expression levels and phenotypic aggressiveness. Differential expression analysis was performed to identify the intracellular protein targets modulated by miR-423-5p overexpression in SNU-387 cells. Quantitative mass spectrometry identified a total of 698 differentially expressed proteins (DEPs), of which 340 upregulated and 358 downregulated proteins in response to miR-423-5p overexpression when compared to control cells (pLKO.1. Empty vector), as depicted in the volcano plot (Fig. 1A). A hierarchical clustering heatmap illustrating the top 50 DEPs is shown in Fig. 1B. Upon comparison between miR-423-5p-overexpressing HCC cells and empty plasmid (pLKO.1), a member of RAS oncogene family (RAB3B), emerged as the most upregulated protein, exhibiting a log2 fold change (log2FC) of + 3.24. In contrast, Keratin, type II cytoskeletal 7 (K2C7) was the most downregulated, with a log2FC of -5.16. Comparison between HCC cells overexpressing

miR-423-5p and those transfected with the empty vector (pLKO.1) revealed several proteins significantly downregulated. These include key regulators of DNA replication and cell proliferation such as MCM7 (log2FC -0.75), MCM5 (log2FC -0.70), and RRM2 (log2FC -1.18); metabolic enzymes like ACACA (log2FC -0.648) and SRM (known as SPEE) log2FC -1.54; signaling molecules including DVL3 (log2FC -2.32) and IMPDH1 (log2FC -1.55); as well as other functionally relevant proteins such as SPNS1 (log2FC -0.6). These marked alterations underscore the profound impact of miR-423-5p on the intracellular protein profile. The complete list of differentially expressed proteins (DEPs) is provided in Table S2.

To gain deeper insight into the biological significance of these proteomic changes, we performed pathway and Gene Ontology (GO) enrichment analyses on the subset of significantly modulated proteins. GO enrichment analysis of downregulated proteins in transduced SNU-387 cells highlights the potential role of miR-423-5p in modulating proteins involved in cellular metabolism. Several enriched biological processes suggest that miR-423-5p may impair RNA degradation and stability, protein biogenesis, DNA replication, and the metabolic response to chemical stimuli. The downregulation of factors involved in mRNA catabolic processes and RNA stabilization points to a broad suppression of RNA turnover and processing. In parallel, the inhibition of ribosome biogenesis and cytoplasmic translation indicates a potential reduction in global protein synthesis. Furthermore, the suppression of key DNA replication pathways and nucleotide biosynthetic processes suggests that miR-423-5p may contribute to cell cycle control or the arrest of cell proliferation. Finally, the modulation of cellular responses to amino acids and acidic compounds supports a role for miR-423-5p in regulating cellular adaptation to metabolic stress. Together, these findings support a key regulatory function of miR-423-5p in hepatic cancer cell metabolism (Fig. 2A). The enrichment of downregulated proteins in specific cellular compartments suggests a broad and targeted effect of miR-423-5p on

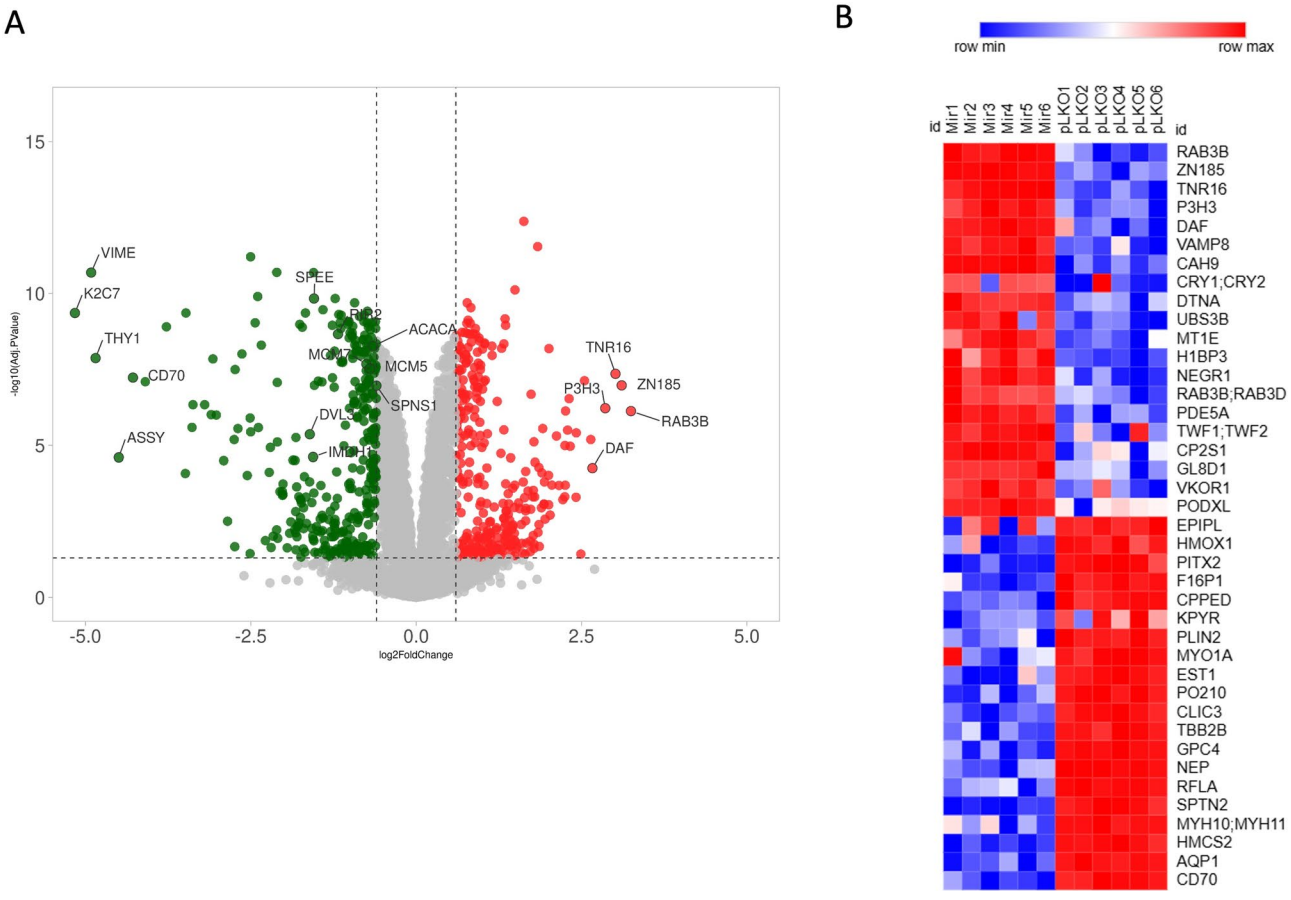


Fig. 1 **A** Volcano plot showing differentially expressed proteins (DEPs) in miR-423-5p-overexpressing SNU-387 cells compared to empty plasmid (pLKO.1). The top five upregulated and downregulated proteins are highlighted. Red dots represent significantly upregulated proteins (log2FC >0.6), green dots indicate significantly downregulated proteins (log2FC < -0.6), and grey dots denote proteins that did not meet the significance criteria (adjusted p-value ≥ 0.05 or |log2FC| ≤ 0.6). **B** Heatmap of the top 50 Differentially Expressed Proteins (DEPs) in miR-423-5p-overexpressing SNU-387 cells compared to empty plasmid (pLKO.1)

the structural and functional organization of the cell. Several enriched cellular components, including "collagen-containing extracellular matrix," "cell-substrate junction," and "focal adhesion," suggest that miR-423-5p affects cell-matrix interactions and adhesion, potentially impairing cell migration and invasiveness. Additionally, the downregulation of components such as the "spindle," "chromosomal region," and "DNA replication preinitiation complex" indicates a suppression of mitotic and DNA replication machinery, consistent with reduced proliferative capacity. The enrichment of nuclear and RNA-associated compartments, including "nuclear envelope," "ribonucleoprotein granule," "P-body," and "polysome," further suggests that miR-423-5p may interfere with mRNA transport, processing, and translation. Moreover, the repression of actin-associated structures such as "contractile fiber," "lamellipodium," and "cell leading edge" points to cytoskeletal remodeling, which may underlie changes in cell morphology and motility. Overall, these data support a multifaceted role for miR-423-5p in regulating cellular metabolism, proliferation, and cytoskeletal dynamics in HCC cells (Fig. 2B). The analysis of molecular functions among downregulated proteins in transduced SNU-387 cells indicates that miR-423-5p exerts a broad inhibitory effect on key biochemical activities related to RNA/DNA metabolism, energy utilization, and structural dynamics. The most significantly enriched molecular function includes "ATP hydrolysis activity," "catalytic activity acting on RNA," and "ATP-dependent activity acting on DNA/RNA," suggesting that miR-423-5p impairs essential enzymatic functions required for nucleic acid processing and cellular energy homeostasis. Moreover, the enrichment of RNA-binding and helicase-related terms such as "single-stranded RNA binding," "mRNA 3′-UTR binding," "ribonucleoprotein complex binding," and "RNA helicase activity" points to a strong suppression of proteins involved in post-transcriptional regulation, RNA stability, and translation. These findings are consistent with a regulatory role of miR-423-5p in modulating gene expression at the post-transcriptional level. Notably, terms related to structural integrity and cell–matrix interactions such as "extracellular matrix structural constituent" and "collagen binding" are also downregulated, demonstrating that miR-423-5p may reduce cellular adhesion and motility, potentially contributing to a less invasive phenotype. Finally, the stress-related molecular functions, including "heat shock protein binding" and "oxidoreductase activity," suggest that miR-423-5p could suppress the cellular capacity to survive with oxidative and proteotoxic stress (Fig. 3A). KEGG pathway analysis revealed significant downregulation of several critical processes involved in cell proliferation and survival. Simultaneous downregulation of motor proteins suggests impaired intracellular transport and cytoskeletal dynamics, which are essential for cell migration and invasion. The observed decrease in proteins related to tight junction's points to a potential stabilization of cell-cell adhesion, reducing permeability and possibly limiting metastatic spread. Importantly, our data highlight a strong downregulation of multiple metabolic pathways, including nucleotide, pyrimidine, and glutathione metabolism. The suppression of nucleotide

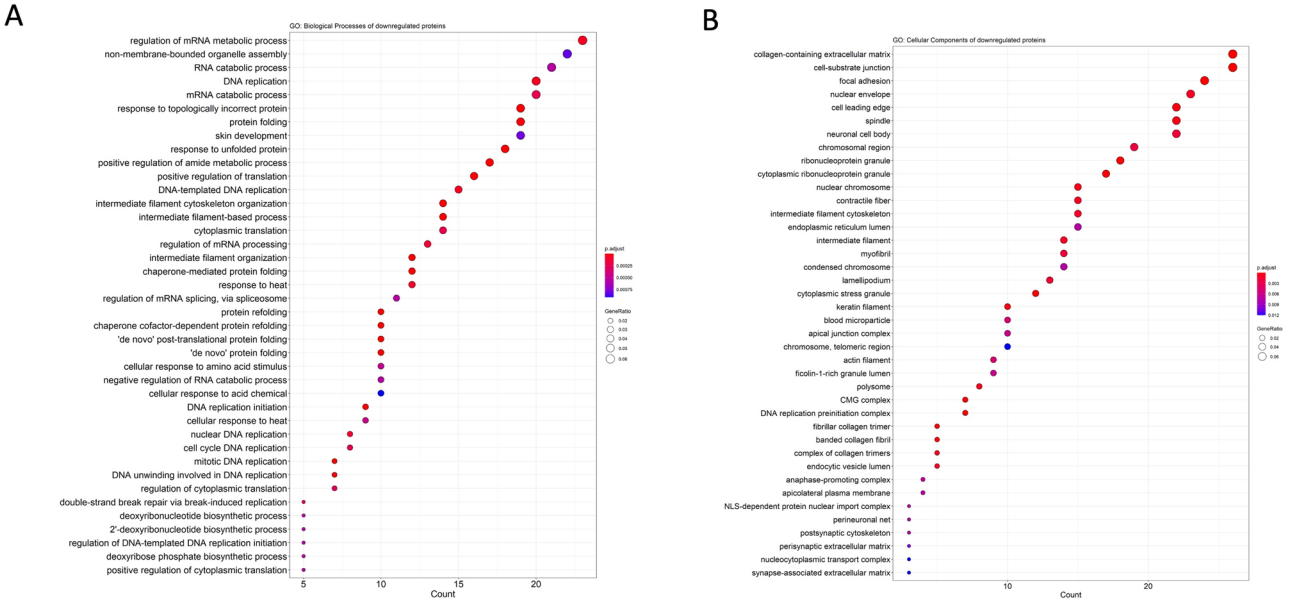


Fig. 2 Dot plot of enriched Gene Ontology (GO) categories selected for proteins down-regulated by miR-423-5p compared to pLKO.1. **A** Biological Processes (GO: BP) **B** Cellular Components (GO: CC). Dot sizes represent the number of downregulated proteins associated with the GO term and dot colors represent the p-value from the over-represented Fisher's test

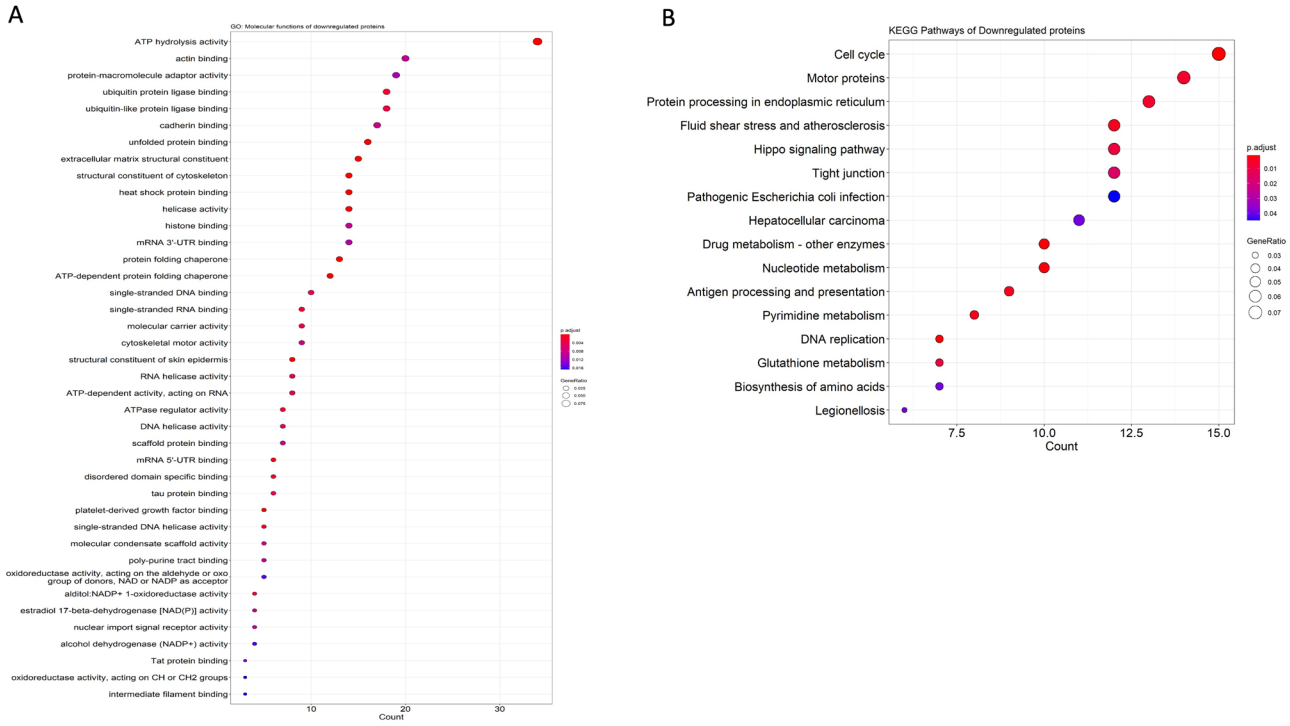


Fig. 3 Enriched Gene Ontology (GO) analysis of down-regulated proteins in miR-423-5p overexpressing SNU-387 cells compared to pLKO.1. **A** Molecular Function (GO: MF) **B** KEGG pathways Dot sizes represent the number of down-regulated proteins and dot colors indicate significance (p-value, Fisher's exact test)

and pyrimidine metabolism likely restricts the availability of building blocks required for DNA synthesis and repair, thus impairing cell proliferation. Moreover, the reduction in glutathione metabolism may contribute to tumor cells being more vulnerable to oxidative stress by weakening their antioxidant defenses (Fig. 3B). Additional functional analysis revealed that the upregulated proteins were primarily associated with molecular functions such as phospholipid and actin binding, GTPase activity, and phosphatidylinositol binding (Fig. S1). Corresponding biological processes included small GTPase-mediated signal transduction, cell-substrate adhesion, and negative regulation of protein modification processes (Fig. S2). Overall, these findings emphasize that miR-423-5p shows a multifaceted regulatory role, particularly impacting metabolic pathways crucial for tumor growth and survival. This metabolic reprogramming underlines the potential of miR-423-5p as a therapeutic target to disrupt the metabolic adaptability and progression of hepatocellular carcinoma.

Identification of experimental and predicted targets of hsa-miR-423-5p in HCC cells

To investigate the downstream targets of miR-423-5p in HCC cells, target prediction analyses were conducted with four independent bioinformatic tools: mirDIP, mirDB, TargetScanHuman, and DIANA-microT. Targets predicted by at least three out of four tools were selected for further analysis. A comprehensive summary of the targets predicted by each tool, along with the overlapping targets, is shown in Fig. 4A; Table 1. Furthermore, using miRTarBase, 348 unique experimentally validated targets of miR-423-5p were identified. These validated targets are listed in Table S3. To determine which of these predicted and validated targets are modulated in miR-423-5p overexpressing HCC cells, we compared them with DEPs obtained from MS analysis of miR-423-5p overexpressing SNU-387 cells. This comparison revealed 43 DEPs (11 upregulated and 32 downregulated) that matched predicted or experimentally validated targets of hsa-miR-423-5p (Fig. 4B). The complete list of these 43 DEPs is provided in Table S4.

Gene expression profiling interactive analysis of miR-423-5p target genes

To further explore the clinical relevance of intracellular targets modulated by miR-423-5p overexpression, we analysed publicly available datasets using the GEPIA2 platform. Expression patterns of the identified miRNA target genes (miTGs) were evaluated by comparing tumour and normal liver tissues from the TCGA-LIHC dataset, integrated with GTEx data. In addition, to explore the clinical relevance of miTG expression Kaplan–Meier survival analyses were performed to assess

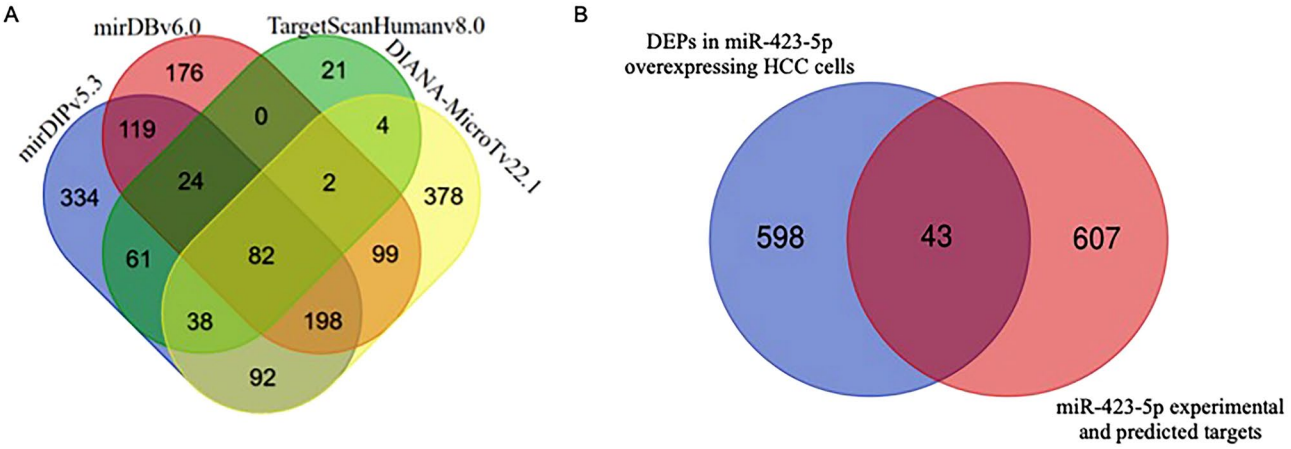


Fig. 4 TGs prediction analysis and comparison with DEPs. **A** Predicted targets of hsa-miR-423-5p identified using four independent target prediction tools: miRDIP (v5.3), mirDB (v6.0), TargetScanHuman (v8.0), and DIANA-microT (v22.1) depicted in the Venn diagram **B** Comparison between experimentally validated and computationally predicted targets of miR-423-5p and DEPs identified in response to miR-423-5p overexpression in SNU-387 hepatocellular carcinoma (HCC) cell line

Table 1 Comprehensive statistics of target gene prediction analysis for hsa-miR-423-5p obtained using four distinct MiRNA target prediction tools

Target gene prediction tool	Count of unique miTGs predicted
DIANA microT-miRbase v22.1	893
TargetScan_Human_v8.0	232
mirDIP v5.3	948
mirDB v6.0	700
Targets predicted by all 4 prediction tools	82
Targets predicted by min. 3/4 prediction tools	262
Experimentally validated targets identified using mirTarBase release v9.0	348

their association with overall survival (OS) and disease-free survival (DFS) in patients from the TCGA-LIHC cohort. Therefore, seven miR-423-5p targets ACACA, ANKRD52, DVL3, MCM5, MCM7, RRM2, SPNS1, SRM were significantly associated with patient prognosis (log-rank p-value < 0.05 (Table 2; Fig. 5). The seven selected targets displayed significantly altered expression (p-value < 0.05) in tumour samples compared to GTEx and/or TCGA datasets (Fig. 6). Notably, while these targets were downregulated in miR-423-5p overexpressing HCC cells, they were upregulated in TCGA-LIHC tumor tissues, suggesting a potential role for hsa-miR-423-5p in the regulation of HCC pathogenesis (Table 2). Stage-specific expression profiling demonstrated significant variation in target expression across disease stages. Interestingly, expression levels of all seven targets increased progressively from stage I to stage III, followed by a marked decrease at stage IV in TCGA-LIHC patient samples (Fig. 6).

Validation of predicted miRNA targets with Western blotting

Based on the obtained results, we additionally validated the impact of miR-423-5p by assessing the expression levels of MCM7, SPEE, IMPDH1, and DVL3 proteins through Western blotting in SNU-387 and Hep-3B cells transduced with miR-423-5p (Fig. 7). The overexpression of miR-423-5p induced a reduction of MCM7 (-50%), IMPDH1 (-60%) and DVL3 (-60%) and a significant decrease of SPEE (-70%) in SNU-387 cells compared to empty plasmid (plKO.1.) Conversely, in Hep-3B cells, all proteins showed a 30–40% decrease compared to the control, except for SPEE, which was reduced by 60%. Notably, SPEE expression exhibited a pronounced decrease in both cell lines overexpressing miR-423-5p, underscoring its possible involvement in the molecular effects mediated by miR-423-5p. These data confirmed the results of quantitative proteomics and suggest that the coordinated downregulation of key enzymes involved in lipid and polyamine biosynthesis contributes to the inhibition of proliferation and survival of HCC cells.

Discussion

Emerging evidence indicates that microRNAs not only modulate signaling pathways and cell cycle regulation, but also play a crucial role in the metabolic reprogramming of cancer cells, a well-established hallmark of tumor progression [35]. In this study, we investigated the impact of miR-423-5p overexpression in HCC cells by integrating quantitative proteomic analysis with bioinformatic predictions, clinical datasets, and experimental validation. Our findings support a tumor-suppressive role for miR-423-5p in HCC, primarily mediated through the down-regulation of proteins involved in cell proliferation, nucleotide and amino acid metabolism, and oncogenic

Table 2 GEPIA2 analysis of differentially expressed proteins (DEPs) identified in miR-423-5p-overexpressing SNU-387 cells

UniProt protein ID	Protein name	Gene symbol	Description	log2FC [t_Mir_vs_pLKO]	Expression status [t_Mir_vs_pLKO]	log-rank *p*-value		Expression status	
						Overall survival	Disease-free survival	TCGA LIHC tumor vs. TCGA + GTEx normal	TCGA LIHC tumor vs. TCGA normal
Q13085	ACACA	ACACA	Acetyl-CoA carboxylase 1	-0.648086463	Downregulated	0.0066	0.0083	Upregulated	Upregulated*
Q8NB46	ANR52	ANKRD52	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C	-2.100271718	Downregulated	0.0036	0.0023	Upregulated	Upregulated*
Q92997	DVL3	DVL3	Segment polarity protein dishevelled homolog DVL-3	-1.608614926	Downregulated	0.0074	0.0034	Upregulated	Upregulated*
P33992	MCM5	MCM5	DNA replication licensing factor MCM5	-0.701634685	Downregulated	0.048	0.032	Upregulated*	Upregulated*
P33993	MCM7	MCM7	DNA replication licensing factor MCM7	-0.753247569	Downregulated	0.00032	0.011	Upregulated*	Upregulated*
P31350	RIR2	RRM2	Ribonucleoside-diphosphate reductase subunit M2	-1.181484629	Downregulated	0.00058	0.00032	Upregulated*	Upregulated*
Q9H2V7	SPNS1	SPNS1	Protein spinster homolog 1	-0.600723146	Downregulated	0.0015	0.013	Upregulated*	Upregulated*
P19623	SPEE	SRM	Spermidine synthase	-1.544832421	Downregulated	0.0014	0.16	Upregulated	Upregulated*

The listed deps show significant differential expression (p-value ∗< 0.05) in TCGA-LIHC tumor samples compared to normal liver tissues from TCGA and GTEx datasets. Additionally, overall survival (OS) and disease-free survival (DFS) analyses indicate a significant association of these deps with patient outcomes (log-rank p-value ∗< 0.05)

signalling pathways. Proteomic profiling revealed 698 differentially expressed proteins (DEPs), among which RAB3B and K2C7 emerged as the most significantly up- and downregulated, respectively. Elevated expression of RAB3B has been implicated in the progression of multiple cancer types through mechanisms that include enhanced vesicle-mediated transport of oncogenic factors [36, 37]. Despite its recognized involvement in malignancies, the precise oncogenic role of RAB3B in HCC remains insufficiently characterized and warrants further investigation, especially considering its significant upregulation upon miR-423-5p overexpression as observed in our study. Significantly, we identified 43 DEPs that overlapped with predicted or experimentally validated targets of miR-423-5p, strengthening the causal link between their overexpression and the observed proteomic shifts. Among these, seven proteins (ACACA, ANR2, DVL3, MCM5, MCM7, RRM2, SPSN1) showed significant associations with poor prognosis in TCGA-LIHC patients and displayed a consistent inverse expression pattern both in vitro and in clinical samples, additionally supporting their oncogenic relevance. Recent studies have identified upregulation of ACACA in various human cancers, where it promotes *de novo* lipogenesis to support the increased metabolic request associated with rapid cell growth and proliferation [38]. In PCa, the expression of ACACA, which encodes acetyl-CoA carboxylase 1 (ACC1), is essential for maintaining mitochondrial function and lipid metabolism. ACACA depletion impairs mitochondrial β-oxidation, leading to decreased ATP production, altered NADP/NADPH ratio, and apoptosis. Interestingly, similar alterations in lipid metabolism have been observed during lung pre-metastatic niche (PMN) formation in breast cancer, where these metabolic changes contribute to niche establishment and metastatic progression [39]. In HCC, ACACA has emerged as a key prognostic factor since it may promote malignant behavior by activating of the Wnt/β-catenin signaling pathway [40]. In our study, we observed a downregulation of ACACA expression, suggesting a possible association with the lack of Wnt pathway activation. ANKRD52 encodes a putative regulatory subunit of protein phosphatase 6 (PP6), which is potentially involved in recognizing of phosphoprotein substrates. ANKRD52 may serve as a therapeutic target by disrupting a pathway essential for CRC progression and cancer stem cell maintenance [41, 42]. However, its role in HCC remains unexplored, and no data are currently available regarding its function or expression in this context. Dishevelled-3 (DVL3) is a regulator of both Wnt/β-catenin and Notch signaling pathways, which are critically involved in cancer progression, chemoresistance, and the maintenance of stem cell-like properties. However, the precise biological functions of DVL3 remain

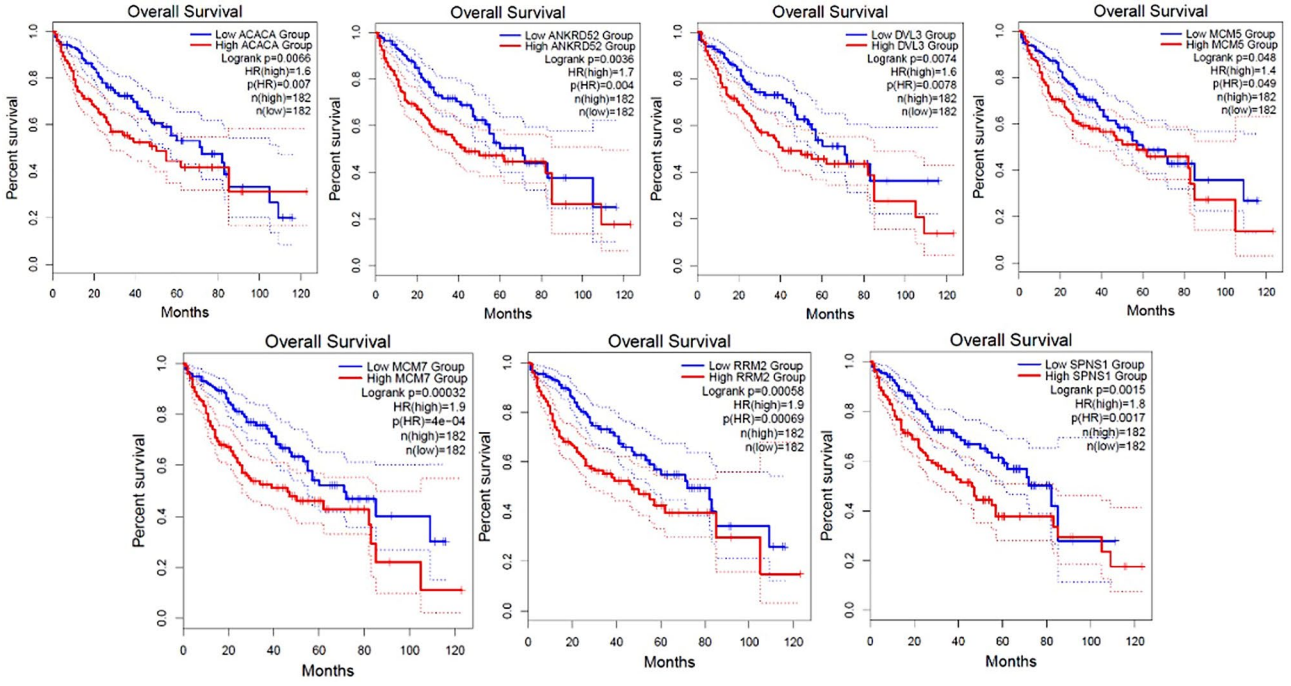


Fig. 5 Kaplan -Meier curves for overall and disease-free survival of differentially expressed proteins (DEPs) in miR-423-5p overexpressing SNU-387 cells, showing significant correlation with patients' outcomes in TCGA-LIHC dataset (log-rank p-value < 0.05)

incompletely understood. Zhao Q., et al. have demonstrated that DVL3 acts as a key regulator of CRC stemness and chemoresistance, suggesting that it can work as a potential therapeutic target in CRC [43]. Moreover, another study reported that DVL3 promotes RAC1-PCP-JNK signaling, providing a potential target for clinical intervention in NSCLC treatment [44]. Additionally , it can interact with Prickle-1 to activate the Wnt/beta-catenin pathway in HCC [45]. Although we did not investigate its role in HCC specifically, the miRNA-mediated downregulation of this target aligns with potential cancer-preventive mechanisms. MCM5 and MCM7 are core components of the minichromosomal maintenance (MCM) complex, which functions as a replicative helicase essential for the initiation and progression of DNA replication. Dysregulation of these proteins has been increasingly linked to cancer development, progression, and prognosis in CRC and HCC [46, 47]. We confirmed the regulation of this target and validated its involvement, demonstrating the role of the miRNA in our HCC model. Ribonucleotide reductase regulatory subunit M2 (RRM2) has been identified as both a prognostic biomarker and a therapeutic target in HCC in sorafenib treated cells [48] or exerting an anti-ferroptotic role in liver cancer cells through GSH synthesis [49]. SPNS1 (Spinster homolog 1) is a lysosomal proton-dependent transporter involved in the efflux of lysophospholipids [50] and contributes to cellular adaptation under metabolic stress by facilitating the recycling of lysophospholipids, thereby supporting autophagy [51]. Although the role of RRM2 and SPNS1 in HCC were not directly investigated, the miRNA-mediated downregulation of these targets is consistent with a potential cancer-preventive effect. Spermidine/Spermine N^1-acetyltransferase (SPEE, also known as SRM) a key enzyme in spermidine biosynthesis, essential for polyamine metabolism plays a central role in promoting cell growth, DNA stability, and autophagy processes that are often hijacked in cancer to support tumor progression [52]. Elevated spermidine levels have been linked to poor prognosis in various cancers, including HCC, where its metabolism is frequently dysregulated to sustain increased biosynthetic demands [53]. Recent evidence indicated that targeting polyamine biosynthesis can impair HCC cell proliferation and sensitize tumors to chemotherapy agents [54]. Wang S., et al. demonstrated that SPEE mediated depletion of polyamines not only reduced tumor cell proliferation, but also markedly inhibited the migration and invasion of HCC and CRC cells [55]. Therefore, we observed a significant reduction of SPEE protein expression in both HCC models overexpressing miR-423-5p, additionally supporting its tumour suppressive activity and regulatory impact on polyamine metabolism. Our previous study demonstrated SPEE's involvement in amino acid metabolism and tumorigenesis in prostate cancer cells, reinforcing the tumor-suppressive function of miR-423-5p through metabolic interference [15]. Based on this evidences, our data

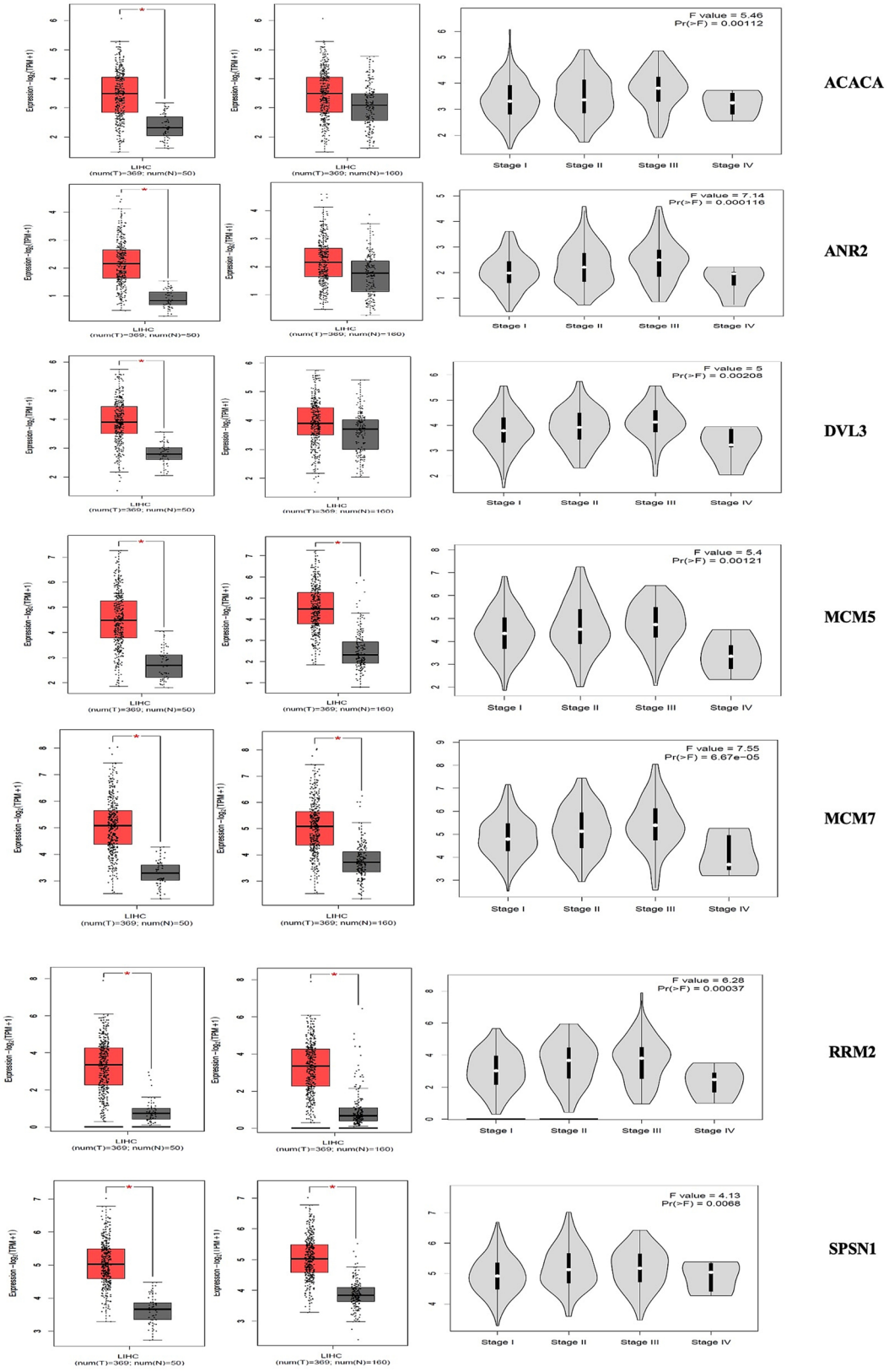


Fig. 6 Box plots and stage-wise expression plots showing the expression patterns of seven target genes (TGs) identified in miR-423-5p-overexpressing SNU-387 cells. These TGs exhibit significantly altered expression in TCGA-LIHC tumor samples compared to normal liver tissues from the TCGA and GTEx datasets

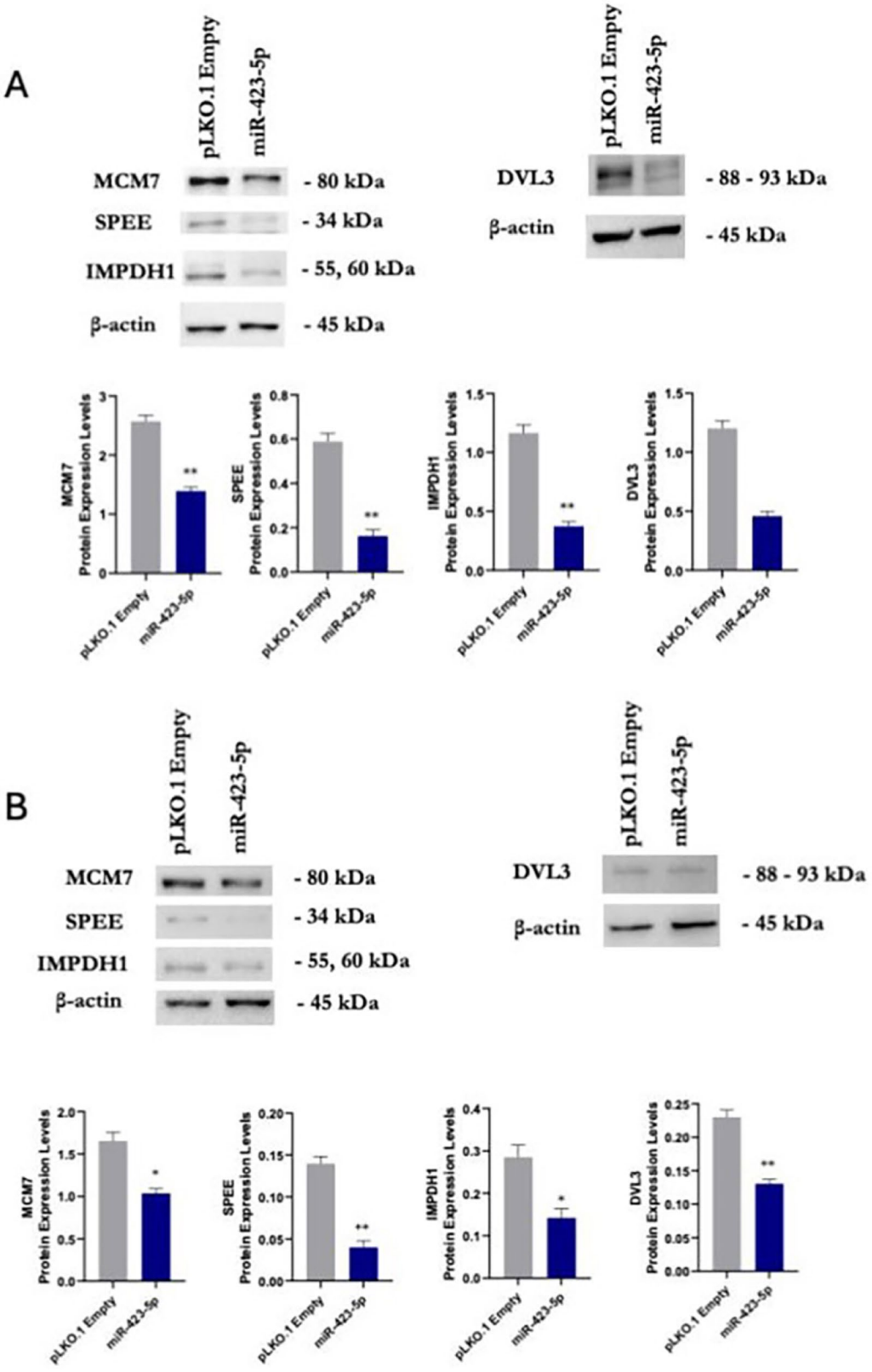


Fig. 7 Western blot analysis of transduced HCC cell models. **A** SNU-387 cells lysates were incubated with MCM7, SPEE, IMPDH1 and DVL3. **B** Hep-3B cells lysates were incubated with MCM7, SPEE, IMPDH1 and DVL3. β-actin served as a loading control. Each experiment was independently repeated at least three times, yielding consistent similar results. Bar graphs represent the band intensities expressed in arbitrary units. Error bars indicate standard deviations (SD). Statistical significance was determined by ANOVA: $*p < 0.01$; $**p < 0.05$; $***p < 0.005$; $****p < 0.0001$

suggest that miR-423-5p actively contributes to the remodelling of cellular metabolism by downregulating key enzymes involved in lipid and polyamine biosynthesis, two fundamental processes that support sustained tumor growth. Specifically, the observed downregulation of ACACA, a central enzyme in *de novo* fatty acid biosynthesis, implies that miR-423-5p may limit endogenous fatty acid production, which is essential for membrane biogenesis and intracellular signaling in rapidly proliferating cells. In parallel, the reduction of SPEE indicates a potential inhibitory effect of miR-423-5p on the polyamine metabolic pathway, which is closely linked to DNA stability, protein synthesis, and autophagy. This dual interference with essential biosynthetic routes suggests that miR-423-5p functions as a modulator of metabolic plasticity in HCC, restricting the tumor cells' ability to meet the high bioenergetic and biosynthetic demands associated with malignancy. By identifying the miR-423-5p-mediated repression of targets such as ACACA, SPEE, and IMPDH1, we demonstrated that miR-423-5p not only impacts DNA replication and signalling but also interferes with key metabolic programs that sustain tumor growth. The observed down-regulation of MCM7, IMPDH1, and DVL3 upon miR-423-5p overexpression supports the hypothesis that miR-423-5p acts as a tumor suppressor by targeting key nodes of pro-tumorigenic signaling in HCC.

Conclusion

Overall, our findings reveal that miR-423-5p exerts broad tumour-suppressive effects by targeting multiple oncogenic effectors. MiR-423-5p and its downstream effectors may be promising candidates for prognostic and therapeutic applications in HCC. By demonstrating that miR-423-5p modulates key biosynthetic and energetic pathways, including fatty acid and polyamine metabolism, we provide evidence that this miRNA acts as a metabolic gatekeeper in HCC, with potential translational implications for targeted therapy. These findings complement existing knowledge about miR-423-5p in other cancer models, reinforcing its broader role in tumour suppression through metabolic interference. Certainly, some aspects need to be considered, as our functional validation was performed on in vitro HCC models, and in vivo studies are needed to confirm these effects within the tumour microenvironment. Although the proteomic profile revealed numerous DEPs, not all predicted targets of miR-423-5p have been experimentally validated, and off-target effects cannot be completely ruled out. Although clinical data correlations support the relevance of the selected targets, prospective patient-based studies are needed to establish direct prognostic or therapeutic significance.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Author contributions

AL, AMC, SZ, MC: conceptualization; AMC, MPR: validation; MB, MRDI: cell models; DJB, CC: MA and proteomic analysis; MST: bioinformatic analysis; AMC, MST, DJB, CC: data curation; AL, MB, AMC, RS, LM: investigation; EAMV: Protocols; GM, EAMV, MC, SZ: resources; AMC: major contributor in writing original draft preparation; MB, AL: writing original draft; AMC, GV, IS, SZ, MC: review and editing; SZ, MC, EAMV: supervision. All authors have read and agreed to the published version of the manuscript.

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Data availability

All LC-MS/MS data generated in this study have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD064869 [56]. The other data supporting the findings of this study are available within the article and its supplementary materials.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

All authors declare no conflicts of interest.

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