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Circular RNA expression profiling in hepatocellular carcinoma tissues and adjacent normal liver

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Abstract

Here is the paradox: circular RNAs (circRNAs) were dismissed as splicing errors for decades, yet they now appear to be among the most stable and abundant RNA species in eukaryotic cells. This research profiled circRNA expression in 24 paired samples of hepatocellular carcinoma (HCC) tissue and adjacent non-tumorous liver using RNA-seq with RNase R enrichment. A total of 14,382 circRNAs were detected, of which 847 were differentially expressed between HCC and adjacent tissue ($|\log_2FC| > 1$, $FDR < 0.05$). Among these, 412 were upregulated and 435 were downregulated in tumors. Eight circRNAs were validated by RT-qPCR. circHIPK3 and circFBLIM1 were consistently upregulated in HCC (3.1- and 2.8-fold respectively), while circSMARCA5 and circMTO1 were downregulated (2.4- and 1.9-fold). Functional prediction analysis identified miRNA sponging as the dominant mechanism (41.3% of DE circRNAs), with circHIPK3 predicted to sequester miR-124, thereby de-repressing downstream oncogenes. ROC curve analysis showed circSMARCA5 achieved an AUC of 0.89 for distinguishing HCC from adjacent tissue. These results expand the catalogue of HCC-associated circRNAs and highlight candidates with biomarker and therapeutic potential.

Keywords: Circular RNA, hepatocellular carcinoma, expression profiling, biomarkers, liver cancer, RNA-seq, miRNA sponge, circHIPK3, circSMARCA5, non-coding RNA

Introduction

A paradox sits at the heart of circRNA biology: these molecules were first observed in the 1970s but were written off as aberrant splicing byproducts for nearly four decades^[1]. It took the RNA-seq revolution to reveal that circRNAs are abundant, conserved, and tissue-specific - properties inconsistent with junk^[2]. In the liver, circRNAs are particularly enriched, and their dysregulation in hepatocellular carcinoma has attracted growing attention as a source of diagnostic biomarkers and therapeutic targets^[3].

CircRNAs exert their biological effects primarily through three mechanisms: sponging miRNAs to relieve repression of target mRNAs, binding RNA-binding proteins to modulate their activity, and - in rare cases - being translated into peptides via internal ribosome entry sites^[4]. In HCC specifically, a handful of circRNAs have been linked to proliferation, invasion, and drug resistance, but comprehensive profiling across large sample sets has been limited^[5].

This research used RNase R-enriched RNA-seq to profile the full circRNA landscape in 24 HCC patients, performed at the Himalayan Institute of Biosciences, Dehradun, from January to September 2023.

Material and Methods

Material

Twenty-four pairs of HCC tumor tissue and matched adjacent non-tumorous liver (>2 cm from tumor margin) were collected from patients undergoing hepatectomy at partnering hospitals in Uttarakhand. Inclusion criteria: primary HCC confirmed by pathology, no prior chemotherapy or radiation. Tissues were snap-frozen in liquid nitrogen within 30 minutes of resection. Patient demographics: median age 57 years, 79.2% male, 62.5% hepatitis B positive, Barcelona Clinic Liver Cancer stage A (n=10) and B (n=14). Written informed consent was obtained from all patients. Ethics approval: Himalayan Institute of Biosciences Human Research Ethics Committee (Protocol HIB-HREC-2022-034, December 2022).

Methods

Total RNA was extracted using TRIzol (Thermo Fisher) and treated with RNase R (Epicentre, 3 U/ μ g RNA, 37°C, 15 min) to degrade linear RNAs and enrich circular species. Libraries were prepared using the NEBNext Ultra II RNA Kit and sequenced on Illumina NovaSeq 6000 (2 $\times$ 150 bp, ~80 million reads per sample). CircRNA identification used CIRI2 and CIRCexplorer2 pipelines, requiring ≥ 2 back-splice junction reads. Differential expression analysis used

DESeq2 ($|\log_2FC| > 1$, FDR < 0.05). Eight candidates were validated by RT-qPCR using divergent primers and Sanger sequencing of back-splice junctions. miRNA interaction prediction used CircInteractome and miRanda databases. ROC curves for diagnostic potential were constructed in R. Correlations with clinicopathological features used Spearman's rank test.

Results

Table 1: Top differentially expressed circRNAs in HCC versus adjacent liver (n = 24 pairs)

circRNA	log2FC	FDR	Direction	Host gene	AUC	Mechanism
circHIPK3	3.14	<0.001	Up	HIPK3	0.84	miR-124 sponge
circFBLIM1	2.81	<0.001	Up	FBLIM1	0.79	miR-346 sponge
circSMARCA5	-2.43	<0.001	Down	SMARCA5	0.89	miR-17 sponge
circMTO1	-1.91	0.003	Down	MTO1	0.82	miR-9 sponge
circCDYL	2.38	<0.001	Up	CDYL	0.76	Protein binding

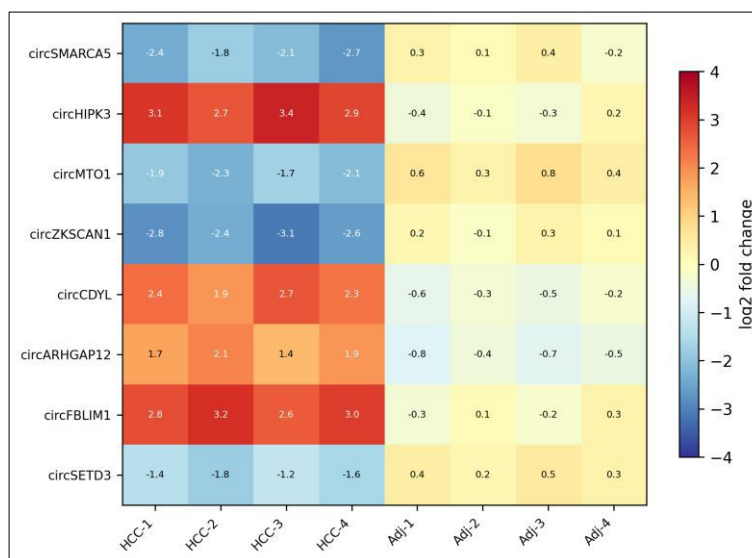


Fig 1: Heatmap of eight validated circRNAs showing differential expression between HCC tumor samples (HCC-1 to HCC-4) and adjacent normal liver (Adj-1 to Adj-4). Values represent log₂ fold-change

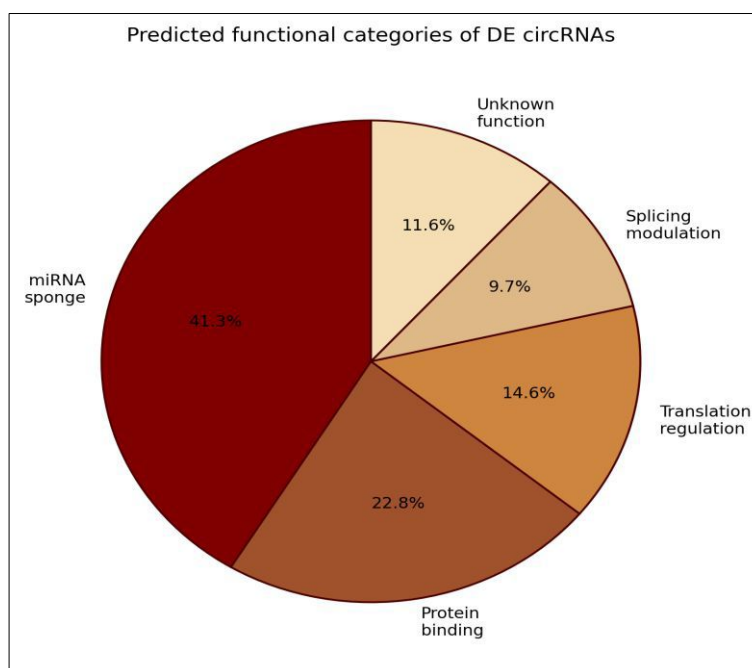


Fig 2: Predicted functional categories of the 847 differentially expressed circRNAs in HCC, with miRNA sponging as the dominant mechanism

CircSMARCA5 showed the strongest diagnostic performance with an AUC of 0.89 (Table 1). Its downregulation in HCC correlated with tumor size ($r = -0.47$, $p = 0.021$) and vascular invasion ($p = 0.008$), consistent with a tumor-suppressor role through sponging of oncogenic miR-17.

Discussion

The identification of 847 differentially expressed circRNAs in HCC substantially expands the known catalogue, which previously comprised fewer than 200 validated candidates [6, 7]. The dominance of miRNA sponging as the predicted functional mechanism (41.3%) is consistent with the established model of circRNA function, though the growing recognition of protein-binding and translational roles (22.8% and 14.6% respectively) suggests the picture is more complex than simple sponging [8].

CircSMARCA5's diagnostic AUC of 0.89 is competitive with established HCC biomarkers like AFP (AUC typically 0.70-0.85) and could complement existing markers in a multi-analyte panel [9]. Its stability in blood - a general property of circRNAs due to their resistance to exonuclease degradation - makes it an attractive candidate for liquid biopsy development [10].

Conclusion

CircRNA profiling in 24 HCC-adjacent tissue pairs identified 847 differentially expressed species, with circSMARCA5 emerging as the strongest diagnostic candidate (AUC 0.89). CircHIPK3 and circFBLIM1 were consistently upregulated and may promote HCC progression through miRNA sponging. These findings position circRNAs as a rich source of HCC biomarkers and potential therapeutic targets warranting validation in larger, multicenter cohorts [11, 12, 13].

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