Regulatory Rewiring in Adrenocortical Carcinoma: Tumor-Suppressive microRNAs Modulate Cell Cycle, ER Stress, and Sterol Metabolism Axes

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Abstract

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy with poor prognosis and limited therapeutic strategies. In this study, transcriptomic profiles from TCGA, GTEx 2025, and miRNATissueAtlas 2025 were integrated to construct ceRNA networks specific to tumor and normal adrenal tissues, leveraging a novel integrative analytical framework specifically developed in this study. Network topology revealed substantial rewiring in tumors, with miR-940 emerging as a tumor-exclusive hub, and miR-375 and miR-326 losing centrality despite marked downregulation. Experimentally validated and negatively correlated miRNA-mRNA interactions were identified, highlighting suppression of key oncogenes. Functional enrichment and PPI analyses demonstrated that the targets of these miRNAs are involved in pathways such as cell cycle regulation, EMT, and transcriptional activation. Survival analysis of target genes confirmed their prognostic relevance. Notably, miR-940 exhibited increased expression in radiation-treated patients, indicating therapy-responsive regulatory response, while miR-375 and miR-326 showed stable profiles. Cross-cancer expression analysis further supported the tumor-suppressive roles of all three miRNAs across multiple malignancies. These findings collectively establish miR-940, miR-375, and miR-326 as context-dependent tumor suppressors in ACC with potential therapeutic and biomarker utility.

Keywords: Adrenocortical Carcinoma (ACC), Tumor Suppressor microRNAs, ceRNA Network, Transcriptomic Analysis

1. Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive adrenal malignancy with an incidence of 0.7-2 cases per million per year [1], showing a bimodal age distribution in young children and adults aged 40-60 years [2]. Although surgery is the primary treatment, five-year survival remains poor (16-47 percent) due to frequent recurrence and metastasis [3]. ACC development is driven by key molecular alterations, including dysregulated Wnt/β-catenin signaling, IGF2 overexpression, and germline TP53 mutations, particularly in Li-Fraumeni syndrome [4, 5]. The rarity of the disease has substantially hindered therapeutic development, as noted by The Cancer Genome Atlas Research Network.

Non-coding RNAs, including miRNAs, lncRNAs, and circRNAs, regulate transcriptional and post-transcriptional programs in cancer [6]. Their interactions are captured by the ceRNA hypothesis, in which transcripts sharing microRNA response elements compete for miRNA binding [7]. Experimentally validated ceRNA activity, such as lncRNA HOTAIR and circRNA CDR1as, and broader ceRNA-network dysregulation have been linked to multiple cancers and poor prognosis [8-10]. Within ceRNA networks, specific miRNAs function as topological hubs that regulate broad gene-expression programs by interacting with multiple RNA species through shared MREs [11, 12]. MiR-21 and miR-155 exemplify such hubs across breast, lung, and colorectal cancers, with roles in apoptosis, immunity, and proliferation [13]. Comparative and pan-cancer analyses reveal extensive miRNA-driven network rewiring between normal and malignant tissues, identifying hub targeting and lncRNAs such as LINC01929 as potential therapeutic regulators of metastasis [14-16].

In ACC, prognostic relevance has been assigned to miR-195 and miR-483-5p, whose dysregulated expression has been correlated with adverse clinical outcomes [17]. Genome-wide profiling has also shown pronounced down-regulation of miR-139 and other tumour-suppressive miRNAs relative to benign adenomas, implying growth-restraining functions [18]. A multi-omics analysis by TCGA has delineated an aggressive C1A subtype characterised by elevated miR-483-5p and reduced miR-195/497 expression, reaffirming the value of miRNA signatures in tumour stratification [5].

MiR-940, miR-375, and miR-326 exhibit tumour-suppressive functions across multiple malignancies. MiR-940 down-regulation has been reported in hepatocellular carcinoma, oesophageal squamous cell carcinoma, and triple-negative breast cancer, where its restoration inhibits proliferation, invasion, and improves survival through targets including CXCR2 and ZNF24 [19-21]. MiR-375 suppresses epithelial-mesenchymal transition, proliferation, and drug resistance, limiting tumour aggressiveness via repression of RON and CIP2A, and attenuating metastasis through silencing oncogenic ceRNA hubs even when overexpressed [22-25]. MiR-326 loss is associated with enhanced tumour growth, while its re-expression suppresses progression in

cervical, breast, and colorectal cancers by targeting ELK1, SOX12, and NOB1 [26-28]. However, the roles of these miRNAs in adrenocortical carcinoma remain unexplored.

Recent ceRNA-based investigations have underscored the value of systems-level regulatory modeling for biomarker discovery in adrenocortical carcinoma. Synergistic oncogenic ceRNA modules centered on CKS2 and ACAT2 have been characterized, linking cell-cycle progression with metabolic reprogramming and demonstrating strong diagnostic and prognostic relevance [29]. In addition, miR-466, miR-507, and miR-665 have been identified as central miRNA hubs that mediate tumor-specific regulatory rewiring and exhibit tumor-suppressive potential [30, 31]. Complementing these findings, an integrative review has consolidated multi-omics and network-based biomarker discoveries, highlighting critical translational gaps in ACC [32].

Accordingly, a first-of-its-kind, comprehensive systems-level analysis was conducted to evaluate the tumor-suppressive potential of miR-940, miR-375, and miR-326 within the ceRNA network of ACC. For the first time, these miRNAs are identified and functionally characterized as key regulatory components in ACC, using integrated large-scale transcriptomic datasets from TCGA [33] as well as newly released GTEx 2025 [34] and miRNATissueAtlas 2025 [35], in combination with experimentally validated miRNA-mRNA interactions and advanced network-based topological methods. While their tumor-suppressive roles have been established in other malignancies, their discovery and systematic analysis in the context of ACC is entirely novel. This framework enabled the elucidation of their regulatory centrality, context-specific expression patterns, and mechanistic impact in ACC, providing new and unprecedented insights into their potential as diagnostic and prognostic biomarkers and their involvement in the molecular pathogenesis of this rare adrenal tumor.

2. Materials and Methods

2.1 Data Acquisition and Preprocessing

Transcriptomic data for ACC were retrieved from The Cancer Genome Atlas (TCGA) [33] and Genotype-Tissue Expression (GTEx 2025) [34] databases to ensure comprehensive representation of tumor and normal adrenal tissues. Specifically, RNA-Seq and miRNA-Seq datasets from 78 ACC tumor samples were downloaded from TCGA-ACC, encompassing both gene and microRNA expression profiles.

To represent the normal tissue counterpart, RNA-Seq and miRNA-Seq data from 250 adrenal gland samples were obtained from GTEx 2025 [34]. For miRNA data, additional filtering was required to remove redundant entries and retain only mature human miRNAs. The miRNA data has been reported recently in GTEx 2025 Preprocessing steps included data harmonization, format standardization, and the generation of unified case-by-feature matrices for both RNA and miRNA datasets.

Given the limited availability and recent inclusion of miRNA expression data in GTEx 2025 [34], especially for adrenal gland tissues, a complementary dataset was utilized to enhance the analysis of hsa-miR-466. Specifically, normalized miRNA expression profiles from the miRNATissueAtlas 2025 [35] were incorporated, which includes harmonized RPMM-based data from 630 normal adrenal gland samples. This dataset provided broader coverage and improved statistical power for downstream correlation and network analyses.

2.2 Quality Control and Filtering Strategies

Quality control and expression filtering were applied uniformly across mRNA and miRNA datasets. To remove transcriptionally inactive or near-zero expression genes, features with expression values below 1 in more than 80% of samples were excluded. This permissive threshold was chosen to retain weakly expressed but potentially informative transcripts, given the limited sample size of the ACC dataset. Gene expression distributions were visually inspected after log-transformation to assess consistency between tumor and normal samples and to evaluate normalization performance, with no major deviations or technical artifacts observed. No samples showed evidence of being technical outliers. All filtering steps were implemented using custom R scripts with consistent criteria across mRNA and miRNA data, reducing technical noise while preserving biologically relevant signals for downstream analyses.

2.3 Differential Expression Analysis

Differential expression analysis between normal and tumor adrenal tissue samples was conducted using the limma-voom pipeline [36] in R, which combines linear modeling with empirical Bayes moderation after precision weighting of log-transformed expression values through the voom method. This approach has been widely adopted for RNA-Seq transcriptomic data analysis. Raw RNA-Seq count data for both tumor (TCGA-ACC [33]) and normal (GTEx 2025 [34]) samples were first curated to retain only valid gene entries and to remove transcript version numbers. The two datasets were then merged by gene ID, and the unstranded count values were extracted to construct a unified count matrix representing all samples. To reduce noise and improve model stability, genes with low expression were filtered out prior to transformation. To account for library size differences and prepare the data for linear modeling, the filtered count matrix was transformed into log2-counts per million using the voom function, which also estimated the mean-variance trend and assigned precision weights.

A design matrix was then constructed to define the two experimental groups, normal and tumor, with the normal group set as the reference level. Linear modeling was performed with lmFit, followed by empirical Bayes shrinkage via the eBayes function to improve variance estimation. Differentially expressed genes (DEGs) were extracted using the topTable function, ranking results by adjusted p-values using the Benjamini-Hochberg FDR correction. DEGs were selected based on an adjusted p-value < 0.05 and an absolute $|\log2 \text{ fold change}| > 1$. Final results were saved with corrected gene identifiers for downstream analyses.

2.4 Integration of miRNA-mRNA Interactions and ceRNA Network Construction

A comprehensive ceRNA network was established based on transcriptomic data derived from TCGA-ACC tumor samples and GTEx normal adrenal tissue samples. Experimentally validated miRNA-mRNA interactions were obtained using the multiMiR R package [37], which integrates high-confidence interactions from databases including miRTarBase, TarBase, and miRecords. Only interactions supported by at least five independent sources across the aggregated databases were retained to increase confidence. To ensure functional relevance within the adrenal context, miRNA-mRNA pairs were evaluated against tumor- and normal-specific expression profiles, and only those exhibiting significant negative correlation ($p < 0.05$) were included. This two-stage approach, integrating experimental validation with tissue-specific expression evidence, enhanced the biological robustness of the resulting network. The final network was restricted to miRNA-mRNA interactions, consistent with established practices in ceRNA network analysis.

3. Results and Discussion

3.1 ceRNA Network in ACC vs Adrenal Gland

Analysis of the ceRNA networks constructed from normal and tumor transcriptomes of ACC reveals substantial differences in regulatory architecture between physiological and cancerous states [30, 31]. In the normal adrenal network, hsa-miR-375 and hsa-miR-326 were among the top six hub nodes. These miRNAs exhibit discrete, modular connectivity patterns, suggesting that post-transcriptional regulation in normal tissue is compartmentalized and functionally localized. In contrast, the tumor-specific network demonstrates a markedly more interconnected and compact topology, indicative of extensive regulatory rewiring in ACC. Within this network, the dominant hubs include hsa-miR-940 which is not central or even active in the normal network. The emergence of these tumor-exclusive hubs reflects a significant shift in the balance and influence of miRNA-driven regulation under oncogenic pressure.

These observations are further substantiated by quantitative analysis, which compares betweenness centrality values of the most influential miRNAs across normal and tumor networks [30, 31]. In the comparative analysis of the ceRNA networks, substantial shifts in hub miRNAs are observed between normal and tumor conditions. In the tumor network, miR-940 exhibits the highest betweenness centrality, reflecting a pronounced reorganization of regulatory control toward tumor-specific modules. Conversely, in the normal network, miR-326 and miR-375 serve as the principal hubs, while both displays reduced or negligible centrality in the tumor context. These reciprocal changes highlight the dynamic rewiring of miRNA-mediated interactions in ACC and reveal context-specific hub miRNAs with potential utility in biomarker discovery and therapeutic targeting.

The constructed ceRNA network consisted of over 72 nodes and 57 edges in normal tissues and expanded to more than 267 nodes and 210 edges in tumor samples, indicating substantial topological complexity in the malignant state. In GTEx 2025-derived normal samples [34], several

hub miRNAs identified in tumor tissues were not detected. This absence, however, is likely attributable to the stringent preprocessing pipeline and database-specific filtering applied in GTEx 2025 [34], rather than a true lack of biological expression. Supporting this, data from the miRNATissueAtlas 2025 [35] confirm that many of these miRNAs are indeed expressed in normal adrenal tissues, underscoring the importance of integrating complementary datasets to obtain a more comprehensive view of miRNA expression.

Building upon the comparative analysis of expression patterns and network centrality among hub miRNAs in normal and tumor ceRNA networks, three candidates, miR-940, miR-375, and miR-326 emerged as potential tumor suppressors warranting further investigation. Notably, miR-940 appears as a dominant hub exclusively in the tumor-specific network, despite exhibiting relatively low expression levels, suggesting a structurally central yet potentially inhibitory regulatory role in the malignant state. Conversely, miR-375 and miR-326, which occupy prominent central positions in the normal network, show markedly reduced centrality in the tumor context. This downshifting may reflect oncogenic suppression of their physiological regulatory functions. Collectively, these findings highlight the context-dependent regulatory roles of these miRNAs and support their candidacy as tumor suppressors in ACC, meriting comprehensive functional validation in future studies.

3.2 Expression Profile of Central miRNAs

Figure 1a presents the expression profiles of three candidate tumor-suppressive miRNAs, miR-940, miR-375, and miR-326 in normal versus tumor adrenal tissues. In all three cases, a marked downregulation is observed in tumor samples, as measured by $\log_2(\text{RPM} + 0.01)$, with statistically distinct distribution patterns. For the comparisons shown in **Figure 1**, p-values were calculated using the limma package, which applies a linear modeling framework combined with an empirical Bayes moderated t-test for differential expression analysis. Statistical significance is represented by asterisks: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns not significant. Notably, miR-375 exhibits the highest expression in normal tissue, followed by a significant decline in tumors, suggesting loss of a strong regulatory influence. Similarly, miR-326 and miR-940 display consistent downregulation in tumors, despite their emergence as structurally central nodes in the tumor-specific ceRNA network. These inverse relationships between expression level and network centrality reinforce their potential roles as tumor suppressors in ACC and underscore the importance of further mechanistic validation.

The expression landscape of miR-940, miR-375, and miR-326 is further clarified through violin plot analysis, revealing consistent and statistically significant downregulation in tumor tissues relative to normal counterparts (***, $P < 0.001$). The distinct narrowing and leftward shift in distribution observed for each miRNA in the tumor group suggests both suppression of expression and reduced heterogeneity under malignant conditions. These expression patterns, coupled with

their network centrality dynamics, strengthen the case for their functional roles as tumor-suppressive regulators in the context of ACC.

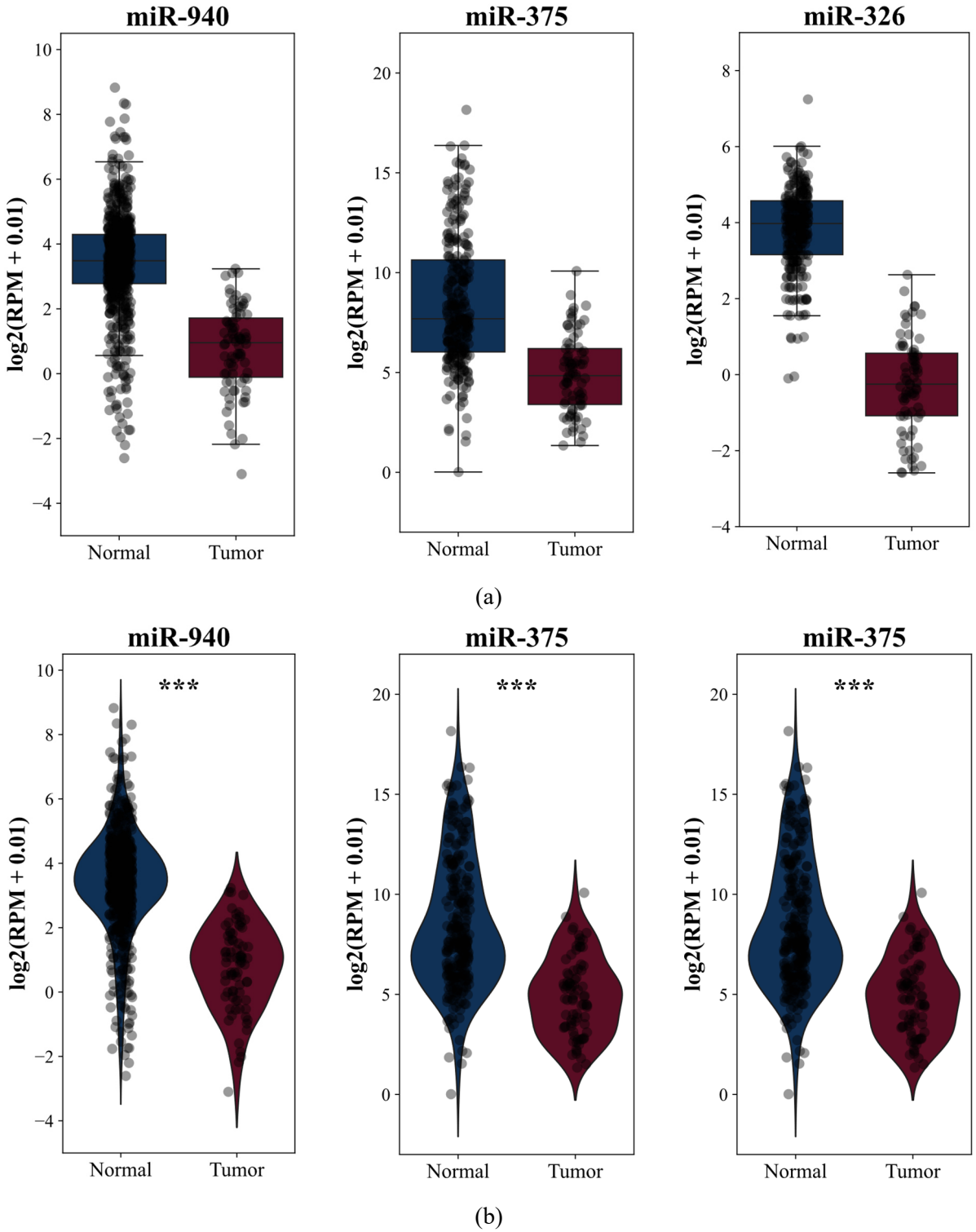

Figure 1. Box plots and violin plots of central miRNAs expression in Normal vs. Tumor tissue samples. These miRNAs exhibit statistically significant expression differences and are considered central regulators

in the dataset. (Tumor: ACC TCGA [33], Normal: miR-940: miRNATissueAtlas 2025 [36], Normal: miR-375: GTEx 2025 [35], Normal: miR-375: GTEx 2025 [35])

3.3 Target Prediction of Central miRNAs

Experimentally validated and computationally predicted mRNA targets of miR-940, miR-375, and miR-326 were systematically compiled from miRTarBase [38] and miRDB databases [39]. All human targets (hsa) from miRTarBase were included regardless of the validation method, encompassing luciferase reporter assays, qRT-PCR, and Western blotting. In cases where the number of validated targets exceeded 200, prioritization was performed based on the number of supporting experimental records, and the top 200 genes were retained. For miR-940, supporting evidence ranged from 6 to 35 entries in miRTarBase [38], while miRDB [39] predictions were filtered using a score threshold > 80. Target sets for miR-375 and miR-326 were filtered with thresholds of 2-43 and 3-38 experimental records, and miRDB score cutoffs > 55 and > 75, respectively. This integrated approach allowed the selection of robust candidate targets combining empirical evidence and predictive confidence.

Subsequent correlation analysis was carried out using transcriptomic profiles from TCGA-ACC [33] to assess the regulatory relevance of the selected miRNA-mRNA pairs in the ACC context. Pearson correlation coefficients were calculated for each pair, and only interactions showing statistically significant inverse correlations ($r < -0.3$, $p < 0.05$) were retained. The resulting high-confidence target genes: miR-940: OGFRL1, IRAK3, CYP4V2, MPP7, CLCN5, PIK3C2B, CNOT4, ZBTB7A, SIGLEC8, PLEKHA2, THNSL1, PNPO, GNG7, GM2A, GK5, ZC4H2, PCCB, NFASC, HS3ST3B1, PRKN, FAM104B, PARD3B, FHIT, CKMT2; ; miR-375: COL4A4, FGF7, ITPKB, ARID5B, SOX12, RBM39, MATR3, PHF6, RBM12B, DRAM1, PRDM1, XAF1, JUND, PHACTR2, PHC3, FBXO32, MSL2, EXOC6, ITPKB, CLEC5A, NR5A2, DNALI1, MBNL1, COLCA2, CPNE8, SGK3, MXI1, NOX4, PIGL, BAHCC1, SAMD8, TSPAN2, MGAT4A; miR-326: ELFN2, HOOK3, XKR9, CPA4, KLHDC10, ZNF772, ATXN7L3B, ELMO2, GGA2, TMBIM4, SETD1B, ZNF814, GLYR1, TOM1L2, USH1G, MAVS, CBX5, SLC27A1, DPF2, VLDLR, EPHX2, NPAS3, NLK, FBXO21, ZNF197, CFAP70, TK2, DNM1L, JADE1, RNF150, SOBP, PNISR, RALGAPA1, CDK15, which represent a context-specific subset with putative functional relevance. A considerable proportion of these genes have been previously characterized as oncogenic drivers in other cancer types, further supporting the role of miR-940, miR-375, and miR-326 as potential tumor suppressors in adrenocortical carcinoma.

Given that oncogenes are typically overexpressed in cancer and exhibit a $\log_2$ fold change (logFC) > 1, this section aimed to evaluate the extent to which miR-940, miR-375, and miR-326 target upregulated genes in ACC. An initial differential expression analysis was performed using the limma package, and the resulting data were visualized through a volcano plot (**Figure 2**). This analysis identified 2,465 genes in the upregulated region of the plot, representing potential oncogenic candidates in ACC. Pearson correlation analysis was subsequently applied between

each miRNA and this subset of genes, with interactions filtered at a significance threshold of $r < -0.2$ and $p < 0.05$ to capture plausible inhibitory relationships. The results revealed that miR-940 negatively correlated with 122 upregulated genes, miR-375 with 31, and miR-326 with 81, indicating varying degrees of suppressive targeting among these miRNAs. These findings are presented in **Figure 2** and summarized in **Table 1**, where the top 10 most significantly correlated genes for each miRNA are also listed.

Table 1. Summary of significantly correlated upregulated target genes for miR-940, miR-375, and miR-326 among 2,465 genes identified through differential expression analysis in TCGA ACC [33]. Correlation thresholds were set at $r < -0.2$ and $p < 0.05$. The top 10 genes per miRNA are shown with their respective logFC and p-value ranges.

Central miRNA	Regulation Type	% of Targeted Genes	r Range	p-value Range	Top 10 Selected Target Genes	logFC Range	p-value Range
miR-940	Up (2465 total)	4.95% (no 122)	< -0.2	< 0.05	BCL2L1, JAK3, GDF15, RASGRP1, IQGAP2, GRPR, ANGPTL6, CD82, CLGN, CLCN4	1.0007 6.4017	1.11e-157 0.0017
miR-375	Up (2465 total)	1.26% (no 31)	< -0.2	< 0.05	HSPB1, IL3RA, TNFRSF4, PLD4, LOXHD1, HSPB1P1, HSPB1P2, SEPTIN1, CDKL2, SNRPB	1.0007 6.4017	1.11e-157 0.0017
miR-326	Up (2465 total)	3.41% (no 84)	< -0.2	< 0.05	TWIST1, BMP4, DLK1, BSG, PRKACA, ILVBL, GPR143, CLCN4, SUB1P1, PLA2G4D	1.0007 6.4017	1.11e-157 0.0017

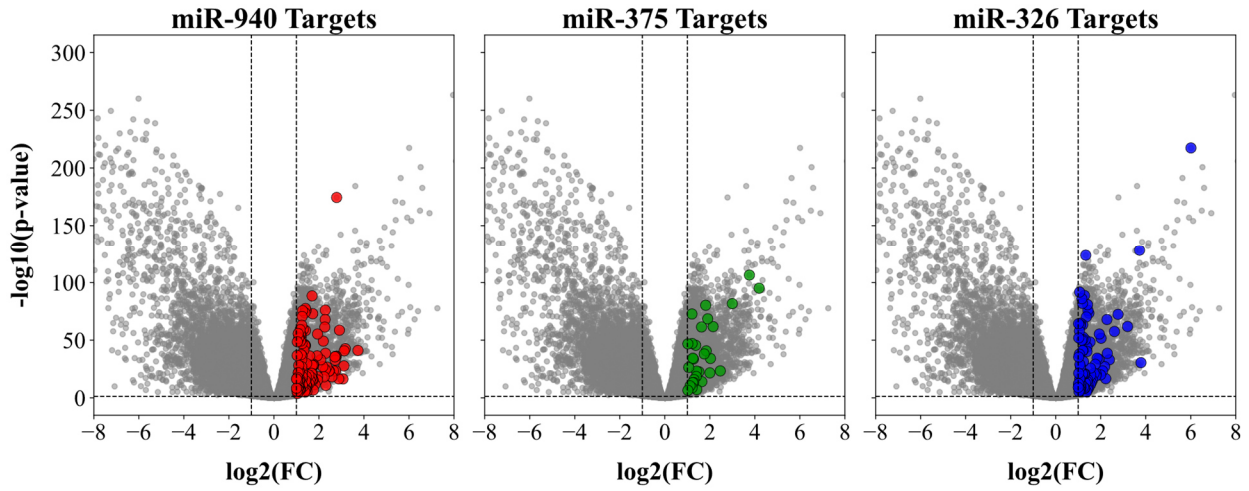

Figure 2. Volcano plot illustrating the upregulated genes in ACC ($logFC > 1$, $p < 0.05$). Target genes significantly correlated ($r < -0.2$, $p < 0.05$) with miR-940, miR-375, and miR-326 are highlighted.

The volcano plot in **Figure 2** further highlights the subset of upregulated genes targeted by each miRNA, providing a visual representation of their potential roles in modulating oncogene

expression in ACC. The expression profiles and differential expression magnitudes of the top candidate genes from **Table 2** are illustrated in **Figure 3**, which includes boxplots across tumor and normal tissues as well as $\log_2$ fold change values, providing additional support for their functional relevance in ACC. Interestingly, the analysis revealed that the gene INSYN2B is concurrently targeted by all three central miRNAs, miR-940, miR-375, and miR-326 suggesting a shared regulatory mechanism and potential functional importance in ACC. In addition, BLVRB and PLD4 were found to be jointly targeted by miR-940 and miR-375, while ACADL and ABHD11-AS1 were co-targeted by miR-940 and miR-326. The convergence of multiple tumor-suppressive miRNAs on these genes underscores their likely involvement in key oncogenic pathways and highlights them as promising candidates for future functional studies aimed at elucidating the molecular mechanisms underlying ACC progression.

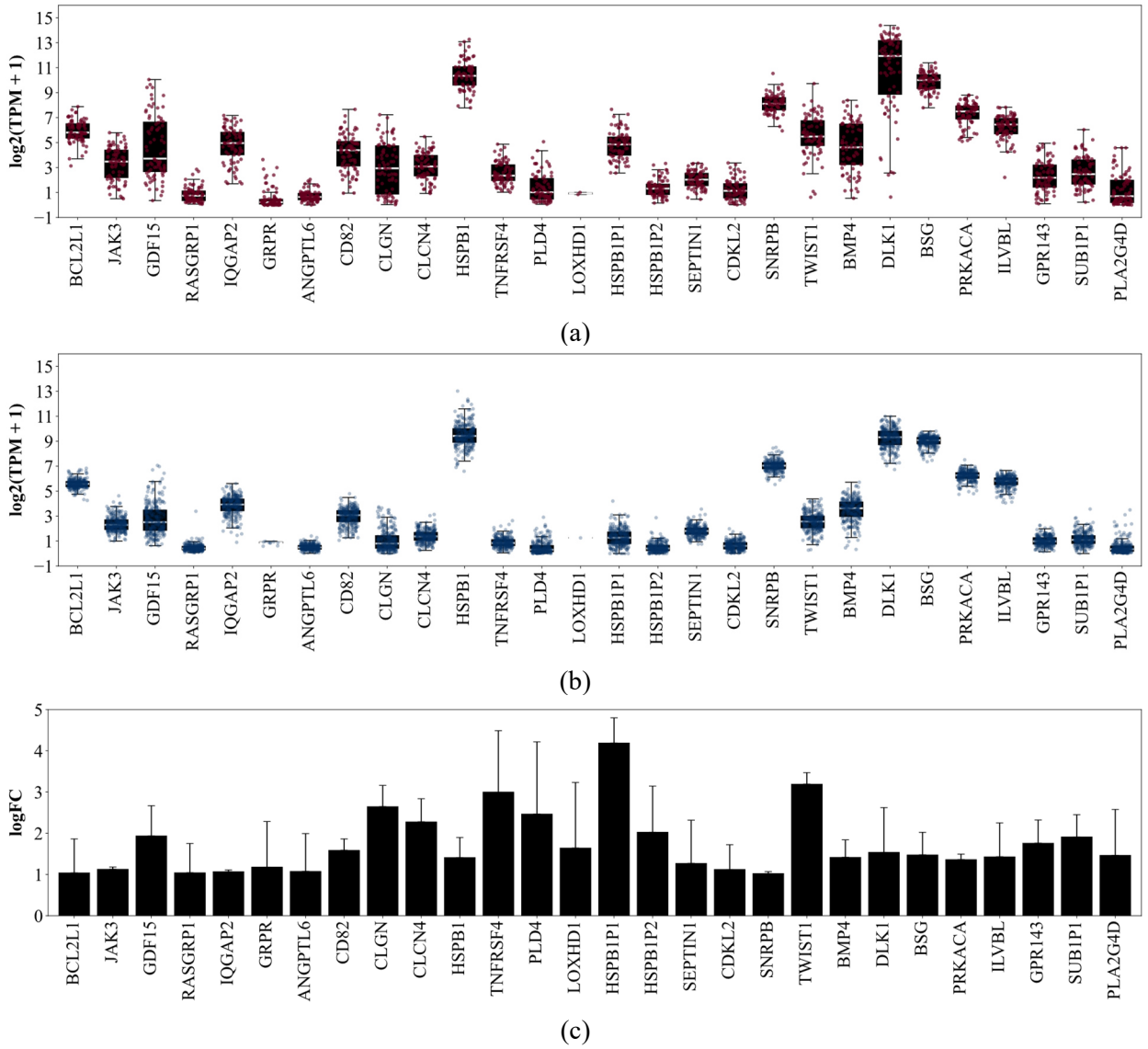

Figure 3. Expression analysis of representative target genes of miR-507 and miR-665 in ACC. (a-b) Boxplots showing expression levels across TCGA-ACC tumor samples [33] and GTEx 2025 normal

adrenal tissues [34]; (c) log2 fold changes highlighting differential expression direction and magnitude. Targets include mitotic regulators, apoptotic mediators, and transcriptional modulators.

3.4 Target Prediction of Central miRNAs with Changing the Regulatory

To identify context-dependent regulatory shifts, a focused analysis was performed on miR-375 and miR-326 target upregulated genes that exhibited substantial changes in correlation patterns between normal and tumor conditions. Differential correlation analysis was based on Pearson's correlation coefficients (r) and associated p-values, using TCGA-ACC data [33] for tumor samples and GTEx 2025 normal adrenal tissues [34] for the normal state. miRNAs were correlated with their predicted or validated mRNA targets, and pairs showing significant changes in correlation ($p < 0.05$ in both conditions) were retained. Two major criteria were applied for prioritization: (1) targets with a large absolute Δr (difference in correlation) between tumor and normal, and (2) targets that demonstrated a directional switch, specifically changing from a positive correlation in normal to a negative correlation in tumor, suggestive of a gain of post-transcriptional repression. This analysis was not extended to miR-940 due to unavailability of corresponding mrna seq profiles in miRNATissueAtlas 2025 [35] and absence of this miRNA in the GTEx 2025 miRNA dataset [34]. A curated list of representative target genes that met these criteria is summarized in **Table 2**.

Table 2. List of target genes that exhibit a substantial shift in miRNA-mRNA correlation between normal and tumor samples.

Central miRNA	Target Genes	r (Normal)	p-value (Normal)	r (Tumor)	p-value (Tumor)	Δr	Direction Change	Evidence Source
miR-375	NAPSB	0.25235	0.000029	-0.32142	0.00387	0.57377	TRUE	TCGA Data Analysis
	PLD4	0.27415	0.000005	-0.25784	0.02178	0.53199	TRUE	
	TMCC3	0.25906	0.000017	-0.23123	0.04033	0.49029	TRUE	
	SEPTIN1	0.23801	0.000083	-0.25012	0.02621	0.48813	TRUE	
	LOXHD1	0.15468	0.011222	-0.32384	0.01024	0.47852	TRUE	
	SNX32	0.19189	0.001600	-0.24108	0.03349	0.43296	TRUE	
	TNFRSF4	0.14763	0.015570	-0.26109	0.02012	0.40873	TRUE	
	INSYN2B	-0.12235	0.045373	-0.27500	0.01854	0.15264	FALSE	
	HSPB1	-0.12259	0.044948	-0.26777	0.01704	0.14518	FALSE	
	BLVRB	-0.18249	0.002711	-0.22176	0.04952	0.03927	FALSE	
miR-326	LRRC3-DT	0.17822	0.003418	-0.25101	0.03895	0.42923	TRUE	
	ZNF404	0.13529	0.026786	-0.25215	0.03139	0.38744	TRUE	
	C19orf48	0.12080	0.048210	-0.23134	0.04892	0.35214	TRUE	
	SMIM10L2A	-0.15496	1.11e-02	-0.35142	0.00230	0.19645	FALSE	
	FRG1JP	-0.13338	2.90e-02	-0.32505	0.00501	0.19168	FALSE	
	HYDIN	-0.12601	3.93e-02	-0.28251	0.03663	0.15650	FALSE	
	CCDC96	-0.13248	3.01e-02	-0.28883	0.01320	0.15635	FALSE	
	IQGAP2	-0.23891	7.81e-05	-0.38318	0.00082	0.14427	FALSE	
	ATXN7L3B	-0.38891	4.15e-11	-0.24626	0.03571	0.14264	FALSE	
	ZNF768	-0.17929	3.23e-03	-0.31159	0.00729	0.13230	FALSE	

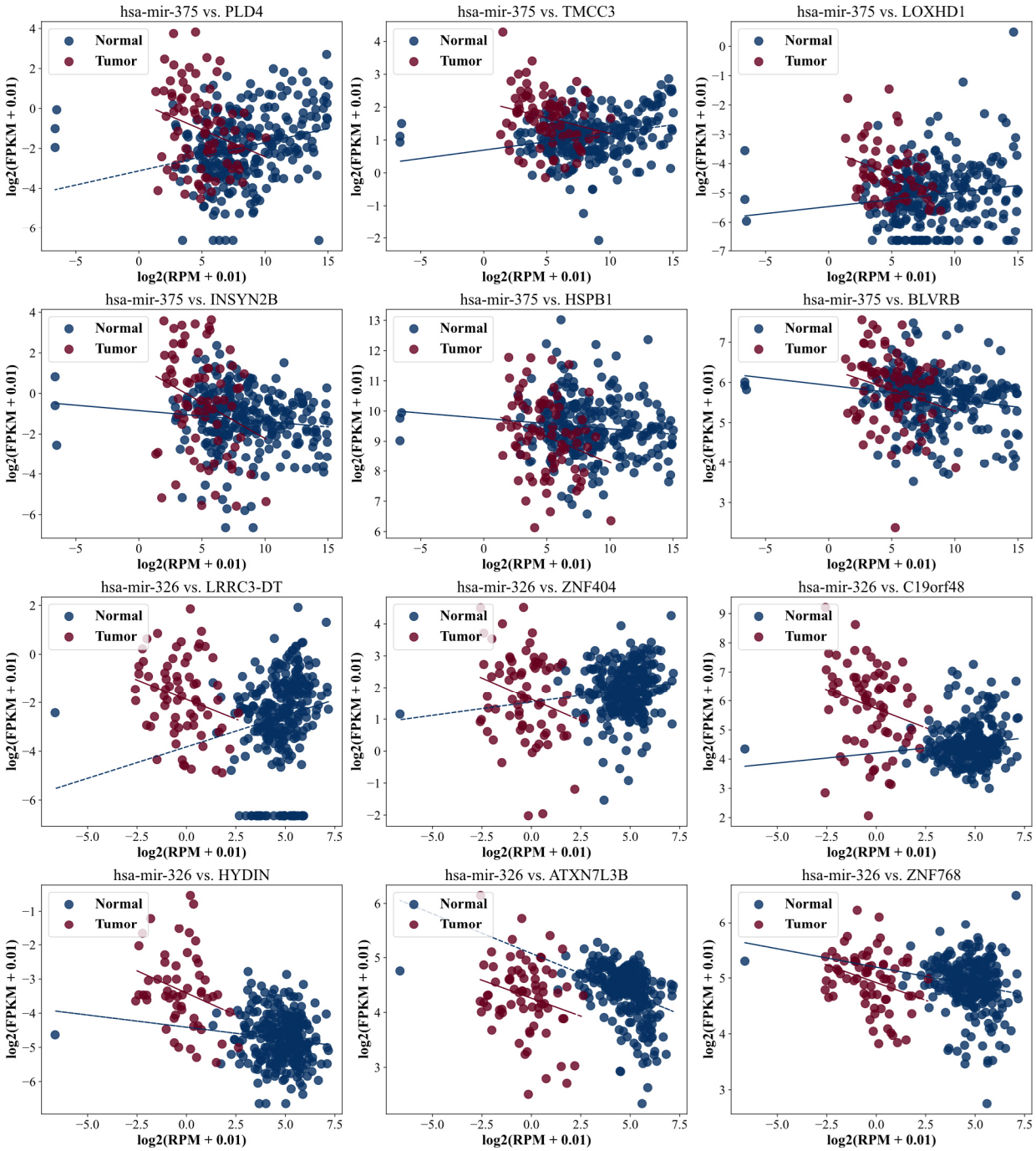

Figure 4. Scatter plots illustrating representative miRNA-mRNA pairs for miR-375 and miR-326 that exhibit a reversal in correlation direction or substantial Δr between normal (GTEx 2025 [34]) and tumor (TCGA-ACC [33]) samples, highlighting dynamic regulatory shifts in ACC.

The results revealed a subset of genes for which miRNA-mRNA correlations shifted dramatically between physiological and malignant contexts, implying dynamic regulatory rewiring. Notably, several targets of miR-375, including PLD4, TMCC3, and LOXHD1, exhibited strong positive correlations in normal tissue that became significantly negative in tumor samples, with Δr values

exceeding 0.4. Similarly, miR-326 showed pronounced regulatory changes with targets such as LRRC3-DT, ZNF404, and C19orf48. These findings are illustrated in **Figure 4**, which depicts scatter plots of selected gene-miRNA pairs that exemplify either a reversal of correlation direction or a marked increase in negative regulatory strength. Together, these results support the notion that miR-375 and miR-326 engage in tumor-specific regulatory interactions that are not evident under normal physiological conditions, reinforcing their role as context-dependent repressors in ACC.

3.5 Prognostic Value of Central miRNAs

Despite their central positions in the ceRNA regulatory network, miR-940, miR-375, and miR-326 did not individually demonstrate statistically significant associations with patient survival based on Kaplan-Meier analysis, likely due to their post-transcriptional regulatory nature and the limited dynamic range of their expression. To overcome this limitation and indirectly assess their prognostic potential, survival outcomes were instead evaluated for selected key target genes of each miRNA, based on both validated interactions and biological relevance to tumor progression. Patients were stratified into high and low expression groups using the median cutoff ($n = 38$ per group), and Kaplan-Meier plots were generated using GEPIA2 [40] (**Figure 5**). The analysis focused on targets expected to be oncogenic if de-repressed, as would be the case with loss of tumor-suppressive miRNA regulation.

The survival data provided strong indirect evidence of tumor-suppressive activity for all three miRNAs. For miR-940, overexpression of targets such as CEP63, FRY, HEXB, and CPNE8 was associated with reduced overall survival (e.g., $HR = 4.1$ for HOXD3, $p = 0.00088$). Similarly, targets of miR-375 including HOXD3, JUND, LDHB, and RBM39 showed significant hazard ratios ($HR > 2.5$), supporting their oncogenic potential. For miR-326, targets such as SNRPB, DPF2, DYNLL1, and PNISR also exhibited strong survival associations, further reinforcing their role as biologically relevant effectors. These findings, visualized in **Figure 5**, highlight the inverse relationship between target gene overexpression and patient survival, collectively supporting the functional role of miR-940, miR-375, and miR-326 as putative tumor suppressors in ACC.

3.6 Functional Analysis of Central miRNAs Target Genes

Enrichment analysis of the combined target genes of miR-940, miR-375, and miR-326 was performed using the DIANA-miRPath platform [41], revealing a set of significantly overrepresented biological pathways associated with tumor suppression. The results, summarized in **Figure 6**, show strong enrichment ($p < 0.05$ across all terms) in pathways such as the MLL5-L complex, transcriptional repressor complex, innate immune response, cell death, and mitotic cell cycle regulation, all of which are recognized for their roles in suppressing tumor progression. Additional suppressed pathways include intrinsic apoptotic signaling, transforming growth factor beta receptor signaling, Fc-gamma receptor-mediated phagocytosis, and transcriptional corepressor activity, further supporting the notion that these miRNAs cooperatively regulate genes

involved in key anti-oncogenic processes. The coordinated targeting of such pathways provides functional evidence consistent with the tumor-suppressive roles of these miRNAs in ACC.

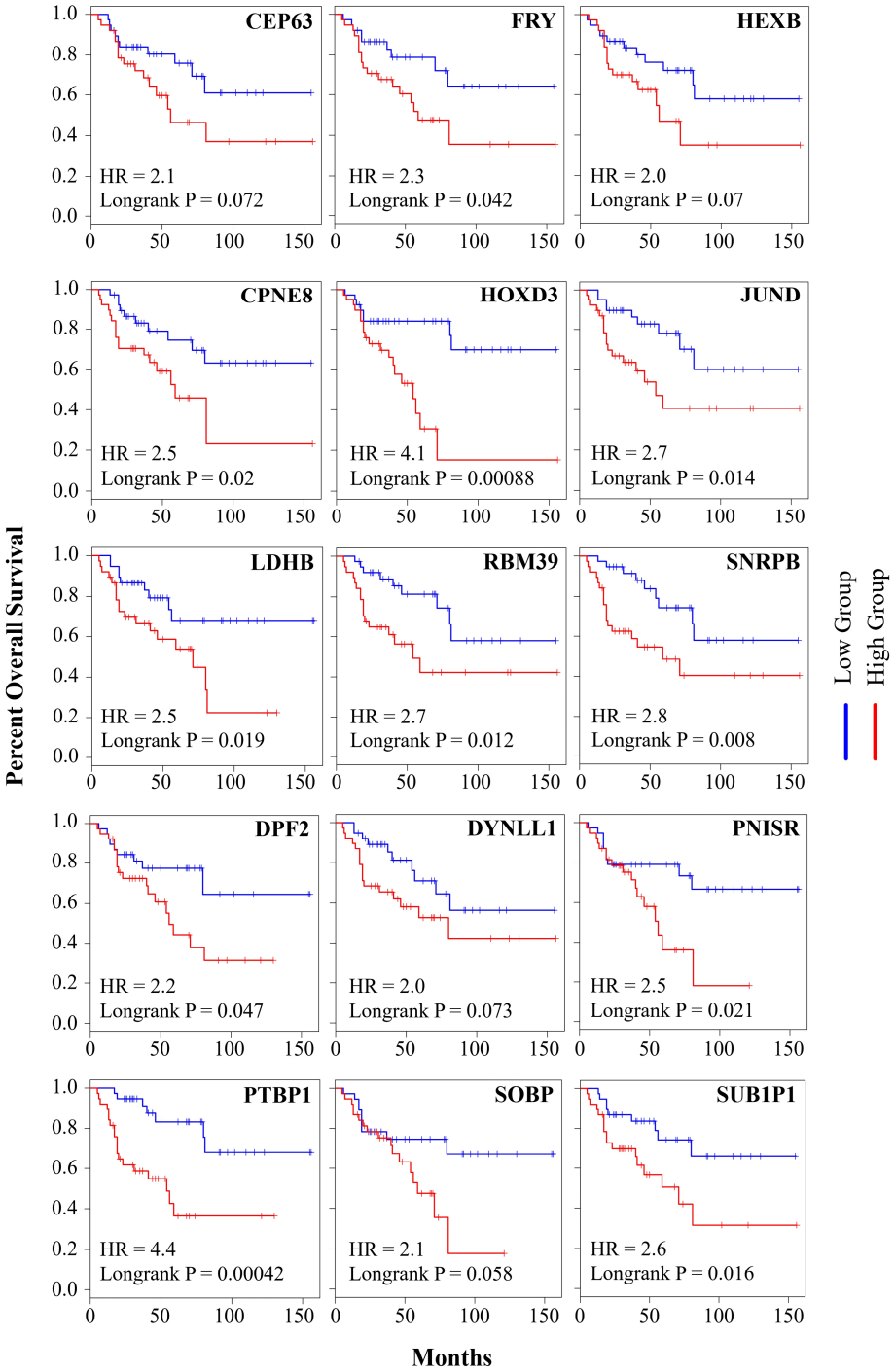

Figure 5. Kaplan-Meier survival GEPIA2 analyses [40] of central miRNAs' their key target genes in ACC. For each central miRNA, two biologically relevant target genes were selected based on validated interactions and functional importance. Patients were stratified into high and low expression groups based on the median expression level (number of patients in the high group was 38, and in the low group was 38).

To gain mechanistic insights into the biological roles of the target genes regulated by miR-940, miR-375, and miR-326, gene ontology (GO) enrichment analysis was conducted using the DAVID functional annotation platform [42]. GO terms were identified across the Biological Process (BP) category, with statistical significance thresholds of $p < 0.05$. MiR-940 targets were enriched in processes such as cell adhesion, steroid hormone signaling, toll-like receptor signaling, and multiple forms of ubiquitination, all known to be implicated in cancer progression and immune evasion. Similarly, targets of miR-375 were involved in transcriptional activation, epithelial cell proliferation, EGF signaling, and Wnt pathway regulation, underscoring their role in sustaining tumor growth. For miR-326, enriched GO terms included positive regulation of angiogenesis, cell migration, actin cytoskeleton remodeling, and DNA replication control, which are central to metastasis and cell cycle dysregulation.

These enriched biological functions suggest that the genes regulated by each miRNA are critically involved in processes that facilitate oncogenesis. The suppression of such genes by miR-940, miR-375, and miR-326 thus reinforces their functional classification as tumor-suppressive miRNAs in ACC. While the current discussion emphasizes BP categories, comprehensive GO profiling also includes significantly enriched Cellular Component (CC) and Molecular Function (MF) terms, many of which are likewise aligned with tumorigenic mechanisms, including chromatin-binding, transcriptional coactivator activity, and components of nuclear signaling complexes. Together, these data provide a coherent functional framework linking the regulatory activity of central miRNAs to the suppression of oncogenic programs in ACC.

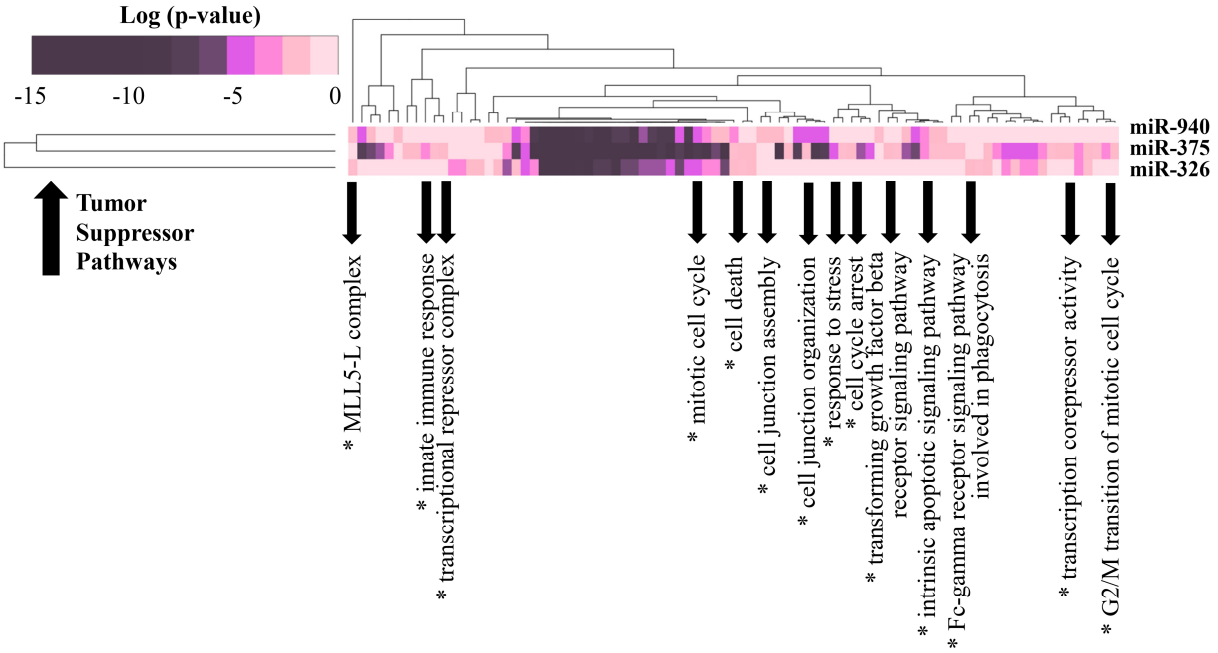

Figure 6. Hierarchical clustering of significantly enriched biological pathways based on DIANA-miRPath analysis [41] of target genes regulated by miR-940, miR-375, and miR-326. Highlighted pathways are associated with tumor-suppressive functions, supporting the coordinated regulatory role of these miRNAs in ACC.

To further elucidate the functional relevance of the target genes, Reactome pathway enrichment analysis using the DAVID functional annotation platform [42] was performed individually for miR-940, miR-375, and miR-326. The analysis revealed significant enrichment ($p < 0.05$) of oncogenic signaling pathways across all three miRNA target sets. For miR-940, pathways such as cell-cell junction organization, adherens junction interactions, and the RHOJ GTPase cycle were identified, which are known to be dysregulated during tumor invasion and metastasis. miR-375 targets were enriched in pathways including transcriptional activation, ERBB2 signaling, TP53 regulation, and PI3K/Akt signaling, all of which are critically involved in proliferation and evasion of apoptosis. Likewise, miR-326 target genes mapped to highly cancer-associated pathways such as Rho GTPase signaling, NTRK signaling, TP53 acetylation, and extracellular matrix organization, each contributing to aggressive tumor behavior when upregulated. Collectively, these findings provide functional evidence that the repression of these oncogenic pathways by miR-940, miR-375, and miR-326 is consistent with a tumor-suppressive role in ACC.

3.7 Therapeutic Implications

Expression dynamics of miR-940, miR-375, and miR-326 were investigated under different therapeutic regimens to assess their potential treatment-related regulation in ACC. Tumor miRNA expression data and clinical metadata were obtained from TCGA-ACC [33], and patients were stratified into two cohorts based on treatment modality: those receiving pharmaceutical therapy alone, and those receiving pharmaceutical therapy combined with radiation (hereafter referred to as the radiation group). To address distributional disparities between treatment arms, min-max normalization was applied within each group. The mean normalized expression values were then compared across the two cohorts by computing Δ values (Δ = mean_pharma − mean_radiation) for each miRNA, capturing the direction and magnitude of expression shift.

The resulting distribution of Δ values is shown in **Figure 7a**, where each bar represents a single miRNA's expression change between treatment groups. Notably, miR-940 exhibited a substantial negative Δ, indicating decreased expression in the pharmaceutical group compared to radiation. Conversely, miR-326 and miR-375 showed slightly elevated expression in pharmaceutical-treated patients, though the differences were modest. These trends were further confirmed in the radar plot (**Figure 7b**), which visualizes average expression levels across groups. A clear divergence in miR-940 expression was observed, with higher levels under radiation therapy, while miR-375 and miR-326 demonstrated comparable or slightly higher expression in pharmaceutical-only patients. These patterns suggest that miR-940 may exhibit radiation-sensitive expression, aligning with its proposed tumor-suppressive function and supporting its relevance in radiation-modulated regulatory programs.

Direct comparison of raw expression profiles is provided in **Figure 7c**, where $\log_{10}$-transformed expression levels of the three miRNAs are plotted across the two treatment groups. miR-940 displayed significantly reduced expression in the pharmaceutical group relative to radiation,

further emphasizing its differential responsiveness to therapy. For miR-375, slightly higher expression was noted under radiation therapy, whereas miR-326 showed relatively uniform expression across both conditions. These observations collectively suggest that miR-940 may represent a radiation-responsive tumor suppressor, while miR-375 and miR-326 exhibit more stable or pharmacologically influenced expression patterns, offering additional insights into the context-specific regulatory dynamics of central miRNAs in ACC treatment.

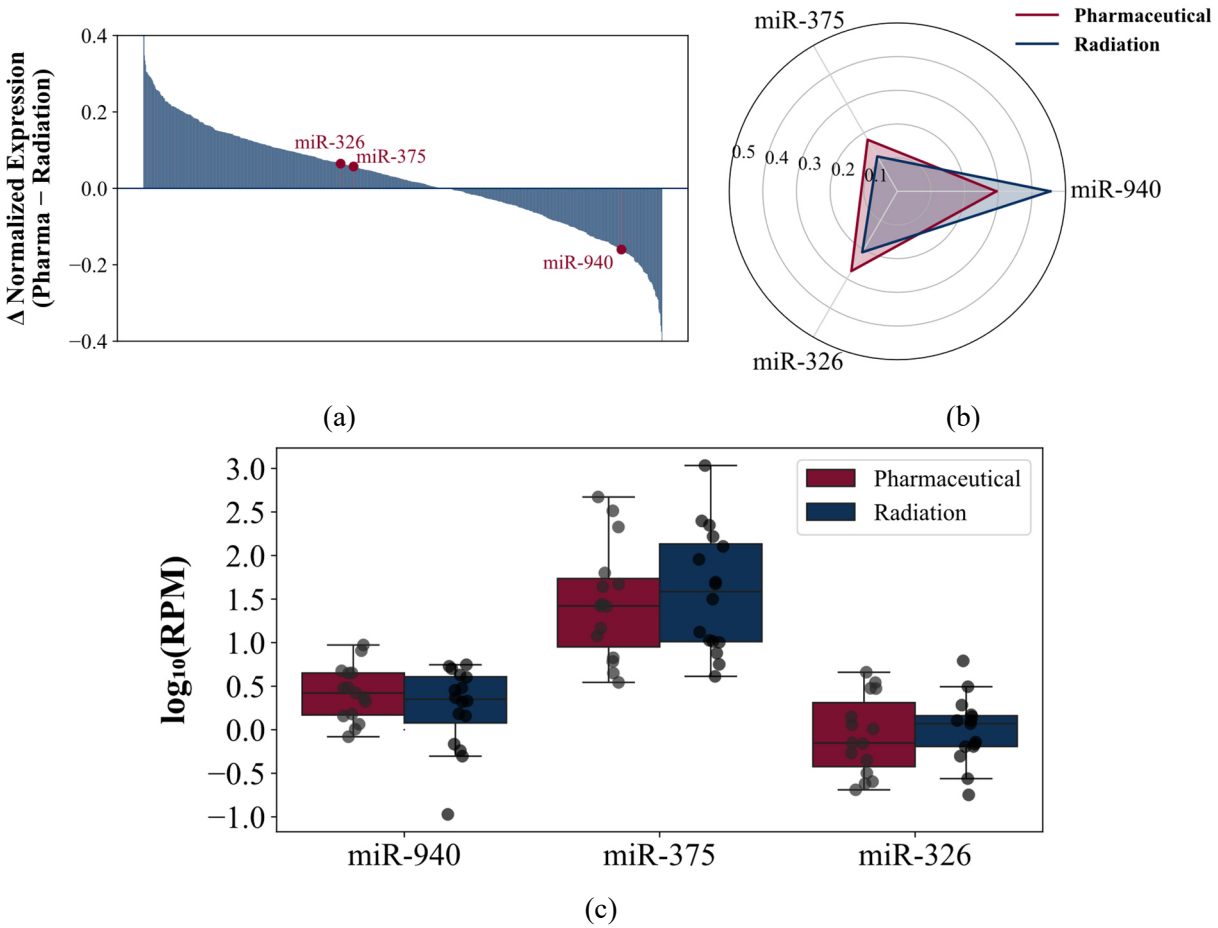

Figure 7. Differential expression of miR-940, miR-375, and miR-326 across treatment groups. (a) Waterfall plot of Δ normalized miRNA expression (pharmaceutical − radiation), highlighting the three central miRNAs. (b) Radar plot comparing mean normalized expression levels between pharmaceutical and radiation therapy groups. (c) Boxplots of $\log_{10}$-transformed expression values (excluding zero-expression samples) for miR-940, miR-375, and miR-326 in ACC tumors across treatment groups.

3.8 Discussion and Future Works

In this study, miR-940, miR-375, and miR-326 were characterized as context-dependent tumor suppressors within the ceRNA landscape of ACC, providing the first integrative evidence of their regulatory prominence in this rare endocrine malignancy. To the best of our knowledge, this is the

first integrative study to characterize the tumor-suppressive roles and regulatory centrality of miR-940, miR-375, and miR-326 in ACC using a ceRNA network-based systems biology approach.

The findings related to miR-940 are consistent with earlier reports on other cancer types. In hepatocellular carcinoma, overexpression of miR-940 was found to inhibit cell growth, migration, and invasion, primarily through repression of CXCR2 [19]. Similarly, in esophageal squamous cell carcinoma, it was reported that miR-940 restoration suppressed proliferation and induced apoptosis via downregulation of SRCIN1, supporting a tumor-suppressive function [20]. In triple-negative breast cancer, miR-940 was shown to target ZNF24 and reduce oncogenic activity [21]. These observations parallel the current study, where miR-940 emerged as a tumor-specific hub in the ACC ceRNA network, displaying significant negative correlations with multiple oncogenic targets and showing increased expression in radiation-treated patients, suggesting a responsive regulatory mechanism under therapeutic pressure.

MiR-375 has similarly been characterized as a tumor suppressor in various malignancies. In oral squamous cell carcinoma, Jung et al. demonstrated that miR-375 repressed CIP2A and destabilized MYC, thereby reducing proliferation and invasion [24]. A systematic review [22] emphasized the broad inhibitory roles of miR-375 across gastric, hepatic, and lung cancers, noting its impact on epithelial-mesenchymal transition and resistance pathways. Additionally, miR-375 has been shown to repress the RON oncogene and induce G1 cell cycle arrest in gastric cancer [23]. In prostate cancer, miR-375 was found to suppress migration and invasion in both AR-positive and AR-negative cells, forming a central node in ceRNA-based networks associated with aggressive disease [25]. These findings are congruent with the current observation that miR-375 lost centrality in the tumor-specific ACC network despite being highly expressed in normal adrenal tissue, reinforcing its role as a repressed regulatory element during tumorigenesis.

MiR-326 has also been extensively documented as a tumor suppressor. In colorectal cancer, it was shown to inhibit cell proliferation, migration, and invasion by targeting NOB1 [28]. Similar findings were reported in non-small-cell lung cancer, where miR-326 repressed the oncogenic transcription factor SP1 and suppressed EMT [27]. In prostate carcinoma, miR-326 reduced proliferation and metastasis through MUC1 regulation and modulated the PI3K/AKT pathway [26]. The present study confirms and extends these insights by positioning miR-326 as a central node in the normal adrenal ceRNA network and documenting its significant loss of centrality in tumor samples, consistent with oncogenic suppression. Furthermore, several targets of miR-326 exhibited dynamic correlation shifts between normal and tumor states, highlighting context-dependent regulatory rewiring and suggesting that the integrative analytical framework used in this study may be applicable to other rare cancers for systematic discovery of non-coding RNA-based regulatory programs.

Together, these cross-cancer comparisons support the notion that miR-940, miR-375, and miR-326 belong to a conserved class of tumor-suppressive miRNAs that are actively downregulated or

functionally suppressed in cancer-specific ceRNA networks. The novelty of the current work lies in uncovering their regulatory role in ACC, where their expression profiles, network topologies, correlation reversals, and target enrichment analyses collectively support their suppressive influence on oncogenic programs.

Despite these insights, additional research is required to confirm and extend these findings. Functional validation through in vitro assays and in vivo models is necessary to establish the causal role of these miRNAs in ACC tumorigenesis. The specific regulatory impact of each miRNA on their negatively correlated oncogenic targets, such as HOXD3, LDHB, and SNRPB, should be experimentally dissected through loss- and gain-of-function studies in ACC cell lines. Furthermore, therapeutic modulation of these miRNAs using mimics or nanoparticle delivery systems may offer promising avenues for future interventions. The observed therapy-responsive behavior of miR-940 particularly merits further investigation, as its upregulation following radiation suggests a potential role as a predictive biomarker for treatment response.

Given the context-specific nature of miRNA activity, exploration of the upstream regulatory mechanisms that drive their suppression in ACC may also be valuable. Epigenetic silencing, ceRNA competition, or dysregulation of miRNA-processing enzymes could each contribute to the observed loss of centrality and expression. Finally, pan-cancer profiling highlighted a shared pattern of downregulation for all three miRNAs in colorectal and thyroid malignancies, suggesting broader applicability for their use as diagnostic or prognostic indicators.

In summary, while miR-940, miR-375, and miR-326 have been well-studied in other cancers, their tumor-suppressive roles in ACC were identified for the first time in this study. The integration of ceRNA network topology, correlation-based filtering, differential expression analysis, and functional enrichment supports their central roles in ACC progression and underscores their potential as both therapeutic and prognostic tools, laying the groundwork for future translation of miRNA-based diagnostics or targeted therapeutics in the clinical management of ACC.

4. Conclusion

In this study, three miRNA hubs that had been identified in the authors' previous integrative network analyses were investigated in order to characterize their roles in ACC, with the results supporting their tumor-suppressive functions [29-31]. Transcriptomic datasets from TCGA [33], GTEx 2025 [34], and miRNATissueAtlas 2025 [35] were integrated and analyzed to construct competing endogenous RNA (ceRNA) networks representing both normal and tumor adrenal tissues. Through this approach, significant topological differences were identified between the two states, and a pronounced rewiring of post-transcriptional regulatory networks was observed in ACC.

Validated and predicted miRNA-mRNA interactions were evaluated, and significant negative correlations were identified between the three miRNAs and numerous upregulated oncogenes,

including HOXD3, LDHB, and SNRPB. Several of these targets were found to be associated with poor survival, providing indirect evidence for the tumor-suppressive potential of the miRNAs. Additionally, context-specific regulatory shifts were observed through correlation reversal analyses, where targets switched from positive to negative correlation in tumor samples. Functional enrichment analyses revealed that target genes of miR-940, miR-375, and miR-326 are involved in key oncogenic processes such as transcriptional activation, cell proliferation, migration, immune response, and Wnt/PI3K signaling. These pathways were significantly enriched and were also reflected in protein-protein interaction networks, supporting the coordinated repression of oncogenic modules by the miRNAs. Moreover, treatment-related expression analysis indicated that miR-940 was upregulated in radiation-treated patients, suggesting a radiation-responsive regulatory response, while miR-375 and miR-326 showed stable or modest variation. Cross-cancer analysis confirmed that downregulation of these miRNAs is recurrent in other cancers, particularly colorectal and thyroid, suggesting broader relevance. In conclusion, miR-940, miR-375, and miR-326 were identified as context-dependent tumor suppressors in ACC, acting through the repression of oncogenic targets and pathways. Their potential for prognostic application and therapeutic targeting warrants further functional validation and translational research.

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Author Statement

J. O.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review and editing

Ethics Statement

N/A

Conflict of Interest Statement

The author declares no conflicts of interest.

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