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# PIWIL genes in hepatocellular carcinoma: a multi-omics approach uncovering dysregulated expression and ceRNA networks in mice

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**Abstract**

This multi-omics study delves into the expression patterns of PIWIL genes and their correlation with hepatocellular carcinoma (HCC) progression, utilizing whole transcriptome sequencing, bioinformatics, and reverse transcription quantitative polymerase chain reaction (RT-qPCR) in mice. We identified differential expression levels of PIWIL genes between HCC and control tissues and analyzed their roles within the competing endogenous RNA (ceRNA) network related to regulatory non-coding RNA-mediated gene silencing (RNGS). Our findings showed that *Piwil1* and *Piwil4* were overexpressed while *Piwil2* is underexpressed. As ceRNAs, specific lncRNAs, including *Pvt1*, *Gas5*, and *BGIGI10090_38749*, might sponge up miR-351-5p and miR-31-5p, promoting *Piwil1* and *Piwil4* expression, while miR-133b-3p, lacking ceRNA sponge absorption, continues to inhibit *Piwil2*. Through their interactions with PPI proteins encoded by RNGS genes, especially *Dhx9*, *Drosha*, *Mov10*, and *Tdrd1*, PIWI family members might play a multifaceted role in regulating gene expression and metabolic processes, thereby involving the development and progression of HCC. These interactions within the PPI network could influence the stability and activity of PIWIL proteins and contribute to the overall regulation of gene expression and HCC progression. In the RNGS, a diverse array of miRNAs, genes, lncRNAs, circRNAs, and pseudogenes have been observed, which are suggested to intricately interplay, potentially weaving a complex ceRNA regulatory network. Abnormally expressed miRNA-targeted genes in RNGS are associated with key biological processes, such as lipid metabolism and immune responses, crucial for tumor cell survival, and processes supporting tumor growth and invasion, like translation and cytoskeleton organization. This regulation is reflected in distinct KEGG pathways for downregulated and upregulated targets, highlighting the dualistic role of PIWIL genes in modulating HCC progression. The study concludes that PIWI family members have a correlation with HCC progression and play divergent roles in the pathogenesis, with overexpression of the *Piwil1* and *Piwil4* potentially promoting HCC progression and underexpression of *Piwil2* likely suppressing tumor development. The ceRNA mechanism and PPI network are crucial in regulating the expression and function of PIWIL genes, respectively. The intricate ceRNA network potentially regulates the expression of miRNA-targeted genes in RNGS, which might be crucial for

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tumor survival and promotion, with impacts on immune responses and cell growth based on enriching results of dysregulated miRNA-targeted genes in HCC. By shedding light on the molecular intricacies of HCC, this multi-omics study underscores the pivotal roles of epigenetic regulations, especially the influence of PIWI family genes with other genes and ncRNAs in the RNGS process in HCC pathology. The findings offer valuable insights into the molecular mechanisms underpinning HCC, which may inform future research into potential targets for therapeutic intervention. The future research could benefit from integrating a diverse range of methodologies to further elucidate the roles of PIWIL genes in HCC progression, building upon the findings presented here.

**Keywords** PIWIL genes, Regulatory ncRNA-mediated Gene Silencing, Hepatocellular Carcinoma, Multi-omics, Dysregulated expression, ceRNA networks, Pathway associations, Mice

## Introduction

Hepatocellular carcinoma (HCC) is a formidable malignancy, characterized by high incidence and mortality rates worldwide [1]. Its pathogenesis is complex, involving intricate genetic and epigenetic regulatory mechanisms that contribute to the initiation and progression of the disease [2]. Among the various factors implicated in these processes, the PIWI family have emerged as a significant player in the regulation of gene expression and the silencing of transposable elements in germ cells [3, 4], with recent evidence suggesting their involvement in cancer development and progression [2].

Initially discovered in *Drosophila melanogaster* [5], these PIWIL proteins interact with PIWI-interacting RNAs (piRNAs) to regulate gene expression and maintain genome stability during spermatogenesis [3, 4]. The PIWI family, known for their signature PIWI domain, includes four members in humans: PIWIL1 (HIWI), PIWIL2 (HILI), PIWIL3 (HIWI3), and PIWIL4 (HIWI2) [6]. In mice, there are three PIWI family members: Piwil1 (Miwi), Piwil2 (Mili), and piwil4 (Miwi2) [7].

In humans, the PIWI family has been implicated in various cancers, with their abnormal expression patterns often correlating with tumorigenesis and cancer progression [2, 8, 9]. For instance, PIWIL1 has been associated with tumor proliferation and metastasis in pancreatic cancer [9–11], while PIWIL2 has been observed to modulate cancer cell properties, including proliferation, apoptosis, and epithelial-mesenchymal transition (EMT) [8, 10–13]. The dualistic nature of PIWIL proteins in cancer progression is thus evident, with the potential to serve as both promoters and inhibitors of carcinogenesis [8, 9]. Notably, *PIWIL1* can act as a co-activator for the ubiquitin ligase APC/C, facilitating the ubiquitination and degradation of Pinin, a protein pivotal to cell adhesion, thus promoting cancer cell proliferation and metastasis [10]. PIWIL proteins are involved tumor immune evasion by stabilizing PD-L1 through the deubiquitinating enzyme OTUB2 [12]. By modulating PD-L1, PIWIL proteins facilitate to orchestrate critical immune evasion strategies in tumors [14]. In the tumor microenvironment, which is a complex ecosystem composed of tumor cells, immune cells, fibroblasts, vascular cells, and extracellular matrix, PIWIL proteins also are important partners [15]. They influence the function of the tumor microenvironment and immune cells by participating in the metabolism of tumor cells [15]. For example, PIWIL proteins can regulate metabolic pathways in tumor cells, altering energy metabolism and biosynthetic processes, thus providing the materials and energy required for tumor cell growth and invasion [15]. Additionally, PIWIL proteins are also able to modulate the function of T cells, dendritic cells, and macrophages, thereby influencing the immune evasion and the effectiveness of immunotherapy of tumors [16].

Regulatory non-coding RNA-mediated gene silencing (RNGS) is a key biological process (BP) in which PIWIL proteins participate [14]. This process involves a multitude of genes and non-coding RNAs (ncRNAs), including microRNA (miRNA), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), and pseudogenes [15]. The latter three types of ncRNAs, as well as mRNAs, can act as competing endogenous RNA (ceRNA). The ceRNAs regulatory network refers to the competition among mRNAs, lncRNAs, circRNAs, and other ncRNAs within the cell for miRNA binding sites, thereby modulating each other's stability and expression levels [15]. This regulatory mechanism adds an extra layer of complexity to miRNA function and plays a vital role in tumor development. Aberrantly expressed ncRNAs in the RNGS process can modulate pathological progression by regulating the expression of downstream target molecules [17]. The PIWI family members are thought to interact with the proteins in RNGS process to influence the expression of ncRNAs, thereby regulating physiological or pathological processes in which PIWIL proteins are implicated.

Although the PIWIL genes have been studied in various cancers, there has been no research on the participation of PIWIL genes in HCC through RNGS. Moreover, due to the diverse but related functions of each member of the PIWIL family, each PIWIL family member was selected for investigation in this study. We hypothesized that the PIWIL1, PIWIL2, and PIWIL4 proteins in mice may interact with genes and ncRNA in RNGS through their interactions with PPI and ceRNAs, thereby influencing HCC progression. In this study, we aimed to

dissect the intricate functions of PIWIL genes and their potential mechanisms in HCC by identifying differences in the expression levels of PIWIL genes between HCC and control tissues. We also analyzed the Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that PIWIL genes share with other genes and miRNAs, and their involvement in the ceRNA network within RNGS. Through functional enrichment and bioinformatics analysis, we explored the regulatory mechanisms by which PIWIL genes are implicated in HCC. The research encompasses the analysis of differentially expressed miRNAs, their target genes, and ceRNAs, including lncRNAs, circRNAs, and pseudogenes. These analyses aim to elucidate the impact of PIWIL genes on HCC through RNGS and to uncover the underlying possible mechanisms, offering novel perspectives on the biological functions and correlations of the PIWIL genes with HCC.

Our study reveals correlations between PIWIL gene expression and HCC progression, with potential roles in tumor promotion and suppression. It highlights the complex interplay between PIWIL genes, RNGS genes, and ncRNAs, forming a ceRNA network that modulates gene expression and influences tumor development. Functional enrichment and PPI analysis show PIWIL genes' involvement in RNA binding, gene silencing, and metabolic processes crucial to HCC. By shedding light on the molecular intricacies of HCC, this study underscores the pivotal roles of epigenetic regulations, especially the influence of PIWI family genes with other genes and ncRNAs in the RNGS process in HCC pathology. The findings offer valuable insights into the molecular mechanisms underpinning HCC, which may inform future research into potential targets for therapeutic intervention.

## Materials and methods

All experimental procedures involving mice were conducted in accordance with the ARRIVE Guidelines and the principles of the Basel Declaration and Recommendations of the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Experimental Animal Ethics Review Committee of Youjiang Medical University for Nationalities under permit No. 202,304,250.

**Table 1** The sequences of primers used in this study

| Gene name | Primer name | Primer sequence |
|---|---|---|
| *Piwil1* | *Piwil1*-F | TCACAGGCCTGACTGATAAAAT |
| | *Piwil1*-R | CTTGTGGATGTAGTCGATGAGG |
| *Piwil2* | *Piwil2*-F | ACGAAGCAGTTTATCAGTACCA |
| | *Piwil2*-R | TCTTGATACTGATCTCGGCATC |
| *Piwil4* | *Piwil4*-F | CACTCGGCACTGTTGTGGACTC |
| | *Piwil4*-R | TGTGGTCAGGCTTCAAGGCATTG |
| *Gapdh* | *Gapdh*-F | GGTTGTCTCCTGCGACTTCA |
| | *Gapdh*-R | TGGTCCAGGGTTTCTTACTCC |

### Tissue samples collection

A total of 60 specific pathogen-free (SPF) male mice, aged one month and weighing between 20 and 22 g, were procured from Tianqin Biotechnology Co., Ltd (Changsha). The 60 mice were randomly divided into two equal groups, the experimental group and the control group. Following a (3–4)-day quarantine period in the isolation room of the animal center, 0.2 mL of H22 cell suspensions (Pulangsi, CL-0341) was inoculated into the left axillary region of each mouse. The cell suspension was cultured ex vivo, diluted to a concentration of $3\times10^6$ cells per 0.2 mL with saline. Mice injected with an equal volume of saline served as controls. On day 15 post-inoculation, tumor and control tissues were dissected from both groups after they were sacrificed by cervical dislocation.

### Total RNA extraction and reverse transcription

Tissue samples, approximately 100 mg each, were finely ground in liquid nitrogen using a mortar. The powdered tissue was then transferred to a clean EP tube for RNA extraction using the Sangon Biotech RNA extraction kit (R1200), as per the manufacturer's instructions. RNA integrity was assessed by the absorbance ratio measured with a micro-spectrophotometer (Tiangen Biotech, OSE-260-01). High-quality RNA samples were reverse transcribed into cDNA using the BeyoRT cDNA First Strand Synthesis Kit (D7168M) with Oligo (dT)18 as the primer. The PCR reverse transcription conditions were set at 37 °C for 60 min, followed by 80 °C for 5 min, and held at 4 °C thereafter. The cDNA concentration was quantified using an ultra-micro spectrophotometer and then stored for subsequent analysis.

### RT-qPCR analysis of PIWI family members expression in HCC

Primers for the mouse PIWI family members were designed based on their mRNA sequences using Primer7.0 software (https://www.primer-e.com/), with *Gapdh* as the endogenous control. Primer synthesis was carried out by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The sequences of primers were listed in the Table 1. The cDNA was diluted to a concentration of 500 ng/μL with RNase-free ddH2O. The RT-qPCR reaction system was prepared according to the Hiff™ qPCR SYBR® Green Master Mix (1120) protocol and consisted of 10 μL of Master Mix, 0.4 μL of each primer (10 μM), 2 μL of cDNA, and RNase-Free ddH2O to reach a final volume of 20 μL. The qPCR cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s on a Roche 96-well PCR system. The relative expression levels were calculated using

the $2^{-\Delta\Delta Ct}$ method, normalizing to *β-Actin* and comparing treated versus control groups. Statistical comparisons were made using a two-tailed t-test, and a P-value less than 0.05 was considered statistically significant.

#### Whole-transcriptome sequencing

Fifteen tumor tissues and an equal number of control ones were pooled in triplicate groups, with each group comprising a composite of equal amounts from five individual samples, respectively. Total RNA and miRNA were extracted, their quality assessed, and reverse transcription performed according to standard protocols for high-throughput sequencing and analysis. The quality of the extracted RNA was assessed using the RNA Integrity Number (RIN) with an Agilent 2100 Bioanalyzer. Only samples with a RIN value≥7 were selected for sequencing. The library preparation and sequencing were performed by BGI Genomics Co., Ltd., following the manufacturer's recommended protocols.

#### Expression of RNGS genes and ncRNAs

Genes and ncRNAs involved in RNGS process were sourced from the Quick GO database (https://www.ebi.ac.uk/QuickGO/). To compare their expression levels in tumor and control tissues and identify significantly differentially expressed genes (DEGs) and ncRNAs, we utilized a comprehensive workflow that included data preprocessing with SOAPnuke (v1.5.2) (https://github.com/BGI-flexlab/SOAPnuke) to remove low-quality reads and obtain clean reads in FASTQ format. These reads were then mapped to the reference genome using HISAT2 (v2.0.4) (http://www.ccb.jhu.edu/software/hisat/index.shtml), and for small RNA data, Bowtie2 (v2.2.5) (http://bowtiebio.sourceforge.net/Bowtie2/index.shtml) was used for mapping against sRNA databases. Expression levels were normalized across samples using RSEM (v1.2.12) (https://github.com/deweylab/RSEM) for RNA-Seq data, which accounts for library size differences, and unique molecular identifiers (UMIs) for small RNA data. Differential expression analysis was performed using DESeq2 (v1.4.5) (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) with a Q value≤0.05, providing a robust statistical framework for identifying DEGs and ncRNAs. The expression heatmap was generated using pheatmap (v1.0.8) (https://cran.r-project.org/web/packages/pheatmap/index.html) to visualize expression patterns across different samples. All steps, from preprocessing to analysis, were conducted using specific software versions and parameters, ensuring the computational reproducibility of our analysis pipeline. The BGI Dr.Tom platform (https://biosys.bgi.com/) facilitated the generation of the expression heatmap and analysis, integrating these tools and methods to offer a comprehensive analysis of the transcriptome data.

#### Functional enrichment and PPI network construction

The DAVID platform (https://david.ncifcrf.gov/) was employed for functional enrichment analysis of *PIWIL* with other differentially expressed RNGS genes. The value of false discovery rate (FDR) for each selected GO term and KEGG pathway must be less than 0.05, and their enrichment scores must be greater than 2. The PPI network for their encoded proteins was constructed using STRING platform (https://cn.string-db.org/) with the platform's default parameters.

#### ncRNA target relationship analysis

The targeting relationships within the miRNet platform have been experimentally verified. To ensure the reliability of the targeting relationships we identified, we employed this platform for analyzing the interactions among different molecules. The miRNet platform (https://www.mirnet.ca/) was utilized to identify and download target molecules of differentially expressed miRNAs. These targets were then cross-referenced with whole-transcriptome data to identify differentially expressed molecules. Cytoscape software was employed to visualize the interactions between differentially expressed PIWI family members, miRNAs, and ceRNAs, to elucidate their roles in HCC pathology.

#### Functional enrichment of miRNA target genes

The DAVID platform was also used to perform functional enrichment analysis on the target genes of the differentially expressed miRNAs in RNGS. The criteria for selecting GO terms and KEGG pathways in this section are consistent with those applied in the functional enrichment analysis of PIWIL in conjunction with other differentially expressed RNGS genes.

#### Statistical analysis

RT-qPCR data were represented as log2(Mean+1) for graphical illustration of significant differences. Data analysis and graphing were conducted using Excel, with the t-test employed for comparing two types of samples. A P-value less than 0.05 was considered statistically significant. For whole-transcriptome data, the threshold for identifying DEGs and ncRNAs was set at |log2(Fold Change)| > 1 and an FDR value less than 0.05. Functional enrichment for *PIWIL* and RNGS genes included BP, cellular component (CC), molecular function (MF), and KEGG pathways, with an FDR value threshold of less than 0.05. For ncRNA target genes, functional enrichment included only BP and KEGG pathways, with an FDR value less than 0.05, too, and the top 30 GO terms were selected based on gene count.

It was noted that while our analysis provides valuable insights, it is limited by the reliance on in silico methods. Future work should involve functional assays, such

as knockdown or overexpression studies, to validate the bioinformatics findings.

## Results

### Expression dynamics of PIWI family in HCC

Our RT-qPCR analysis has revealed significant differences in the expression levels of *Piwil1*, *Piwil2*, and *Piwil4* in HCC tissue samples when compared to the controls in mice. Specifically, *Piwil1* and *Piwil4* exhibited a significant increase in expression levels with P-values of less than 0.01, suggesting their potential roles in HCC development (Fig. 1). In contrast, *Piwil2* showed a notable decrease in expression with a *P*-value less than 0.05 (Fig. 1), indicating it may act as an inhibitor of tumor progression. These findings showed that PIWI family members might have divergent roles in HCC.

Position of Fig. 1.

### DEGs in RNGS and abnormally expressed ncRNAs through Multi-omics Data

Our whole transcriptome sequencing captured the expression profiles across a spectrum of molecules, including mRNAs, miRNAs, lncRNAs, circRNAs, and pseudogenes. After eliminating duplicate entries from the initial 2079 RNGS molecules sourced from QuickGO, our refined dataset comprised 121 genes, 624 miRNAs, and 170 diverse molecular entities. The subsequent analysis pinpointed 25 differentially expressed genes (DEGs) in tumor tissues, with 18 upregulated and 7 downregulated, excluding those of the PIWI family (Fig. 2A). Additionally, 51 miRNAs displayed differential expression, with 33 upregulated and 18 downregulated (Fig. 2B). Notably, 10 miRNAs were identified to have target relationships with the upregulated *Piwil1* and *Piwil4*, while 4 targeted the downregulated *Piwil2*.

Furthermore, the study identified 8 upregulated and 1 downregulated lncRNAs, 2 downregulated circRNAs, and 1 downregulated pseudogene, all of which are implicated as ceRNAs. These molecules potentially modulate

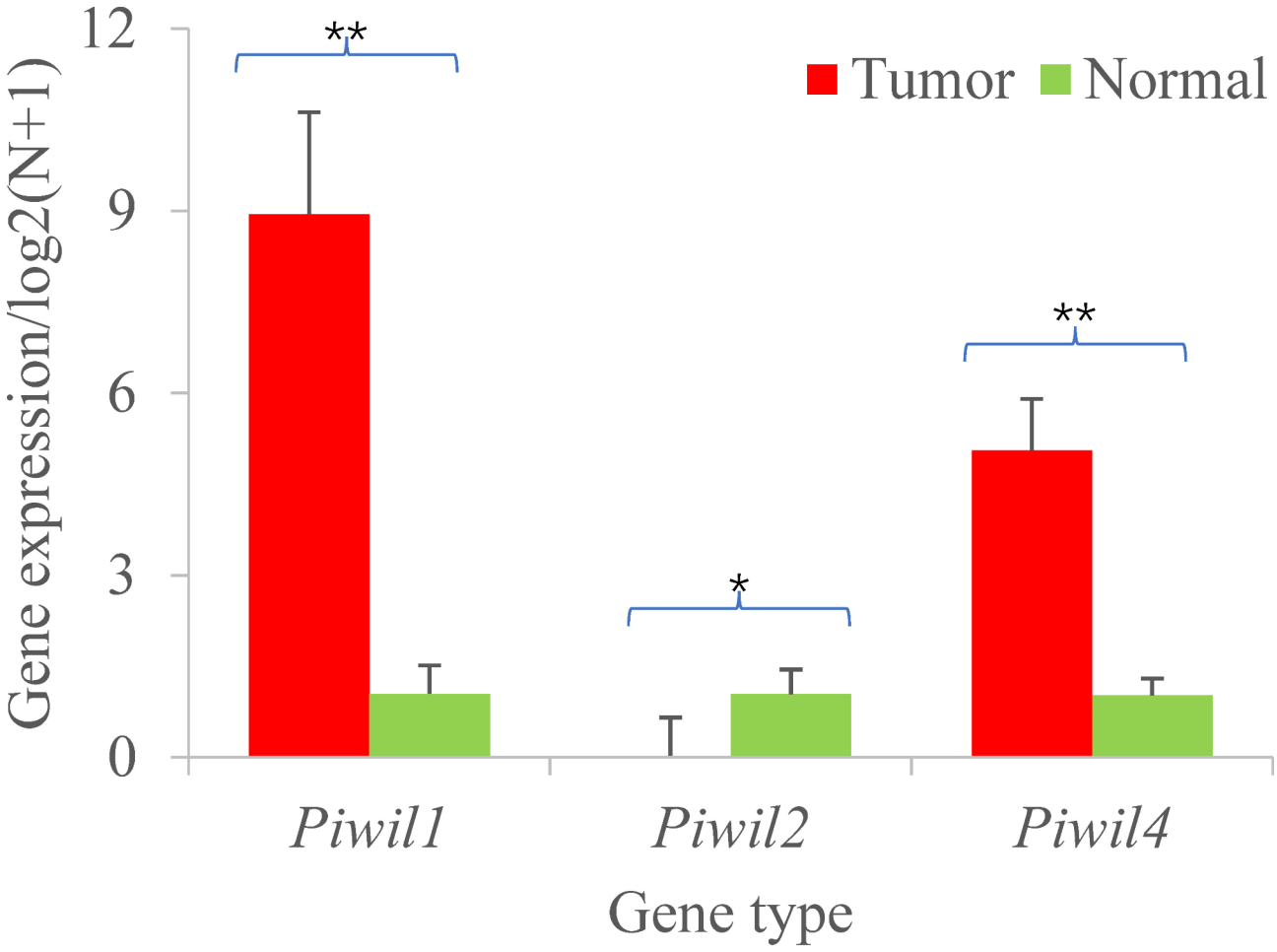


**Fig. 1** Relative expression of the three PIWIL genes in mouse HCC tissue and control. *Note*: * indicates $P < 0.05$, ** indicates $P < 0.01$

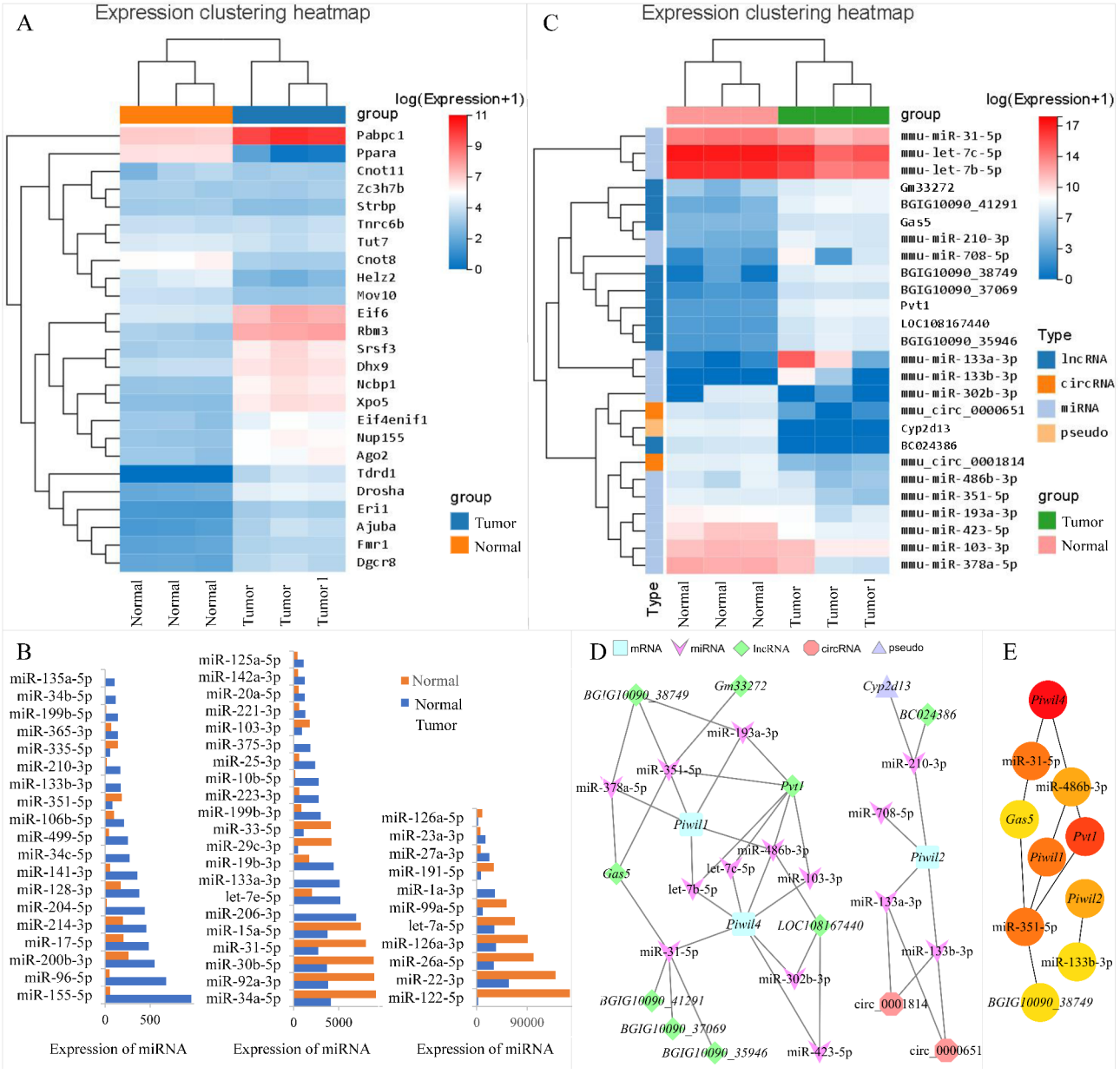


**Fig. 2** Expression of differentially expressed RNGS genes and ncRNAs in HCC tissue and the ceRNA regulatory network of PIWIL genes. *Note*: **A**, Clustering heat map of differentially expressed RNGS genes; **B**, Differential expression levels of RNGS ncRNAs; **C**, Clustering heat map of expression levels of ceRNA members regulating PIWIL genes; **D**, ceRNA regulatory network of PIWIL genes; **E**, Top 10 core members in the PIWIL gene ceRNA regulatory network

the expression of PIWIL genes through miRNA binding, thus constructing a regulatory network around the PIWIL genes (Fig. 2C and D). Cytoscape's CytoHubba analysis identified miR-351-5p, miR-31-5p, and miR-133b-3p as central regulators of *Piwil1*, *Piwil4*, and *Piwil2*, respectively. miR-486b-3p was found to have a regulatory influence over both *Piwil1* and *Piwil4*. The lncRNAs *Pvt1*, *Gas5*, and *BGIGI10090_38749* emerged as pivotal ceRNAs within this network.

### GO terms and PPI network of proteins encoded by *PIWIL* with RNGS genes

The integration of functional enrichment and PPI analysis has shed light on the roles of PIWIL genes in conjunction with other RNGS genes. These genes are predominantly associated with MFs such as RNA binding and are localized to CCs like the cytoplasm and nucleus. Their involvement spans a range of BPs, including gene silencing by RNA and engagement in a multitude of metabolic processes (Fig. 3). Among the PPI network, four core proteins, *Dhx9*, *Drosha*, *Mov10*, and *Tdrd1*, were

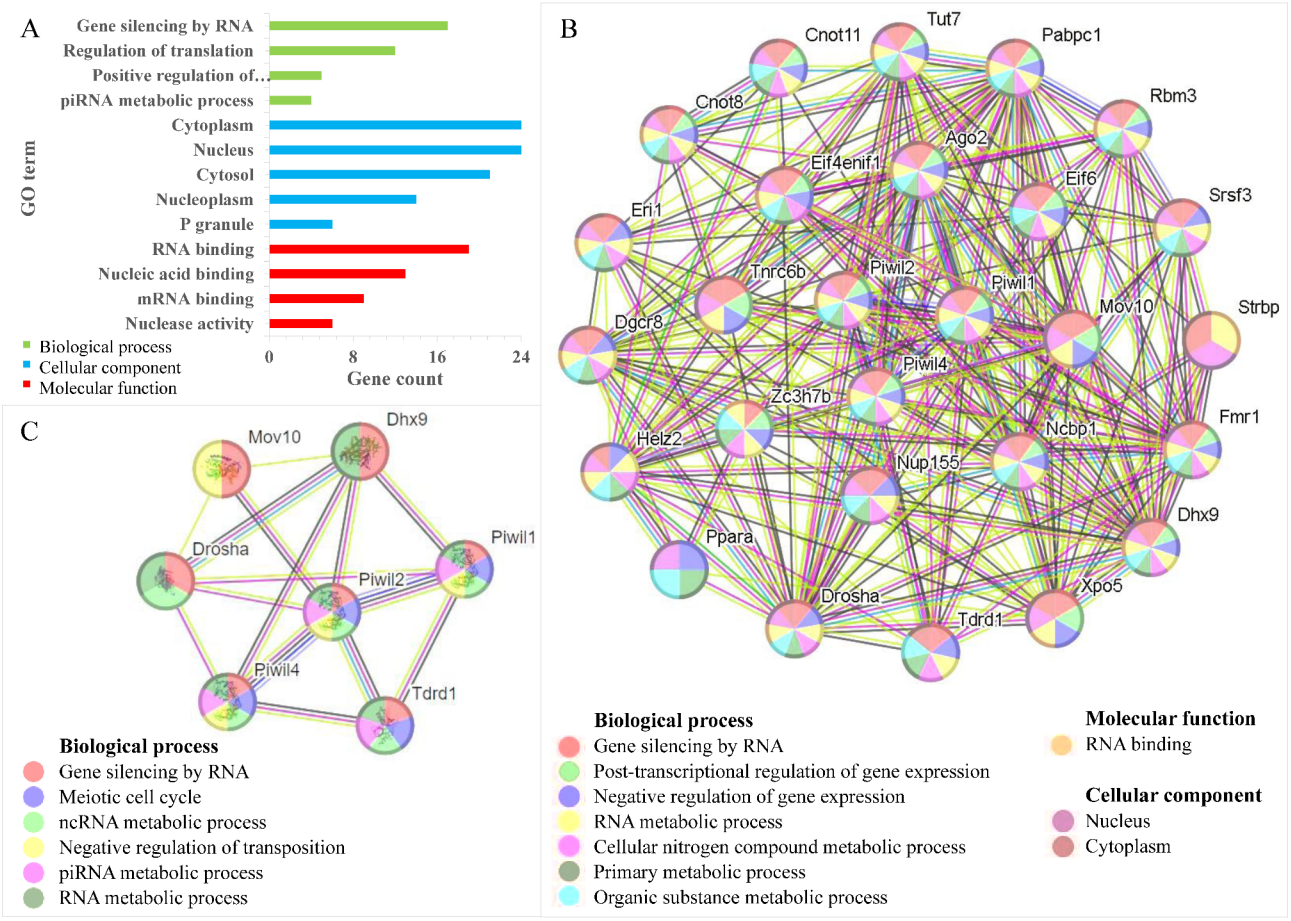


**Fig. 3** Functional enrichment of *Piwil* and RNGS genes and the PPI of their encoded proteins. *Note*: **A**, Functional enrichment results of *Piwil* and RNGS genes; **B**, PPI network of the proteins encoded by *Piwil* and RNGS genes; **C**, Core proteins directly interacted with PIWIL proteins in RNGS

directly associated with PIWIL proteins. The core proteins have a propensity to synergize with PIWIL proteins in engaging with the BPs of RNA-mediated gene silencing, RNA metabolism process, and so on (Fig. 3C). This further indicated that the primary processes in which RNGS is involved in HCC might be RNA-mediated gene silencing and RNA metabolism processes.

### ceRNA regulatory network of miRNA targets

Employing miRNet for analysis, we discovered that among the 33 upregulated miRNAs in tumor tissues, 11 had 150 negatively correlated target genes at the expression level, along with 12 lncRNAs, 2 circRNAs, and 1 pseudogene that may function as ceRNAs (Fig. 4A). Conversely, of the 18 downregulated miRNAs, 14 were linked to 144 upregulated target genes, accompanied by 3 lncRNAs and 4 circRNAs with the potential to act as ceRNAs (Fig. 4B).

### BPs and KEGG pathways of differentially expressed miRNA TARGET GENES

Functional enrichment analysis has brought to light that the target genes of the differentially expressed miRNAs in RNGS are enriched in a plethora of BPs and KEGG pathways. Downregulated target genes were particularly enriched in processes like lipid metabolism (27 genes), cholesterol metabolism (12), complement activation (9), and acute-phase response (6), with KEGG pathways including metabolic pathways (50), complement and coagulation cascades (19), and bile secretion (7) (Fig. 5A). On the other hand, upregulated target genes were enriched in BPs such as translation (16), RNA splicing (11), and actin cytoskeleton organization (10), with KEGG pathways like regulation of actin cytoskeleton (14), ribosome biogenesis (13), and focal adhesion (10) (Fig. 5B). These findings underscore the multifaceted impact of the dysregulated miRNA landscape on the molecular machinery of HCC.

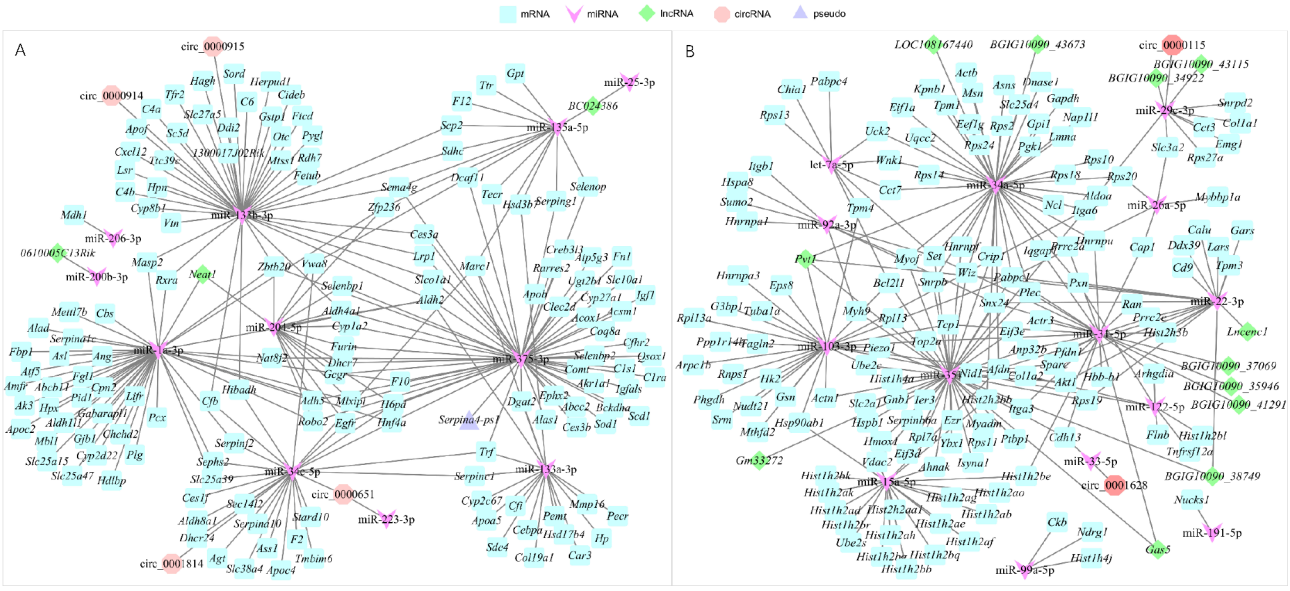


**Fig. 4** The ceRNA regulatory network composed of differentially expressed miRNAs in RNGS and their corresponding differentially expressed target genes. *Note*: **A**, The ceRNA regulatory network formed by upregulated miRNAs and their corresponding downregulated target genes in HCC compared with normal tissues; **B**, The ceRNA regulatory network formed by downregulated miRNAs and their corresponding upregulated target genes in HCC compared with normal tissues

## Discussion

The molecular mechanisms underlying the development and progression of HCC encompass both genetic mutations and epigenetic regulation, including the expression control of ncRNAs. The PIWI family, integral to ncRNA biogenesis, possesses a range of functions that regulate cell proliferation, differentiation, and apoptosis, thereby influencing reproductive processes or the advancement of cancer.

### The divergent roles of PIWI family members in HCC Pathogenesis

Our investigation has revealed a stark contrast in the expression levels of PIWI family members within HCC tissues. Specifically, *Piwil1* and *Piwil4* are significantly overexpressed, in contrast to *Piwil2*, which shows a decrease. This pattern aligns with a growing body of research highlighting the multifaceted influence of the PIWI family on cancer dynamics. A multitude of studies have demonstrated the diverse roles those various members of the PIWI family play in the biogenesis, progression, or metastasis of cancer [16]. Recently, research has shown that PIWIL1 expression induces tumorigenesis in cervical cancer [9]. The researchers discovered that PIWIL1 fosters the malignant progression of papillary thyroid carcinoma by inducing the expression of EVA1A. Similarly, PIWIL4's influence in lung cancer is significant, particularly through its regulation of the YY1/PIWIL4 axis driven by PLIC1 [18]. Conversely, PIWIL2 has been observed to modulate cancer cell properties, including proliferation apoptosis, colony formation, epithelial-mesenchymal transition (EMT), and stem cell-like properties in cancer cells [12, 13]. The presence of miRNAs that target PIWIL2, such as miR-1267 and miR-2276, has been associated with reduced expression in breast cancer [7], and diminished levels of all three PIWI family members correlate with poorer outcomes in renal cell carcinoma [19]. The dualistic nature of PIWIL proteins in cancer progression is thus evident, with the potential to serve as both promoters and inhibitors of carcinogenesis [8, 9]. It is suggested that PIWI family members might promote the occurrence and progression of cancer, or they might facilitate or inhibit the proliferation and migration of cancer cells through the piRNA-PIWI axis [20–23]. Our findings suggest a correlation between the expression levels of PIWI family members and HCC progression, with *Piwil1* and *Piwil4* being overexpressed and potentially fuel HCC progression, while *Piwil2* is underexpressed and could play a suppressive role [8].

### The multifaceted functions of PIWIL genes with RNGS genes and ncRNAs in HCC

In recent years, ncRNAs, particularly miRNAs, lncRNAs, circRNAs, and pseudogenes, have garnered significant attention due to their important roles in gene expression regulation, especially through the ceRNA regulatory mechanism [21–2324]. Our study suggests that PIWI family members may be involved in the ncRNA metabolic process and function of small RNAs, such as piRNA metabolic process, gene silencing, post-transcriptional regulation. Concurrently, ncRNAs might modulate

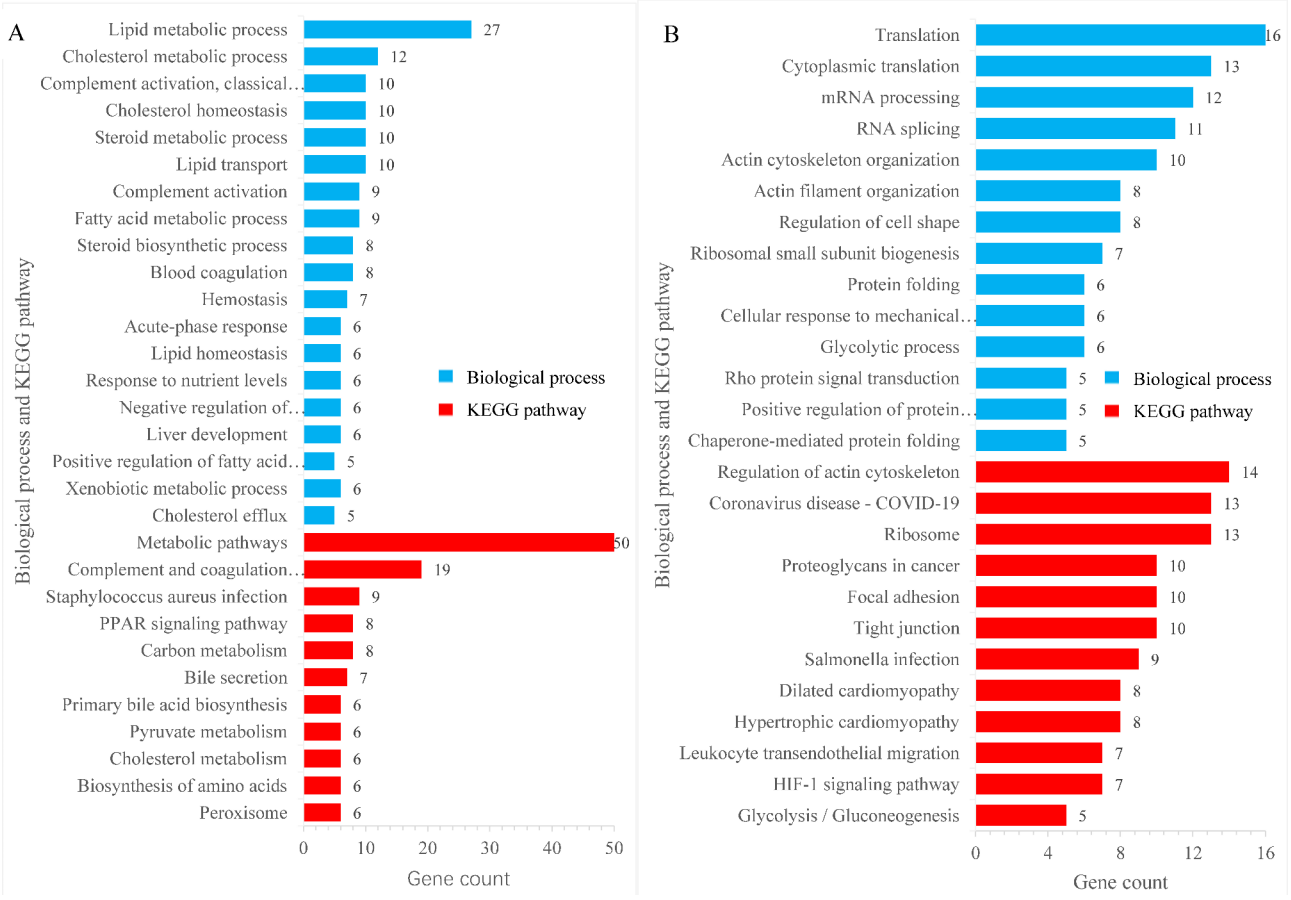


**Fig. 5** BP and KEGG pathways involved in the target genes of differentially expressed miRNAs in RNGS. *Note*: Fold enrichment of all BPs and KEGG pathways are more than 2; FDR of all BPs and KEGG pathways are less than 0.05. **A**, GO terms of target genes downregulated by miRNA in HCC compared with normal tissues; **B**, GO terms of target genes upregulated by miRNA in HCC compared with normal tissues

gene expression through RNGS mechanisms, impacting lipid metabolism, complement activation, acute-phase response, and metabolic pathways, as well as complement and coagulation cascades. RNGS, as identified through data analysis on the QuickGO platform, includes a variety of functional molecules like mRNAs, miRNAs, lncRNAs, circRNAs, and more. In HCC, specific lncRNAs, circRNAs, and pseudogenes might act as ceRNAs, interacting with miRNAs to regulate the expression of PIWIL genes. In addition to the PIWIL genes, 25 genes and 51 miRNAs are significantly differentially expressed in HCC. They potentially form a complex regulatory network that might collectively affects the proregression of HCC. For examples, the expressions of miR-351-5p, miR-31-5p, and miR-133b-3p were significantly negatively correlated with the expressions of *Piwil1*, *Piwil4*, and *Piwil2*, respectively and significantly positively correlated with the expressions of some ncRNAs, such as lncRNAs *Pvt1*, *Gas5*, and *BGIGI10090_38749*, in HCC.

Functional profiling and PPI analysis have shed light on the varied roles of *PIWIL* and RNGS genes in HCC. The primary functions of these genes were the coordination with other members of RNGS, especially *Dhx9*, *Drosha*, *Mov10*, and *Tdrd1*, in the cytoplasm or nucleus to regulate gene silencing by RNA and participate in a wide range of metabolic processes through RNA binding and other MFs. This suggests that the PIWI family might control HCC progression by modulating RNA stability and translation. RNA binding, a core function of PIWIL with RNGS genes, involves the interaction of these genes-encoded proteins with RNA, participating in RNA processing, modification, stability regulation, and translation. Abnormalities in RNA binding in HCC could alter the expression of genes pivotal to tumor behavior, thereby affecting the proliferation, migration, and invasion of tumor cells [25]. The distribution of *PIWIL* with RNGS gene-encoded proteins in the cytoplasm and nucleus reflected their biological roles in different cellular compartments. In the nucleus, these molecules may be involved in the regulation of gene expression, while in the cytoplasm, they may participate in the stability regulation and post-translational control of mRNA, as well as a broad range of metabolic processes. Gene silencing, an important function of *PIWIL* with RNGS genes, can

affect the expression of tumor suppressor genes or oncogenes, thus promoting the development of tumors. Moreover, metabolic processes play a crucial role in the energy supply and biosynthesis of tumor cells, and PIWIL genes with RNGS genes might regulate the metabolic activity of tumor cells by affecting the expression of metabolism-related genes.

In summary, the expression of PIWIL genes may be regulated by the ceRNA mechanism, whereby lncRNAs *Pvt1*, *Gas5*, and *BGIGI10090_38749* could sponge up miR-351-5p and miR-31-5p, thereby promoting the expression of *Piwil1* and *Piwil4*; whereas miR-133b-3p, due to the lack of ceRNA sponge absorption, cannot diminish its inhibitory effect on its target molecule *Piwil2*, resulting in upregulated expression of *Piwil1* and *Piwil4* and downregulated expression of *Piwil2* in HCC. The highly expressed *Piwil1* and *Piwil4* then potentially collaborate with RNGS genes, such as *Dhx9*, *Drosha*, *Mov10*, and *Tdrd1*, to participate in metabolic processed and gene expression regulation.

#### The intricate ceRNA network of miRNA targets in RNGS in HCC

The progression of HCC is governed by intricate molecular regulatory networks. miRNAs, a class of small ncRNAs, play a pivotal role in the initiation and progression of cancer by modulating the expression of target genes [26]. The concept of ceRNA has emerged as a novel perspective for deciphering the regulatory mechanisms of miRNA. ceRNA, such as lncRNAs, circRNAs, and pseudogenes, can compete with mRNA for miRNA response elements (MREs), thereby sharing the binding of miRNAs. Utilizing miRNet analysis, a bioinformatics tool designed to identify interactions between miRNAs, their target genes, and ceRNAs, we identified a plethora of aberrantly expressed target genes and ceRNAs of differentially expressed miRNAs in RNGS in HCC. We discovered that miR-375-3p, miR-34 C-5p, miR-34a-5p, lncRNA *LOG108167440*, circRNA_0001814, pseudogene *Serpina4-ps1*, and so on, along with numerous target genes of these miRNAs, interweave to form complex networks that regulate the expression and functionality of target genes. Our findings underscored the significance of the ceRNA regulatory network for miRNA targets in HCC. These networks, through their intricate interplay, potentially govern gene expression and, consequently, influence the progression of HCC.

#### The functions of RNGS miRNA targets in HCC

Our study reveals significant alterations in various BPs and KEGG pathways in the tumor tissues of HCC compared to those in the normal tissues. These alterations encompass changes in lipid, amino acid, and carbohydrate metabolism, as well as in innate and adaptive immune responses. Despite the complexity of the known pathological mechanisms of HCC [27], our findings suggest that PIWIL genes are intricately involved in the regulation of small RNAs within the RNGS process. This involvement leads to the modulation of BPs and KEGG pathways associated with the targets of NRGS-involved miRNAs. Our functional enrichment analysis of the differentially expressed RNGS-involved miRNA target genes has shown that downregulated target genes are notably linked to BPs such as lipid metabolism, complement activation, and liver development. Conversely, upregulated target genes are associated with processes like translation, cytoskeleton organization, and glycolysis. The KEGG pathways enriched for downregulated target genes include metabolic pathways, complement and coagulation cascades, bile secretion, and amino acid biosynthesis. In stark contrast, the KEGG pathways for upregulated target genes involve regulation of the actin cytoskeleton, ribosome biogenesis, glycolysis and gluconeogenesis, and more. These results underscore the tight interplay between the upregulation or downregulation of specific miRNAs in the RNGS process, in which PIWIL genes are implicated, and the associated BP and KEGG pathways that are either suppressed or activated in HCC. The downregulation of miRNA target genes in HCC suggests a dampening of various metabolic and immune response mechanisms, facilitating the survival of tumor cells. For instance, the suppression of complement activation may weaken immune responses and the clearance of tumor cells within the tumor microenvironment, thereby aiding in immune evasion [28]. Disruptions in liver development and lipid homeostasis may lead to liver structural and functional anomalies, while the downregulation of cholesterol metabolic processes could enable tumor cells to bypass normal growth controls. The roles of downregulated miRNA target genes in the RNGS process in HCC encompass energy metabolism, immune responses, cell proliferation, metabolic reprogramming, and signal transduction. The overarching mechanism for the downregulation of these BPs and KEGG pathways involves the upregulation of *Piwil1* and *Piwil4* and the downregulation of *Piwil2*, which increases the synthesis of corresponding miRNAs in the RNGS process. This, in turn, enhances the degradation or translational repression of their target gene mRNAs, leading to the downregulation of genes implicated in the BPs and KEGG pathways. However, the specific miRNAs that regulate different BPs and KEGG pathways vary, indicating that their fine regulatory mechanisms are not uniform.

Upregulated miRNA target genes in HCC reflect the tumor cells' adaptation to rapid growth and invasive behavior through a variety of biological mechanisms. These include dynamic cytoskeleton regulation, enhanced protein synthesis, metabolic pathway

activation, altered cell-cell interactions, stress response, and immune evasion. For example, the upregulation of translation and ribosome biogenesis supports the tumor cells' protein synthesis capabilities, fostering rapid proliferation. The upregulation of RNA splicing impacts the differentiation and survival of tumor cells. Enhancements in actin cytoskeleton organization and Rho protein signal transduction facilitate tumor cell migration and invasion. The upregulation of glycolytic processes and the HIF-1 signaling pathway are key to metabolic reprogramming, angiogenesis, and hypoxia adaptation in tumor cells. The BPs and KEGG pathways influenced by upregulated miRNA target genes in HCC demonstrate the tumor cells' adaptations to rapid growth and invasive behavior through diverse biological mechanisms. These include cytoskeleton dynamics, protein synthesis enhancement, metabolic pathway activation, cell-cell interaction alterations, stress response, and immune evasion. The upregulation of *Piwil1* and *Piwil4* and the downregulation of *Piwil2* can decrease the synthesis of corresponding miRNAs in the RNGS pathway, reducing their degradation or translational repression of target gene mRNAs, and thus upregulating genes involved in the BPs and KEGG pathways. As with the downregulated target genes, the specific miRNAs that regulate different BPs and KEGG pathways differ, suggesting that their precise regulatory mechanisms are not identical.

The findings offer valuable insights into the molecular mechanisms underpinning HCC, which may inform future research into potential targets for therapeutic intervention.

## Conclusion

The study provides a comprehensive analysis of the molecular dynamics in HCC, highlighting the complex roles and mechanisms of the PIWI family and ncRNAs.

(1) PIWI family members might play divergent roles in the pathogenesis of HCC. Overexpression of *Piwil1* and *Piwil4* may promote HCC progression, while underexpression of *Piwil2* may suppress tumor development.

(2) The ceRNA mechanism and the PPI network are crucial in regulating the expression and the function of PIWIL genes, respectively. Specific lncRNAs could sponge up certain miRNAs, thereby affecting the expression of PIWIL genes. For instance, lncRNA *Pvt1*, *Gas5*, and *BGIGI10090_38749* may promote the expression of *Piwil1* and *Piwil4* by sponging miR-351-5p and miR-31-5p, while miR-133b-3p, due to the lack of ceRNA sponge absorption, cannot diminish its inhibitory effect on its target molecule *Piwil2*, leading to upregulated expression of *Piwil1* and *Piwil4* and downregulated expression of *Piwil2* in HCC. The interaction of PIWIL proteins encoded by RNGS genes with other components in the RNGS, such as *Dhx9*, *Drosha*, *Mov10*, and *Tdrd1*, is critical for their function in RNA processing, modification, stability regulation, and translation. These interactions within the PPI network not only influence the stability and activity of PIWIL proteins but also contribute to the overall regulation of gene expression and the progression of HCC.

(3) The intricate ceRNA network regulates the expression of miRNA-targeted genes in RNGS through miRNA, which is vital for HCC development. These networks may be key regulators of gene expression and could significantly impact HCC progression.

(4) The functions of abnormally expressed miRNA-targeted genes in RNGS in HCC are associated with key biological processes, such as lipid metabolism and immune responses, which are crucial for tumor cell survival, while upregulated targets are associated with processes that support tumor growth and invasion, like translation and cytoskeleton organization. This regulation is reflected in the enrichment of distinct KEGG pathways for downregulated and upregulated targets, highlighting the dualistic role of PIWIL genes in modulating HCC progression. The suppression of certain miRNA targets may contribute to immune evasion and metabolic reprogramming in HCC, while their upregulation reflects the cellular adaptations necessary for rapid proliferation and invasion. These findings underscore the complex interplay between miRNA regulation, PIWIL gene expression, and the dysregulation of biological pathways in HCC, offering insights into potential therapeutic targets and biomarkers within this multifaceted disease landscape.

(5) The present study's reliance on bioinformatics analysis and RT-qPCR validation limits its scope, and future research could benefit from integrating a diverse range of methodologies to further elucidate the roles of PIWIL genes in HCC progression, building upon the findings presented here.

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**Author contributions**

S.H. wrote the main manuscript text and data analysis; S.H. and R.L. prepared Figs. 1 and 2 and S.P., S.H., Y.M. and G.L. data analysis and prepared Figs. 3, 4 and 5 and R.L. and G.L. funding acquisition and project administration; G.L. conceptualization, methodology, supervision, and writing - review. All authors reviewed the manuscript.

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**Data availability**
All data generated or analyzed during this study will be available upon request, such as sequencing data and RT-qPCR data, please contact ligenliang@ymun.edu.cn. The high-throughput sequencing data were deposited in the SRA database of NCBI (https://www.ncbi.nlm.nih.gov/sra). The numbers of whole transcriptome sequencing included SRR21422257, SRR21423889, SRR21423874, SRR21422457, SRR21422501, and SRR21422503, and the numbers of miRNA sequencing included SRR21397261, SRR21397260, SRR21397259, SRR21397099, SRR21397098, and SRR21397097.

## Declarations

**Ethics approval and consent to participate**
All experiments (mice) were performed in accordance with the ARRIVE Guidelines (https://arriveguidelines.org) for the reporting of animal experiments and the principles of the Basel Declaration and Recommendations of the Guide for the Care and Use of Laboratory Animals (http://grants1.nih.gov/grants/olaw/references/phspol). The protocol was approved by the Experimental Animal Ethics Review Committee of Youjiang Medical University for Nationalities under permit No. 202304250.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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