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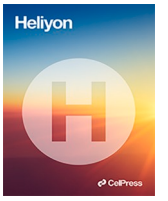

Research article

Serum tRF-33-RZYQHQ9M739P0J as a novel biomarker for auxiliary diagnosis and disease course monitoring of hepatocellular carcinoma

Xian Li [a,b,1], Yang Li [a,1], Jie Yuan [a,b], Weiwei Zhang [b], Tianxin Xu [a], Rongrong Jing [b,**], Shaoqing Ju [b,*]

[a] Medical School of Nantong University, Nantong, Jiangsu, 226001, China
[b] Department of Laboratory Medicine, Affiliated Hospital of Nantong University, Nantong, Jiangsu, 226001, China

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ABSTRACT

Objective: In most cases, patients with hepatocellular carcinoma (HCC) develop advanced disease when diagnosed. Finding new molecules to combine with traditional biomarkers is crucial for HCC early diagnosis. In cancer development, tRNA-derived small RNAs (tsRNA) play a crucial role. Here, we aimed to identify a novel biomarker among tsRNAs that can facilitate HCC diagnosis and monitor its prognosis.

Methods: We screened candidate tsRNAs in 3 pairs of HCC and adjacent tissues through high-throughput sequencing. tRF-33-RZYQQ9M739P0J was screened in tissues, sera, and cells through quantitative real-time polymerase chain reaction (qRT-PCR) for further analysis. tRF-33-RZYQHQ9M739P0J was characterized using agarose gel electrophoresis, Sanger sequencing, and nuclear and cytoplasmic RNA isolation. Experiments at room temperature and repeated freeze-thaw cycles were conducted to evaluate the detection performance of tRF-33-RZYQHQ9M739P0J. We measured the levels of differential expression of tRF-33-RZYQHQ9M739P0J in sera using qRT-PCR. We applied the chi-square test to evaluate the correlation between tRF-33-RZYQHQ9M739P0J expression levels and clinicopathological features, and assessed its prognostic value by plotting Kaplan-Meier curves. The diagnostic efficacy of tRF-33-RZYQHQ9M739P0J was evaluated using the receiver operating characteristic (ROC) curve. Finally, the downstream genes related to tRF-33-RZYQHQ9M739P0J were explored through bioinformatics prediction.

Results: tRF-33-RZYQHQ9M739P0J was highly expressed in HCC tissues and sera, and its expression was correlated with metastasis, TNM stage, BCLC stage, and vein invasion. Expression of tRF-33-RZYQHQ9M739P0J were decreased after surgery in patients with HCC. High serum tRF-33-RZYQHQ9M739P0J levels are associated with low survival rates, and they can predict survival times in patients with HCC according to the Kaplan-Meier analysis. Combining tRF-33-RZYQHQ9M739P0J with serum alpha-fetoprotein and prothrombin induced by vitamin K absence II can improve the diagnostic efficiency of HCC, suggesting its potential as a biomarker for HCC.

* Corresponding author. Department of Laboratory Medicine, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong, 226001, Jiangsu, China.
** Corresponding author.
E-mail addresses: ntfyjrr@ntu.edu.cn (R. Jing), jsq_jyk@ntu.edu.cn (S. Ju).
[1] Xian Li and Yang Li are co-first authors and contributed equally to this work.

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Conclusion: tRF-33-RZYQHQ9M739P0J may not only be a promising non-invasive marker for early diagnosis, but also a predictor of liver cancer progression.

1. Introduction

Most primary liver cancers are hepatocellular carcinomas (HCC), which account for about 80 % of cases [1,2]. Ultrasonography can diagnose most HCC cases. However, in most HCC cases, the diagnosis is made at an advanced stage by imaging, with poor prognosis and short survival. Notably, screening serum markers early is crucial for detecting HCC early. Serum alpha-fetoprotein (AFP) is an essential biomarker for HCC screening, diagnosis, and disease progression monitoring. However, AFP levels in 30 % of liver cancer patients are normal [3], and the number of HCC patients with normal AFP levels and early-stage HCC is on the rise [4]. Research has shown that the sensitivity of AFP combined with B-ultrasound examination in early detection of liver cancer is only 63 % [5]. Therefore, it is crucial to discover novel potential biomarkers for HCC that can enhance diagnostic efficacy and provide more patients with the opportunity for surgical treatment.

Human genomes encode only about 2 % of proteins, and 80 % are non-coding RNAs [6]. In cancer, ncRNAs can promote tumor growth and suppress tumor growth. They encompass rRNAs, tRNAs, miRNAs, long ncRNAs (lncRNAs), and circular RNAs (circRNAs). ncRNAs play a crucial role in both normal physiological development and pathological processes in humans [7]. tRNAs participate in protein translation, and changes in their abundance or function can lead to changes in protein expression and disease [8]. Small RNAs derived from tRNA were first discovered in cancer patients' urine and believed to be random degradation products [9]. Later, high-throughput sequencing technology revealed that when cells are exposed to stress conditions like hypoxia, starvation, oxidative stress, and high temperatures, precursor or mature tRNAs were specifically cleaved to generate new small non-coding RNAs ranging from 18 to 40 nucleotides in length [10]. There is no unified nomenclature for tsRNAs at the moment. tsRNAs are classified into different species according to origin and cleavage site. The first type of RNA consists of tRNA halves (tiRNAs), whose length ranges from 30 to 40 nucleotides. Angiogenin, Dicer, and other RNases cleave tRNA into 5′ and 3′ fragments, called 5′- and 3′-tiRNAs. tRNA-derived fragments (tRFs) are the second type; they are 14–36 nucleotides long and can be categorized as 1-tRF, 2-tRF, 3-tRF, 5-tRF, and i-tRF [11,12].

tsRNAs are involved in cancer pathogenesis and development, including cancer cell proliferation, metastasis, and invasion [13–15]. Recently, whether tsRNAs, a novel type of ncRNA, can be used as tumor markers has attracted attention. Many studies have suggested that tsRNAs may regulate the development of breast [16], ovarian [17], prostate [18], and other cancers [19]. As tsRNAs bind competitively to RNA-binding proteins, they can control tumor progression, according to research on tsRNA regulatory mechanisms. For example, tRFs can competitively adhere to YBX1 mRNA and stabilize oncogene transcription to inhibit the growth and metastasis of breast cancer cells [20]; there are also reports that tsRNAs can exert RNA silencing similar to miRNAs, adhere to Argonaute protein, and form RNA-induced silencing complexes to silence mRNA, thereby regulating target genes and affecting the emergence and progression of tumors [21]. tsRNAs, however, remain largely unknown for their clinical application.

In this study, high-throughput sequencing revealed that tRF-33-RZYQHQ9M739P0J expression levels in HCC tissues were higher than those in normal tissues. To investigate the potential of tRF-33-RZYQHQ9M739P0J as a value indicator for HCC, we detected its expression levels in HCC tissues, sera, and cell lines. We explored its combined application with routine clinical diagnostic indicators for patients with HCC. We found that patients with HCC expressed more tRF-33-RZYQHQ9M739P0J in their sera than healthy donors, and that increased expression correlated closely with tumor stage and metastasis. The combination of tRF-33-RZYQHQ9M739P0J with AFP and prothrombin induced by vitamin K absence II (PIVKA-II) can improve HCC diagnosis efficiency. In addition, tRF-33-RZYQHQ9M739P0J can effectively play a dynamic monitoring role in HCC development. Therefore, serum tRF-33-RZYQHQ9M739P0J may be an innovative biomarker for HCC diagnosis.

2. Methods and materials

2.1. Samples collected from clinical trials

In the study, serum samples were collected at the Department of Laboratory Medicine at Nantong University Hospital, these samples included 122 preoperative and 31 patients with HCC, 98 healthy individuals, and 80 patients with benign liver lesions. All preoperative patients received no treatment. Additionally, 39 pairs of HCC tissues (16 pairs from the 122 preoperative patients with HCC) as well as adjacent non-tumor tissues were from the Department of Pathology. In this study, patients with benign liver lesions were diagnosed by clinical doctors and patients with HCC were diagnosed by two or more pathological experts. The clinical and pathological stage of HCC patients was determined based on the tumor-node-metastasis (TNM) and Barcelona Clinic Liver Cancer (BCLC) staging systems. All participants provided voluntary informed consent prior to the study. The protocol was approved by the ethics committee.

2.2. Sequencing with high throughput

We used Trizol to extract RNA from tissues, Qubit®2.0,Agilent 2200 were utilized to assess RNA quantity and integrity.

Approximately 1 μg of RNA per sample was prepared by NEBNext® multiplexing for Illumina library construction. Sequencing was performed on the HiSeq 2500, and MINTmap was utilized to compare clean reads with MINTbase, predicting novel tsRNAs. EdgeR was used to analyze tsRNA variations. DESeq 2.0 was applied to define differentially expressed tRFs with a Log2 (fold change) > 1 or < -1, P value < 0.05.

2.3. Cell cultivation

We purchased HuH-7, Hep3B, HCCLM3, MHCC97-L, Sk-Hep1, MHCC97-H, and HepG2, normal liver cell QSG-7701 cell lines from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and Beyotime Biotechnology (Shanghai, China). All cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (Corning, Manassas, VA, USA). 1 % penicillin-streptomycin mixture (HyClone, Logan, UT, USA) and 10 % fetal bovine serum (FBS) were added to all media and cultivated in a Humidity-controlled Incubator at 37 °C with 5 % CO2.

2.4. Nucleoplasmic separation assay

Sk-Hep1, HepG2, and HCCLM3 cells were cultured and digested with trypsin. At least 5×10^6 cells were collected. 60 μL of nuclear and cytoplasmic RNA was extracted using the Paris™ kit (Thermo Fisher Scientific) and stored at −80 °C. After that, qRT-PCR was used

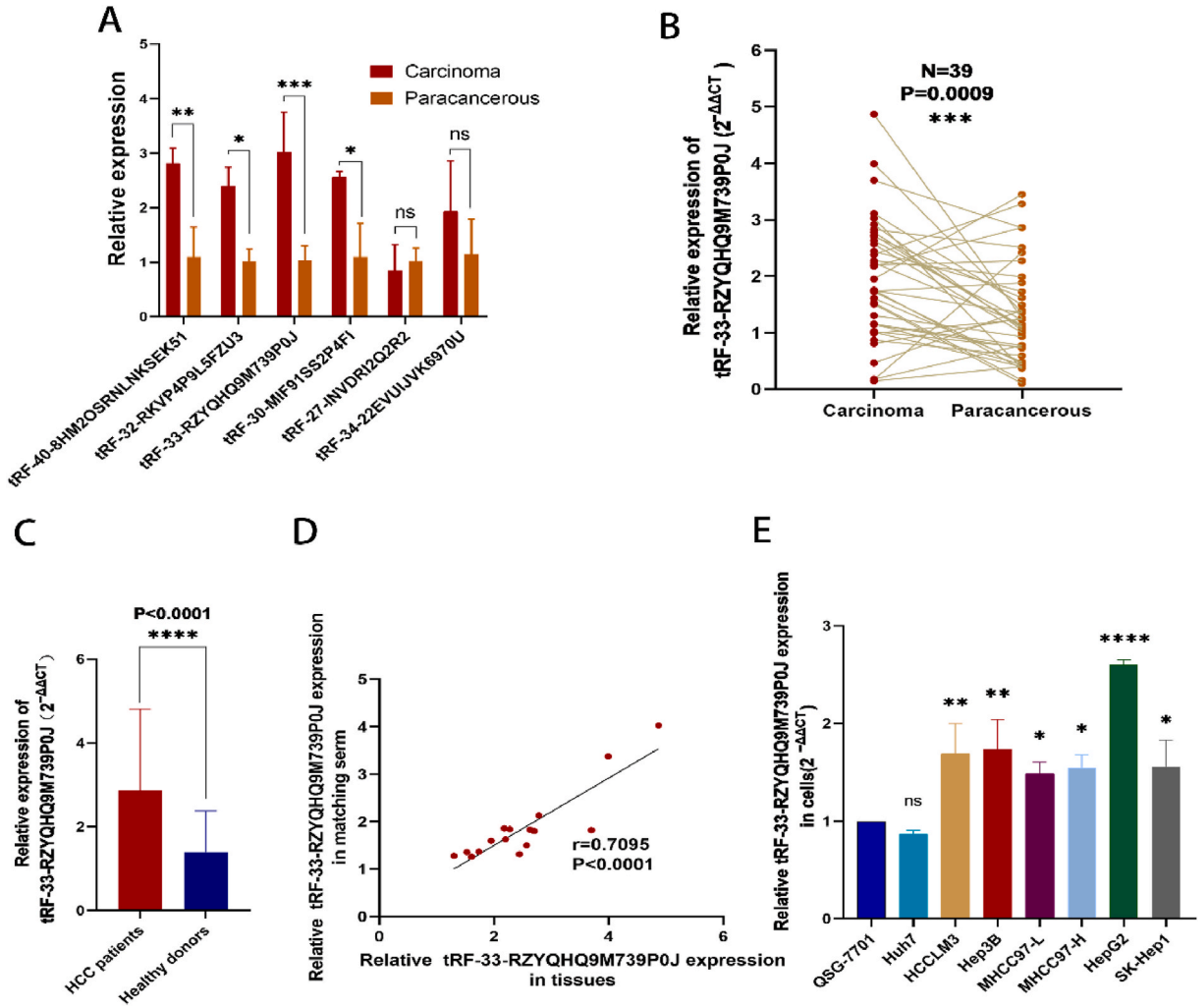

Fig. 1. Validation of tRF-33-RZYQHQ9M739P0J expression in HCC tissues, serum, and HCC cell lines. (A) The expression levels of six tsRNAs in 3 adjacent non-tumor tissues, HCC tissues. (B) qRT-PCR quantified expression of tRF-33-RZYQHQ9M739P0J in 39 HCC tissues compared to adjacent non-tumor tissues. (C) Expression levels of tRF-33-RZYQHQ9M739P0J in serum samples from HCC patients (n = 46) and healthy controls (n = 30). (D) Correlation analysis of 16 HCC tissues and serum samples in terms of tRF-33-RZYQHQ9M739P0J expression levels. (E) Expression of tRF-33-RZYQHQ9M739P0J in HCC cells and QSG-7701. Statistical significance: ****<0.0001, ***<0.001, **<0.01, *<0.05; ns = non-significant.

to determine tsRNA in the nucleus and cytoplasm. As reference genes for the nucleus and cytoplasm, U6 and 18S were used.

2.5. Extraction of total RNA, reverse transcription into cDNA, and quantitative RT-PCR

The total RNA from non-serum was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted from 300 mL of serum by separation kit (BioTeke, Wuxi, Jiangsu, China). We used tRF-33-RZYQHQ9M739P0J as the target molecule and RNU6B as the internal reference. We prepared 10 μL of cDNA using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, USA) and specific primers (Ribobio Corporation, Guangzhou, China), including tRF-33-RZYQHQ9M739P0J RT and RNU6B RT. The prepared 10 μL mixture was amplified at 42 °C for 1 h and inactivated at 70 °C for 5 min. We used 20 μL reaction system on ABI QuantStudio 5 for qRT-PCR; the reaction system contained 10 μL Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, Jiangsu, China), 5 μL cDNA, 1 μL forward primer, 1 μL reverse primer, and 3 μL enzyme-free water. The data were analyzed using the $2^{-\Delta\Delta CT}$ calculation method.

2.6. Experiments at room temperature, repeated freeze-thaw cycles

Two groups were formed by randomly mixing 20 serum samples. One of the groups was kept at room temperature (25 °C) for 0, 6, 12, 18, and 24 h. A second serum mixture was repeatedly frozen and thawed for 0, 1, 3, 5, and 10 times at −80 °C and room temperature. RNA was extracted and cDNA was synthesized for tRF-33-RZYQHQ9M739P0J expression analysis.

2.7. Statistical analysis

SPSS Statistics version 20.0 (IBM SPSS Statistics, Chicago, IL, USA), MedCalc 20.1 (Medcalc Statistics Software, Belgium), and GraphPad Prism 8.0 (GraphPad Software, San Jose, CA, USA) were all employed for statistical analysis in this study. All data were tested for normality with GraphPad Prism 8.0 to exclude a normal distribution. The expression of tRF-33-RZYQHQ9M739P0J in different groups was represented as mean ± standard deviation. The Mann–Whitney *U* test was used to compare two independent groups. Multiple independent groups were compared using the Kruskal–Wallis H test. The Wilcoxon matched-pairs signed-rank test was applied to assess tRF-33-RZYQHQ9M739P0J expression levels between patients' serum before and after surgery, as well as between HCC and adjacent tissues. The $\chi 2$ test was used to analyze the correlation between tRF-33-RZYQHQ9M739P0J expression levels and clinical pathological parameters. The log-rank test was applied to determine survival data significance.

3. Results

3.1. Screening of candidate serum tsRNAs in patients with HCC

Based on tsRNA sequencing, we selected six up-regulated tsRNAs (log2(fold change) > 1, $P < 0.05$). (tRF-40-

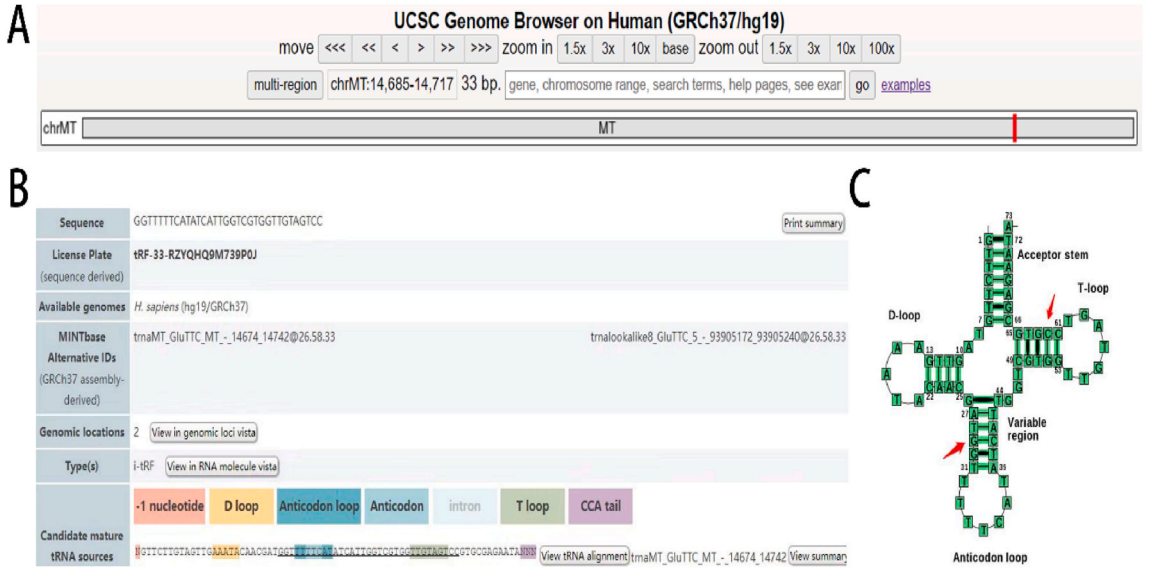

Fig. 2. The tRF-33-RZYQHQ9M739P0J is a type of i-tRF. (A) The tRF-33-RZYQHQ9M739P0J was located on ChrMT with coordinates 14,685–14,717. (B) The tRF-33-RZYQHQ9M739P0J was an i-tRF with a length of 33 nt (5′-GGTTTTTCATATCATTGGTCGTGGTTGTAGTCC -3′) in the MINTbase v2.0. (C) There is a cleavage site on the T-loop stem and the Anticodon (TTTTCAT) stem in the Transfer RNA Database.

8HM2OSRNLNKSEK51, tRF-32-RKVP4P9L5FZU3, tRF-33-RZYQHQ9M739P0J, tRF-30-MIF91SS2P4FI, tRF-27-INVDRI2Q2R2, tRF-34-22EVUIJVK6970U). Validation of the expression of six tsRNAs was performed in three HCC tissues and non-tumor tissues adjacent to the tumors, and the results indicated that tRF-33-RZYQHQ9M739P0J was significantly highly expressed in HCC tissues (Fig. 1A). Then, we conducted qRT-PCR assays in 39 pairs of HCC tissues and adjacent non-tumor tissues, the results showed that tRF-33-RZYQHQ9M739P0J expression levels were significantly higher in HCC tissues compared to adjacent non-tumor tissues ($P = 0.0009$) (Fig. 1B). Meanwhile, we collected serum samples from 46 patients with HCC and 30 healthy donors, and qRT-PCR results showed that the expression levels of tRF-33-RZYQHQ9M739P0J in the sera of patients with HCC were significantly higher than that in healthy donors($P < 0.0001$) (Fig. 1C), and a significant linear correlation was found between the serum results of the 16 HCC tissues and the serum results of the 16 cancer tissues. (Fig. 1D). Finally, qRT-PCR analysis of the cell lines showed that tRF-33-RZYQHQ9M739P0J expression levels were significantly higher in most HCC cell lines than QSG-7701 (Fig. 1E). The above results indicated that tRF-33-RZYQHQ9M739P0J may be valuable in HCC diagnosis, so it was selected as a potential molecule for further study.

3.2. tRF-33-RZYQHQ9M739P0J is categorized as an i-tRF

As reported by the UCSC Genome Browser (https://genome.ucsc.edu/), tRF-33-RZYQHQ9M739P0J was located on ChrMT. With coordinates of 14,685–14,717, and a length of 33 bp (Fig. 2A). MINTbase v2.0 (http://cm.jefferson.edu/MINTbase/) database showed that tRF-33-RZYQHQ9M739P0J was an i-RF with 33 nt (5′-GGTTTTTCATATCATTGGTCGTGGTTGTAGTCC-3′) (Fig. 2B). The basic information about tRF-33-RZYQHQ9M739P0J can be seen in the database. On the T-loop stem and Anticodon stem, there are cleavage sites (http://trna.bioinf.unileipzig.de/) (Fig. 2C).

3.3. An evaluation of tRF-33-RZYQHQ9M739P0J as a biomarker for HCC

To comprehensively evaluate the detection method of tRF-33-RZYQHQ9M739P0J and explore its value in clinical applications, we first evaluated the repeatability of mixed serum detection. Results revealed that CV (coefficient of variation) was acceptable both within and between assays, at 1.43 % and 2.08 %, respectively (Table 1). The test results after the samples were placed at room temperature and under repeated freeze-thaw cycles indicate the stability of the detection method (Fig. 3A and B). The amplification process was detected by agarose gel electrophoresis (AGE), which showed a single band of about 80–90 bp (Fig. 3C). After recovering and TA (Tail Assembly) cloning, Sanger sequencing confirmed that the product contained the full-length sequence of tRF-33-RZYQH9M739P0J, indicating the accuracy of qRT-PCR products (Fig. 3C and D). In HCCLM3, Sk-Hep1, and HepG2 cell lines, we detected tRF-33-RZYQH9M739P0J by nuclear and cytoplasmic RNA separations, indicating predominantly cytoplasmic expression (Fig. 3E). The above experiments can preliminarily demonstrate that this detection method has high reproducibility and stability, and tRF-33-RZYQH9M739P0J can be used as a biomarker for detection in serum.

3.4. Assessment of serum tRF-33-RZYQHQ9M739P0J in diagnosis and prognosis, and its correlation with clinical pathological parameters

To verify the value of tRF-33-RZYQHQ9M739P0J in the clinical diagnosis of HCC, we used qRT-PCR to detect expression levels of tRF-33-RZYQHQ9M739P0J in the serum of 122 patients with HCC, 80 patients with benign liver lesions, and 98 healthy controls. The results showed that the expression levels of tRF-33-RZYQHQ9M739P0J in patients with HCC were significantly higher than that in patients with benign liver lesions ($P = 0.0124$) and healthy donors ($P < 0.0001$) (Fig. 4A), however, there was no statistically significant difference between the benign liver lesions group and the healthy control group ($P = 0.1520$) (Fig. 4A). In addition, 80 patients with benign liver lesions were composed of 40 patients with chronic hepatitis and 40 patients with cirrhosis, there was no significant difference in serum expression between the chronic hepatitis and cirrhosis groups ($P = 0.0758$) (Fig. 4B). Subsequently, we divided 122 HCC patients into two groups based on the median expression level of tRF-33-RZYQHQ9M739P0J: the relatively high expression group (expression level >1.865856865, $n = 61$) and the relatively low expression group (expression level ≤ 1.865856865, $n = 61$). Clinical and pathological parameters were related to serum tRF-33-RZYQHQ9M739P0J expression levels by the $\chi 2$ test, which showed that expression levels of tRF-33-RZYQHQ9M739P0J in serum of patients with HCC were significantly correlated with metastasis ($P = 0.001$), TNM stage ($P = 0.001$), BCLC stage ($P < 0.0001$)Vein invasion ($P = 0.004$), however, there was no significant correlation with age, sex, HBV infection, cirrhosis, tumor differentiation, or tumor size ($P > 0.05$) (Table 2). The clinical and pathological parameters of patients with liver cancer are closely related to the malignancy and progression rate of liver cancer. Tumor markers are substances secreted by tumor cells or shed into body fluids or tissues, which affect the development of tumor cells. In this study, tRF-33-

Table 1
CVs for intra- and inter-assays of tRF-33-RZYQHQ9M739P0J.

	tRF-33-RZYQHQ9M739P0J	RNU6B
Intra assay		
CV (%)	1.43	1.67
Inter assay		
CV (%)	2.08	2.14

CV =SD/Mean × 100 %.

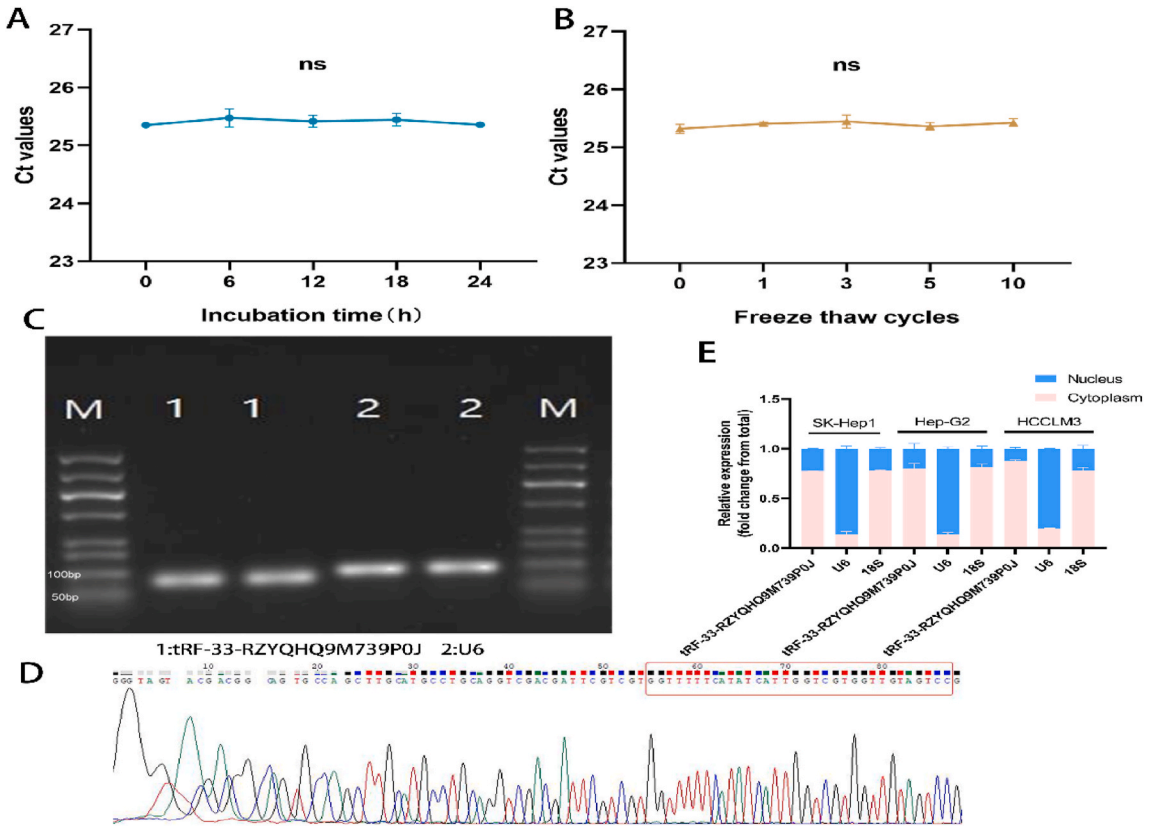

Fig. 3. Methodological evaluation of tRF-33-RZYQHQ9M739P0J. (A, B) Stability of tRF-33-RZYQHQ9M739P0J during incubation at room temperature and repeated freeze-thaw cycles. (C) Agarose gel electrophoresis demonstrated a single band of approximately 80–90 bp for the qRT-PCR product of tRF-33-RZYQHQ9M739P0J. (D) Sanger sequencing confirmed the product of qRT-PCR, which contained the full-length sequence of tRF-33-RZYQHQ9M739P0J. (E) Nuclear and cytoplasmic RNA separation assay for detection of tRF-33-RZYQHQ9M739P0J in Sk-Hep1, HepG2 and HCCLM3 cell lines.

RZYQHQ9M739P0J is closely associated with metastasis and staging of HCC, suggesting that it may provide valuable references for the presence, development, metastasis, and recurrence of tumors.

Next, according to TNM stage, we divided HCC patients into I-II and III-IV groups. The expression levels of tRF-33-RZYQHQ9M739P0J between groups I-II and III-IV did not show significant differences ($P = 0.8238$), but both were significantly higher than those of healthy donors ($P < 0.05$) (Fig. 4C). The expression levels of tRF-33-RZYQHQ9M739P0J in 0, A, B group and C, D group were not significantly different ($P = 0.5668$), however, they were all significantly higher than those from healthy donors($P < 0.05$). In addition, we collected post-operative serum from 31 patients with HCC. The results showed that the expression of tRF-33-RZYQHQ9M739P0J in the serum of HCC patients after surgery decreased significantly ($P < 0.0001$) (Fig. 4E). In addition, Kaplan-Meier analysis showed that the survival rate of HCC patients with low preoperative tRF-33-RZYQHQ9M739P0J expression levels was higher than that of HCC patients with high preoperative expression levels (Fig. 4F), which indicated that tRF-33-RZYQHQ9M739P0J may serve as a prognostic biomarker for HCC and dynamically monitor it.

3.5. The diagnostic value of serum tRF-33-RZYQHQ9M739P0J for HCC

To investigate the clinical value of tRF-33-RZYQHQ9M739P0J, we compared it with other HCC diagnostic markers. AFP and PIVKA-II are common diagnostic markers for HCC. We established ROC curves for the expression levels of tRF-33-RZYQHQ9M739P0J, AFP, and PIVKA-II in the serum of 122 patients with HCC and 98 healthy donors, and analyzed the area under the curve (AUC) (Fig. 5A–C), we found that the AUC for tRF-33-RZYQHQ9M739P0J was 0.702 (95 % CI,0.637–0.762) (Fig. 5A), The AUC (95 % CIs) of AFP and PIVKA-II in serum were 0.849 (0.795–0.894) and 0.877 (0.827–0.918), respectively (Fig. 5B and C), meanwhile, tRF-33-RZYQHQ9M739P0J had 78.7 % SEN and 56.1 % SPE in differentiating patients with HCC from healthy donors when the cut-off value was1.2542 and the Youden index was 0.348(Table 3). AUC reached the highest value of 0.932 (95 % CI,0.891–0.962) when the three biomarkers were combined (Fig. 5D), and the sensitivity increased to 93.4 % (Table 3). Although the specificity of serum tRF-33-RZYQHQ9M739P0J was not as good as that of serum AFP and PIVKA-II, its sensitivity was higher than that of serum AFP and PIVKA-II, suggesting that serum tRF-33-RZYQHQ9M739P0J may be a new potential diagnostic biomarker for HCC. Our results

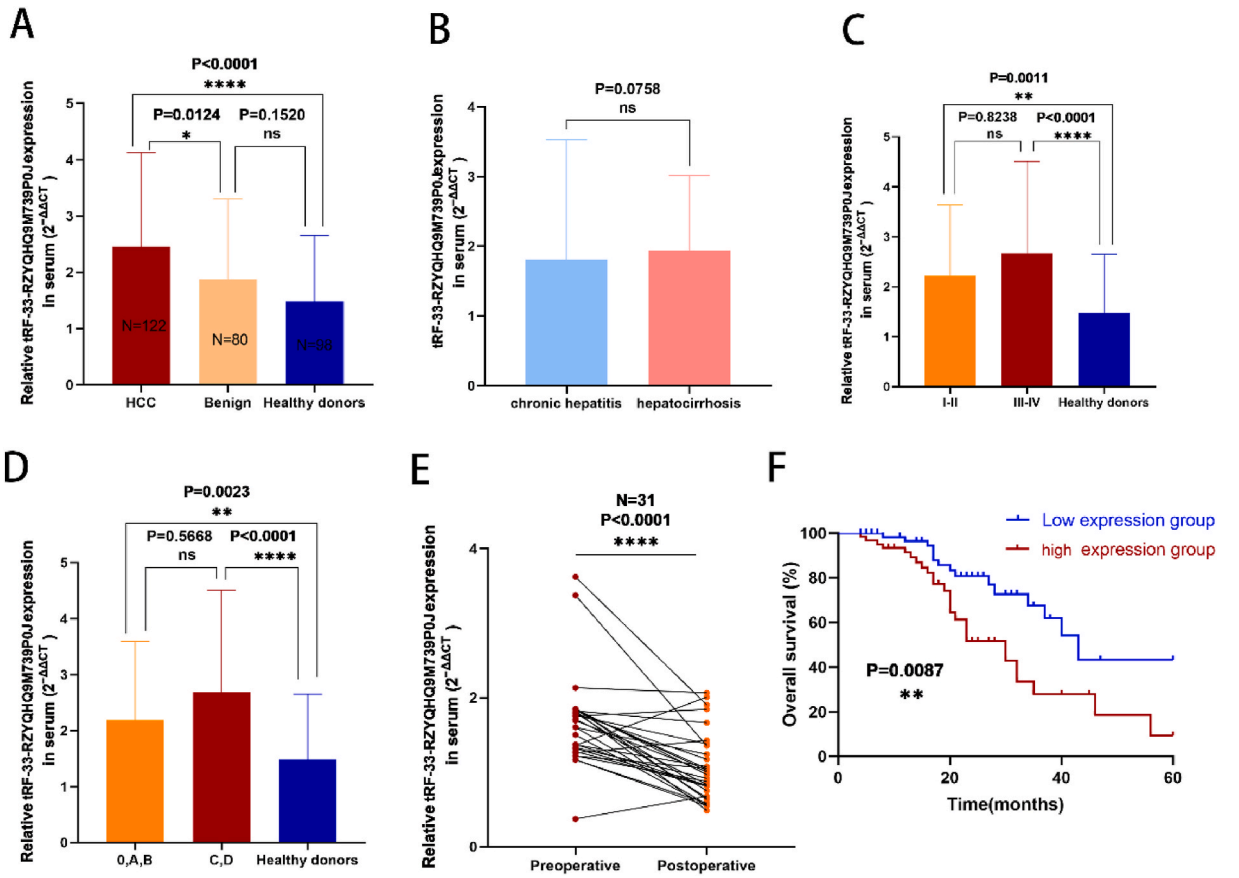

Fig. 4. The prognostic and diagnostic potential of serum tRF-33-RZYQHQ9M739P0J. **(A)** Detection of serum expression of tRF-33-RZYQHQ9M739P0J in HCC patients (n = 122), healthy controls (n = 98) and patients with benign liver lesions (n = 80). **(B)** Detection of serum tRF-33-RZYQHQ9M739P0J expression in 40 patients with chronic hepatitis and 40 patients with cirrhosis. **(C)** The expression levels of serum tRF-33-RZYQHQ9M739P0J in patients with stage I-II (n = 57), patients with stage III-IV (n = 65), and healthy donors (n = 98). **(D)** The expression levels of serum tRF-33-RZYQHQ9M739P0J in patients with stage 0, A, B (n = 55), patients with stage C, D (n = 67), and healthy donors (n = 98). **(E)** The expression level of serum tRF-33-RZYQHQ9M739P0J in the serum of 31 patients with HCC before and after surgery. **(F)**A Kaplan-Meier analysis of preoperative serum tRF-33-RZYQHQ9M739P0J expression levels and survival in HCC patients.

suggested that tRF-33-RZYQHQ9M739P0J combined with AFP and PIVKA-II could improve HCC diagnostic efficiency.

3.6. Downstream prediction of tRF-33-RZYQHQ9M739P0J

Based on bioinformatics database analysis (miRanda, RNAhybrid, and TargetScan), we predicted potential target genes in HCC to explore its potential mechanism. The results showed that the 2016 genes that intersect in the databases are the likely target genes of tRF-33-RZYQHQ9M739P0J (Fig. 6A). KEGG signaling pathway enrichment analysis was performed on potential target genes, and we found significant enrichment of pathways in metabolism, autophagy, and cancer. GO functional enrichment indicated that tRF-33-RZYQHQ9M739P0J was significantly enriched in protein kinase regulator activity and protein binding regulation (Fig. 6B and C). As tRF-33-RZYQHQ9M739P0J is mainly located in the cytoplasm, combined with bioinformatics results, it provides a general direction for future experiments.

4. Discussion

Globally, HCC ranks second in cancer-related mortality and fifth in frequency. Studies have shown that patients with advanced HCC have a median survival of 1 year. However, the 5-year survival of HCC patients can reach 70 % if diagnosed early [22]. Compared with diagnostic imaging, biomarkers in serum are more beneficial for the early detection of HCC. Currently, AFP is the most common biomarker for HCC. However, it lacks specificity at low levels (20 ng/dL) [23–25]. Another study showed that the sensitivity and specificity of PIVKA-II in the diagnosis of HCC were 71 % and 84 %, respectively, It also showed that increased levels of PIVKA-II were correlated with a high prevalence of portal vein invasion [26]. To improve early HCC diagnosis, it is urgent to identify potential biomolecular markers and optimize their rational combinations.

Table 2
Clinicopathological analysis of the tRF-33-RZYQHQ9M739P0J expression level.

Parameter	No. of patients	(high)	(low)	*P*-value
Sex				
Male	75	39	36	0.577
Female	47	22	25	
Age(year)				
≤64	62	27	35	0.147
>64	60	34	26	
HBV infection				
Negative	54	28	26	0.715
Positive	68	33	35	
Hepatocirrhosis				
Absent	69	38	31	0.201
Present	53	23	30	
Tumor differentiation				
Well + Moderate	43	17	26	0.088
Poor	79	44	35	
Tumor size, cm				
≤5	59	25	34	0.103
>5	63	36	27	
Metastasis				
Negative	65	23	42	0.001
Positive	57	38	19	
TNM stage				
I-II	57	19	38	0.001
III-IV	65	42	23	
Vein invasion				
No	79	32	47	0.004
Yes	43	29	14	
BCLC stage				
0, A, B	55	17	38	<0.0001
C, D	67	44	23	

Note: Note: Statistical analyses were conducted using Pearson $\chi 2$ test.

More ncRNAs are being discovered as RNA sequencing develops rapidly. miRNAs [27,28], circRNAs [29,30], lncRNAs [31,32], and enhancer RNAs [33,34] have been studied in the context of hepatocarcinogenesis mechanism and development, providing the basis for the exploration of new HCC diagnostic markers. The tRNA is one of the most abundant ncRNAs, accounting for 4%–10 % of all cellular RNA [35,36]. Its role in certain cancers has been explored, but research on tsRNA as a tumor diagnostic marker and its biological function in HCC is still limited [15,37,38], suggesting that further research on the diagnostic value and mechanism of tsRNAs in HCC is particularly important. A positive correlation was found between tRF-33-RZYQHQ9M739P0J expression and TNM, BCLC and metastasis stages. This suggests that tRF-33-RZYQHQ9M739P0J may promote the progression of HCC, indicating its potential to become a new biomarker for HCC.

To investigate whether tsRNAs are differentially expressed in HCC tissues, high-throughput sequencing was performed on three pairs of HCC tissues with their corresponding control tissues and validated in serum, HCC cells, and HCC tissues; we selected tRF-33-RZYQHQ9M739P0J, which showed significant differences, for further analysis. According to MINTbase v2.0, tRF-33-RZYQHQ9M739P0J is an i-tRF derived from trnaMT_GluTTC, with a length of 33 nucleotides. Considering the significance of sample stability in clinical practice for liquid biopsy, we evaluated the detection method of tRF-33-RZYQHQ9M739P0J, which exhibited satisfactory intra-assay and inter-assay CV. Through experiments at room temperature and repeated freeze-thawing, it was observed that the detection method had a certain degree of resistance to external interference. To ensure the integrity and accuracy of qRT-PCR products, we verified the PCR amplification products through agarose gel electrophoresis (AGE) and Sanger sequencing. The above experimental results indicated that the stability and detection accuracy of tRF-33-RZYQHQ9M739P0J met the prerequisites for its clinical application. Subsequently, we detected expression levels of tRF-33-RZYQHQ9M739P0J in additional serum samples. We found that the serum expression levels of tRF-33-RZYQHQ9M739P0J were significantly higher in patients with HCC than in patients with benign liver lesions and healthy donors, suggesting that tRF-33-RZYQHQ9M739P0J can help distinguish between HCC and chronic liver disease as well as healthy individuals. We also found that its expression in serum is correlated with some pathological parameters. In addition, the expression of tRF-33-RZYQHQ9M739P0J significantly decreased in the serum of postoperative patients with HCC, indicating that tRF-33-RZYQHQ9M739P0J can help estimate treatment efficacy in these patients. High expression of tRF-33-RZYQHQ9M739P0J was associated with a low survival rate, suggesting that tRF-33-RZYQHQ9M739P0J can be used to assess HCC patients' prognosis. Furthermore, ROC curve analysis indicated that the combination of tRF-33-RZYQHQ9M739P0J with AFP and PIVKA-II could further improve diagnostic value in HCC. tsRNA is abundant in body fluids, and serum is an easily accessible and manageable body fluid that can be used for large-scale screening and diagnostic testing. Tissue samples provide more direct cellular and molecular information, but tissue specimens are difficult to collect, so this article first screens and validates using partial tissue samples, and then further validates using a large number of samples. By comparing these two sample types, we can more fully understand the role of tsRNA fragments in HCC. However, this study had the following limitations: 1) The procedure for detecting tsRNAs

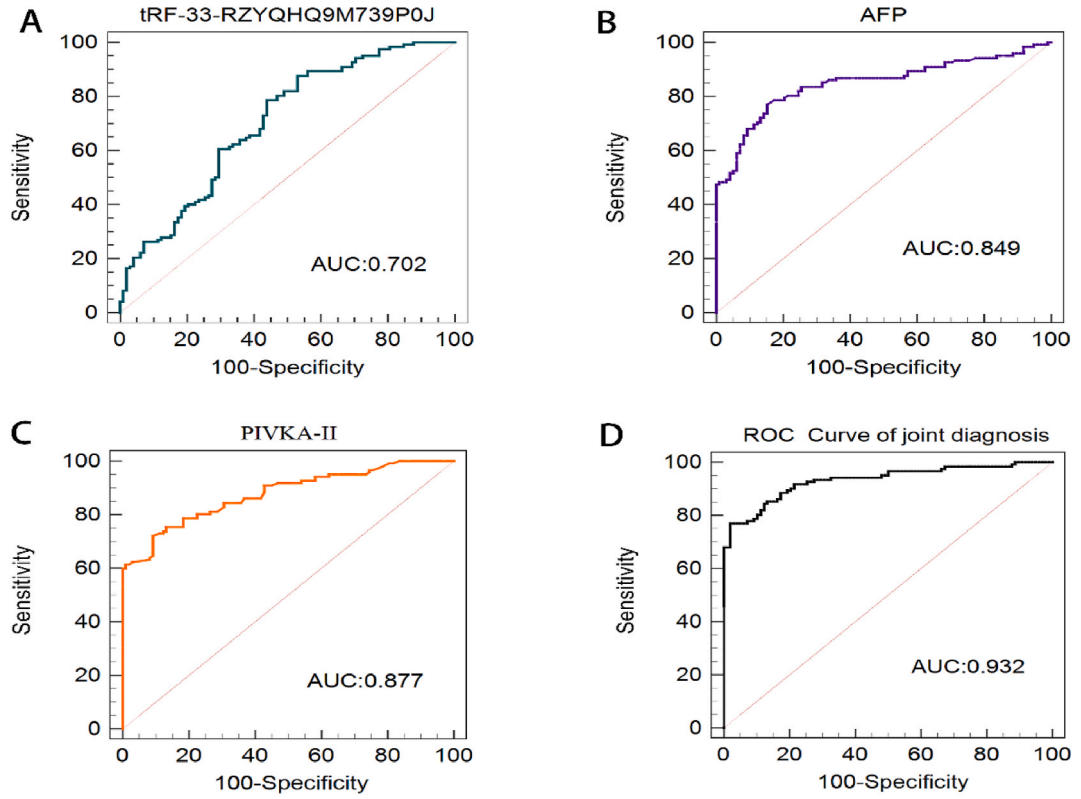

Fig. 5. Evaluation of the diagnostic value of tRF-33-RZYQHQ9M739P0J in HCC. (A–D) Construction of ROC curve to compare the diagnostic performance of serum tRF-33-RZYQHQ9M739P0J AFP, PIVKA-II and joint diagnosis to discriminate HCC from healthy controls.

Table 3
Diagnostic value of serum tRF-33-RZYQHQ9M739P0J, serum AFP and PIVKA-II for HCC.

Biomarker	SEN (%)	SPE (%)	ACCU (%)	PPV (%)	NPV (%)
tRF-33-RZYQHQ9M739P0J	78.7(96/122)	56.1(55/98)	68.6(151/220)	69.1(96/139)	67.9(55/81)
AFP	76.2(93/122)	84.7(83/98)	80.0(176/220)	86.1(93/108)	74.1(83/112)
PIVKA-II	72.1(88/122)	90.8(89/98)	80.5(177/220)	90.7(88/97)	72.4(89/123)
tRF-33-RZYQHQ9M739P0J + AFP	90.2(110/122)	46.9(46/98)	70.9(156/220)	67.9(110/162)	79.3(46/58)
tRF-33-RZYQHQ9M739P0J + PIVKA-II	87.7(107/122)	53.1(52/98)	72.3(159/220)	69.9(107/153)	77.6(52/67)
tRF-33-RZYQHQ9M739P0J + AFP + PIVKA-II	93.4(114/122)	45.9(45/98)	72.3(159/220)	68.3(114/167)	84.9(45/53)

SEN stands for sensitivity; SPE stands for specificity; ACCU represents overall accuracy; PPV represents positive predictive value; NPV is for negative predictive value.

through qRT-PCR needs further standardization; 2) Due to limitations in conditions, the results of this study cannot be representative of other regions; 3) The sample size is relatively small, in future studies, a large cohort is needed to verify the expression of tRF-33-RZYQHQ9M739P0J in HCC. Therefore, to fully explore the clinical application value of serum tRF-33-RZYQHQ9M739P0J, it is necessary to expand the cohort and optimize the detection method of tsRNAs.

MINTbase v2.0 showed that tRF-33-RZYQHQ9M739P0J is derived from trnaMT_GluTTC, which is a tsRNA derived from tRNA in mitochondria. Moreover, we observed that tRF-33-RZYQHQ9M739P0J is mainly located in the cytoplasm through separating nuclear and cytoplasmic RNA. Interestingly, we predicted the downstream target genes of tRF-33-RZYQHQ9M739P0J through bioinformatic prediction, which suggested that it was associated with liver metabolism, autophagy, and protein binding. The liver is a significant metabolic organ and is mainly responsible for removing toxins and balancing the uptake of glucose, lipids, and amino acids. Notably, metabolic reprogramming plays an important role in liver cancer. At the same time, tumorigenesis and progression are often accompanied by rearrangements in metabolic pathways, leading to metabolic imbalance in order to meet and sustain the energy and metabolic requirements of liver cancer cell proliferation. This reprogramming involves a series of complex biochemical reactions involving glucose, nucleic acids, lipids, and other metabolites [39–41]. In the future, we will investigate the impact of tRF-33-RZYQHQ9M739P0J on cell proliferation, metastasis, cell cycle, metabolism, as well as its impact on mitochondrial function in HCC cells. To explore the mechanism of tRF-33-RZYQHQ9M739P0J in the regulation of HCC-related genes, in subsequent studies, we will transfect a tRF-33-RZYQHQ9M739P0J inhibitor and its inhibitor control group (inhibitor NC) into HCC cells and perform

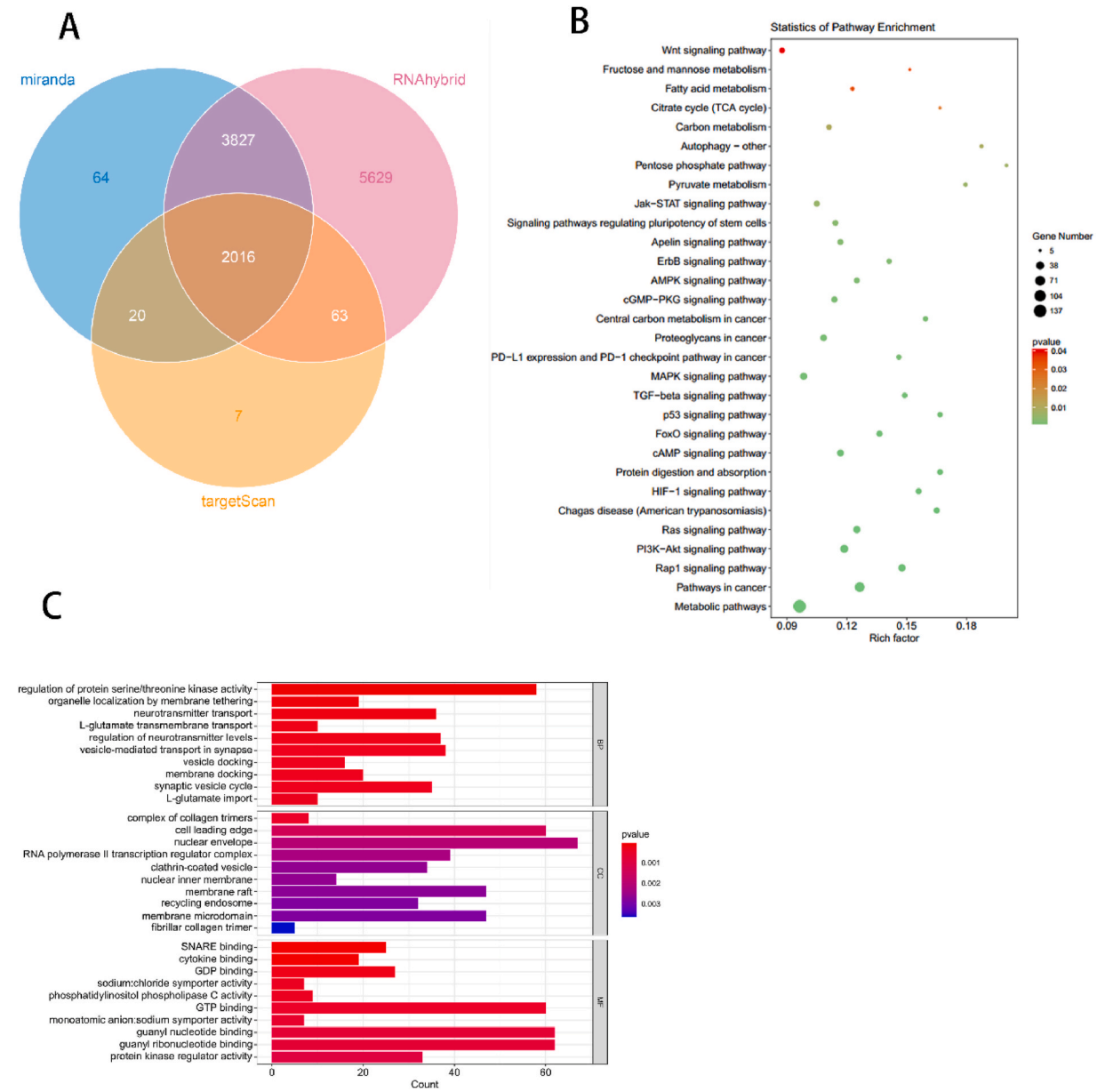

Fig. 6. Prediction of tRF-33-RZYQHQ9M739P0J target gene and target gene KEGG and GO enrichment analysis. **(A)** tRF-33-RZYQHQ 9M739P0J target gene prediction. **(B)** Bubble plot of KEGG analysis of tRF-33-RZYQHQ9M739P0J downstream target genes. **(C)** GO enrichment analysis of tRF-33-RZYQHQ9M739P0J target genes.

transcriptome sequencing. Our research direction will be based on these results and cell function validation.

5. Conclusion

In conclusion, tRF-33-RZYQHQ9M739P0J may be a diagnostic and prognostic biomarker for HCC. Next, we will explore further the effect of tRF-33-RZYQHQ9M739P0J on the malignant progression of HCC and its molecular mechanism.

Approval and consent for ethics participation

This study was performed in line with the Helsinki Declaration principles. This experiment has been approved by the Ethics Committee of the Affiliated Hospital of Nantong University, with the ethics number of 2018-L006.

Data availability statement

The data will be available upon request.

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CRediT authorship contribution statement

Xian Li: Writing – original draft, Data curation, Conceptualization, Visualization, Writing – review & editing. **Yang Li:** Formal analysis, Data curation, Methodology. **Jie Yuan:** Data curation, Visualization. **Weiwei Zhang:** Formal analysis, Software, Visualization. **Tianxin Xu:** Data curation, Software. **Rongrong Jing:** Writing – original draft, Writing – review & editing. **Shaoqing Ju:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

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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