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RESEARCH ARTICLE

Serum mitochondrial tsRNA serves as a novel biomarker for hepatocarcinoma diagnosis

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Abstract **Hepatocellular carcinoma (HCC), which makes up the majority of liver cancer, is induced by the infection of hepatitis B/C virus. Biomarkers are needed to facilitate the early detection of HCC, which is often diagnosed too late for effective therapy. The tRNA-derived small RNAs (tsRNAs) play vital roles in tumorigenesis and are stable in circulation. However, the diagnostic values and biological functions of circulating tsRNAs, especially for HCC, are still unknown. In this study, we first utilized RNA sequencing followed by quantitative reverse-transcription PCR to analyze tsRNA signatures in HCC serum. We identified tRF-Gln-TTG-006, which was remarkably upregulated in HCC serum (training cohort: 24 HCC patients vs. 24 healthy controls). In the validation stage, we found that tRF-Gln-TTG-006 signature could distinguish HCC cases from healthy subjects with high sensitivity (80.4%) and specificity (79.4%) even in the early stage (Stage I: sensitivity, 79.0%; specificity, 74.8%; 155 healthy controls vs. 153 HCC patients from two cohorts). Moreover, *in vitro* studies indicated that circulating tRF-Gln-TTG-006 was released from tumor cells, and its biological function was predicted by bioinformatics assay and validated by colony formation and apoptosis assays. In summary, our study demonstrated that serum tsRNA signature may serve as a novel biomarker of HCC.**

Keywords tsRNA; biomarker; hepatocarcinoma

Introduction

Hepatocellular carcinoma (HCC) is the most primary liver cancer; it is the third most frequent cause of cancer-related deaths and ranks sixth in incidence worldwide [1]. The abysmal prognosis of HCC is due to late detection, the aggressive nature of the tumor, and the resistance to chemotherapy and radiotherapy [2]. Given the lack of sensitive diagnosis methods or biomarkers, most HCC patients are diagnosed in the advanced stage, when either the tumor has invaded the surrounding major vessels or distant metastases have occurred; thus, the opportunity to perform curative resection is lost [3]. Sensitive and effective screening of diagnostic biomarkers to detect HCC in the early stage is the best strategy to improve the effectiveness of HCC therapies.

Cell-free DNAs or RNAs are released into and circulate in the serum, plasma, and other body fluids [4–8]. The signatures of circulating nucleic acids in body fluids may represent a gold mine of noninvasive biomarkers for diseases. tRNA-derived small RNAs (tsRNAs) are newly defined noncoding RNAs of approximately 18–40 nucleotides (nt) in length; these are generated from pre-tRNAs and mature tRNAs [9–11]. Based on the different parts where tsRNAs were derived, they can be further divided into tRNA fragments (tRFs) that are about 18–22 nt long and generated by nucleases RNase Z or Dicer [12];

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tRNA-derived stress-induced RNAs (tiRNAs) are the product of specific cleavage, which occurs with the help of ribonucleases Rny1 or angiogenin (ANG) [13,14]. The tsRNAs have special signatures in various cancers, including breast cancer, lung cancer, and B cell lymphoma, thereby suggesting that tsRNAs may participate in the generation and development of these diseases [15–18]. Although a general pattern of tsRNA function in cancer genesis is still under investigation, several mechanisms have been proposed. Some tsRNAs that can target 3′ untranslated region (3′UTR) and specifically inhibit gene expression to influence cancer cell functions, such as proliferation and apoptosis, have been reported [18–20]. For example, ts-3676, which was downregulated in B cell chronic lymphocytic leukemia (CLL), could target 3′UTR of TCL1; thus, ts-3676 functions as a tumor suppressor [19]. Moreover, tsRNAs can bind RNA binding protein YBX1 and disturb its combination with many oncogene transcripts, thereby suppressing the oncogene expression at the post-transcriptional level in breast cancer [21]. Also, tsRNAs derived from androgen receptor (AR)-positive prostate cancer cell and estrogen receptor (ER)-positive breast cancer were regulated by sex hormones and their receptors and can regulate cell proliferation [15]. Further studies are needed to uncover underlying mechanisms. Notably, tsRNAs were identified to be stable in serum, and circulating tsRNA could be a valuable non-invasive biomarker for several diseases, including pancreatic cancer [20], breast cancer, and squamous cell carcinoma of the head and neck [22–24]. However, the profiling and potential diagnostic value of tsRNA in HCC remain enigmatic.

To validate that serum tsRNA profile can be used as a biomarker to diagnose HCC, we used RNA sequencing and qRT-PCR. Results identified HCC-associated tsRNA signatures in human serum and tumor cancer cell lines. The mitochondrial tsRNA in serum showed great promise as a novel biomarker for HCC diagnosis.

Materials and methods

Clinical serum samples

Thirty HCC patients and 30 healthy controls were enrolled for next-generation sequencing from the First People's Hospital of Lianyungang. During the same period of time, other 24 HCC patients and 24 healthy controls were enrolled in the training cohort from the same hospital. In the validation phase, we tested the significantly increased serum tsRNAs in two independent cohorts from the First People's Hospital of Lianyungang (80 HCC patients and 79 healthy controls) and the Affiliated Drum Tower Hospital of Nanjing University Medical School (73 HCC patients and 76 healthy controls). All the serum samples were collected before any therapeutic procedures. The study was approved by the Medical Ethics Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School and the First People's Hospital of Lianyungang. All serum samples were collected and stored using standard procedures. A total of 2–5 mL of peripheral venous blood was obtained from each patient to acquire the serum or plasma samples. The serum was collected using a vacuum tube with separation gel and then centrifuged at 3000× *g* for 10 min at room temperature to remove cell fractions. The final supernatants were transferred to a new tube and stored at −80 °C until analysis. The clinical pathological characteristics of the patients are described in Table 1. The study was conducted in accordance with the *Declaration of Helsinki* and approved by the institutional review boards of all participating institutions.

Small RNA sequencing

The tsRNA sequencing was performed as we previously described [20]. Before the sequencing procedure, the integrity and quantity of each RNA sample were tested using agarose gel electrophoresis and Nanodrop. The serum samples were pooled from 30 HCC patients or 30 healthy controls. Samples were mixed with TRIzol reagent and homogenized, after which RNA extraction was performed. Considering that tsRNAs are heavily decorated by RNA modifications, we performed the following treatments before library construction: 3′-aminoacyl (charged) deacylation to 3′-OH for 3′-adaptor ligation; 3′-cP (2′,3′-cyclic phosphate) removal to 3′-OH for 3′-adaptor ligation; 5′-OH (hydroxyl group) phosphorylation to 5′-P for 5′-adaptor ligation; and m1A and m3C demethylation. Library construction and deep sequencing were then performed by Aksomics (Shanghai, China). Sequencing libraries were size-selected for the RNA biotypes and qualified using Agilent BioAnalyzer 2100. For standard small RNA sequencing on Illumina NextSeq instrument, the sequencing type was 50 bp single read.

Quantitative RT-PCR for tsRNAs

Total RNA extraction and tsRNA quantitation were performed as we previously described [5,20]. Dilution was first made by adding 300 μL DEPC-treated water per 100 μL of serum. RNA was extracted by three steps of phenol/chloroform purification and dissolved in 10 μL of DEPC-treated water. TaqMan probe-based RT-qPCR assay was performed using the following procedure. Total RNA (2 μL) was reversely transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and the specific stem-loop RT primer (synthesized by Applied Biosystems, USA). The reaction system was incubated at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Subsequently, qRT-PCR was performed on a Roche

LightCycler96 RT-PCR System (Roche, Germany) using TaqMan-custom miRNA probes (Applied Biosystems) following the instructions. The qPCR procedure for tsRNA was as follows, 95 °C, 5 min, followed by 45 cycles at 95 °C for 15 s and at 60 °C for 1 min. The threshold cycle (C_t) values were determined using the fixed threshold settings. For the absolute quantitative analysis of tsRNAs, a series of synthetic tsRNA standards (dissolved in DEPC water) of known concentrations (from 1 pmol/L to 10 nmol/L) were also reverse-transcribed and amplified to generate a standard curve. The absolute amount of tsRNA was then calculated by referring to the standard curve; the corresponding concentration was converted to the tsRNA concentrations in serum. Considering the lack of current consensus on housekeeping tsRNAs for qRT-PCR analysis of serum tsRNAs, the expression levels of tsRNAs were directly normalized to serum volume and then to the control group.

ELISA assay

Serum alpha fetoprotein (AFP) protein levels in patients with HCC and healthy controls were assessed using the Human alpha-fetoprotein/AFP ELISA Kit (Abclonal, RK00355) according to the manufacturer's directions.

Cell culture and supernatants collection

HepG2 and HEK-293T were obtained from ATCC (Manassas, USA). HuH-7 cell line was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The three cell lines were cultured in DMEM supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere with 5% CO_2. For tsRNA *in vitro* secretion analysis, cells were seeded at different densities (0, 2.5×10^5, 5×10^5, 1×10^6, and 2×10^6) in 10 cm^2 dishes and cultured for 48 h. The culture medium was collected and centrifuged at 2000× *g* for 10 min at 4 °C to remove cell debris before storing at −80 °C.

Colony formation assay

HepG2 and HuH-7 cells that were transfected with scrambled negative control RNAs (mimic nc) or tRF-Gln-TTG-006 mimic were suspended in 2 mL 10% DMEM at a density of 500 cells/mL and seeded in a 6-well plate. The culture media was changed every 3 days. Synthetic tRF-Gln-TTG-006 mimic and mimic nc were purchased from RiboBio (Guangzhou, China). After 16 days, the colonies were fixed with 4% paraformaldehyde and stained with 0.05% crystal violet (Beyotime, China). Then, the dish was rinsed softly with ddH_2O to remove the redundant crystal violet. Colonies were captured by photo microscopy (BX51 Olympus, Japan), and the numbers were counted by ImageJ.

Cell apoptosis assay

HepG2 and HuH-7 cells were transfected with mimic nc or tRF-Gln-TTG-006 mimic. Cells were harvested at 36 h after transfection, and the apoptosis of HuH-7 cells was detected by the Apoptosis Detection Kit I (556547; BD Biosciences, USA) following the manufacturer's instructions, as described previously [25].

Wound healing assay

HuH-7 cells were seeded in a 6-well plate and transfected with mimic nc or tRF-Gln-TTG-006 mimic when cell confluence reached 70%. The next day, the tip of a 200 μL pipette was used to make a "wound" on the 6-well plate. The plates were washed twice with PBS buffer to remove exfoliated cells. Then, 2 mL of 2% DMEM was added. Pictures of the wounds were captured at the same position at 0, 24, and 48 h. Percentage of wound recovery was calculated by (24 h or 48 h scratch area − 0 h scratch area)/0 h scratch area × 100%.

tsRNA target prediction

RNAhybrid [26] was used to predict potential target genes and binding sites of tsRNA tRF-Gln-TTG-006. For the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of tsRNA-target genes, KOBAS [27] was utilized, and the bubble plot was generated by the website of bioinformatics.com.cn.

Statistical analysis

Statistical analysis was performed by SPSS 24.0 statistical software and GRAPHPAD PRISM 8.0 (Graphpad, San Diego, CA, USA) using the *t*-test. When the group was > 2, one way ANOVA was performed followed by Kruskal–Wallis multiple comparison test. Data were presented as mean ± SEM. Differences were considered statistically significant at $P < 0.05$.

Results

Analysis of differentially expressed serum tsRNAs in HCC

Tumor-derived small non-coding RNA in the serum or plasma are emerging as novel blood-based fingerprints for the detection of human cancers especially in the early stage [28,29]. tsRNAs were demonstrated to circulate in a highly stable, cell-free form in the serum [22]. To validate whether the tsRNA profile in the serum could serve as biomarkers for the blood-based detection of HCC, a strategy that included tsRNA next-generation sequencing followed by qRT-PCR validation on individual samples

was used. The study design was presented in Fig. 1. First, total RNA from pooled serum of 30 HCC and 30 age- and sex-matched healthy controls was extracted and sequenced to screen the candidate tsRNAs. No significant difference in length distribution between the two groups (Fig. S1) was found. A total of 232 tsRNAs were identified with counts per million of total aligned reads (CPM) of > 10. The tsRNAs with a fold-change of > 2 between HCC and healthy controls were considered to be differentially expressed. Based on the criteria, the scatter plot showed that 110 of identified 232 tsRNAs were dysregulated (59 were upregulated, and 51 were downregulated) in the serum of patients with HCC (Fig. 2A). To avoid deviation, we further considered tsRNAs that satisfied two conditions that indicate significant differential expression, as follows: CPM of > 10 in the serum by sequencing detection; and fold-change of > 10 between two groups. The analysis found 17 significant differentially expressed tsRNAs in the serum of patients with HCC compared with that of healthy controls (Fig. 2B and Table S1).

Validation of RNA sequencing by RT-qPCR in training phase

In the training phase, we used probe-based RT-qPCR assay to validate the sequencing results. Absolute quantitative analysis was used to validate the expression of five candidates from the top 10 upregulated tsRNAs using individual serum sample from 24 healthy controls and 24 patients with HCC. Only five tsRNAs were assessed by probe-based RT-qPCR due to the length limitation. To test whether this method was reliable and reproducible for detecting serum tsRNA, whether the standard curve for different concentrations of synthetic tsRNA standards was linear from 1 pmol/L to 10 nmol/L was determined. There is satisfactory consistency between results from RT-qPCR assay and the real concentrations (Fig. S2A). The qPCR products were also cloned into the pClone007 vector and sequenced to confirm the correctness and specificity of the reaction. Absolute quantification identified that two tsRNAs (tRF-Pro-AGG-005 and tRF-Gln-TTG-006) were significantly elevated in the serum from HCC patients compared with that from healthy controls (Fig. 2C–2G). Based on these results, tRF-Pro-AGG-005 and tRF-Gln-TTG-006 were identified as candidates for the satisfactory discrimination of the serum from patients with HCC and that from the control group.

Diagnostic value of identified tsRNAs in the validation phase

To further validate the above results, the expression profiles of tRF-Pro-AGG-005 and tRF-Gln-TTG-006 were further determined in two independent larger cohort of serum samples (validation set: 80 HCC patients vs. 79 healthy controls from the First People's Hospital of Lianyungang (cohort 1) and 73 HCC patients vs. 76 healthy controls from the Affiliated Drum Tower Hospital of Nanjing University Medical School (cohort 2)). Patients' information is listed in Table 1. The serum

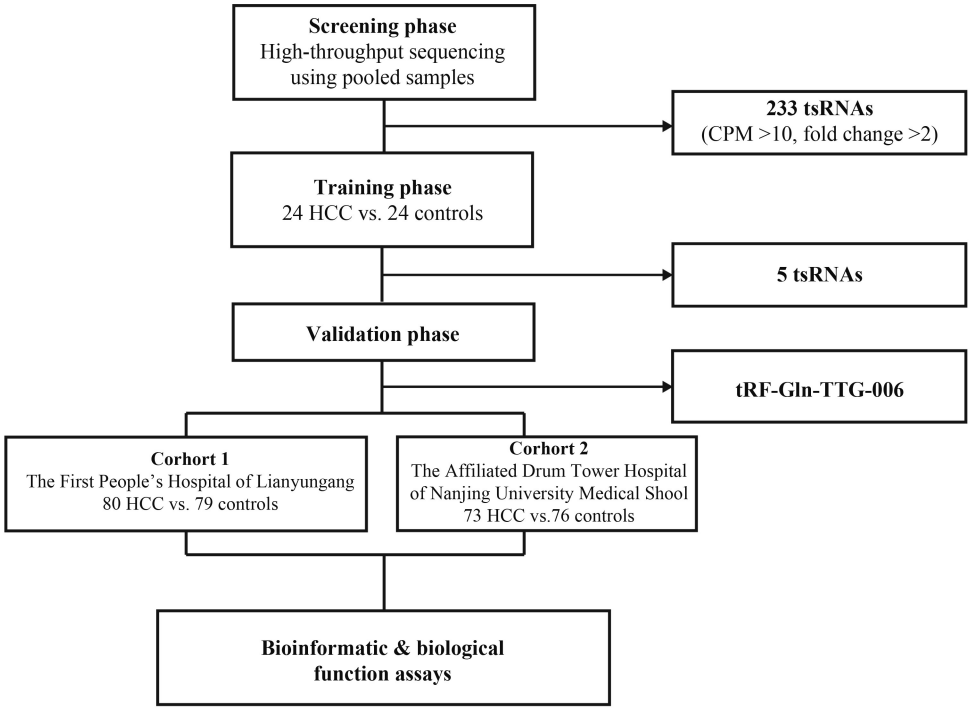

Fig. 1 Flowchart of the study design.

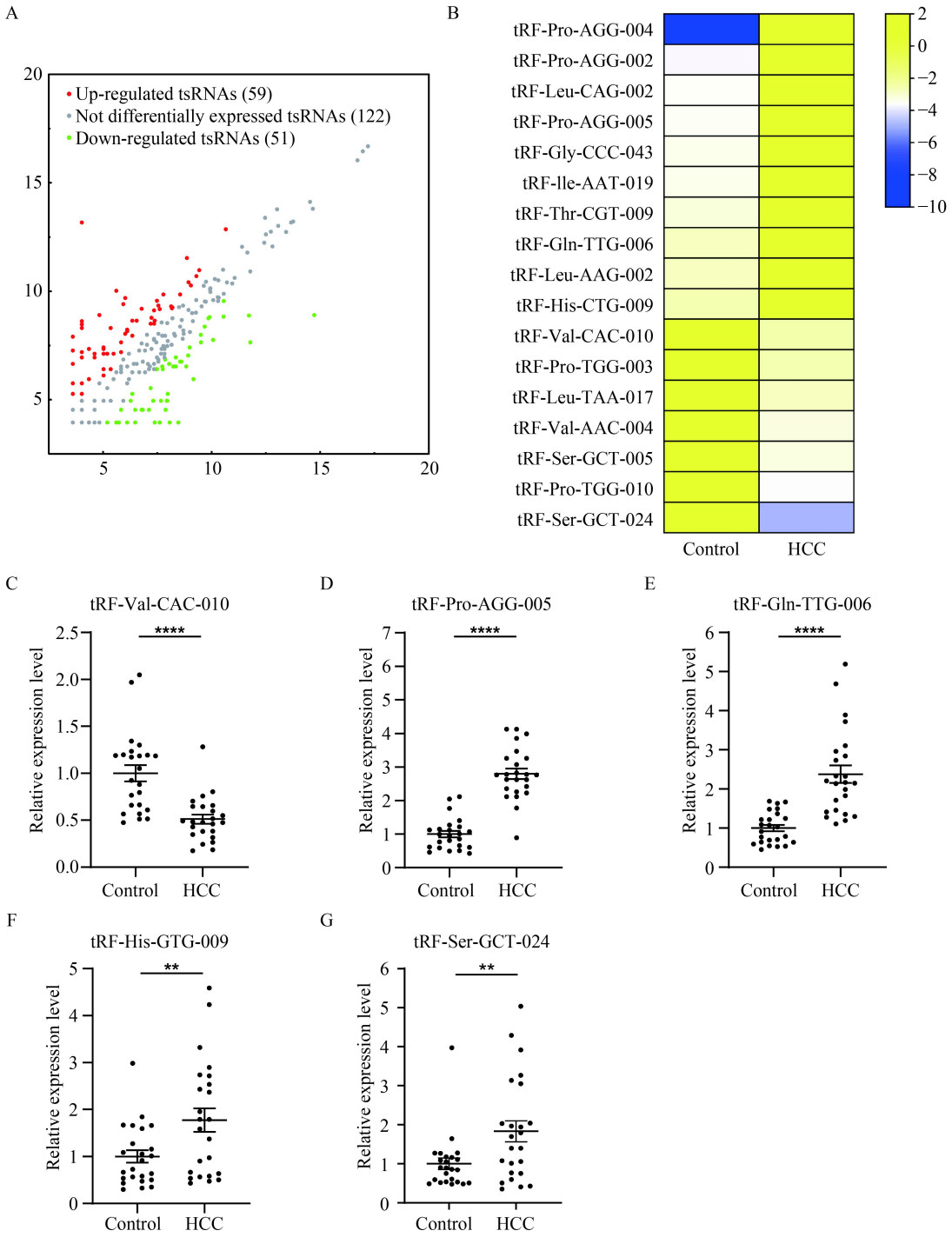

Fig. 2 Analysis of differentially expressed serum tsRNAs in HCC. (A) The scatter plot expressed the changes in tsRNAs expression. (B) Heat map of 17 significant differentially expressed tsRNAs. (C–G) qRT-PCR data on the tsRNA expression levels in the screen phases (n_{HCC} = 24, $n_{control}$ = 24) of tRF-Val-CAC-010 (C), tRF-Pro-AGG-005 (D), tRF-Gln-TTG-006 (E), tRF-His-GTG-009 (F), and tRF-Ser-GCT-024 (G). **P < 0.01; ****P < 0.0001.

concentration of tRF-Gln-TTG-006 was significantly increased in HCC patients compared with controls (P < 0.0001) (Fig. 3A and 3B), whereas tRF-Pro-AGG-005 showed no difference between the two groups (Fig. S3). To further measure the diagnostic values of tRF-Gln-TTG-006, ROC analysis was performed for evaluating the sensitivity and specificity of prediction based on the risk scores. tRF-Gln-TTG-006 showed high diagnostic use with an AUC of 0.919 (95% CI 0.879–0.959), sensitivity of 80.0%, and specificity of 88.6% in patients

Table 1 Information of the HCC patients of the two hospitals cohorts

	The First People's Hospital of Lianyungang			The Affiliated Drum Tower Hospital of Nanjing University Medical School
	Screening cohort	Training cohort	Validation cohort 1	Validation cohort 2
Number	30	24	80	73
Age (year)	59.07 ± 11.48	61.67 ± 9.98	59.63 ± 10.14	58.45 ± 10.02
Sex				
Male	21	19	65	67
Female	9	5	15	6
Viral status ($B^-C^-/B^+C^-/B^-C^+/B^+C^+$)[a]	7/11/4/8	6/7/3/5	15/50/6/9	13/58/1/1
Tumor stage				
I	19	16	41	40
II	6	4	19	19
III	3	2	16	10
IV	2	2	4	4

The data of patients'age are expressed as the mean ± standard deviation (SD).
[a]Negative HBs-Ag, positive HBs-Ag, negative anti-HCV Ab, and positive anti-HCV Ab were defined as B^-, B^+ , C^-, and C^+, respectively.

with HCC compared with healthy controls from the First People's Hospital of Lianyungang. In the serum samples from the Affiliated Drum Tower Hospital (Fig. 3C and 3D), these values were as follows: AUC of 0.825 (95% CI 0.758–0.893); sensitivity of 76.7%; and specificity of 75.0%. Combining the two cohorts, tRF-Gln-TTG-006 showed diagnostic value with AUC of 0.875 (95% CI 0.836–0.914), sensitivity of 80.4%, and specificity 79.4%. We compared the diagnostic effects of tRF-Gln-TTG-006 with clinical HCC biomarker AFP. ROC analysis

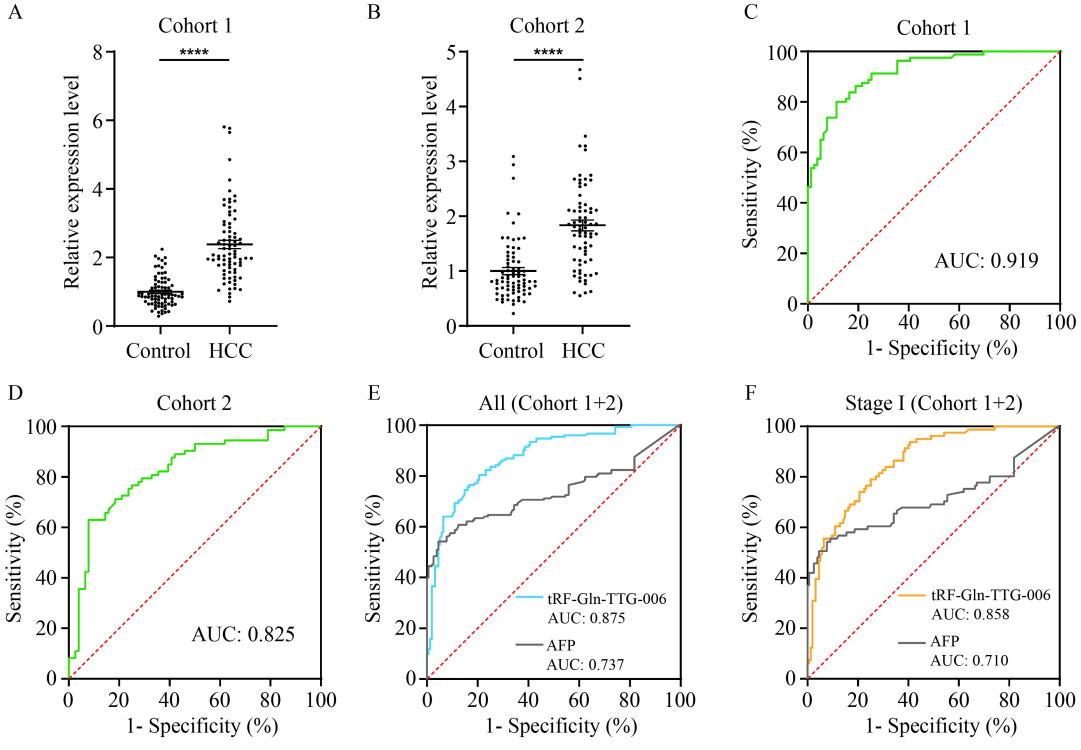

Fig. 3 Training phase of tRF-Gln-TTG-006 in the two cohorts from different hospitals. (A and B) qRT-PCR shows the concentrations of tRF-Gln-TTG-006 in serum from The First People's Hospital of Lianyungang (cohort 1) (A) and The Affiliated Drum Tower Hospital of Nanjing University Medical School (cohort 2) (B). (C–E) ROC curves analysis compared the diagnosis value of tRF-Gln-TTG-006 and AFP concentration in serum in the cohort 1 (green line, AUC = 0.919) (C), cohort 2 (green line, AUC = 0.825) (D), cohort 1 + 2 (blue line, AUC = 0.875; gray line AUC = 0.737) (E). (F) ROC curves of tRF-Gln-TTG-006 and AFP concentration in serum from clinical stage I cases and controls in all sets (orange line, AUC = 0.858; gray line, AUC = 0.710). ****$P < 0.0001$.

indicated that tRF-Gln-TTG-006 had better diagnostic effect than AFP (Fig. 3E). Intriguingly, tRF-Gln-TTG-006 demonstrated a significantly superior diagnostic accuracy for patients with early-stage HCC (stage I) with an AUC of 0.858 (95% CI 0.810–0.905), sensitivity of 79.0%, and specificity of 74.8%, and these results were better than those obtained for AFP (AUC of 0.710, sensitivity of 54.3%, and specificity of 92.3%) (Fig. 3F). HBV and HCV infections are the leading cause of HCC. We further explored whether virus infection is related to abnormally high tRF-Gln-TTG-006 expression in serum from patients with HCC. HCC patients were further divided into four groups according to HBV/HCV infection status, and their corresponding tRF-Gln-TTG-006 expression levels were analyzed (Fig. S4A). The tRF-Gln-TTG-006 expression pattern showed no relationship with the different HBV/HCV infection statuses, which suggested that HBV/HCV infection may not be the main cause of tRF-Gln-TTG-006 elevation in HCC. Moreover, no significant correlation was found between tRF-Gln-TTG-006 expression pattern and patients' age and sex (Fig. S4B and S4C). The levels of serum tRF-Gln-TTG-006 may serve as a promising diagnostic indicator of human HCC, especially in the early stage.

Biological function analysis of tRF-Gln-TTG-006 in HCC

To determine whether the high expression of tRF-Gln-TTG-006 in serum was correlated with the HCC tissue, we analyzed the tsRNA expression level in HCC tissue and paired serum of 11 HCC patients. We observed a positive correlation in the tRF-Gln-TTG-006 expression level between HCC tissue and paired serum (Fig. 4A). To further explore whether the upregulated tRF-Gln-TTG-006 was released from HCC cells, HepG2 cells (human HCC cell line) were cultured, and tsRNA expression was checked in the culture medium of cells in various numbers. HepG2 cells released the tRF-Gln-TTG-006 into the culture medium, and its expression was directly proportional to cell number, which suggested that the tsRNA was derived from HCC cells (Fig. 4B). Some tsRNAs have miRNA-like structure and function; they directly bind to target mRNA 3′ untranslated region (3′UTR) and repress gene expression at post-transcriptional level [16,21,30]. MINTbase [31] alignment revealed that tRF-Gln-TTG-006 was matched to a mitochondrial tRNA (trna-MT_GlnTTG_MT_-_4329_4400). As shown in Fig. 4C, tRF-Gln-TTG-006 was located in position 1–22 of the predicted secondary structure of the tRNA. We inferred that tRF-Gln-TTG-006 may also have the miRNA-like function. To explore the potential function, we used RNAhybrid [32] to predict target genes of tRF-Gln-TTG-006. A total of 143 potential target genes were generated, and Gene Ontology (GO) enrichment analysis indicated that the target genes had enriched cadherin and beta-catenin binding (Fig. S5A). KEGG analysis found that the target genes were closely related to ECM-receptor-interaction, Jak-STAT signaling pathway, and others (Fig. S5B). Cadherin binding, beta-catenin binding, ECM-receptor-interaction, and Jak-STAT signaling pathways have been implicated in most steps of tumor progression, especially cancer invasion, metastasis, and immunoregulation [33–41].To further elucidate the function of tRF-Gln-TTG-006 on HCC, we transferred tRF-Gln-TTG-006 mimics into HepG2 and HuH-7 cells. To confirm the transfection efficiency, the expression of tRF-Gln-TTG-006 in cells was monitored by RT-qPCR (Fig. S6). To our surprise, tRF-Gln-TTG-006 decreased the colony formation ability of both HCC cell lines (Fig. 4D−4F). Apoptosis assays showed that tRF-Gln-TTG-006 induced higher apoptosis ratio in HuH-7 cells (Fig. 4G and 4H). We also performed wound healing assay on HuH-7 cells, and the result suggested that tRF-Gln-TTG-006 may not affect the migration ability of HCC cells (Fig. S7). These results indicated that tRF-Gln-TTG-006 may act as a tumor suppressor and is released by HCC cells.

Discussion

HCC early detection and surveillance increase the success of treatment. Early-stage HCC can be treated curatively by local ablation, surgical resection, or liver transplantation [42]. In recent studies, liquid biopsies based on biomarkers in the circulation provide one of the most promising ways, in principle, to detect cancers at an early stage. In the best currently available blood test in HCC, AFP exhibits low sensitivity and specificity, particularly in early-stage HCC [43–45]. Circulating microRNAs (miRNAs) [5,46] or tumor DNAs [7,47] have also been explored as biomarkers in HCC. Circulating miRNAs are the most studied circulating nucleic acids in HCC; their expression pattern in serum and functions in tumor progress were largely investigated, and it was found that few miRNAs could identify HCC from a very early stage [48–50]. Recent reports indicated that circulating tumor DNA had positive effects on early-stage HCC diagnosis [51,52]. Nevertheless, new diagnostic biomarkers with higher sensitivity, stability, and specificity are still urgently needed for HCC early detection.

tsRNAs, a novel type of small non-coding RNA generated from cleavage of tRNA or pre-tRNA, were found to be stable in circulation due to various RNA modifications (inherited from their tRNA precursors) [9,11,12,53,54]. Furthermore, tsRNAs' signature may sensitively change under stress stimulation, such as hypoxia or oxidation [13,55,56], in the environment to which the tumor cells are commonly exposed. This finding indicated that tsRNA signature possibly reflects the status of the tumor in a highly sensitive manner. Although dysregulation and function of tsRNAs have

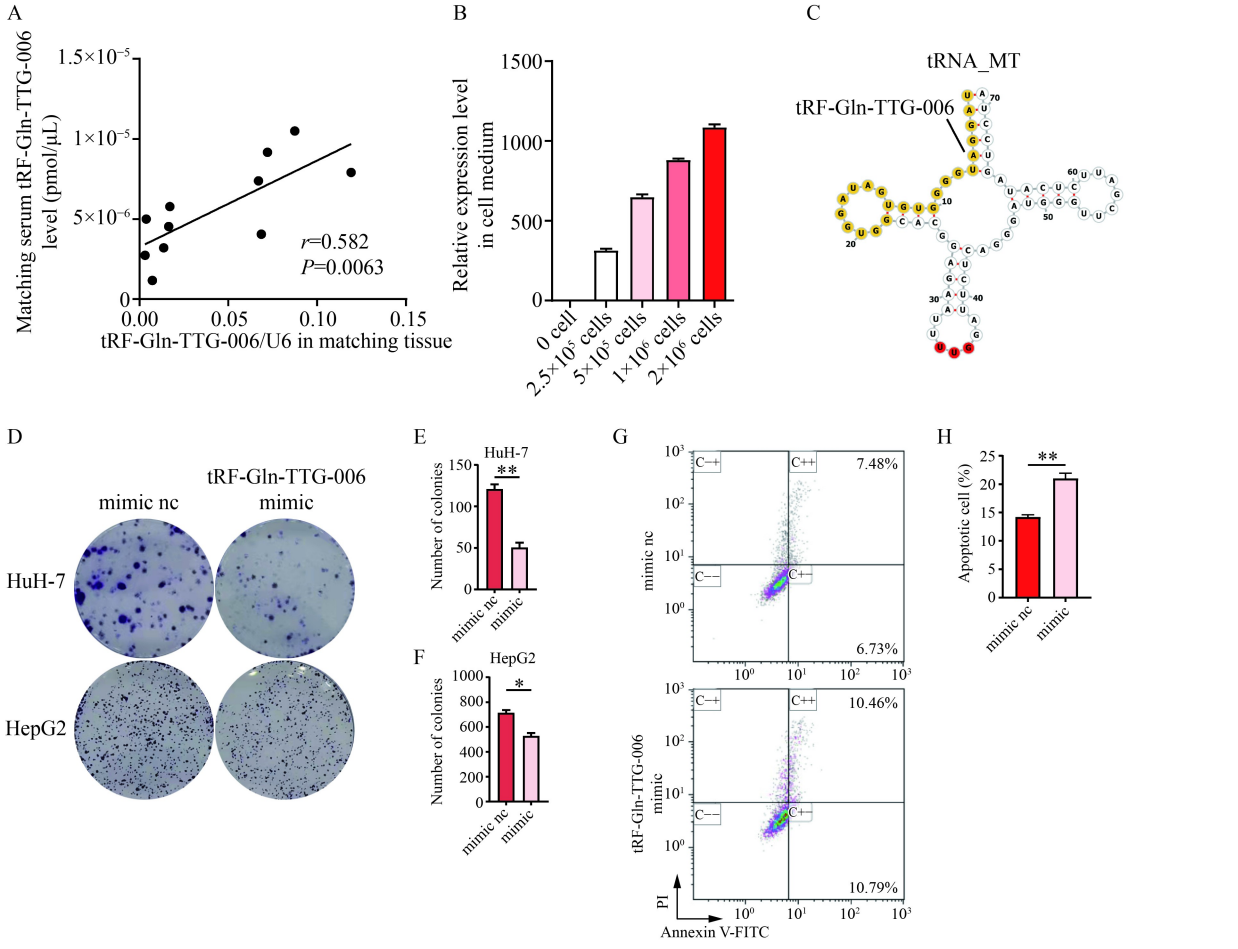

Fig. 4 Biological functions of tRF-Gln-TTG-006. (A) Quantitative analysis of tRF-Gln-TTG-006 expression in HCC tissues and paired serum from same patients (n = 11). (B) Expression status of tRF-Gln-TTG-006 in HepG2 cell culture medium. The amount of tRF-Gln-TTG-006 excreted in the culture medium increased depending on the cell number. (C) Position of tRF-Gln-TTG-006 in the cloverleaf secondary structure of trnaMT_GlnTTG_MT_-_4329_4400. (D) Colony formation assay was performed on HuH-7 and HepG2 cells transfected with mimic nc or tRF-Gln-TTG-006 mimic. (E and F) Quantitative analysis of the colony number of HuH-7 (E) and HepG2 cells (F). (G and H) tRF-Gln-TTG-006 inhibited apoptosis in HuH-7 cells (G) and quantitative analysis of apoptosis (H). *P < 0.05; **P < 0.01.

been observed in some malignancies, the profiling and role of tsRNAs in HCC has not been previously studied. In this study, we provided a "proof-of-principle" approach to identify a particular HCC-specific serum tsRNA profile for the first time. This approach included a high throughput RNA sequencing of pooled serum samples followed by multiple qRT-PCR validation sets at the individual level. We first determined tsRNA signatures in the serum of HCC patients and identified a mitochondrial tsRNA signature (tRF-Gln-TTG-006) that had an excellent ability to distinguish patients with HCC from controls with 80.4% sensitivity and 79.4% specificity. The same excellent results were found even in the early stage with a sensitivity of 79.0% and specificity of 74.8%.

The biological functions of circulating tsRNAs remain enigmatic. Accumulating evidence suggested that non-coding RNAs secreted by cells through exosomes contribute significantly to cell-to-cell communication, revealing a complex interplay between tumor cells, tumor microenvironment cells, and immune cells [57–59]. GO and KEGG analyses indicated that the tRF-Gln-TTG-006 was not only predictive biomarker but also functional regulator of HCC progression. Colony formation assay and apoptosis assay both showed that tRF-Gln-TTG-006 has an inhibitory effect on HCC cells. Hence, we supposed that HCC cells maintain their survival by excreting tsRNAs to the serum, because tsRNAs may be harmful to the cells; this could partly explain the significant upregulation of tRF-Gln-TTG-006 in HCC serum. A more detailed study is needed to uncover the underlying mechanism of this interesting observation. Also, whether the function of tRF-Gln-TTG-006 was carried out by directly targeting specific cancer-related gene needs further investigation. Taken together, our data suggested that the circulating tsRNAs may play a crucial role in HCC progression.

We defined a distinctive serum tsRNA signature in

HCC patients from high throughput RNA sequencing followed by multiple sets of individual qRT-PCR evaluations. In particular, we identified a novel serum tsRNA in HCC patients, and this serum tsRNA signature can possibly serve as a valuable biomarker for HCC diagnosis. Our study also reveals the potential biological function of the selected tsRNA. Randomized clinical trials are needed to evaluate the possible application of the tsRNAs signature in the early diagnosis and prognosis of HCC.

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Compliance with ethics guidelines

Shoubin Zhan, Ping Yang, Shengkai Zhou, Ye Xu, Rui Xu, Gaoli Liang, Chenyu Zhang, Xi Chen, Liuqing Yang, Fangfang Jin, and Yanbo Wang declare that they have no conflict of interest. All the procedure of this study was approved by the Medical Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School and the First People's Hospital of Lianyungang.

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11684-022-0920-7 and is accessible for authorized users.

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