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ORIGINAL ARTICLE

tRFs as Potential Exosome tRNA-Derived Fragment Biomarkers for Gastric Carcinoma

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SUMMARY

Background: **Gastric Carcinoma (GC) is one of the common diseases induced by the interaction of genes and environment. Exosomes are potential markers for several health problems, which contain lipids, proteins, long non-coding RNAs, microRNAs (miRNAs), and tRNA-derived fragments (tRFs). The roles of mRNAs and miRNAs in GC have been studied comprehensively; however, little research was focused on the function of plasma exosomal tRFs.**
Methods: **We collected plasma samples from fifty healthy controls and fifty GC patients, and all exosomes were isolated with a combined centrifugation and characterized by electron microscopy, western blot, and flow cytometry. The small RNA sequence was performed to detect the plasma exosomal tRFs, and tRFs markers were validated by real-time quantitative PCR. Three exosomal diagnostic tRFs were confirmed by receiver operating characteristic analyses.**
Results: **In this study, we found higher plasma exosomal tRF-25, tRF-38, tRF-18 expression in GC than in controls. Plasma exosomal tRF-25, tRF-38, and tRF-18 showed better accuracy for GC diagnosis.**
Conclusions: **Our results suggest that plasma exosomal tRF-25, tRF-38, and tRF-18 were biomarkers for GC detection; tRF-25, tRF-38 and tRF-18 might be predictive of GC prognosis.**
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KEY WORDS

gastric carcinoma, plasma, exosome, tRF, ROC, diagnostic biomarker

INTRODUCTION

Gastric carcinoma (GC) is one of the highest cancer-related deaths in China, and the incidence rate and mortality rate of GC is very high [1,2]. Despite the most effective treatment for GC being surgical removal of the tumor-baring stomach, more and more GC patients are diagnosed at later stages [3,4]. Several studies have found that the immune system is closely related to GC [5,6]. In addition, other studies have shown that deregulation of mRNA and microRNA (miRNA) expression can affect GC [7,8]. Therefore, a better understanding of the molecular mechanisms of GC is critical for the identification of biomarkers and novel therapeutic agents for

GC.

Exosomes are characterized as 30 - 150 nm diameter membrane vesicles that originate from multivesicular bodies [9,10]. Exosomes have been isolated from various cells and body fluids, such as endothelial cells, immune cells, blood platelets, smooth muscle cell, blood, saliva, urine, and cerebrospinal fluid [10]. Exosomes have been proven to be potential markers for many health problems, which contain cellular plasma, lipids, proteins, and nucleic acid species including genomic DNA, miRNAs, lincRNAs, rRNAs, and tRNA-derived fragments (tRFs) [11-14].

tRF, a new type of small non-coding RNAs originating from tRNAs [15-17], are related to several cell life activities [18-20]. Several studies found that tRFs are closely associated with posttranscriptional gene expression by directly inhibiting protein synthesis with the eIF4G translation initiation factor [21-23]. In B-cell lymphoma cells, one 3'-derived tRF shows the guide RNA function that inhibits propagation [24]. tRFs competitively combine with RNA-binding protein YBX1, which inhibits the activity of YBX1 to suppress cell growth and invasion [25].

The function of mRNAs and miRNAs in GC has been well studied [7,8]; however, little research is observed on the role of plasma exosomal tRFs in GC. tRFs are considered small biologically interesting molecules, whose abundance and composition in the transcriptome depends on disease, disease subtype, gender, and tissue [26]. This result indicates that tRFs can be identified as new biomarkers of disease [26,27]. In the present study, we collected plasma samples from 50 healthy controls and GC patients, and all exosomes were isolated and characterized. The small RNA sequence was performed to detect the plasma exosomal tRFs and tRF markers were validated by real-time quantitative PCR and receiver operating characteristic analyses. Our findings indicated that plasma exosomal tRF-25, tRF-38, and tRF-18 were biomarkers for GC detection; tRF-25, tRF-38, and tRF-18 may be predictive of GC prognosis.

MATERIALS AND METHODS

Plasma from GC patients

All plasma was obtained from 50 healthy normal people and GC patients. With green-top vacutainer tubes, venous blood was obtained and then was centrifuged at 2,000 g for 15 minutes. The plasma was removed and divided for storage at -80°C.

Exosome isolation from plasma

Plasma exosomes were isolated by the ExoQuick™ Plasma Prep and Exosome Precipitation Kit (Cat# EXOQ5TM-1). First, the supernatant of dissolved plasma samples was added with 63 µL ExoQuick Exosome Precipitation Solution after centrifuging at 3,000 g for 15 minutes. Second, the ExoQuick/plasma mixture was centrifuged at 1,500 g for 30 minutes. Third, the residual ExoQuick solution was spun down by centrifugation at 1,500 g for 5 minutes. After that, all exosomes are at the bottom of the vessel. Lastly, the exosome pellet was resuspended in 100 µL PBS [28].

Transmission electron microscopy (TEM)

The exosomes were fixed in 2% paraformaldehyde solution, processed into ultrathin sections, and labeled with anti-CD63 antibody. All sections were examined by transmission electron microscope, and their images were recorded with an AMT 2k CCD camera.

Western blot

Plasma exosomes were lysed with a RIPA buffer, resuspended in the loading buffer, boiled at 100°C for 5 minutes and then electrophoresed on SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membrane, which was blocked with 5% non-fat dry milk in Tris buffered saline/Tween 20. Immunodetection was performed with anti-CD63 antibody (Santa Cruz-sc-5570) and anti-TSG101 antibody (Abcam-ab-125011) at a dilution of 1:500 followed by incubation with appropriate HRP-conjugated secondary antibody (1:5,000). Bands were revealed using Pierce ECL Plus Western Blotting Substrate.

RNA extraction, small RNA library preparation and sequencing

RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Purified RNA was then sent to JMDNA (Shanghai, China) for constructing small RNA libraries and carrying out small RNA sequencing analysis. Briefly, the small RNA was bound with 3'- and 5'-adapters, and cDNA constructs were created with reverse transcription followed by PCR. The small RNA fragments (15 - 40 nt) were excised and purified, and the purified libraries were quantified and validated. All six libraries were sequenced on an Illumina HiSeq 2500. The raw sequencing data should filter out low quality reads and short reads (< 15 nt). To identify known small non-coding RNAs, all small RNA clean reads were aligned to miRBase database (http://www.mirbase.org/), piRNA database, NCBI, Genomic tRNA database (http://gtrnadb.ucsc. edu/), tRFdb (http://genome.bioch.virginia.edu/ trfdb/), and MintBase. In the expression of small non-coding RNAs between GC and healthy control, the clean data was analyzed as follows: 10 copies by sequencing in two groups, and a two-fold difference in copy numbers between GC and healthy control. The functions and pathways of almost all tRFs were classified by Gene Ontology (GO) assignment and Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Real-time quantitative PCR (RT-PCR)

A total of 100 ng RNA was reverse-transcribed to cDNA using NEBNext® Multiplex Small RNA Library Prep Kit (NEB #E7560S). First, 4 µL RNA, 1 µL 3' SR adaptor and 2 µL nuclease-free water were mixed and

were incubated at 70°C for 2 minutes. Then 10 µL 3' ligation reaction buffer (2 x) and 3 µL 3' ligation enzyme mix were added and incubated for 1 hour at 25°C. Second, the ligation mixture of 4.5 µL nuclease-free water and 1 µL SR RT primer was added, heated for 5 minutes at 75°C, and transferred to 37°C for 15 minutes, followed by 15 minutes at 25°C. Thirdly, 4 µL of samples, 1 µL of 5' SR adaptor, and 2 µL of nuclease-free water were mixed and incubated at 70°C for 2 minutes. Then, 1 µL 5' SR adaptor, 1 µL 5' ligation reaction buffer (10 x), and 2.5 µL 5' ligation enzyme mix were added to the ligation mixture and incubated for 1 hour at 25°C. Lastly, 30 µL adaptor ligated RNA, 8 µL first strand synthesis reaction buffer, 1 µL murine RNase inhibitor, and 1 µL ProtoScript II reverse transcriptase were mixed in a nuclease-free tube and incubated for 60 minutes at 50°C. RT-PCR was performed on ABI Q6 detection system (Applied Biosystems Inc., USA).

Statistical analysis

For the differential expression data, the $2^{-\Delta\Delta CT}$ method was used to obtain the tRFs by RT-PCR. The predicted probability of being diagnosed with GC was analyzed by operating characteristic (ROC) curve. The ROC analysis was performed using the MedCalc (Version 10.4.7.0; MedCalc, Mariakerke, Belgium). $p < 0.05$ was considered significant.

RESULTS

Characterization of the isolated plasma exosomes by transmission electron microscopy, western blot, and flow cytometry

To obtain the purified exosomes, all plasma from GC and healthy control was subjected to sequential low- and high-speed centrifugation. The plasma exosomes were measured by TEM, western blot, and flow cytometry, which revealed that the exosomes were 100 - 150 nm spherical particles with a complete membrane structure (Figure 1). This result indicated the successful isolation of plasma exosomes from GC and healthy control.

Small RNA sequencing of plasma exosomes from GC and healthy controls

For the six sequencing libraries, an average of 11.7 million raw reads per library were obtained, where reads with insert lengths > 15 nt were approximately 9.8 million (83.29%) (Table S1). Among the mapped reads, miRNAs were the most common with an average of 2,844,884 reads (28.97%), tRFs had an average of 40,284 reads (0.41%) (Table S2 and Table S3). The main types of small non-coding RNA in GC and healthy control were miRNA, piRNA, and tRF (Figure 2A). In this study, we detected 738 miRNAs, 58 piRNAs, and 288 tRFs between GC and healthy control, 63 differentially expressed miRNAs, 3 differentially expressed piRNAs, and 29 differentially expressed tRFs between GC and healthy control (Table 1). There were six types of tRF in GC and healthy control, including 3'-half, 5'-half, i-tRF, tRF-1, tRF-3, and tRF-5 (Figure 2B). As shown in Table 1, compared with healthy control, there were 12 upregulated miRNAs, 11 upregulated tRFs, 51 downregulated miRNAs, 3 downregulated piRNAs, and 18 downregulated tRFs in GC.

Differentially expressed tRFs of plasma exosomes from GC and healthy controls

More and more studies demonstrate that tRFs play an important role in gene expression. In our present study, we found 288 total tRFs and 29 differentially expressed tRFs (11 upregulated and 18 downregulated) between GC and healthy control (Table 2 and Figure 2). By use of GO analysis, we found that a large number of their targeted genes were involved in many processes including transcription, axon guidance, neurotrophin TRK receptor signaling pathway, neuron migration, cytoplasm, cell junction, protein binding, DNA binding (Figure 3A). After mapping all of targeted genes to terms in KEGG database, we found that the targeted genes participated in "Proteoglycans in cancer", "ErbB signaling pathway", "PI3K-Akt signaling pathway", "FoxO signaling pathway", "cGMP-PKG signaling pathway", "Wnt signaling pathway", "MAPK signaling pathway", "Ras signaling pathway", "Calcium signaling pathway", and "Endocytosis" (Figure 3B).

Validation of differentially expressed tRFs from plasma exosomes

In the next step, in order to validate the differential expression, three high candidate tRFs (tRF-25, tRF-38, and tRF-18) were measured by RT-PCR in 47 GC samples and 47 healthy controls. Our results indicated that tRF-25, tRF-38, and tRF-18 showed a difference between GC patients and controls (Figure 4A). Thus, these tRFs were excluded from further analysis. We found several potential targets of tRF-25, tRF-38, and tRF-18 using Miranda (http://www.microrna.org/) and RNAhybrid. There were 25, 192, and 38 targeted genes of tRF-25, tRF-38, and tRF-18, respectively (Figure 5).

Diagnostic accuracy of plasma tRF-25, tRF-38, and tRF-18 in GC

ROC curve analysis was used to measure the diagnostic accuracy of plasma tRF-25, tRF-38, and tRF-18. ROC curve analyses revealed that plasma tRF-25, tRF-38, and tRF-18 could serve as biomarkers for differentiating GC with the AUC of 0.825, 0.803, and 0.817, respectively (Figure 4B). At the cutoff value for plasma tRF-25, tRF-38, and tRF-18, the sensitivity and the specificity were 87.56%, 82.17%, and 82.51%, respectively. These findings suggested that plasma tRF-25, tRF-38 and tRF-18 might reflect GC dynamics and were available as new biomarkers for monitoring GC.

Table 1. The number of small RNA in GC.

	Control group 1	Control group 2	Control group 3	AR 1	AR 2	AR 3	Total	No. of different
miRNA	633	629	627	558	620	686	738	63
piRNA	39	54	50	42	43	58	58	3
tRF	263	285	281	273	263	279	288	29

Table 2. The differentially expressed small RNA in GC compared with healthy control.

The differentially expressed small RNA
1. Upregulated miRNAs in GC (12)
hsa-miR-7843, hsa-miR-7151, hsa-miR-6869, hsa-miR-6852, hsa-miR-6772, hsa-miR-6770, hsa-miR-548a, hsa-miR-4488, hsa-miR-3187, hsa-miR-3127, hsa-miR-1909, hsa-miR-1247
2. Downregulated miRNAs in GC (51)
hsa-miR-98, hsa-miR-96, hsa-miR-7705, hsa-miR-6805, hsa-miR-6747, hsa-miR-601, hsa-miR-582, hsa-miR-582, hsa-miR-548ar, hsa-miR-542, hsa-miR-5091, hsa-miR-5010, hsa-miR-500a, hsa-miR-490, hsa-miR-483, hsa-miR-4785, hsa-miR-4778, hsa-miR-4732, hsa-miR-4695, hsa-miR-4646, hsa-miR-4645, hsa-miR-4303, hsa-miR-424, hsa-miR-3944, hsa-miR-3934, hsa-miR-3911, hsa-miR-374a, hsa-miR-3610, hsa-miR-3605, hsa-miR-3192, hsa-miR-3138, hsa-miR-29c, hsa-miR-2681, hsa-miR-215, hsa-miR-2115, hsa-miR-194, hsa-miR-184, hsa-miR-183, hsa-miR-181c, hsa-miR-16-1, hsa-miR-15a, hsa-miR-149, hsa-miR-144, hsa-miR-133a, hsa-miR-1278, hsa-miR-1277, hsa-miR-10a, hsa-let-7i, hsa-let-7g, hsa-let-7f, hsa-let-7a
3. Downregulated piRNAs in GC (3)
pir-36378, pir-36225, pir-33468
4. Upregulated tRFs in GC (11)
tRF-19, tRF-24, tRF-18, tRF-17, tRF-25, tRF-26, tRF-29, tRF-2, tRF-28, tRF-38, tRF-13
5. Downregulated tRFs in GC (18)
tRF-34, tsRNA-1035, tRF-22, tRF-287, tRF-26Z, tRF-33, tRF-43, tRF-45, tRF-46, tRF-30, tRF-40, tRF-36, tRF-47, tRF-38, tRF-31, tRF-44, tRF-50, tRF-41

DISCUSSION

With the development of sequencing technologies, numerous studies on small non-coding RNAs have discovered the novel small RNA classes, especially tRFs [28]. tRFs are confirmed as important components of gene regulatory networks to control many critical pathophysiologic processes and risk factors, such as cancer, neurodegenerative disease, inherited metabolic disorder [29]. Several recent studies have revealed that tRFs are non-invasive diagnostic biomarkers for various diseases, especially in cancer [30]. Exosomes play important roles in intercellular communication through delivery of proteins, genes, and small non-coding RNAs [30]. Small non-coding RNAs in exosomes have been investigated as valuable biomarkers of various diseases [30].

In our present study, we studied the expression of tRFs in plasma exosomes from GC and health control by small RNA sequencing. We found that all plasma tRFs in the size range from 15 - 50 nt showed the small class of tRFs. While classifying tRFs into 3'- or 5'-derived tRFs is very common [31], we found that six different classes (tRF-1, tRF-3, tRF-5, i-tRF, 3'-half-tRF, and 5'-half-tRF) were present across our samples (Figure 2B). There were 1, 9, 9, 8, and 2 different expressed tRFs derived from tRF-1, tRF-3, tRF-5, i-tRF, and 5'-half-tRF, respectively (Table 3). Here, we found that the levels of tRF-25, tRF-38, and tRF-18 in plasma exosomes from GC were significantly higher than those from healthy subjects. Our results demonstrated that plasma exosomal tRF-25, tRF-38, and tRF-18 levels were significantly increased in all patients.

In GC and healthy control, there were 288 total tRFs and 29 differentially expressed tRFs. Using GO analysis, the targeted genes of these differentially expressed tRFs were involved in transcription, axon guidance, neuron migration, cytoplasm, cell junction, protein binding, DNA binding. Mapping all targeted genes to KEGG database showed that they participated in "Insulin signaling pathway", "ErbB signaling pathway", "PI-3K-Akt signaling pathway", "FoxO signaling pathway", "Neurotrophin signaling pathway", "cGMP-PKG sig-

Table 3. The differentially expressed tRFs in GC compared with healthy control.

tRFs	Fragment sequence	Type	Anticodons	Length
tRF-19	AATCCGGCTCGGAGGACCA	tRF-3	TyrGTA	19
tRF-24	GAAAATGTTTAGACGGGCTCACAT	i-tRF	PheGAA	24
tRF-18	TCCCGGGCGGAAACACCA	tRF-3	ValCAC	18
tRF-182	ATCCGGCTCGGAGGACCA	tRF-3	TyrGTA	18
tRF-25R	ATGTTTAGACGGGCTCACATCACCC	i-tRF	PheGAA	25
tRF-25	AATGTTTAGACGGGCTCACATCACC	i-tRF	PheGAA	25
tRF-29	TCCCCGGTTCGAAACCGGGCGGAAACACC	tRF-3	ValCAC, ValAAC	29
tRF-23	GTTCGAAACCGGGCGGAAACACC	tRF-3	ValCAC, ValAAC	23
tRF-256	GGTTCGAAACCGGGCGGAAACACCA	tRF-3	ValCAC, ValAAC	25
tRF-38	GCGAAAGGTCCCCGGTTCGAAACCGGGCGGAAACACCA	tRF-3	ValCAC, ValAAC	38
tRF-186	AACCGGGCGGAAACACCA	tRF-3	ValCAC, ValAAC	18
tRF-346	GGCCGGTTAGCTCAGTTGGTTAGAGCGTGGTGCT	5'-half	IleAAT	34
tsRNA-1035	GATATCCAACCTTCGGCTATAGGGTGGAGACTTTTT	tRF-1	ThrCGT	36
tRF-22	TCAGTTGGTTAGAGCGTGGTGC	i-tRF	IleAAT	22
tRF-237	GTTGGTCTAGGGGTATGATTCTCGCTTT	i-tRF	ProTGG	28
tRF-268	TTGGTCTAGGGGTATGATTCTCGCTT	i-tRF	ProAGG, ProCGG, ProTGG	26
tRF-33	GTTTCCGTAGTGTAGTGGTTATCACGTTTGCCT	5'-half	ValAAC	33
tRF-43	GCCTTCAAAGCCCTCAGTAAGTTGCAATACTTAATTTCTGCCA	tRF-3	TrpTCA	43
tRF-45	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGATTAA	tRF-5	LysTTT	45
tRF-46	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGATTAAG	tRF-5	LysTTT	46
tRF-304	CTCGTTGGTCTAGGGGTATGATTCTCGCTT	i-tRF	ProAGG, ProCGG, ProTGG	30
tRF-40	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAG	tRF-5	LysTTT	40
tRF-36	GTTTCCGTAGTGTAGCGGTTATCACATTCGCCTCAC	tRF-5	ValCAC	36
tRF-47	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGATTAAGA	tRF-5	LysTTT	47
tRF-38	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAA	tRF-5	LysTTT	38
tRF-239	GCTCGTTGGTCTAGGGGTATGATTCTCGCTT	i-tRF	ProAGG, ProCGG, ProTGG	31
tRF-44	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGATTA	tRF-5	LysTTT	44
tRF-50	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGATTAA GAGAA	tRF-5	LysTTT	50
tRF-41	CACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGA	tRF-5	LysTTT	41

naling pathway", "Wnt signaling pathway", "MAPK signaling pathway", "Ras signaling pathway", "Calcium signaling pathway" and "Endocytosis". Dysregulated tRFs and their target genes are involved in several signaling pathways (Calcium [31], MAPK [31], PI3K-Akt and Wnt [32]) of GC.

In this study, we found that plasma exosomal tRF-25, tRF-38, and tRF-18 were significantly up regulated in the patients with GC compared to healthy controls. We developed a model including the three tRFs (tRF-25, tRF-38, and tRF-18) to diagnose GC with an average AUC of 0.815. The tRF panel had higher diagnostic

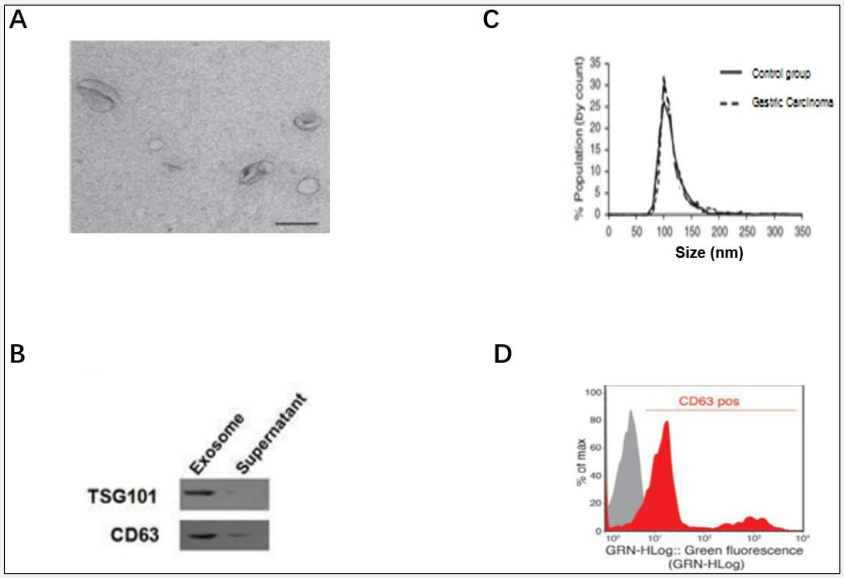

Figure 1. Verification of exosomes from plasma in GC and healthy control.

The morphology of exosomes was analyzed by TEM (scale bar, 200 nm).

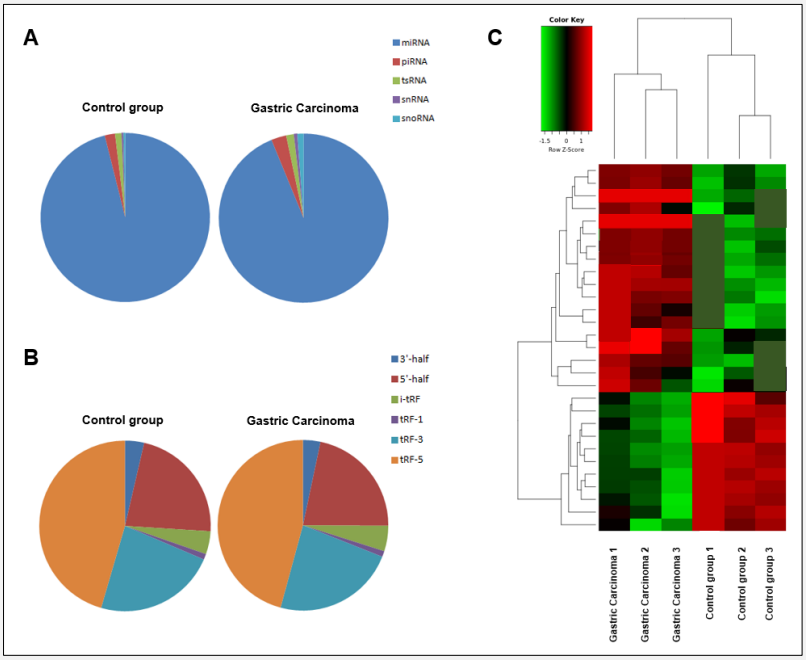

Figure 2. Differential expression analysis of tRFs in GC compared with healthy control.

(A) The types of small non-coding RNAs in plasma exosomes from GC and healthy control. (B) The types of tRFs in plasma exosomes from GC and healthy control. (C) Heatmap depicting the expression of the 29 different expressed tRFs across all six samples.

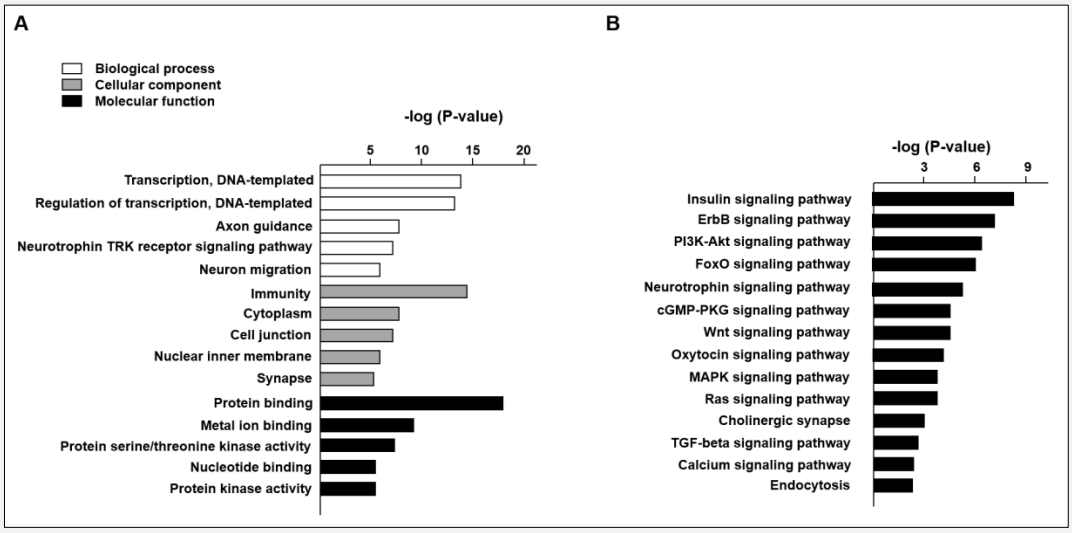

Figure 3. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) classification for targeted genes of differentially expressed tRFs in GC compared with healthy control.

GO results are summarized in three main categories: biological process, cellular component, and molecular function.

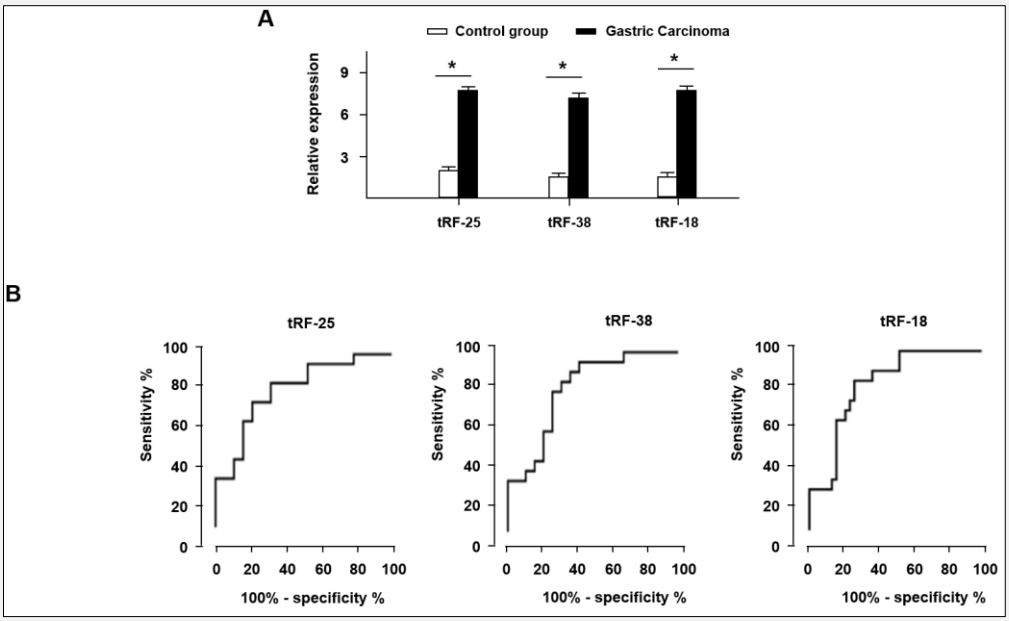

Figure 4. Verification of tRFs between GC and healthy control by RT-PCR and ROC.

The expression of three highly expressed tRFs between GC and healthy control were compared by paired sample with *t*-test method (n = 47, $p < 0.05$).

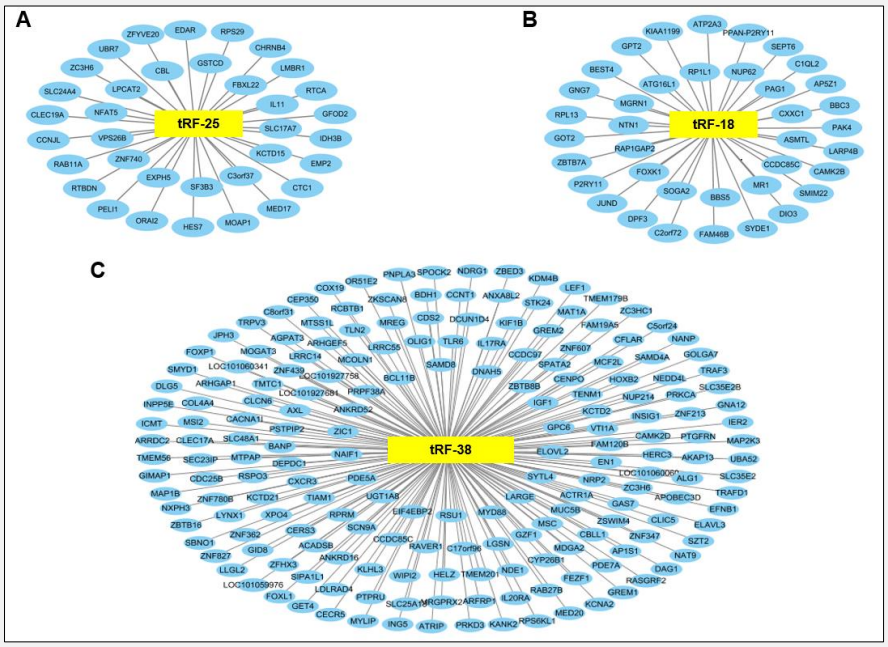

Figure 5. The potential targets of tRF-25, tRF-38, and tRF-18.

sensitivity and specificity for GC compared to not only each of the three tRFs biomarkers independently but also some miRNA biomarkers that are widely used in clinical testing [33,34]. Additionally, our validated tRFs were found primarily in plasma exosomes and could be protected from endogenous RNase degradation.

There are some limitations in our study. The sample size is small and long-term follow-up is required to confirm the relationship between exosomal tRF-25, tRF-38, and tRF-18 expression and patient outcome. In addition, the potential mechanisms of exosomal tRF-25, tRF-38, and tRF-18 in GC still need further studies.

CONCLUSION

Our findings suggest that plasma exosomal tRF-25, tRF-38, and tRF-18 may serve as novel serological biomarkers with significant accuracy in predicting GC patients. Exosomal tRF-25, tRF-38, and tRF-18 may help discriminate GC patients with a high risk of recurrence and poor prognosis.

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Ethics Approval and Consent to Participate:
This study was conducted based on our protocols approved by the Ethical Committee of Fujian Provincial Hospital. All patients signed written informed consent documents prior to this study.

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Authors' Contributions:
CFL designed the experiments. CFL and LWZ performed the experiments and acquired the data. RLH, GHY, JYC and HQL analyzed the data. CFL prepared the manuscript. All authors read and approved the final manuscript.

Declaration of Interest:
The authors declare no conflict of interest.

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