Screening and potential role of tRFs and tiRNAs derived from tRNAs in the carcinogenesis and development of lung adenocarcinoma

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Abstract. Accumulating evidence has indicated that a group of novel molecules, known as transfer RNA (tRNA)-derived fragments (tRFs) and tRNA halves (tiRNAs), which are derived from tRNAs, serve an essential role in numerous types of human disease, in particular solid tumors. However, to the best of our knowledge, the underlying mechanisms of the effect of tRFs and tiRNAs in lung adenocarcinoma have not been reported. The present study aimed to determine the differential expression levels of tRFs and tiRNAs in lung adenocarcinoma and adjacent tissues using a NextSeq system, and further investigated their potential target genes via bioinformatics analysis. Kyoto Encyclopedia of Genes and Genomes signaling pathway and Gene Ontology functional term enrichment analyses were performed to investigate the function of these target genes in the occurrence and development of lung adenocarcinoma. In patients with lung adenocarcinoma, 338 types of tRFs and tiRNAs were detected via sequencing, 284 of which were not previously reported in the tRF database. Compared with the adjacent tissues, 17 types of tRFs and tiRNAs comprising 34 subtypes were found to be abnormally expressed in lung adenocarcinoma tissues, 20 of which were upregulated and 14 downregulated. Reverse transcription-quantitative PCR verification revealed that the expression levels of tiRNA-Lys-CTT-002, tRF-Val-CAC-010 and tRF-Val-CAC-011 were significantly upregulated, while those of tRF-Ser-TGA-005 were downregulated in lung adenocarcinoma tissues. Bioinformatics analysis identified that tRF-Ser-TGA-005 participated in the 'cellular response to transforming growth factor β stimulus' and tRF-Val-CAC-010 and tRF-Val-CAC-011 participated in the 'Hedgehog signaling pathway'. In conclusion, the results of the present study suggested that tRFs and tiRNAs may be closely associated with the pathogenesis and development of lung adenocarcinoma, providing a novel insight for further studies into lung adenocarcinoma.

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Introduction

Lung cancer remains the leading cause of cancer-associated morbidity (~787,000 cases) and mortality (~622,000 deaths) in China in 2015 (1). The mortality rate of male patients is particularly high in the Yunnan province, where tobacco is a mainstay of the local economy (2). Overall, ~85% of patients with lung cancer are diagnosed with non-small cell carcinoma (NSCLC), which comprises large cell carcinoma, lung adenocarcinoma and squamous cell carcinoma subtypes (3,4). More than 80% of patients are dead by 5 years (5-7). Due to the poor prognosis and ineffective screening methods available for lung adenocarcinoma, the clinical cure rate for lung adenocarcinoma remains low.

Transfer RNA (tRNA) is involved in protein synthesis by recognizing and transporting specific amino acids (8). Previous research has focused on investigating the additional functions of tRNA. For example, several studies have identified tRNA-derived fragments (tRFs) and tRNA halves (tiRNAs), which are classes of non-coding small RNA fragments of <40 nucleotides in length derived from tRNA transcripts (9,10). In the context of stress, mature tRNA or precursor tRNA can be precisely cleaved by specific nucleases (such as Dicer and angiogenin) into specific-sized fragments

with regulatory functions, instead of undergoing random tRNA degradation (11-13). Stress-induced tRFs and tiRNAs have been discovered to serve a role in cancer, metabolic diseases and nervous system disorders (14). tRFs and tiRNAs are enriched in biological body fluids, and their composition and quantity are highly dependent on the cell type and disease state (15). Consequently, tRFs and tiRNAs may represent novel non-invasive biomarkers for disease diagnosis (16).

Previous studies have categorized tRFs into the following types: tRF-1, tRF-2, tRF-3 and tRF-5 (9,17-19). In addition, there are currently two known types of tiRNAs, namely 5'-tiRNAs and 3'-tiRNAs (20). tRFs and tiRNAs are similar to microRNA (miRNA) in their structure and function (21,22); however, they are more stable and abundant than miRNA. tRFs and tiRNAs have been reported to suppress mRNA translation of ribosomal proteins (23) and to decrease mRNA stability by binding to Y-box-binding protein 1 (24). In addition, tRFs and tiRNAs have been demonstrated to regulate translation initiation and elongation (25,26). A previous study has revealed that tiRNAs can prevent apoptosis by binding to cytochrome *c* (27). These findings provided insight into the role of tRFs and tiRNAs on proliferation, apoptosis, invasion and metastasis in tumor cells (28). Thus, it is of great significance to study the molecular mechanisms of tRFs and tiRNAs with regards to their role in the development of lung adenocarcinoma to identify reliable biomarkers and novel drug targets. However, the regulatory effects and molecular mechanisms of tRFs and tiRNAs remain poorly understood.

High-throughput sequencing and analysis of small RNA fragments can be used to identify tRFs and their corresponding known tRNA sequences. The present study aimed to use RNA sequencing (RNA-seq) technologies to analyze the expression levels of tRFs and tiRNAs in lung adenocarcinoma and adjacent tissues, some of which were reported for the first time. In addition, the biological functions of tRFs and tiRNAs were evaluated using bioinformatics analysis. The current findings may provide a novel perspective into the molecular mechanisms underlying lung adenocarcinoma development.

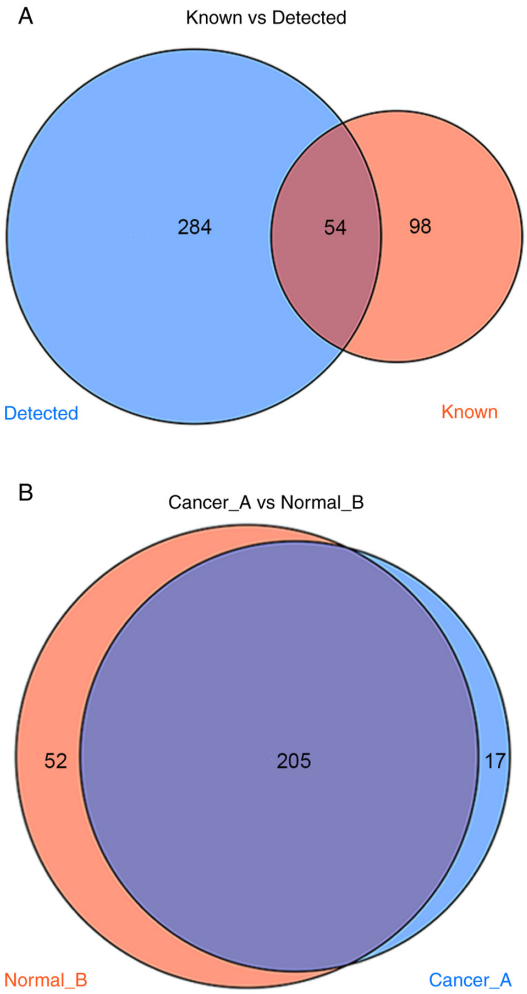

Figure 1. tRFs and tiRNAs screened from lung adenocarcinoma and adjacent tissues. (A) Venn diagram of the number of tRFs and tiRNAs known and stored in the tRF database and those detected in the present study. (B) Venn diagram based on the number of common and differentially expressed tRFs and tiRNAs in lung adenocarcinoma and adjacent tissues. tRFs, transfer RNA-derived fragments; tiRNAs, transfer RNA halves.

Materials and methods

Patient studies. A total of three pairs of lung adenocarcinoma and adjacent tissues were collected from patients in August 2018 at the Department of Pathology in The First People's Hospital of Yunnan Province (Kunming, China). Two patients were female and one was male. All patients were between 51-70 years of age, with a mean age of 58.3 years. The tissues were preserved in RNAlater (Invitrogen; Thermo Fisher Scientific, Inc.) at -80˚C. Both the intraoperative frozen and paraffin-embedded tissues (~3-μm-thick) were diagnosed as infiltrating lung adenocarcinoma. The protocol of the present study was approved by the Ethics Committee of The First People's Hospital of Yunnan Province. All patients provided written informed consent prior to participation.

RNA extraction and quality control. Total RNA was extracted from tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The integrity and quantity of each RNA sample were subsequently analyzed using 1% agarose gel electrophoresis and a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

Pretreatment of tRF and tiRNA. tRFs and tiRNAs have heavy RNA modifications that interfere with small RNA-seq library construction. Therefore, total RNA was pretreated to remove the RNA modifications, including 3'-aminoacyl deacetylation, 2',3'-cyclic phosphate, 5'-OH (hydroxyl group) phosphorylation and m1A and m3C demethylation, using a rtStar™ tRF and tiRNA Pretreatment kit (cat. no. AS-FS-005; Arraystar Inc.). Preprocessed total RNA samples were subsequently used for tRF- and tiRNA-seq library preparation.

Library preparation. Sequencing libraries were size-selected for the RNA biotypes to be sequenced using an automated gel cutter. The sequencing library was quantified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) using an Agilent DNA 1000 chip kit (Agilent Technologies, Inc.). Libraries were mixed in equal amounts and used for sequencing.

Sequencing. The libraries were qualified and absolutely quantified using an Agilent BioAnalyzer 2100 (Agilent Technologies, Inc.). Mixed DNA fragments in the libraries were denatured with 0.1 M NaOH to generate single-stranded molecules, and

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'→3')	Product length, bp
U6	F: GCTTCGGCAGCACATATACTAAAAT	89
	R: CGCTTCACGAATTTGCGTGTCAT	
tiRNA-Lys-CTT-002	F: ATCGCCCGGCTAGCTCAGT	45
	R: TCCGATCTGAGTCTCATGCTCTAC	
tRF-Val-CAC-011	F: CTTCTGTAGTGTAGTGGTTATCACG	46
	R: GTGCTCTTCCGATCTGGC	
tRF-Val-CAC-010	F: CTTCTGTAGTGTAGTGGTTATCACG	45
	R: GTGCTCTTCCGATCTGCG	
tRF-Val-CAC-007	F: CGATCGCTTCTGTAGTGTAGTGG	46
	R: GCTCTTCCGATCTAACGTGATAA	
tRF-His-GTG-008	F: CGATCGCCGTGATCGTATAGT	44
	R: TCCGATCTCGCAGAGTACTAACC	
tRF-Ser-AGA-006	F: AGTCCGACGATCGTAGTCGTG	42
	R: CGATCTCCTTAACCACTCGGC	
tRF-Glu-TTC-027	F: CGACGATCTGACTGGACCTTT	45
	R: GACGTGTGCTCTTCCGATCTAA	
tRF-Lys-CTT-001	F: GATCGCCCAGCTAGCTCAGT	47
	R: GTGCTCTTCCGATCTTATGCTCT	
tRF-Ser-TGA-005	F: GATCGAAGCGGGTGCTCT	41
	R: GACGTGTGCTCTTCCGATCTAT	
tRF-His-GTG-012	F: AGTCCGACGATCTCGAATCC	40
	R: CGATCTTGGTGCCGTGACTC	

tRF, transfer RNA-derived fragment; tiRNA, transfer RNA halves; F, forward; R, reverse.

loaded onto the reagent cartridge at a concentration of 1.8 pM. The sequencing was performed on an Illumina NextSeq 500 system (Illumina, Inc.) using a NextSeq 500/550 V2 kit (cat. no. FC-404-2005; Illumina, Inc.) according to the manufacturer's protocol. The sequencing type was 50-bp single-read.

Data analysis. The tRFs and tiRNAs were identified by mapping to tRFdb (http://genome.bioch.virginia.edu/trfdb/). Image analysis and base calling were performed using Solexa Pipeline v1.8 software (Off-Line Base Caller software; Illumina, Inc.). Sequencing quality was first examined using FastQC v0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and trimmed reads were aligned to allow for only one mismatch to the mature tRNA sequences. Reads that did not map were aligned to allow for only one mismatch to precursor tRNA sequences using bowtie v1.2.2 software (29). The remaining reads were aligned to allow for only one mismatch to miRNA reference sequences with miRDeep2 v2.0.0.8 (30). The abundance of tRF, tiRNA and miRNA was evaluated using their sequencing counts, which were normalized as counts per million (CPM) of total aligned reads. Differentially expressed tRFs and tiRNAs were screened based on the count value using the R package edgeR (31). Principal component analysis (PCA), Pearson correlation analysis, pie plots, Venn plots, hierarchical clustering, scatter plots and volcano plots were generated in R (https://www.r-project.org/) or Perl (https://www.perl.org/) for statistical computing and visualization of the differentially expressed tRFs and tiRNAs. The cut-off values for differentially expressed tRFs and tiRNAs were log2 fold-change (FC) ≥1.4 and P≤0.05. The differentially expressed tRFs and tiRNAs were selected for further analysis.

Reverse transcription-quantitative PCR (RT-qPCR). Preprocessed total RNA samples of the three lung adenocarcinoma and adjacent tissues were reverse transcribed into cDNA using a rtStar™ First-Strand cDNA Synthesis kit (cat. no. AS-FS-003; Arraystar, Inc.) according to the manufacturer's protocol, and 2X SYBR Green qPCR master mix (cat. no. AS-MR-005; Arraystar, Inc.) was used for qPCR analysis. Primers were designed using Primer v5.0 (Premier Biosoft International) and synthesized by Aksomics, Inc. Primer sequences are presented in Table I. qPCR was performed on a QuantStudio™ 5 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following thermocycling conditions: Initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 60 sec. Following amplification, the system was slowly heated from 60 to 99˚C at a ramp rate of 0.05˚C/sec to establish the melting curve of the PCR products. Data for relative quantification of tRFs and tiRNAs was obtained by normalization to U6 expression. The expression levels were determined using the $2^{-\Delta\Delta Cq}$ method for relative quantification of gene expression (32).

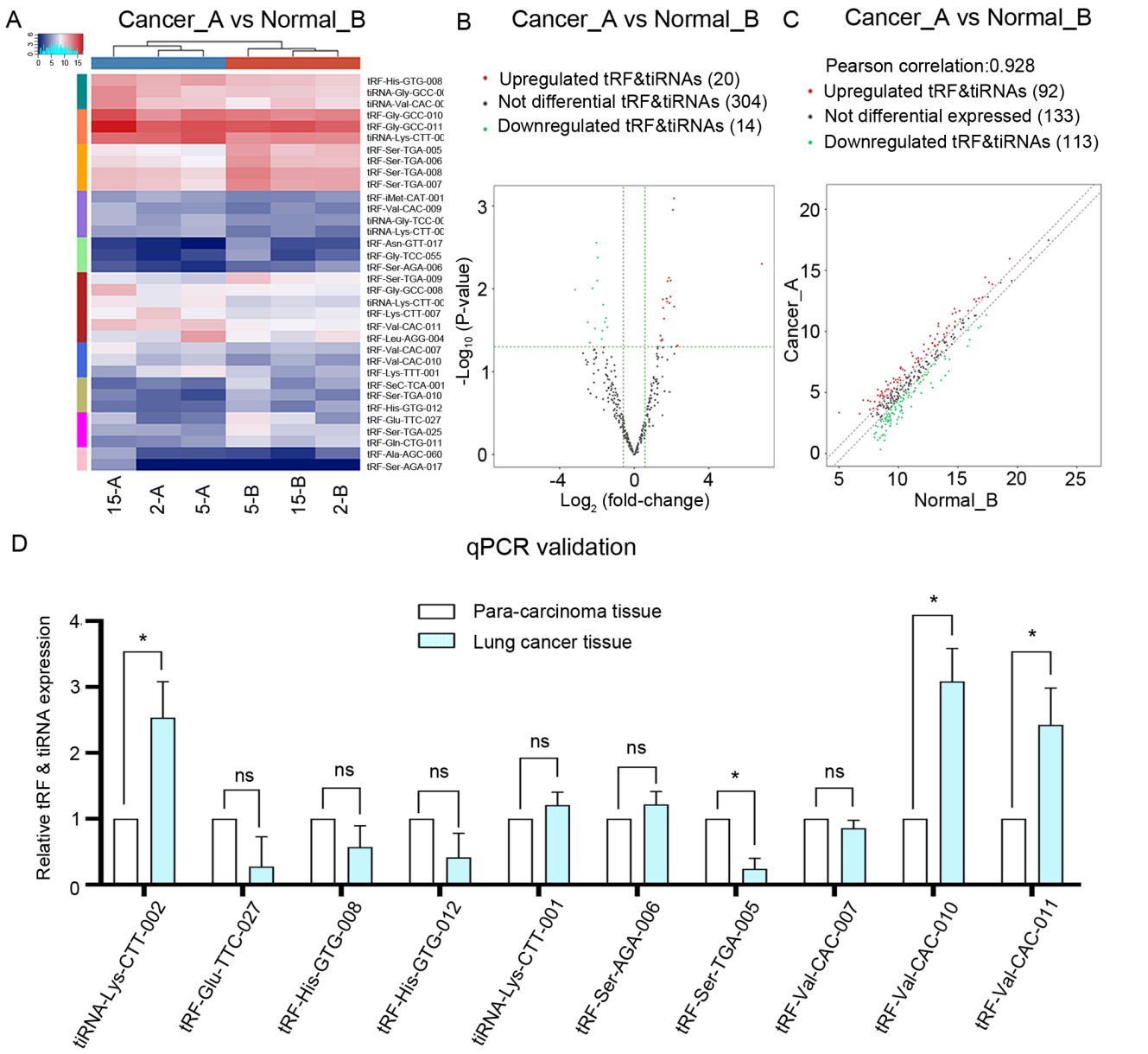

Figure 2. Differently expressed tRFs and tiRNAs identified between lung adenocarcinoma and adjacent tissues. (A) Heat map demonstrating the significant changes in the expression levels of tRFs and tiRNAs between lung adenocarcinoma and adjacent tissues. Red represents high expression; blue represents low expression. (B) Volcano plot showing the number of differentially expressed tRFs and tiRNAs. (C) Scatter plot representing the differentially expressed tRFs and tiRNAs in lung adenocarcinoma and adjacent tissues. (D) Reverse transcription-qPCR analysis of the top 10 differentially expressed tRFs and tiRNAs. *P<0.05. ns, not significant; tRFs, transfer RNA-derived fragments; tiRNAs, transfer RNA halves; qPCR, quantitative PCR.

Bioinformatics analysis. The structures of tRNA were downloaded from the Leipzig tRNA database (http://trna.bioinf.uni-leipzig.de/DataOutput/). As tRFs and tiRNA have been reported to be similar to miRNA in function (21,22), miRanda v3.3a (33) and TargetScan v7.1 software (34) were used to predict the target mRNAs of validated tRFs and tiRNAs (35-37). Gene Ontology (GO) functional term enrichment analysis (http://www.geneontology.org/) was used to determine functional terms of the target genes. Signaling pathway enrichment analysis was used to investigate the significant pathways of the target genes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc.). Results are represented as the mean ± SD of 3 parallel samples. Statistical differences between two groups were determined using a two-tailed paired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

tRF and tiRNA expression profiling in patients with lung adenocarcinoma. There are currently 152 known types of tumor-associated tRFs and tiRNAs that have been reported in the tRF database. A total of 338 types were detected in the sequencing performed in the present study, of which 54 overlapped with the database and the other 284 were novel, to the best of our knowledge (Fig. 1A). Compared with adjacent tissues, 17 differentially expressed tRFs and tiRNAs

Table II. Differentially expressed tRFs and tiRNAs in lung adenocarcinoma.

tRF_ID	Length, nt	log_2FC	FC	P-value	Q-value	Cancer_CPM	Normal_CPM	Regulation
tRF-Ser-AGA-017	32	6.84	114.92	0.00500	0.30239	3.35	-3.49	Up
tRF-Ala-AGC-060	18	2.33	5.02	0.04836	0.42981	4.37	2.04	Up
tRF-Leu-AAG-004	16	2.27	4.83	0.04968	0.42981	10.64	8.37	Up
tRF-Lys-TTT-001	15	2.14	4.41	0.01641	0.30239	8.25	6.11	Up
tiRNA-Lys-CTT-002	34	2.13	4.39	0.00081	0.18848	14.43	12.30	Up
tRF-Val-CAC-011	32	2.08	4.23	0.00112	0.18848	10.44	8.36	Up
tiRNA-Lys-CTT-005	34	1.95	3.86	0.00794	0.30239	5.91	3.96	Up
tRF-Lys-CTT-007	31	1.94	3.85	0.01241	0.30239	9.63	7.69	Up
tiRNA-Val-CAC-001	34	1.90	3.72	0.01487	0.30239	11.44	9.54	Up
tRF-Val-CAC-010	31	1.86	3.64	0.00737	0.30239	7.07	5.20	Up
tiRNA-Lys-CTT-001	34	1.78	3.44	0.00809	0.30239	8.93	7.14	Up
tRF-Val-CAC-007	28	1.77	3.40	0.01435	0.30239	7.98	6.21	Up
tiRNA-Gly-GCC-002	33	1.74	3.34	0.01310	0.30239	11.55	9.82	Up
tRF-Gly-GCC-011	32	1.60	3.03	0.02293	0.36900	15.95	14.35	Up
tiRNA-Gly-TCC-001	34	1.55	2.93	0.01700	0.30239	6.14	4.59	Up
tRF-His-GTG-008	31	1.54	2.91	0.01337	0.30239	11.68	10.14	Up
tRF-Gly-GCC-008	29	1.52	2.87	0.04077	0.42981	9.80	8.28	Up
tRF-Val-CAC-009	30	1.48	2.80	0.04184	0.42981	5.60	4.12	Up
tRF-iMet-CAT-001	30	1.44	2.71	0.04220	0.42981	5.77	4.33	Up
tRF-Gly-GCC-010	31	1.41	2.65	0.03684	0.42981	14.02	12.61	Up
tRF-Asn-GTT-017	15	-3.17	0.11	0.01026	0.30239	0.34	3.51	Down
tRF-Glu-TTC-027	17	-2.49	0.18	0.02539	0.37306	5.40	7.89	Down
tRF-Gly-TCC-055	14	-2.38	0.19	0.04466	0.42981	1.49	3.86	Down
tRF-SeC-TCA-001	15	-2.24	0.21	0.00987	0.30239	3.84	6.08	Down
tRF-Ser-AGA-006	24	-2.10	0.23	0.03021	0.40839	1.86	3.96	Down
tRF-Ser-TGA-005	17	-2.02	0.25	0.00277	0.30239	8.80	10.82	Down
tRF-Ser-TGA-025	15	-1.98	0.25	0.00797	0.30239	5.75	7.74	Down
tRF-Gln-CTG-011	14	-1.96	0.26	0.00421	0.30239	4.95	6.91	Down
tRF-Ser-TGA-010	22	-1.74	0.30	0.04032	0.42981	3.52	5.26	Down
tRF-His-GTG-012	22	-1.70	0.31	0.03203	0.41640	3.45	5.15	Down
tRF-Ser-TGA-006	18	-1.66	0.32	0.01559	0.30239	9.33	10.99	Down
tRF-Ser-TGA-009	21	-1.58	0.33	0.02526	0.37306	7.71	9.29	Down
tRF-Ser-TGA-008	20	-1.52	0.35	0.02253	0.36900	10.42	11.94	Down
tRF-Ser-TGA-007	19	-1.44	0.37	0.02905	0.40839	10.29	11.73	Down

tRF, transfer RNA-derived fragments; tiRNA, transfer RNA halves; FC, fold-change; CPM, counts per million; Up, upregulated; Down, downregulated.

were identified in all three cancer tissues during sequencing (Fig. 1B). Clustering results revealed that there were 34 differently expressed subtypes of tRF and tiRNA between the two groups (Fig. 2A), among which 20 were upregulated and 14 were downregulated (Fig. 2B). The correlation in identified tRFs and tiRNAs between lung adenocarcinoma and adjacent tissues was examined using a scatter plot (Fig. 2C). In both the lung adenocarcinoma and adjacent normal tissues, the three samples were clustered together. The differentially expressed tRFs and tiRNAs are listed in Table II. To validate these changes, ten types of tRF and tiRNA were selected and their expression levels were analyzed using RT-qPCR. As shown in Fig. 2D, four genes out of the 10 measured transcripts demonstrated differential expression between the three lung adenocarcinoma and adjacent tissues, namely tiRNA-Lys-CTT-002, tRF-Ser-TGA-005, tRF-Val-CAC-010 and tRF-Val-CAC-011. In the lung adenocarcinoma group, 14 tRF-1, 1 tRF-2, 28 tRF-3a, 6 tRF-3b, 21 tRF-5a, 3 tRF-5b, 108 tRF-5c, 1 tiRNA-3 and 40 tiRNA-5 were identified (Fig. 3A). In the adjacent tissues, 19 tRF-1, 5 tRF-2, 39 tRF-3a, 9 tRF-3b, 31 tRF-5a, 4 tRF-5b, 110 tRF-5c, 1 tiRNA-3 and 39 tiRNA-5 were identified (Fig. 3B).

Functional enrichment analysis reveals a significant association between tRFs, tiRNAs and carcinogenesis. Several previous studies have demonstrated that tRFs and

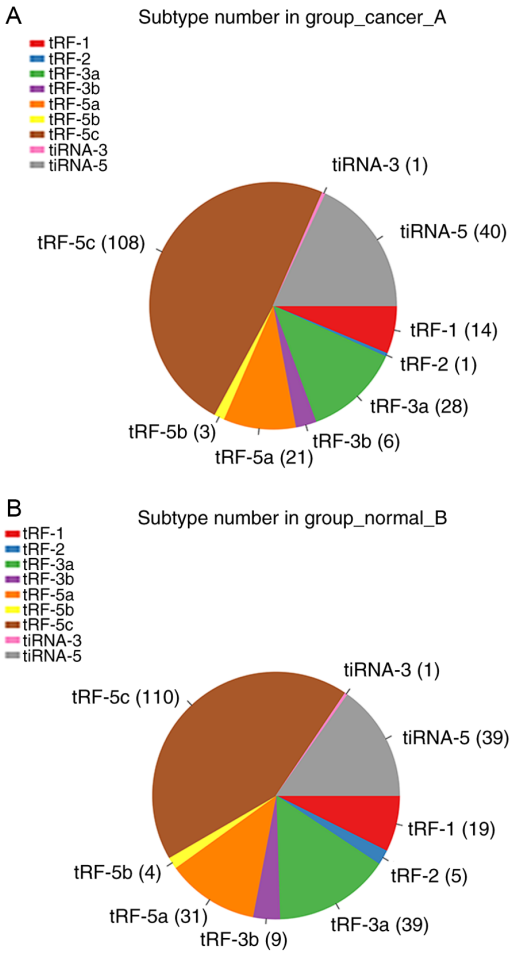

Figure 3. Pie charts of the distribution of tRF and tiRNA subtypes. (A) Lung adenocarcinoma and (B) adjacent tissues. tRFs, transfer RNA-derived fragments; tiRNAs, transfer RNA halves.

tiRNAs are involved in translational regulation and gene silencing (18,38). tRFs and tiRNAs have a miRNA-like structure and function, and can therefore inhibit protein translation (21,22). Based on these characteristics, target genes of tRFs and tiRNAs were identified using miRanda and TargetScan software. Fig. 4A presents the association between tiRNA-Lys-CTT-002, tRF-Ser-TGA-005, tRF-Val-CAC-010 and tRF-Val-CAC-011 and their potential targets, such as cyclin D1 (CCND1), C-X-C motif chemokine ligand (CXCL)1, CXCL2 and glioma-associated oncogene homolog 2 (Gli2). The cleavage site of tiRNA-Lys-CTT-002, tRF-Ser-TGA-005, tRF-Val-CAC-010 and tRF-Val-CAC-011 was further investigated (Fig. 4B). tiRNA-Lys-CTT-002, tRF-Val-CAC-010 and tRF-Val-CAC-011 were generated by the cleavage in or near the tRNA anticodon loop.

To further investigate the possible mechanisms and potential functions of the significant heterogeneity in the expression levels of tRFs and tiRNAs, GO functional term and KEGG signaling pathway analyses based on the predicted target genes were performed. KEGG signaling pathway enrichment analysis revealed that the altered target genes of differentially expressed tRFs and tiRNAs were mostly enriched in 'SNARE interactions in vesicular transport' with tiRNA-Lys-CTT-002 (Fig. 5), 'cushing syndrome' with tRF-Ser-TGA-005 (Fig. 6) and 'Hedgehog signaling pathway' with tRF-Val-CAC-010 and tRF-Val-CAC-011 (Figs. 7 and 8). The significantly enriched GO terms of tiRNA-Lys-CTT-002 included 'vesicle docking' (GO:0048278), 'exocytic process' (GO:0140029) and 'synaptic vesicle fusion to presynaptic active zone membrane' (GO:0031629) (Fig. 9A). The target genes of tiRNA-Lys-CTT-002 were also enriched in 'response to glucose' (GO:0009749), 'response to hexose' (GO:0009746) and 'response to monosaccharide' (GO:0034284) (Fig. 9A). Notably, several proliferation-associated terms of tRF-Ser-TGA-005 were detected, including 'cellular response to transforming growth factor β stimulus' (GO:0071560), 'response to transforming growth factor β' (GO:0071559), 'regulation of cellular response to growth factor stimulus' (GO:0090287), 'transforming growth factor β receptor signaling pathway' (GO:0007179) and 'regulation of transforming growth factor β receptor signaling pathway' (GO:0017015) (Fig. 9B). Several cellular metabolism-associated terms of tRF-Ser-TGA-005 were enriched, including 'negative regulation of cellular metabolic process' (GO:0031324) and 'negative regulation of macromolecule metabolic process' (GO:0010605) (Fig. 9B). The most significantly enriched GO terms of tRF-Val-CAC-010 were 'caveola' (GO:0005901), 'plasma membrane raft' (GO:0044853) and 'cell part' (GO:0044464) (Fig. 9C), which were similar to those of tRF-Val-CAC-011 (Fig. 9D). Moreover, the terms of tRF-Val-CAC-010 and tRF-Val-CAC-011 included 'translation repressor activity' (GO:0030371) and 'lung alveolus development' (GO:0048286) (Fig. 9C and D).

Discussion

The presence of tRFs and tiRNAs has been detected in several types of human cells or tissues, including breast cancer cells (24), ovarian cancer cells (39) and peripheral blood mononuclear cells (40). Due to the lack of oxygen and nutrients in the microenvironment, tumor cells adapt to the microenvironment through different regulatory mechanisms to ensure their survival and proliferation (41). The production of tRFs and tiRNAs from tRNAs under stress is one of the most important pathways of tRF and tiRNA production. tRFs and tiRNAs have been reported to serve a role in lung cancer (42,43). Lung adenocarcinoma represents ~40% of all types of lung cancer and usually evolves from the mucosal glands (44). Despite recent improvements in the early diagnosis and clinical treatment strategies, the prognosis for patients with lung adenocarcinoma remains poor (44). The development of lung adenocarcinoma is influenced by a number of factors, such as post-translational protein modifications, activation of oncogenes and silencing of tumor suppressor genes (45). Current research has increasingly focused on the biological function of small non-coding RNAs in numerous types of disease (42). The results of the present study demonstrated that the expression levels of tiRNA-Lys-CTT-002, tRF-Ser-TGA-005, tRF-Val-CAC-010 and tRF-Val-CAC-011 were significantly different in lung adenocarcinoma tissues compared with in adjacent tissues. The current findings suggested that the altered expression levels of tRFs and tiRNAs may be associated with the tumorigenesis of lung adenocarcinoma. However, despite breakthroughs in determining the roles of tRFs and tiRNAs in previous studies (10,43), the current understanding of the

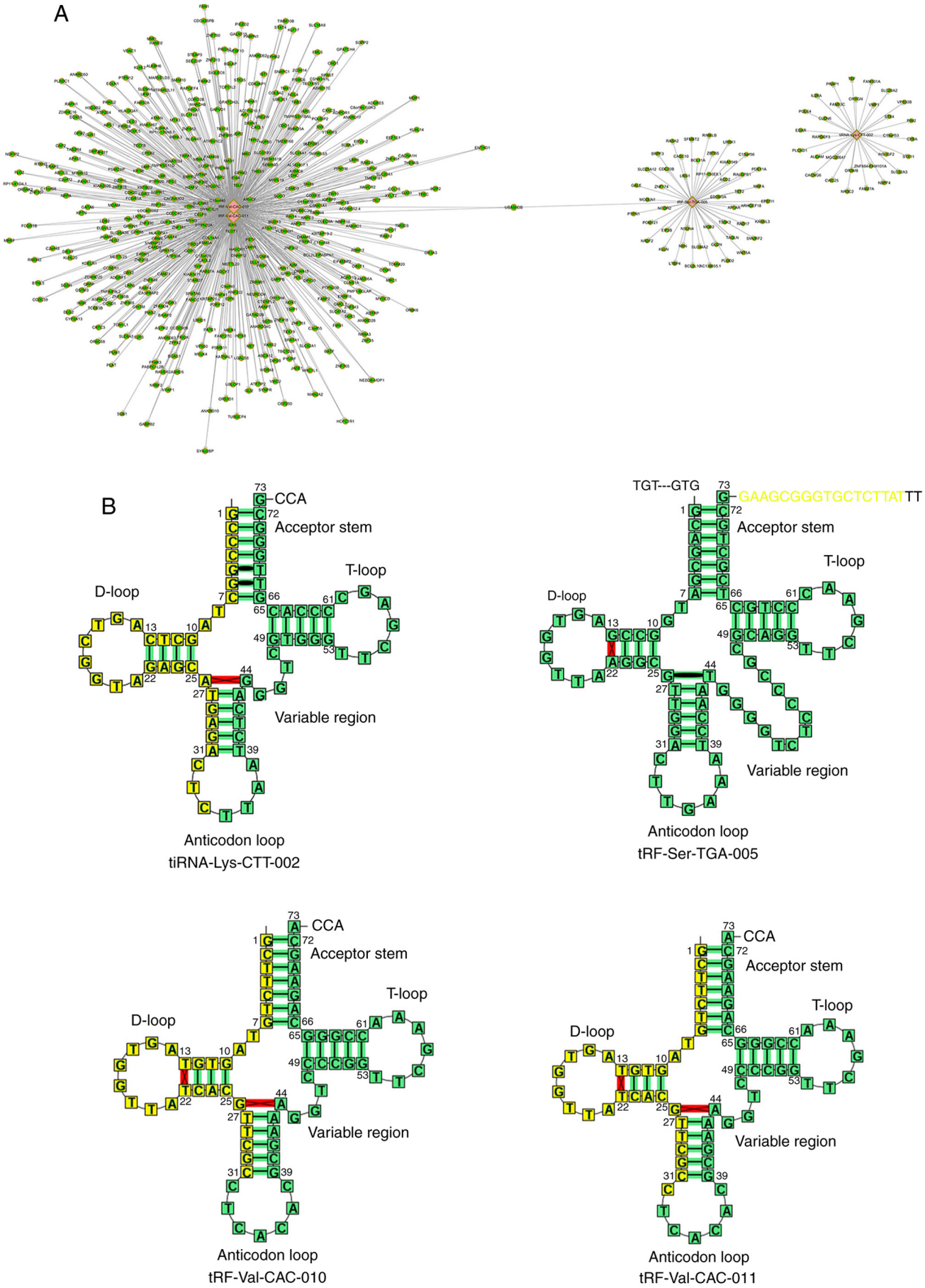

Figure 4. Association and structure of differentially expressed tRFs and tiRNAs. (A) Association between tRFs and target genes. Red represents tRF and tiRNA; green represents the altered target genes. (B) Structure of tRFs and tiRNAs. Yellow represents the sequences of tRF and tiRNA. Green represents the remaining sequences of mature tRNA after cleavage. tRFs, transfer RNA-derived fragments; tiRNAs, transfer RNA halves.

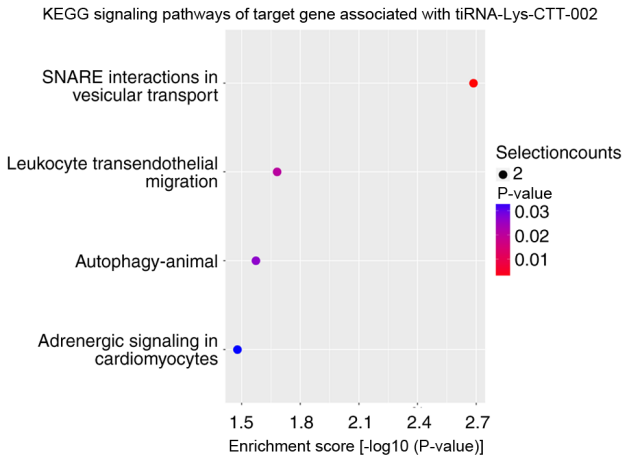

Figure 5. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tiRNA-Lys-CTT-002. tiRNAs, transfer RNA halves; KEGG, Kyoto Encyclopedia of Genes and Genomes.

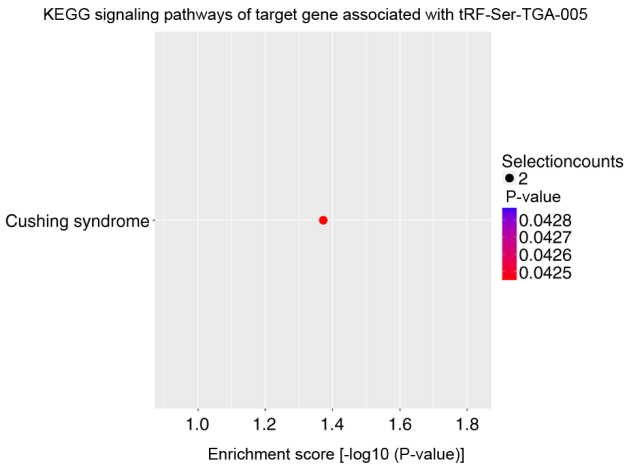

Figure 6. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tRF-Ser-TGA-005. tRFs, transfer RNA-derived fragments; KEGG, Kyoto Encyclopedia of Genes and Genomes.

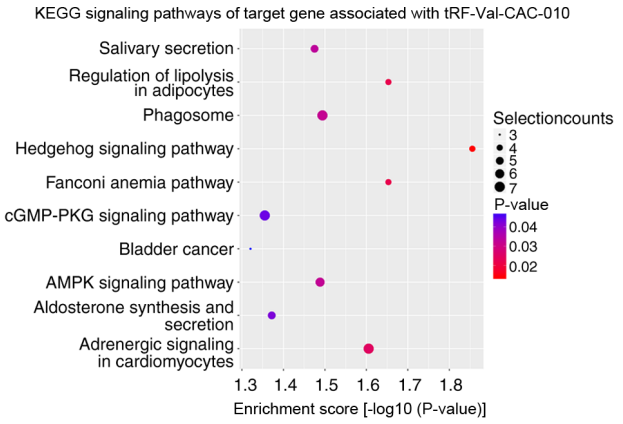

Figure 7. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tRF-Val-CAC-010. tRFs, transfer RNA-derived fragments; KEGG, Kyoto Encyclopedia of Genes and Genomes.

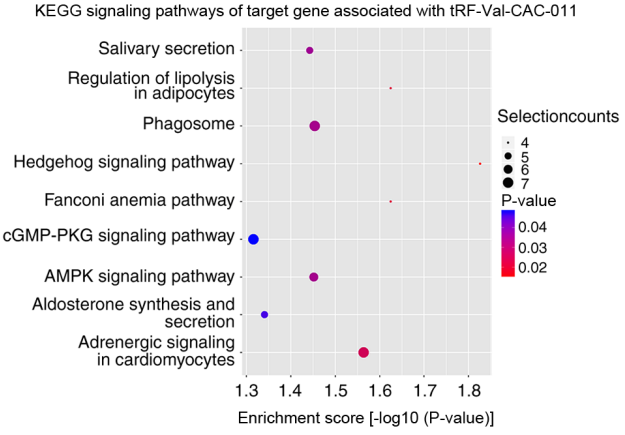

Figure 8. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tRF-Val-CAC-011. tRFs, transfer RNA-derived fragments; KEGG, Kyoto Encyclopedia of Genes and Genomes.

regulatory mechanisms of the majority of tRFs and tiRNAs remains limited.

Previous studies have reported that tRFs and tiRNAs are associated with the occurrence of lung cancer (42,43). To the best of our knowledge, the present study was the first to report that the expression levels of tiRNA-Lys-CTT-002, tRF-Val-CAC-010 and tRF-Val-CAC-011 were significantly upregulated, while those of tRF-Ser-TGA-005 were downregulated in lung adenocarcinoma tissues. Pekarsky *et al* (10) found that the expression levels of ts-4521 (derived from tRNA-Ser) and ts-3676 (derived from tRNA-Thr) were significantly downregulated in lung cancer tissue samples compared with matched normal lung tissue samples. Furthermore, Balatti *et al* (43) demonstrated that the low ts-4521 expression was associated with cell proliferation and apoptosis signaling pathways. The findings of the aforementioned studies indicated that tRFs and tiRNAs may participate in lung carcinogenesis.

To determine the potential role of the altered expression levels of tRFs and tiRNAs, the present study performed GO functional term and KEGG signaling pathway enrichment analyses on the target genes of altered tRFs and tiRNAs. Several proliferation-associated terms were discovered to be associated with tRF-Ser-TGA-005, including 'cellular response to transforming growth factor β stimulus' (GO:0071560), 'response to transforming growth factor β' (GO:0071559), 'transforming growth factor β receptor signaling pathway' (GO:0007179) and 'regulation of transforming growth factor β receptor signaling pathway' (GO:0017015). During the multistep development of tumors, an important hallmark is the ability to modify, or reprogram, cellular metabolism to support cell proliferation (46). The findings of the present analysis revealed that several cellular metabolism-associated terms were enriched. For example, the target genes of tRF-Ser-TGA-005 were enriched in 'negative regulation of cellular metabolic process' (GO:0031324) and 'negative regulation of macromolecule metabolic process' (GO:0010605). Several GO terms of tiRNA-Lys-CTT-002 were also associated with metabolism, such as 'response to glucose' (GO:0009749), 'response to hexose' (GO:0009746) and 'response to monosaccharide' (GO:0034284). Notably, the target genes of tRF-Val-CAC-010 and tRF-Val-CAC-011 were also enriched in 'lung alveolus development' (GO:0048286).

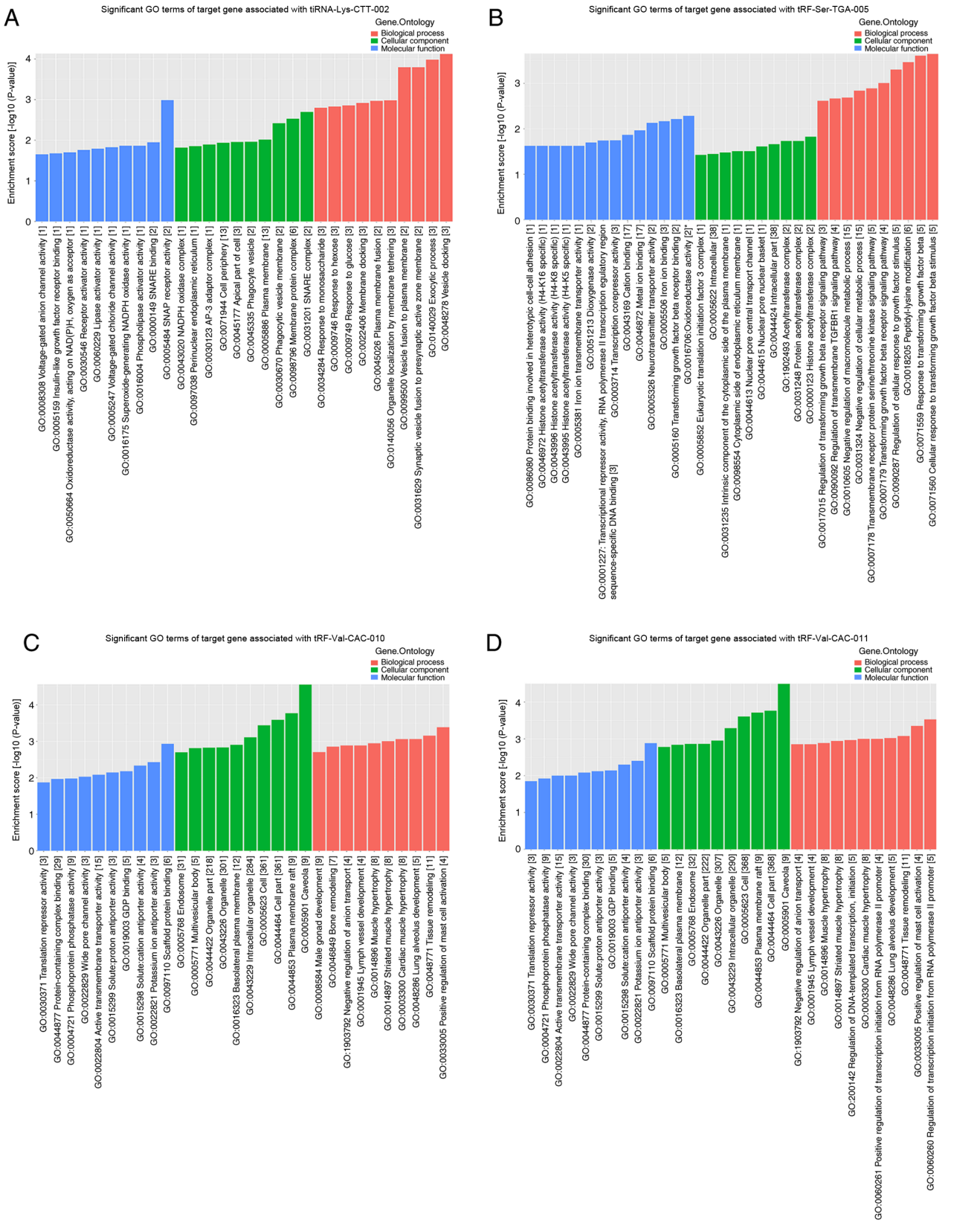

Figure 9. Most significant GO terms of target mRNAs associated with differentially expressed tRFs and tiRNAs. (A) tiRNA-Lys-CTT-002. (B) tRF-Ser-TGA-005 (*GO:0016706: Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors). (C) tRF-Val-CAC-010. (D) tRF-Val-CAC-011. tRFs, transfer RNA-derived fragments; tiRNAs, transfer RNA halves; GO, Gene Ontology.

It has been established that tRFs and tiRNAs exert similar functions to miRNAs, which directly bind to target mRNAs to regulate their stability (21,22). For instance, 3-tRFs derived from tRNA-Leu-CAG were found to inhibit

protein translation or cleave complementary targets in NSCLC cells (42). The present study constructed a network of mRNAs and differentially expressed tRFs and tiRNAs, and identified the tRF-mRNA pairs involved in the regulation of lung adenocarcinoma progression, such as CCND1, CXCL1, CXCL2 and Gli2 associated with tRF-Val-CAC-010 and tRF-Val-CAC-011. CCND1, alongside cyclin-dependent kinases, drives G_1 to S phase progression through retinoblastoma phosphorylation (47). CXCL1 has been closely associated with the formation of tumor blood vessels, and it modulates angiogenesis, tumorigenesis and leukocyte migration (48). The Gli2 protein has been reported to be involved in cancer progression through canonical regulation of the Hedgehog signaling pathway (49). Thus, these genes may also be involved in the development of cancer. The hallmarks of cancer comprise sustained proliferative signaling, induction of angiogenesis and activation of invasion and metastasis, amongst others (46). Further investigations into the underlying mechanisms of differentially expressed tRFs and tiRNAs and their respective targets in cancer development are required.

According to a previous study, tiRNAs may decrease the global translation speed by ~10% (50). Several other studies have reported that tiRNAs assemble into a G-quadruplex structure that competitively binds with eukaryotic translation initiation factor (EIF) 4γ1/EIF4A1 in the translation initiation complex, which in turn inhibits cap-dependent mRNA translation (25,26). In the present study, GO functional term enrichment analysis revealed that target genes of tRF-Val-CAC-010 and tRF-Val-CAC-011 were enriched in 'translation repressor activity' (GO:0030371), which involves EIF4E binding protein 2 (EIF4EBP2). In a previous study, EIF4EBP2 was discovered to regulate EIF4E activity by preventing its assembly into the EIF4A2 complex (51). Therefore, future studies should aim to determine how tRF-Val-CAC-010 and tRF-Val-CAC-011 may regulate the translation process.

In conclusion, the present study investigated tRF and tiRNA profiles in lung adenocarcinoma and adjacent tissues, and identified several dysregulated tRFs and tiRNAs, whose expression may be closely associated with the pathogenesis and development of lung adenocarcinoma. However, the development of lung adenocarcinoma is a complicated multistep process. Therefore, further research is required to elucidate the detailed molecular mechanisms of these tRFs and tiRNAs in lung adenocarcinoma. Additionally, the present study included a small sample size; thus, a larger sample size is required to validate the current findings.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/; project no. GSE168270).

Authors' contributions

JHZ and LHL wrote the paper. JHZ, LHL, LLL, XTY and RL contributed to data interpretation. LLL, XTY and RL contributed to critical revision of the article. JJZ contributed to the acquisition of reagents and materials. YXX collected lung adenocarcinoma fresh tissues. JJZ, YXX, JHZ and LHL performed the experiments. WPW and SYL designed the study and reviewed the manuscript. WPW approved the study. JHZ and WPW confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The First People's Hospital of Yunnan Province (Kunming, China) and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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