

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

JINHUA ZHANG^{1*}, LINHUI LI^{1*}, LILIN LUO¹, XUANTAO YANG¹, JUANJUAN ZHANG¹,
YUXIN XIE¹, RUI LIANG², WANPU WANG¹ and SHUAIYAO LU³

¹Department of Pathology, The First People's Hospital of Yunnan Province, Affiliated Hospital of Kunming University of Science and Technology, Kunming, Yunnan 650032; ²Department of Pathology, The Second Hospital of Tianjin Medical University, Tianjin 300211; ³Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Kunming, Yunnan 650118, P.R. China

Received October 24, 2020; Accepted April 6, 2021

DOI: 10.3892/ol.2021.12767

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.

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

Correspondence to: Dr Wanpu Wang, Department of Pathology, The First People's Hospital of Yunnan Province, Affiliated Hospital of Kunming University of Science and Technology, 157 Jinbi Road, Kunming, Yunnan 650032, P.R. China
E-mail: 82898626@qq.com

Professor Shuaiyao Lu, Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, 935 Jiaoling Road, Kunming, Yunnan 650118, P.R. China
E-mail: 342603482@qq.com

*Contributed equally

Key words: transfer RNA-derived fragments, transfer RNA halves, lung adenocarcinoma, diagnostic biomarker, pathogenesis

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.

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.).

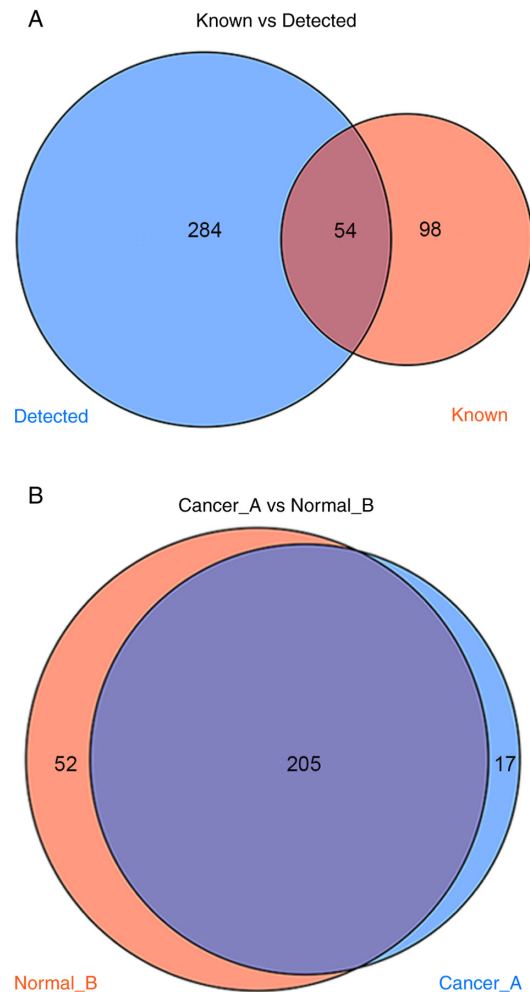


Figure 1. tRFs and tiRNAs screened from lung adenocarcinoma and adjacent tissues. (A) Venn diagram of the number of tRFs and tiRNAs known and stored in the tRF database and those detected in the present study. (B) Venn diagram based on the number of common and differentially expressed tRFs and tiRNAs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

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 R: CGCTTCACGAATTTGCGTGTTCAT	89
tiRNA-Lys-CTT-002	F: ATCGCCCGGCTAGCTCAGT R: TCCGATCTGAGTCTCATGCTCTAC	45
tRF-Val-CAC-011	F: CTTCTGTAGTGTAGTGGTTATCACG R: GTGCTCTTCCGATCTGGC	46
tRF-Val-CAC-010	F: CTTCTGTAGTGTAGTGGTTATCACG R: GTGCTCTTCCGATCTGCG	45
tRF-Val-CAC-007	F: CGATCGCTTCTGTAGTGTAGTGG R: GCTCTTCCGATCTAACGTGATAA	46
tRF-His-GTG-008	F: CGATCGCCGTGATCGTATAGT R: TCCGATCTCGCAGAGTACTAACC	44
tRF-Ser-AGA-006	F: AGTCCGACGATCGTAGTCGTG R: CGATCTCCTTAACCACTCGGC	42
tRF-Glu-TTC-027	F: CGACGATCTGACTGGACCTTT R: GACGTGTGCTCTTCCGATCTAA	45
tRF-Lys-CTT-001	F: GATCGCCCAGCTAGCTCAGT R: GTGCTCTTCCGATCTTATGCTCT	47
tRF-Ser-TGA-005	F: GATCGAAGCGGGTGCTCT R: GACGTGTGCTCTTCCGATCTAT	41
tRF-His-GTG-012	F: AGTCCGACGATCTCGAATCC R: CGATCTTGGTGCCGTGACTC	40

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 $P \leq 0.05$. 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 $2^{-\Delta\Delta C_q}$ method for relative quantification of gene expression (32).

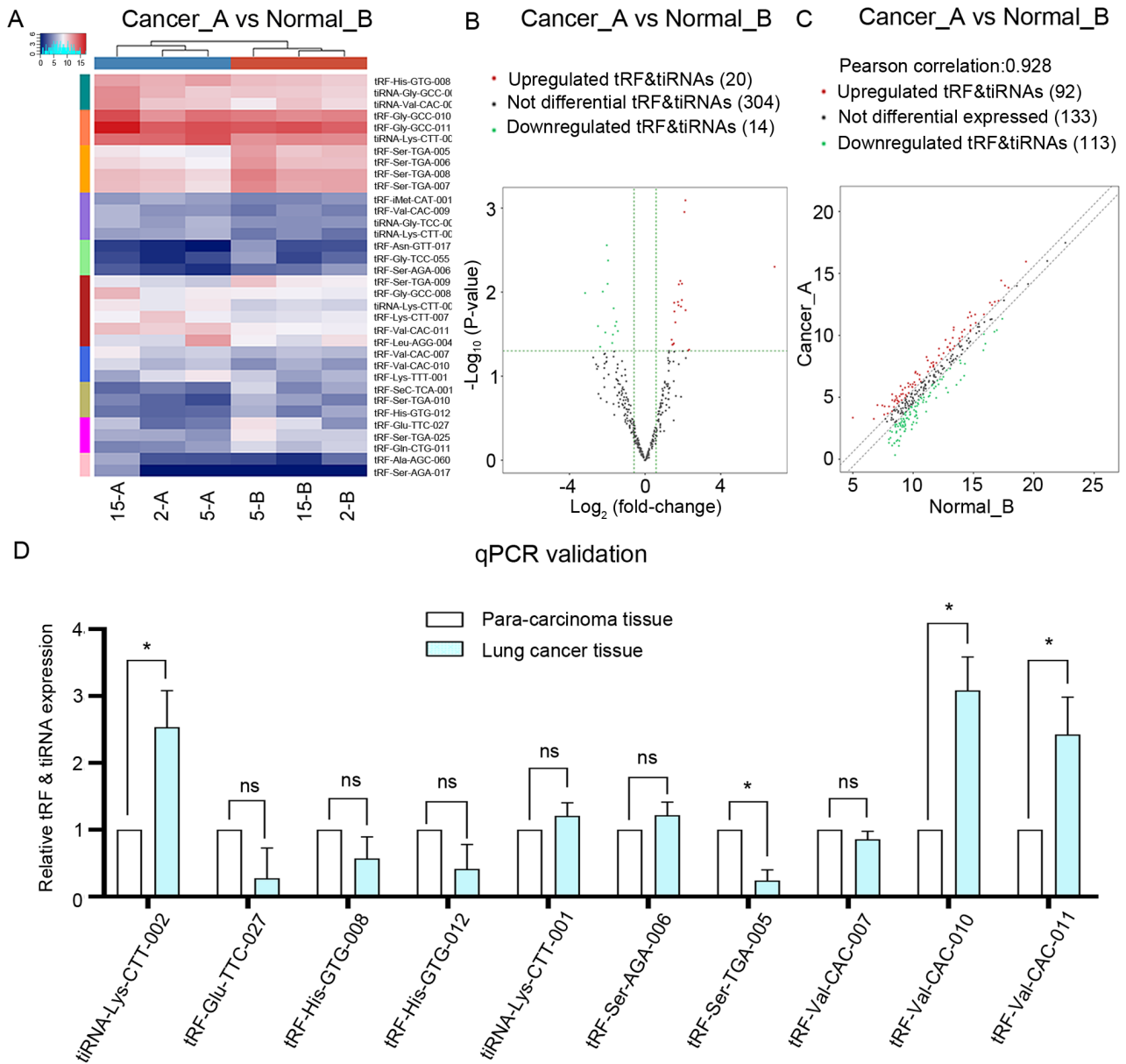


Figure 2. Differently expressed tRFs and tiRNAs identified between lung adenocarcinoma and adjacent tissues. (A) Heat map demonstrating the significant changes in the expression levels of tRFs and tiRNAs between lung adenocarcinoma and adjacent tissues. Red represents high expression; blue represents 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 \pm 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 ₂ FC	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

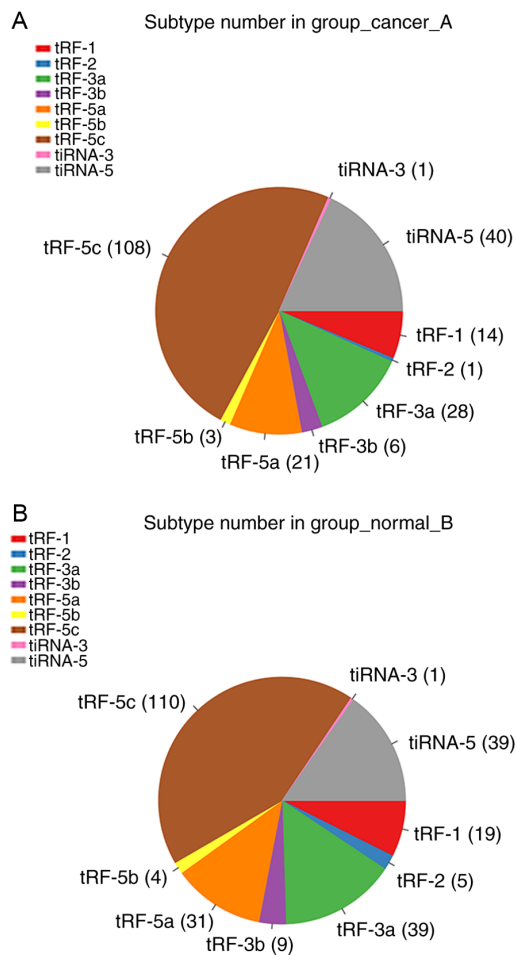


Figure 3. Pie charts of the distribution of tRF and tiRNA subtypes. (A) Lung adenocarcinoma and (B) adjacent tissues. tRFs, transfer RNA-derived fragments; tiRNAs, transfer RNA halves.

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

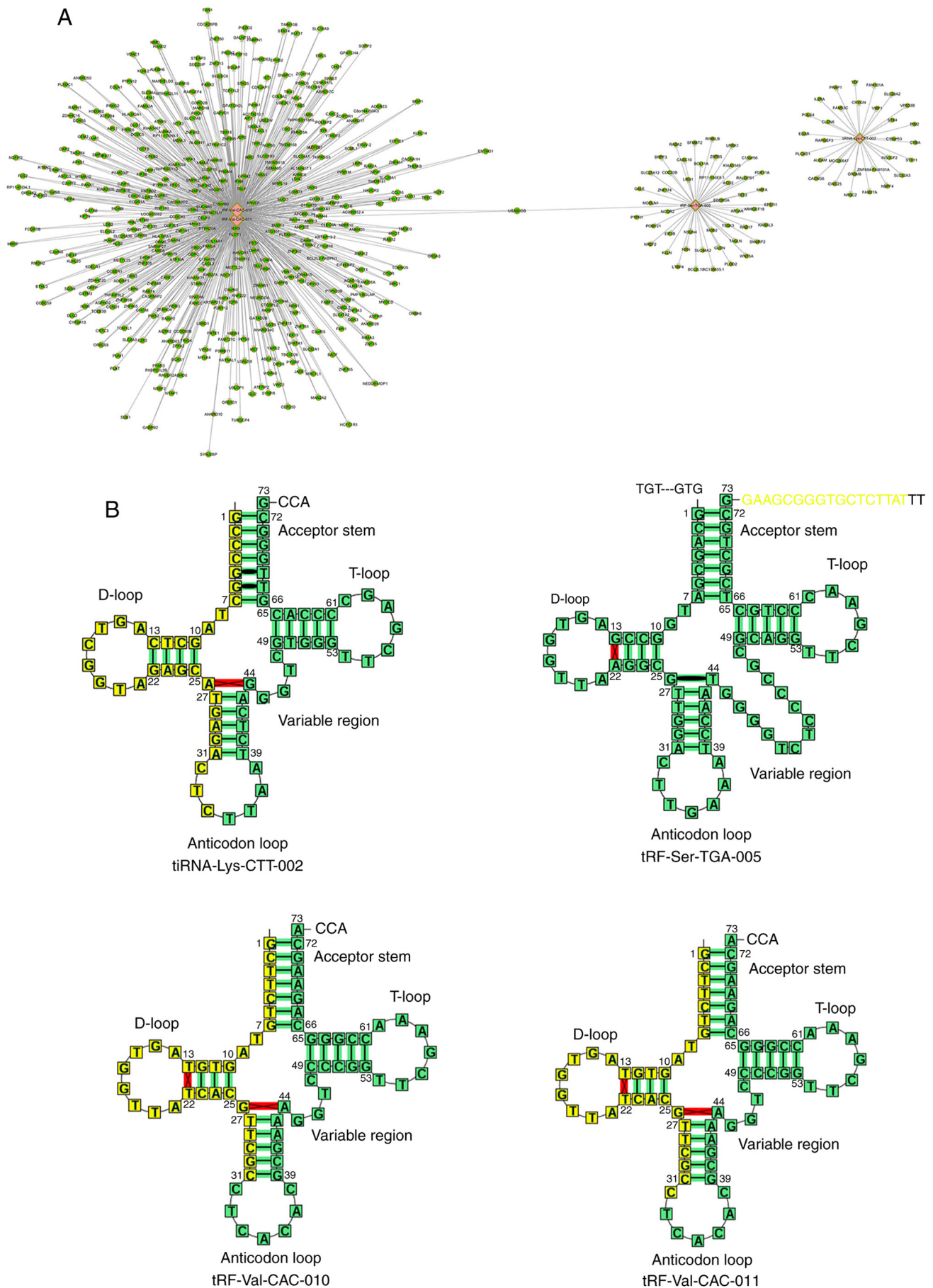


Figure 4. Association and structure of differentially expressed tRFs and tRNAs. (A) Association between tRFs and target genes. Red represents tRF and tRNA; green represents the altered target genes. (B) Structure of tRFs and tRNAs. Yellow represents the sequences of tRF and tRNA. Green represents the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

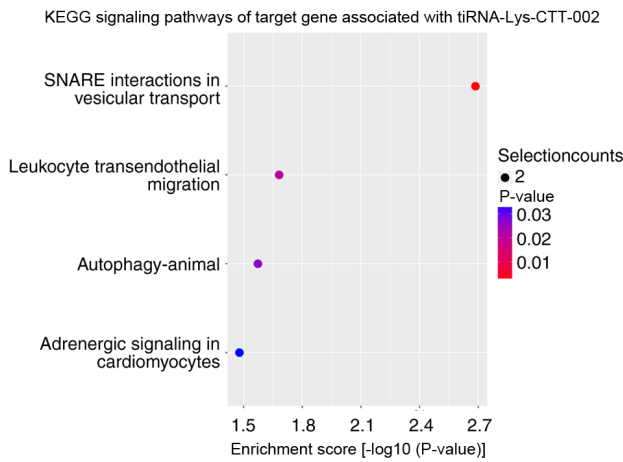


Figure 5. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tiRNA-Lys-CTT-002. tiRNAs, transfer RNA halves; KEGG, Kyoto Encyclopedia of Genes and Genomes.

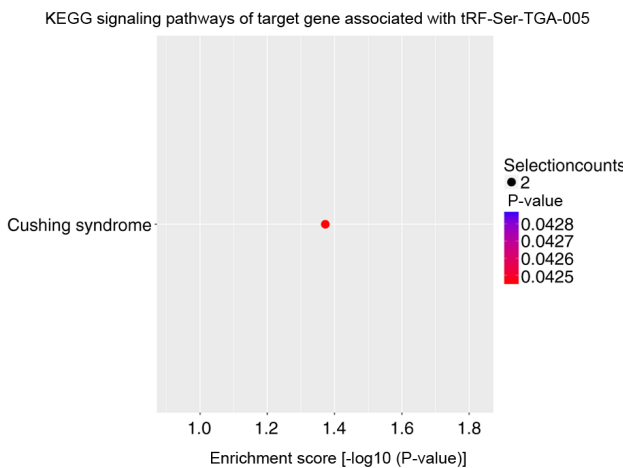


Figure 6. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tRF-Ser-TGA-005. tRFs, transfer RNA-derived fragments; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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 down-regulated 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

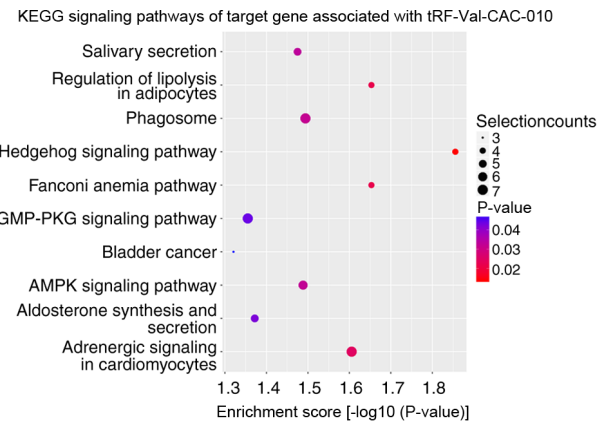


Figure 7. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tRF-Val-CAC-010. tRFs, transfer RNA-derived fragments; KEGG, Kyoto Encyclopedia of Genes and Genomes.

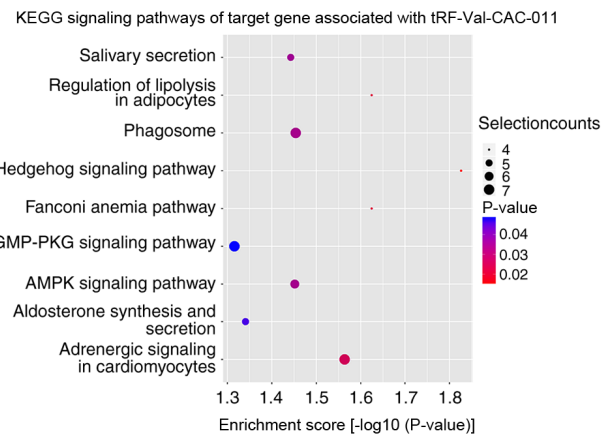


Figure 8. Most significantly enriched KEGG signaling pathways of target mRNAs associated with tRF-Val-CAC-011. tRFs, transfer RNA-derived fragments; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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).

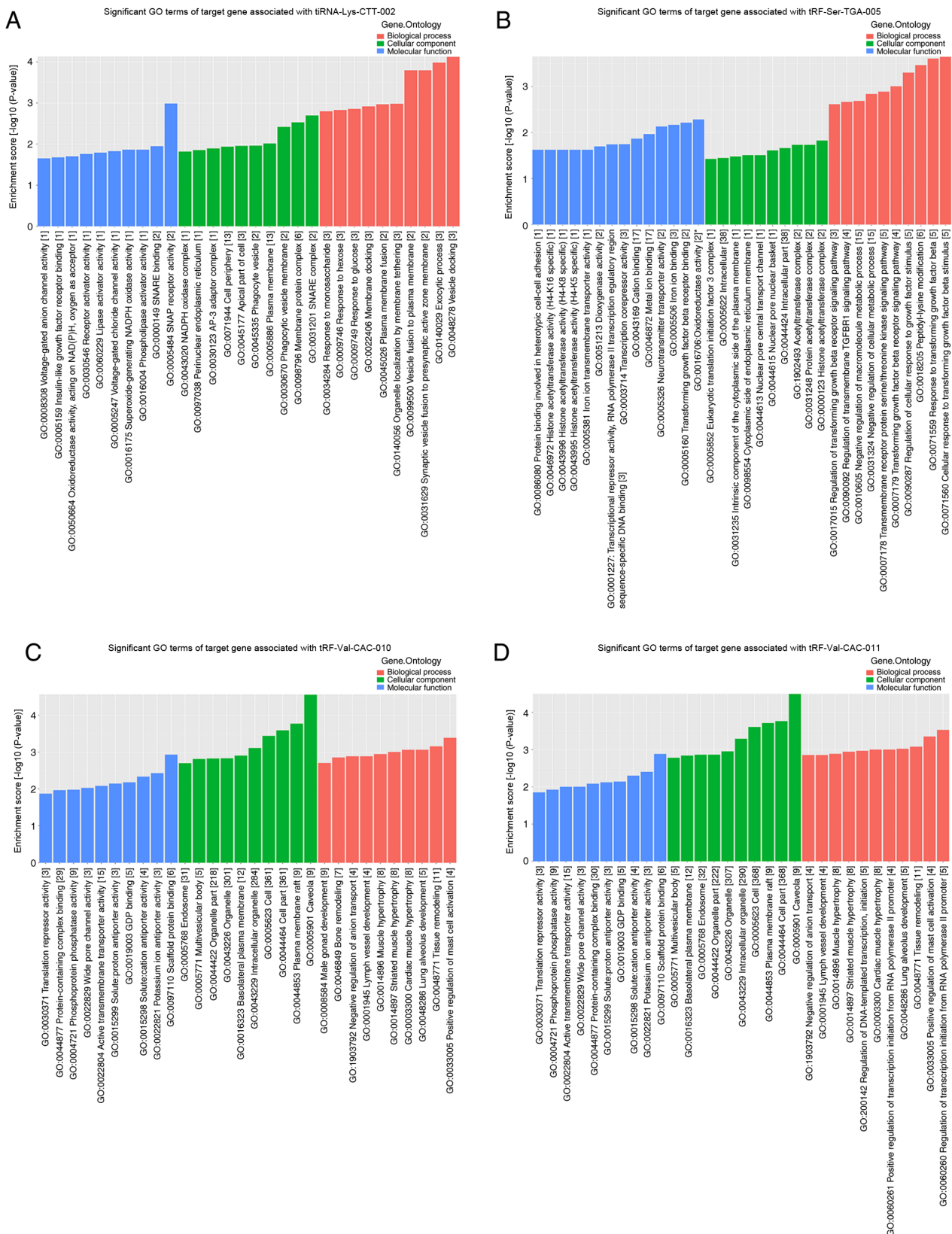


Figure 9. Most significant GO terms of target mRNAs associated with differentially expressed tRFs and tiRNAs. (A) tiRNA-Lys-CTT-002. (B) tRF-Ser-TGA-005 (*GO:0016706: Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and 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 G₁ 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.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Yunnan Health Training Project of High Level Talents (grant no. H-2019001), the National Natural Science Foundation of China (grant no. 81660238), National Natural Science Foundation of China (grant no. 81660302) and Yunnan Province Clinical Center for Hematologic Disease (grant no. 2020LCZXKF-XY13).

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.

References

1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. *CA Cancer J Clin* 66: 115-132, 2016.
2. Chen W, Xia C, Zheng R, Zhou M, Lin C, Zeng H, Zhang S, Wang L, Yang Z, Sun K, *et al*: Disparities by province, age, and sex in site-specific cancer burden attributable to 23 potentially modifiable risk factors in China: A comparative risk assessment. *Lancet Glob Health* 7: e257-e269, 2019.
3. Travis DW, Brambilla E, Burke AP, Marx A and Nicholson AG: WHO classification of tumours of the lung, pleura, thymus and heart. 4th edition. Lyon, France, IARC Press, 2015.
4. Rolfo C, Castiglia M, Perez A, Reclusa P, Pauwels P, Sober L, Passiglia F and Russo A: Liquid biopsy in non-small cell lung cancer (NSCLC). In *Liquid Biopsy in Cancer Patients*. 3rd edition. Giordano A, Russo A and Rolfo C (eds). Springer International Publishing AG, Cham, Switzerland, pp103-115, 2017.
5. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2020. *CA Cancer J Clin* 70: 7-30, 2020.
6. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68: 394-424, 2018.
7. Howlader N, Noone AM, Krapcho M, Miller D, Bishop K, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, *et al* (eds): SEER cancer statistics review, 1975-2013, based on November 2015 SEER data submission, posted to the SEER web site, April 2016, Bethesda, MD, National Cancer Institute, 2016.
8. Banerjee R, Chen S, Dare K, Gilreath M, Praetorius-Ibba M, Raina M, Reynolds NM, Rogers T, Roy H, Yadavalli SS and Ibba M: tRNAs: Cellular barcodes for amino acids. *FEBS Lett* 584: 387-395, 2010.
9. Lee YS, Shibata Y, Malhotra A and Dutta A: A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). *Genes Dev* 23: 2639-2649, 2009.

10. Pekarsky Y, Balatti V, Palamarchuk A, Rizzotto L, Veneziano D, Nigita G, Rassenti LZ, Pass HI, Kipps TJ, Liu CG and Croce CM: Dysregulation of a family of short noncoding RNAs, tsRNAs, in human cancer. *Proc Natl Acad Sci USA* 113: 5071-5076, 2016.
11. Anderson P and Ivanov P: tRNA fragments in human health and disease. *FEBS Lett* 588: 4297-4304, 2014.
12. Pliatsika V, Loher P, Telonis AG and Rigoutsos I: MINTbase: A framework for the interactive exploration of mitochondrial and nuclear tRNA fragments. *Bioinformatics* 32: 2481-2489, 2016.
13. Zheng LL, Xu WL, Liu S, Sun WJ, Li JH, Wu J, Yang JH and Qu LH: tRF2Cancer: A web server to detect tRNA-derived small RNA fragments (tRFs) and their expression in multiple cancers. *Nucleic Acids Res* 44 (W1): W185-W193, 2016.
14. Blanco S, Dietmann S, Flores JV, Hussain S, Kutter C, Humphreys P, Lukk M, Lombard P, Treps L, Popis M, *et al*: Aberrant methylation of tRNAs links cellular stress to neuro-developmental disorders. *EMBO J* 33: 2020-2039, 2014.
15. Maute RL, Schneider C, Sumazin P, Holmes A, Califano A, Basso K and Dalla-Favera R: tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma. *Proc Natl Acad Sci USA* 110: 1404-1409, 2013.
16. Torres AG: Enjoy the silence: Nearly half of human tRNA genes are silent. *Bioinform Biol Insights* 13: 1177932219868454, 2019.
17. Zhu L, Ge J, Li T, Shen Y and Guo J: tRNA-derived fragments and tRNA halves: The new players in cancers. *Cancer Lett* 452: 31-37, 2019.
18. Li S, Xu Z and Sheng J: tRNA-derived small RNA: A novel regulatory small non-coding RNA. *Genes (Basel)* 9: 246, 2018.
19. Kumar P, Mudunuri SB, Anaya J and Dutta A: tRFdb: A database for transfer RNA fragments. *Nucleic Acids Res* 43: D141-D145, 2015.
20. Li S and Hu GF: Emerging role of angiogenin in stress response and cell survival under adverse conditions. *J Cell Physiol* 227: 2822-2826, 2012.
21. Karaikos S, Naqvi AS, Swanson KE and Grigoriev A: Age-driven modulation of tRNA-derived fragments in *Drosophila* and their potential targets. *Biol Direct* 10: 51, 2015.
22. Kumar P, Anaya J, Mudunuri SB and Dutta A: Meta-analysis of tRNA derived RNA fragments reveals that they are evolutionarily conserved and associate with AGO proteins to recognize specific RNA targets. *BMC Biol* 12: 78, 2014.
23. Luo S, He F, Luo J, Dou S, Wang Y, Guo A and Lu J: *Drosophila* tsRNAs preferentially suppress general translation machinery via antisense pairing and participate in cellular starvation response. *Nucleic Acids Res* 46: 5250-5268, 2018.
24. Goodarzi H, Liu X, Nguyen HC, Zhang S, Fish L and Tavazoie SF: Endogenous tRNA-derived fragments suppress breast cancer progression via YBX1 displacement. *Cell* 161: 790-802, 2015.
25. Ivanov P, Emara MM, Villen J, Gygi SP and Anderson P: Angiogenin-induced tRNA fragments inhibit translation initiation. *Mol Cell* 43: 613-623, 2011.
26. Ivanov P, O'Day E, Emara MM, Wagner G, Lieberman J and Anderson P: G-quadruplex structures contribute to the neuro-protective effects of angiogenin-induced tRNA fragments. *Proc Natl Acad Sci USA* 111: 18201-18206, 2014.
27. Saikia M, Jobava R, Parisien M, Putnam A, Krokowski D, Gao XH, Guan BJ, Yuan Y, Jankowsky E, Feng Z, *et al*: Angiogenin-cleaved tRNA halves interact with cytochrome c, protecting cells from apoptosis during osmotic stress. *Mol Cell Biol* 34: 2450-2463, 2014.
28. Chen Q, Yan M, Cao Z, Li X, Zhang Y, Shi J, Feng GH, Peng H, Zhang X, Zhang Y, *et al*: Sperm tsRNAs contribute to inter-generational inheritance of an acquired metabolic disorder. *Science* 351: 397-400, 2016.
29. Langmead B, Trapnell C, Pop M and Salzberg SL: Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 10: R25, 2009.
30. Friedländer MR, Mackowiak SD, Li N, Chen W and Rajewsky N: miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res* 40: 37-52, 2012.
31. Robinson MD, McCarthy DJ and Smyth GK: EdgeR: A bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139-140, 2010.
32. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
33. Betel D, Wilson M, Gabow A, Marks DS and Sander C: The microRNA.org resource: Targets and expression. *Nucleic Acids Res* 36: D149-D153, 2008.
34. Agarwal V, Bell GW, Nam JW and Bartel DP: Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 4: e05005, 2015.
35. Enright AJ, John B, Gaul U, Tuschl T, Sander C and Marks DS: MicroRNA targets in *Drosophila*. *Genome Biol* 5: R1, 2003.
36. Grimson A, Farh KK, Johnston WK, Garrett-Engle P, Lim LP and Bartel DP: MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. *Mol Cell* 27: 91-105, 2007.
37. Friedman RC, Farh KK, Burge CB and Bartel DP: Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19: 92-105, 2009.
38. Fu Y, Lee I, Lee YS and Bao X: Small non-coding transfer RNA-Derived RNA fragments (tRFs): Their biogenesis, function and implication in human diseases. *Genomics Inform* 13: 94-101, 2015.
39. Zhou K, Diebel KW, Holy J, Skildum A, Odean E, Hicks DA, Schotl B, Abrahante JE, Spillman MA and Bemis LT: A tRNA fragment, tRF5-Glu, regulates BCAR3 expression and proliferation in ovarian cancer cells. *Oncotarget* 8: 95377-95391, 2017.
40. Xu H, Chen W, Zheng F, Tang D, Dai W, Huang S, Zhang C, Zeng J, Wang G and Dai Y: The potential role of tRNAs and small RNAs derived from tRNAs in the occurrence and development of systemic lupus erythematosus. *Biochem Biophys Res Commun* 527: 561-567, 2020.
41. Keith B and Simon MC: Hypoxia-inducible factors, stem cells, and cancer. *Cell* 129: 465-472, 2007.
42. Shao Y, Sun Q, Liu X, Wang P, Wu R and Ma Z: tRF-Leu-CAG promotes cell proliferation and cell cycle in non-small cell lung cancer. *Chem Biol Drug Des* 90: 730-738, 2017.
43. Balatti V, Nigita G, Veneziano D, Drusco A, Stein GS, Messier TL, Farina NH, Lian JB, Tomasello L, Liu CG, *et al*: tsRNA signatures in cancer. *Proc Natl Acad Sci USA* 114: 8071-8076, 2017.
44. Myers DJ and Wallen JM: Lung adenocarcinoma. National Institutes of Health 2020.
45. Hanahan D and Weinberg RA: The hallmarks of cancer. *Cell* 100: 57-70, 2000.
46. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. *Cell* 144: 646-674, 2011.
47. Kato J, Matsushima H, Hiebert SW, Ewen ME and Sherr CJ: Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev* 7: 331-342, 1993.
48. Amiri KI and Richmond A: Fine tuning the transcriptional regulation of the CXCL1 chemokine. *Prog Nucleic Acid Res Mol Biol* 74: 1-36, 2003.
49. Hui CC and Angers S: Gli proteins in development and disease. *Annu Rev Cell Dev Biol* 27: 513-537, 2011.
50. Yamasaki S, Ivanov P, Hu GF and Anderson P: Angiogenin cleaves tRNA and promotes stress-induced translational repression. *J Cell Biol* 185: 35-42, 2009.
51. Martineau Y, Azar R, Bousquet C and Pyronnet S: Anti-oncogenic potential of the eIF4E-binding proteins. *Oncogene* 32: 671-677, 2013.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.