Expression profiles and potential roles of serum tRNA-derived fragments in diabetic nephropathy

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Received November 5, 2022; Accepted March 29, 2023

DOI: 10.3892/etm.2023.12010

Abstract. Diabetic nephropathy (DN) is one of the most important causes of end-stage renal disease and current treatments are ineffective in preventing its progression. Transfer RNA (tRNA)-derived fragments (tRFs), which are small non-coding fragments derived from tRNA precursors or mature tRNAs, have a critical role in various human diseases. The present study aimed to investigate the expression profile and potential functions of tRFs in DN. High-throughput sequencing technology was employed to detect the differential serum levels of tRFs between DN and diabetes mellitus and to validate the reliability of the sequencing results using reverse transcription-quantitative PCR. Ultimately, six differentially expressed (DE) tRFs were identified ($P<0.05$; |log2fold change| ≥1), including three upregulated (tRF5-GluCTC, tRF5-AlaCGC and tRF5-ValCAC) and three downregulated tRFs (tRF5-GlyCCC, tRF3-GlyGCC and tRF3-IleAAT). Potential functions and regulatory mechanisms of these DE tRFs were further evaluated using an applied bioinformatics-based analysis. Gene ontology analysis revealed that the DE tRFs are mainly enriched in biological processes, including axon guidance, Rad51 paralog (Rad51) B-Rad51C-Rad51D-X-Ray repair cross-complementing 2 complex, nuclear factor of activated T-cells protein binding and fibroblast growth factor-activated receptor activity. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis indicated that they are associated with axon guidance, neurotrophin signaling, mTOR signaling, AMPK signaling and epidermal growth factor receptor family signaling pathways. In conclusion, the present findings indicated that tRFs were DE in DN and may be involved in the regulation of DN pathology through multiple pathways, thereby providing a new perspective for the study of DN therapeutic targets.

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Key words: tRNA-derived fragments, diabetic nephropathy, expression profiles, GO analysis, KEGG pathway enrichment analysis

Introduction

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes mellitus (DM) and a major cause of the end-stage renal disease (ESRD) worldwide (1). DN is characterized by persistent proteinuria, glomerulosclerosis and progressive decline in renal function. Approximately 30-40% of patients with DM also have DN, which seriously affects their quality of life and causes a substantial economic burden (2). Conventional treatments with strict control of hyperglycemia and hypertension may only delay the progression of DN to a certain extent, but cannot stop or reverse it (3). Therefore, it is crucial to investigate DN pathogenesis and actively search for specific and effective therapeutic targets.

Transfer RNA (tRNA)-derived small RNAs (tsRNAs) are a class of non-coding RNA derived from mature tRNA or tRNA precursors. Depending on their cleavage site and length, tsRNAs are classified into tRNA halves (tiRNAs) and tRNA-derived fragments (tRFs). tRFs, approximately 16-40 nucleotides (nt) in length, are further classified into 5'-tRF, 3'-tRF and tRF-1 series, according to their origin (4). tRFs exist in all types of tissues and cells and are highly conserved, with a stable structure and tissue-specific expression (5). tRFs are involved in regulating processes such as cell proliferation, priming of viral reverse transcriptases, regulation of gene expression, RNA processing, modulation of the DNA damage response, tumor suppression and neurodegeneration (6,7), and are closely associated with various human diseases (8). In particular, tRFs are aberrantly expressed in tumor cells, including those of ovarian (9), non-small cell lung (10), breast (11) and gastric cancer (12), and affect tumor development. For instance, Han *et al* (13)

found that tRF-3008A inhibited the proliferation and migration of colorectal cancer *in vivo* and *in vitro* by destabilizing FOXK1 in an argonaute RISC component 1-dependent manner. Furthermore, circulating tRF-7816 may be a novel potential biomarker for the diagnosis of patients with early-stage non-triple-negative breast cancer (14). However, studies of the potential functions of tRFs in DN are limited. In previous studies by our group, several differentially expressed (DE) tRFs were identified in podocytes using high-throughput sequencing and were confirmed to potentially have a significant regulatory role in the differentiation and damage processes of podocytes (15,16).

In the current study, the DE profiles of serum tRFs in patients with DN and DM were analysed using high-throughput sequencing. The reliability of the sequencing results was verified using reverse transcription-quantitative PCR (RT-qPCR). MiRanda was used to predict the target genes of the DE tRFs. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to predict the potential functions of the DE tRFs. The present study aimed to determine the DE profiles and potential functions of tRFs in the development of DN, thus providing new therapeutic targets for DN.

Materials and methods

Patients and samples. In total, three patients with type 2 DN and three patients with type 2 diabetes mellitus (DM), who visited The Second Affiliated Hospital of Nanjing Medical University (Nanjing, China) between March 2021 and October 2021, were enrolled in the present study. The inclusion criteria were as follows: i) Both groups met the World Health Organization diagnostic criteria for DM, i.e. fasting postprandial glucose ≥7.0 mmol/l, 2-h glucose ≥11.1 mmol/l based on an oral glucose tolerance test, random glucose ≥11.1 mmol/l in patients with typical symptoms or glycosylated hemoglobin type a1c ≥6.5%; ii) the DN group met the criteria of at least two urine albumin to urine creatinine ratio results, i.e. ≥30 mg/g from three measurements performed within 3-6 months. Patients with type 1 DM, malignancy and other comorbid renal diseases, such as chronic nephritis and IgA nephropathy, cardiovascular disease, liver disease or a history of kidney-damaging drug administration, were excluded. A 5-ml peripheral blood sample from each participant was collected and centrifuged to obtain the serum sample, which was stored at -80˚C for subsequent experiments. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Nanjing Medical University (Nanjing, China) and all participants provided written informed consent. The baseline clinical characteristics of the study participants are presented in Table I.

Total RNA extraction and RT-qPCR. Total RNA from the serum of patients with DN and DM was prepared for RT-qPCR. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentration and purity of the RNA were determined using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). According to the instructions of the riboSCRIPT™ Reverse Transcription Kit (cat. no. MR101; Guangzhou RiboBio Co., Ltd.), 10 μl RT reaction system was used to reverse-transcribe the extracted RNA into cDNA. Subsequently, qPCR was performed using 5 μl of 2X SYBR, 0.4 μl of forward and 0.4 μl of reverse primers on the StepOne Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The Bulge-Loop™ micro (mi) RNA qRT-PCR Starter Kit (cat. no. C10211-2; Guangzhou RiboBio Co., Ltd.) was used to quantify tRFs expression using specific stem-loop primers synthesized by Guangzhou RiboBio Co., Ltd. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 5 min, followed by 95˚C for 2 sec, 60˚C for 20 sec and 70˚C for 10 sec for 40 cycles. MiR-39-3p (17) served as the external reference and target gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18). Each set of experiments was performed in triplicate. Details of the primers are presented in Table II.

High-throughput sequencing. Total RNA from the serum of patients with DN and DM was treated using the rtStar™ tRF&tiRNA Pretreatment Kit (cat. no. AS-FS-005; Arraystar, Inc.) to remove excess modifications and ensure efficient RT. The 3' and 5' miRNA adapters were then ligated to the pretreated RNA. cDNA was synthesized and amplified using proprietary reverse transcription and amplification primers (NEBNext® Multiplex Small RNA Library Prep Set for Illumina; cat. no. E7330l; Illumina, Inc.). Subsequently, 134-160 bp PCR fragments, equivalent to 14-40 nt small RNA, were extracted and purified using an automatic gel cutter. The identification and quantitative analysis of the library were performed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Inc.). Thereafter, the qualified library was diluted to a 1.3 ml final volume and 1.8 pM final concentration. Finally, libraries with the same concentration and volume were loaded into the NextSeq 500/550V2 kit (cat. no. FC-404-2005; Illumina, Inc.) and sequenced on an Illumina NextSeq 500 system (Illumina, Inc.), according to the manufacturer's instructions. Correlation analysis, hierarchical clustering, scatter plots and principal component analysis (PCA) were performed using R (v.4.1.3) software (https://www.r-project.org/). $P<0.05$ and |log2 fold change (FC)| ≥1 were considered to indicate a statistically significant difference. The sequences of DE tRFs are listed in Table III.

Target gene prediction and GO and KEGG analyses. MiRanda (http://www.microrna.org/microrna/home.do) was used to predict the target genes of DE tRFs. DAVID (https://david.ncifcrf.gov/) was used to explore the potential biological functions and molecular mechanisms of these target genes via GO and KEGG enrichment analyses and GO functional classification and KEGG pathway classification maps were generated. Cytoscape (3.9.0; https://cytoscape.org/) was used to construct the signaling pathway regulation network for predicted target genes.

Statistical analysis. Statistical analyses were conducted with GraphPad Prism 8 software (GraphPad Software; Dotmatics). The measurement data are expressed as the mean ± standard deviation. The unpaired t-test was used for statistical comparison between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Clinical characteristics of the subjects.

Characteristic	DM (n=3)	DN (n=3)	P-value
Sex			
Male	1	1	-
Female	2	2	-
Age, years	74.0±4.583	70.0±11.27	0.5995
Fasting blood glucose, mmol/l	9.383±3.636	8.217±3.004	0.6904
Systolic blood pressure, mmHg	139.3±4.619	140.3±1.528	0.7398
Diastolic blood pressure, mmHg	80.00±2.00	79.33±10.07	0.9158
Glycosylated hemoglobin type a1c, %	9.233±2.515	8.067±1.955	0.5603
Blood urea nitrogen, mmol/l	5.50±2.485	7.51±2.982	0.4205
Serum creatinine μmol/l	60.27±2.053	120.1±33.51	0.0366[a]
Uric acid μmol/l	236.7±27.21	360.0±49.69	0.0196[a]
Urine albumin to urine creatinine ratio, mg/g	<30	>30	-

[a]P<0.05 vs. DM group. DN, diabetic nephropathy; DM, diabetes mellitus.

Table II. Primers used in the present study and their catalogue numbers.

Primer	Cat. no.
Bulge-Loop tRF5-GlyCCC Primer Set, 200T	MQPS0004671-1-200
Bulge-Loop tRF3-GlyGCC Primer Set, 200T	MQPS0004672-1-200
Bulge-Loop tRF3-IleAAT Primer Set, 200T	MQPS0004673-1-200
Bulge-Loop tRF5-GluCTC Primer Set, 200T	MQPS0004674-1-200
Bulge-Loop tRF5-AlaCGC Primer Set, 200T	MQPS0004675-1-200
Bulge-Loop tRF5-ValCAC Primer Set, 200T	MQPS0004676-1-200
Cel-miR-39-3p Standard RNA	MiRB0000010

All products were from Guangzhou RiboBio Co., Ltd.

Table III. Differentially expressed tRFs in diabetic nephropathy.

tRF identifier	tRF sequence	Log_2(fold change)	P-value	Regulation
tRF5-GluCTC	5'-TCCCTGGTGGTCTAGTGGTTAGGATTCGGCG-3'	2.09	0.003	Up
tRF5-AlaCGC	5'-GGGGGTGTAGCTCAGTGGTAGAGCGCGTGC-3'	2.09	0.012	Up
tRF5-ValCAC	5'-GTTTCCGTAGTGTAGTGGTTATCACGTTCGC-3'	1.80	0.032	Up
tRF5-GlyCCC	5'-GCGCCGCTGGTGTAGTGGTATCATGCAAGA-3'	-1.21	<0.001	Down
tRF3-GlyGCC	5'-TCGATTCCCGGCCCATGCACCA-3'	-1.64	0.040	Down
tRF3-IleAAT	5'TCGATCCCCGTACGGGCCACCA-3'	-3.36	0.035	Down

tRF, transfer RNA-derived fragment.

Results

DE profiles of tRFs between DN and DM. The correlation of expression levels between samples is an important indicator for testing the reliability of the experiment and validating the sample selection. The correlation coefficient for any two samples exhibited a high degree of similarity (Fig. 1A). In addition, PCA was used to reduce the complexity of the data and analyze the variation between samples. The results suggested a distinguishable tRF expression profile among the six samples (Fig. 1B). In total, 30 tRFs were identified as dysregulated in the DN group. Based on the screening conditions of P<0.05 and |log2FC| ≥1, six tRFs were identified as DE, of which three were upregulated and three were downregulated (Fig. 1C and D). The cluster analysis indicated that the samples in the same group had similar clustering

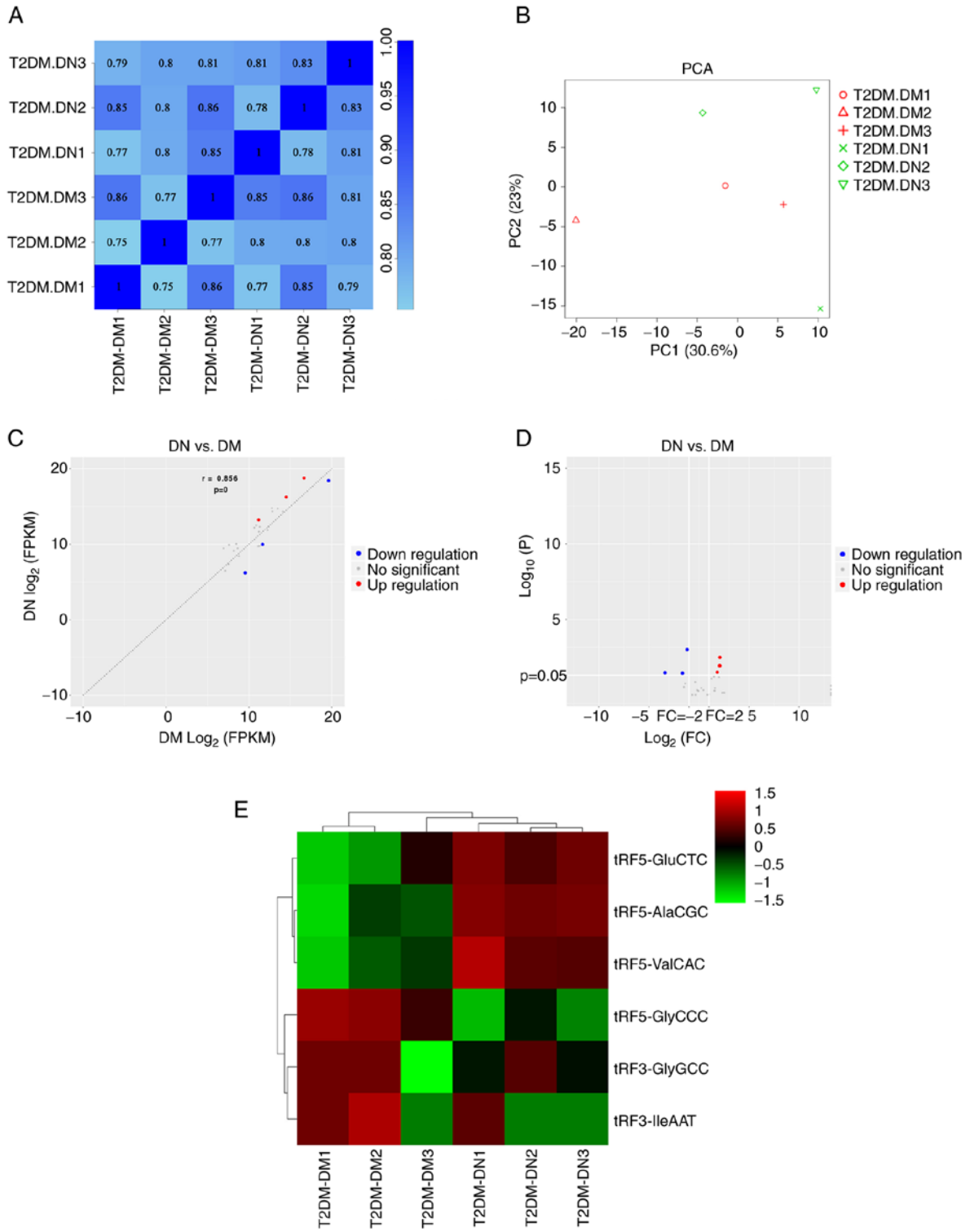

Figure 1. DE profiles of tRFs in DN and DM. (A) Heat map of correlation coefficients for all samples. A darker color indicates higher similarity between samples. The numerical value is the correlation coefficient. (B) Principal component analysis with indicators representing variation between samples. (C) Scatter plot of the DE tRFs. (D) Volcano plot of the DE tRFs. (E) Hierarchical clustering heatmap of the DE tRFs. Each row represents a tRF and each column represents a sample. Red indicates high expression and green indicates low expression. DN, diabetic nephropathy; DM, diabetes mellitus; T2, type 2; DE, differentially expressed; tRF, transfer RNA-derived fragment; PCA, principal component analysis; FPKM, fragments per kilobase of exon per million mapped reads; FC, fold change.

patterns and expression profiles, thereby suggesting that the differences between the samples were small. The expression of tRF5-GluCTC, tRF5-AlaCGC and tRF5-ValCAC was upregulated, whereas that of tRF5-GlyCCC, tRF3-GlyGCC and tRF3-IleAAT was downregulated (Fig. 1E).

Validation of DE tRFs. To verify the reliability of the sequencing results, RT-qPCR was used to detect the expression levels of the DE tRFs in the sera of patients with DN and DM. Compared with that in the DM group, the expression of tRF5-GluCTC, tRF5-AlaCGC and tRF5-ValCAC was

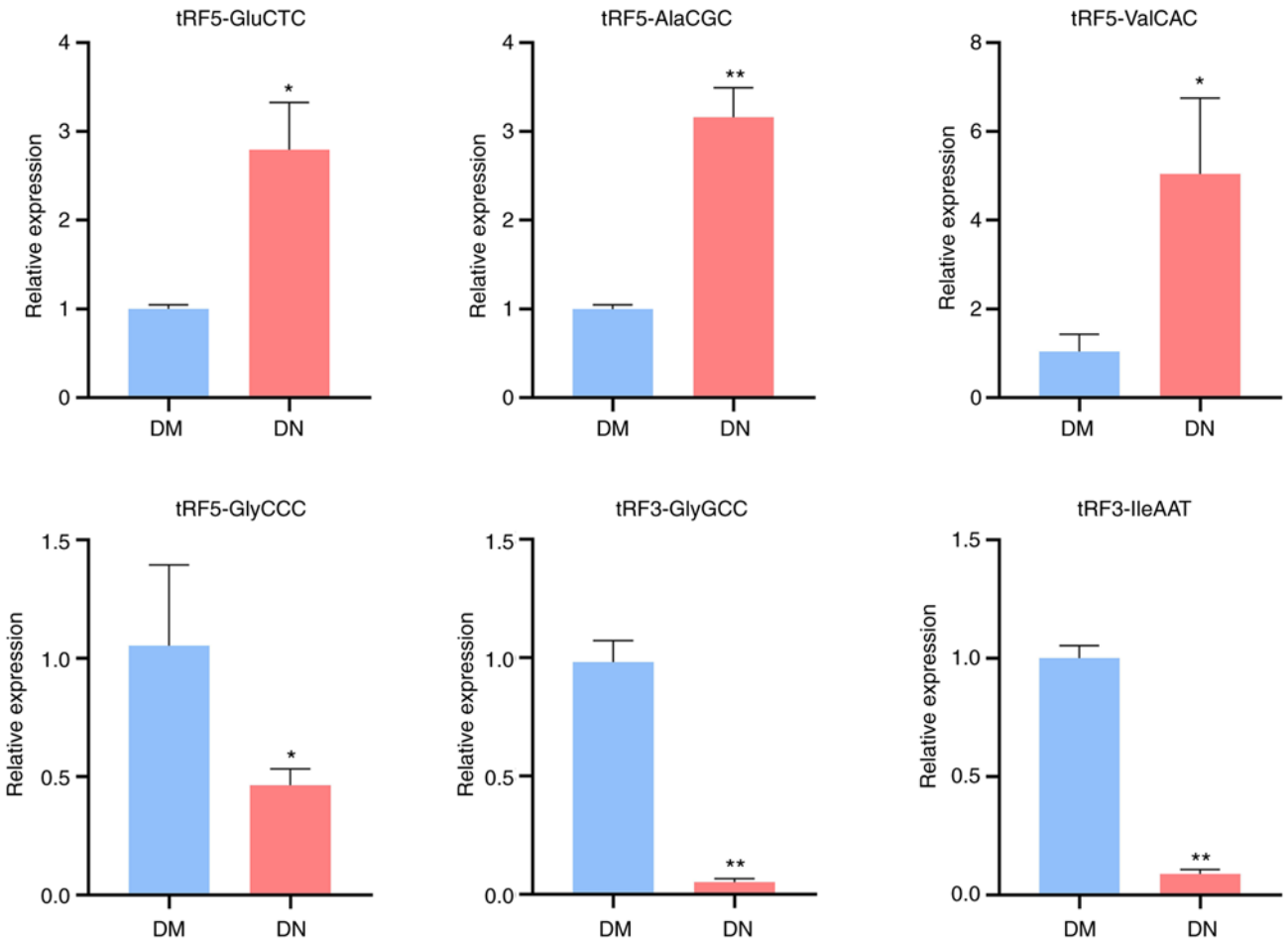

Figure 2. Verification of the expression levels of the differentially expressed tRFs by reverse transcription-quantitative PCR. The expression of tRF5-GluCTC, tRF5-AlaCGC and tRF5-ValCAC was significantly increased in the DN group. The expression of tRF5-GlyCCC, tRF3-GlyGCC and tRF3-IleAAT was significantly decreased in the DN group. *P<0.05 and **P<0.01 vs. DM. DN, diabetic nephropathy; DM, diabetes mellitus; tRF, transfer RNA-derived fragment.

upregulated, while that of tRF5-GlyCCC, tRF3-GlyGCC and tRF3-IleAAT was downregulated in the DN group (Fig. 2). The RT-qPCR results were consistent with the sequencing data.

Prediction of target genes. MiRanda was used to predict the target genes of DE tRFs. The upregulated tRFs, tRF5-GluCTC, tRF5-AlaCGC and tRF5-ValCAC, corresponded with 4,671, 3,813 and 5,234 target genes, respectively. The downregulated tRFs, tRF5-GlyCCC, tRF3-GlyGCC and tRF3-IleAAT, corresponded with 677, 3,448 and 856 target genes, respectively. The top 20 target genes with the target scores for each tRF are listed in Table IV.

GO and KEGG enrichment analysis. To further investigate the potential functions of these target genes, GO analyses, including the categories biological process, cellular component and molecular function, and KEGG pathway enrichment analyses were performed. The top 30 enriched GO terms and top 30 enriched KEGG pathways are presented in Fig. 3. The significantly enriched terms in the category cellular component were micro-ribonucleoprotein complex, the Rad51 paralog (Rad51)B-Rad51C-Rad51D-X-Ray repair cross complementing 2 (XRCC2) complex and ribbon synapse, and the enriched primary molecular function terms were nuclear factor of activated T-cells (NFAT) protein binding and fibroblast growth factor-activated receptor activity (Fig. 3A and B). The KEGG enrichment analysis indicated that the target genes were mainly enriched in axon guidance and neurotrophin, AMPK, mTOR and ErbB signaling pathways (Fig. 3C and D). The target genes associated with the enriched signaling pathways are presented in Fig. 3E.

Discussion

DN pathogenesis is complex and involves various biomolecules and signaling pathways, including advanced glycation end products (19), the renin-angiotensin-aldosterone system (20), oxidative stress and inflammation (21). Traditional treatments such as strict control of hyperglycemia and hypertension cannot effectively delay the progression of DM to ESRD (3). Hence, researchers have been increasingly committed to identifying specific and effective therapeutic targets for DN.

tRNA, an essential molecule responsible for transporting amino acids to the ribosome for protein synthesis, has been neglected in the past. A previous study indicated that tRNAs serve as major sources of small non-coding RNAs (sncRNAs) with unique and diverse functions (22). These tRNA-derived sncRNAs are not the result of random degradation but are produced by precise biogenic processes. Under various stress conditions, tRNA precursors and mature tRNAs are sheared

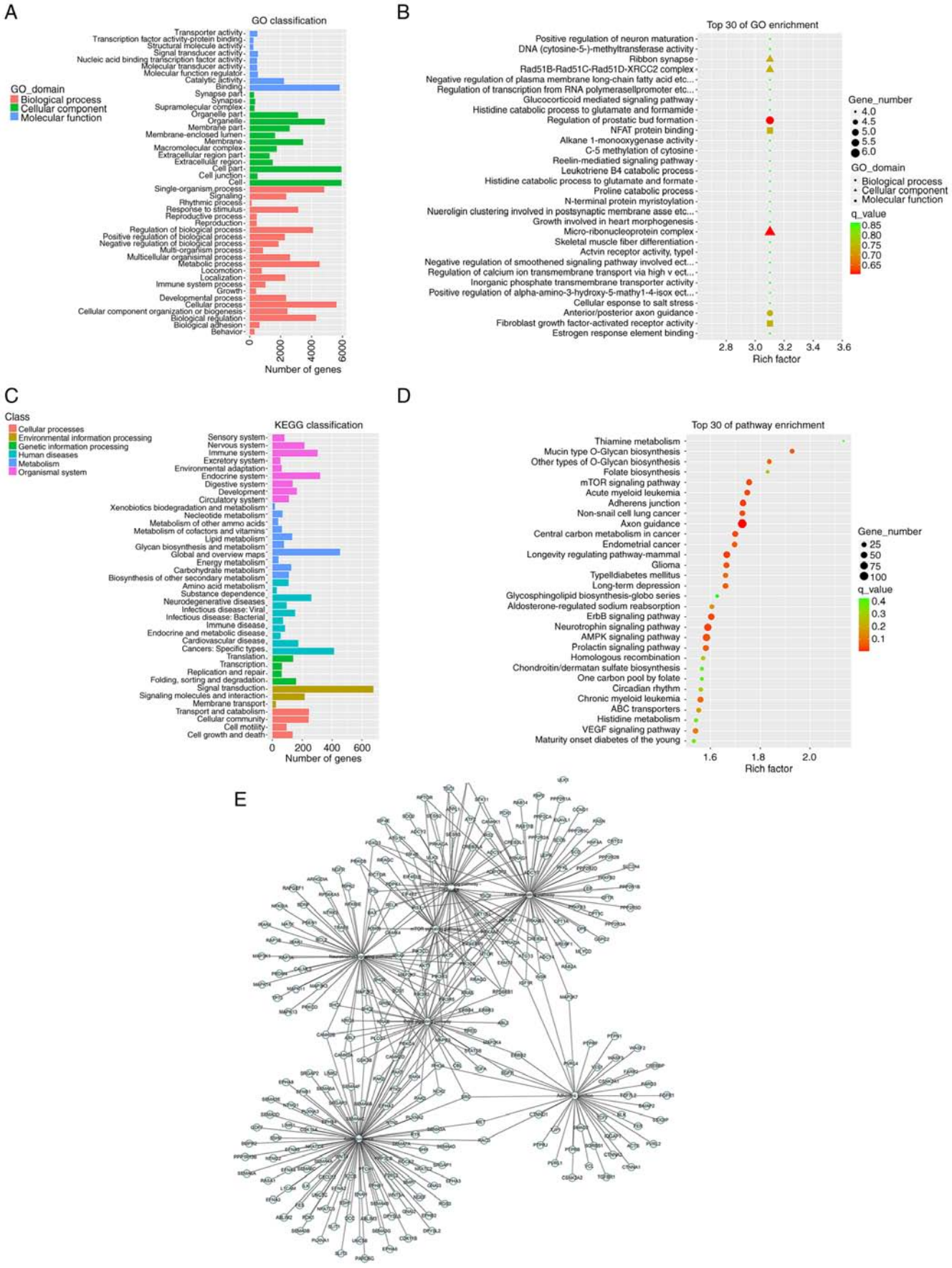

Figure 3. GO and KEGG analysis. (A) GO functional classification map for target genes of DE tRFs. (B) Top 30 GO terms for target gene enrichment analysis. (C) KEGG pathway classification map for target genes of DE tRFs. (D) Top 30 terms from the KEGG pathway enrichment analysis. A larger Rich Factor indicates a greater enrichment. The q-value is the P-value after correction for multiple hypothesis testing and a smaller value indicates more significant enrichment. A larger area of the graph indicates a greater number of genes. (E) Signaling pathway regulation network for predicted target genes. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; tRF, transfer RNA-derived fragment; DE, differentially expressed.

Table IV. Target genes with target scores in the top 20.

tRF	Targets	Log_2(fold change)	P-value
tRF5-GluCTC	C22orf46, ADAM11, KCNC4, TTC34, HEMK1, C1orf95, CD276, POTEC, TAB1, ATP8B3, FAM126B, ZNF395, CWC25, ADAM11, MMP24	2.09	0.003
tRF5-AlaCGC	TTC34, FCER2, ADGRA1, UNC5A, TRIOBP, RP1-37E16.12, SOX12, PSMC1, UBE2L3, MPRIP, UBE2L3, EHD2, C1orf95	2.09	0.012
tRF5-ValCAC	CLSTN2, TAB3, NBPF19, NBPF12, NCBP3, SHPRH, ZFAT, HEATR6, NBPF11, SEMA5A, KIAA1958, PPP1R21, MMACHC, ZNF891, MPV17L, DMTF1, DMTF1, DMTF1	1.80	0.032
tRF5-GlyCCC	SLC25A10, NCK2, KLC2, HNF1A, ESPL1, PPARD, EIF4G1, KIAA0930, CCDC151, STARD3, TSEN54, PCSK6, MDH2, INO80B, CDHR5, CDK18, GTPBP3, SDF4, XPC	-1.21	<0.001
tRF3-GlyGCC	METTL21A, SMAD2, ORAI2, CFLAR, CYP20A1, NFIX, NAT8L, TMOD3, RP11-5A19.5, KLC1, TMEM184A, KLC1	-1.21	<0.001
tRF3-IleAAT	MAFF, ARL2-SNX15, TRPV4, SNRNP70, IQSEC3, FAM57A, YIPF4, DCAF11, ACIN1, MAP3K9, HCLS1, DLG3	-3.36	0.035

tRF, transfer RNA-derived fragment.

by specific nucleic acid endonucleases to produce tRFs and tiRNAs. tRFs have attracted much attention in recent years because of their extensive biological functions. tRFs are abundantly expressed in various body fluids and are second only to miRNAs in abundance (23,24). Studies indicate that tRFs may have an miRNA-like regulatory effect, regulating gene expression by affecting mRNA stability. For instance, Goodarzi *et al* (25) found that tRFs from tRNAGlu, tRNAAsp, tRNAGly and tRNATyr suppress the stability of multiple oncogenic transcripts in breast cancer cells by competitively binding the RNA-binding protein YBX1. In addition, tRFs may regulate the translation process by affecting ribosome biogenesis, binding ribosomes and influencing the translation initiation process, and may interact with cytochrome C to regulate apoptosis and regulate gene expression as new epigenetic regulators (26,27). tRFs may also be involved in a variety of human diseases, such as cancer (28), kidney injury (29), viral infectious (30) and neurodegenerative diseases (31). In particular, tRFs have been confirmed to be dysregulated in a variety of cancer tissues, including ovarian, gastric, pancreatic and colon cancers, and are associated with the proliferation, invasion and migration of tumor cells (32). In the current study, high-throughput sequencing was used to identify six DE tRFs between DN and DM. The expression of tRF5-GluCTC, tRF5-AlaCGC and tRF5-ValCAC was significantly upregulated, whereas that of tRF5-GlyCCC, tRF3-GlyGCC and tRF3-IleAAT was significantly downregulated in patients with DN. RT-qPCR was performed to validate the expression levels and confirmed the results of the DE analysis. It may be hypothesized that the DE tRFs are associated with the development of DN.

GO analysis was performed to explore the potential biological functions of the DE tRFs. In the biological process category, anterior/posterior axon guidance had a high enrichment ratio. Axon guidance is the process by which axons originating from neurons form accurate synapses and is essential for the development of the nervous system. Semaphorin is an important axon guidance factor that contains the semiotic structural domain and semaphoring 3A (Sema3A) is currently the most widely studied semaphorin (33). Aggarwal *et al* (34) found that Sema3A expression was significantly upregulated in podocytes from patients with advanced DN and it was able to disrupt the glomerular filtration barrier, leading to massive proteinuria and renal failure. Further mechanistic studies revealed that Sema3A induced laminin and collagen IV accumulation in the glomerulus via nephrin, αvβ3 integrin and microtubule-associated monooxygenase calponin and LIM domain containing 1 interaction with plexin-A1, thereby resulting in diffuse podocyte peduncle loss and F-actin collapse. In addition, the axon guidance factor Netrin-1 and its receptor unc-5 netrin receptor B are involved in early DN angiogenesis (35). In the cellular component category, the most enriched terms were the micro-ribonucleoprotein complex, ribbon synapse and Rad51B-Rad51C-Rad51D-XRCC2 complex.

Oxidative stress is an independent risk factor for DN development. In patients with DN stimulated by persistent hyperglycemia, the level of oxidative stress is elevated and free radical production increases, leading to increased DNA damage (36). Rad51 protein is the core protein involved in DNA damage repair. The Rad51B - Rad51C - Rad51D - XRCC2 complex is involved in the entire process of DNA repair and is essential for maintaining genome integrity (37). In the molecular function category, NFAT protein binding and fibroblast growth factor (FGFs)-activated receptor activity were the most enriched terms. NFAT is a transcription factor with pleiotropic regulatory functions and is expressed in a variety of immune cells (38). NFAT is also widely expressed in other cells. NFAT is activated in response to high glucose stimulation, which exacerbates podocyte damage (39). FGFs are a group of growth factors with multiple isoforms that participate in angiogenesis, wound healing, embryonic development and various endocrine signaling pathways (40,41). FGF1 and FGF21 are promising therapeutic targets for DN. They delay DN-related renal injury by regulating glucolipid metabolism, inhibiting inflammation, reducing oxidative stress and improving insulin resistance. Conversely, FGF2 and FGF23 have been associated with worsening renal function in DN (42). In conclusion, GO analysis suggested that the DE tRFs may be associated with DN pathology.

KEGG pathway analysis demonstrated that the target genes were enriched in several pathways, including axon guidance, neurotrophin, AMPK, mTOR and ErbB signaling pathways. Neurotrophin signaling pathways are correlated with axon guidance. Each of these neurotrophic factors [nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3-3 and NT-4] may interact with tropomyosin receptor kinase (Trk) or p75NT receptors to activate Ras, PI3K, NF-κB and Jun kinase (43). miR-365 regulates high-fat diet/streptozotocin-induced DN fibrosis by targeting the BDNF-TrkB signaling axis (44). The mTOR and AMPK signaling pathways are the most important nutrient-sensing signaling pathways and are the most common pathways regulating cellular autophagy and disruption of autophagic homeostasis in renal cells affects DN progression (45). A study on the effect of butyrate on DN-induced muscle atrophy implied that butyrate was able to activate free fatty acid receptor 2-mediated PI3K/Akt/mTOR signaling to inhibit autophagy and exert a protective effect on DN-induced muscle atrophy (46). In addition, Li *et al* (47) investigated the role of the vitamin D receptor (VDR) in autophagy in DN and found that VDR deficiency led to a more severe autophagy defect in diabetic mice. However, paricalcitol or VDR overexpression restored autophagy defects. Furthermore, this study reported for the first time that paricalcitol ameliorates autophagy defects in high glucose-induced HK-2 cells, partially through the Ca^{2+}-calcium/calmodulin-dependent protein kinase kinase 2-AMPK pathway. In summary, the present KEGG analysis suggested that these DE tRFs may be involved in the regulation of DN via multiple signaling pathways.

Despite these findings, the current study still faced certain limitations. First, the relatively small sample size may have led to a large statistical difference between the samples; thus, further investigations based on a large sample size are needed. Furthermore, the present results were only based on tRF sequencing analysis and bioinformatics predictions. Thus, further functional validation studies using *in vitro* and *in vivo* models should be performed. Hence, in the future, a more in-depth study will be performed to confirm and further explore the present findings.

In conclusion, the current study examined the expression of serum tRFs in DN and identified six DE tRFs. Bioinformatics analysis indicated that these DE tRFs may be associated with the pathological processes of DN. The present findings provide a new perspective and theoretical basis for the study of therapeutic targets for DN.

Acknowledgements

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (grant no. 81970664), the Natural Science Foundation of Jiangsu Province (grant nos. BK20191082 and BK20211385), 789 Outstanding Talent Program of SAHNMU (grant nos. 789ZYRC202080119 and 789ZYRC202090251), the National Innovation and Entrepreneurship Training Program for College Students (grant no. 202210312038Z) and the Science and Technology Development Foundation of Nanjing Medical University (grant no. NMUB2020049).

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the Sequence Read Archive database under accession no. PRJNA916973 (https://www.ncbi.nim.nih.gov/sra/PRJNA916973).

Authors' contributions

CH and LD wrote the manuscript, prepared the figures and tables and confirm the authenticity of all the raw data. JJ and YQ collected the samples and performed the sequencing. ZX, HS and SZ were responsible for collating and analyzing the data. WG and AZ designed the study and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanjing Medical University [approval no. (2022)-KY-162.01] and all participants 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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