

Tissue tRNA-Derived Fragments (tRFs) as Potential Candidates Correlate with Invasiveness in Colorectal Carcinomas

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Abstract

Background

Colorectal cancer (CRC), which is one of lethal and invasive cancer with metastasis at the time of diagnosis. Despite, advanced therapy and surgical techniques, Patients with metastases CRC have a poor survival rate. Enhanced predictive biomarkers such as non-coding RNAs (ncRNAs) are needed at the time of diagnosis to better therapies. The tRNA- derived fragments (tRF) are non-protein-coding sequences that probably function as oncogenes or tumor suppressor genes, tRFs produced under physiological or stress conditions from enzymatically cleavage in tRNAs.

Methods

Documenting the tRNA-derived fragments (tRF) in colorectal cancer and showed that it is significantly down-regulated in colorectal tissue tumors compared to normal tissue samples. Since data distribution was not normal, Mann-Whitney, Kruskal-Wallis, and Spearman correlation coefficient tests were used for analysis.

Results

Among several tRF, quantitative real-time PCR analyses declared that tRF/miR-1280, tRNA-derived fragment-Asp, tRNA-derived fragment-Val down-regulated in CRC. Notably, its expression was correlated with invasive stage and metastases ($p < 0.05$).

Conclusions

This research can elucidate the association between tRFs with the invasion stage and CRC clinical pathology, our results not only expand better understanding about tRNA-derived fragments species in CRC but also highlight the potential usages of tRFs as a promising biomarker in the prognosis of metastatic cancer.

Introduction

Colorectal cancer (CRC) as one of the most ranked cancer type in the worldwide lead to the fourth most common cause of cancer-related deaths [Cancer \(1\)](#). It is occurred because of unusual growth in the colon or rectum. Epidemiological analysis proved that CRC is determined as a high prevalence rate disease in developed countries and is spread increasingly in underdeveloped countries [\(2\)](#). The Asymptomatic period of CRC usually lasts over five years. During the early stage of cancer transformation, adenomatous polyps can invade over the basement membrane. The accurate detection of CRC stage in order to determine the proper treatment has been considered as a preference in the health system [\(3\)](#).

Recently, according to the introduction of novel biomarkers, we witness that understanding cellular and molecular mechanisms of metastasis have been improved. It is worth to mentioning that a wide range of biomarkers including DNA, RNA, and proteins can play significant roles such as epigenetic and metabolic ones. Accumulating data proved that biomarkers have the promising potential to enhance diagnostic and therapy approaches (4). The advent of deep sequencing technologies has been led to the discovery of Non-coding RNAs (ncRNAs), as a group of ncRNAs named transfer of RNA-derived fragments (tRFs) (5). Based on the cleavage position of tRNA, tRFs are classified into two groups. First, tRNA halves (tiRNAs) that is produced by a specific cleavage in the anticodon loop of mature tRNAs, and the second one is tRNA-derived small RNA fragments (tRFs) which are derived from both mature and immature tRNA, shown in Fig. 1 (6). According to reliable sources, it is reported that the characteristics and function of these tRNA fragments remain largely unknown (7). Some databases, Non-coding RNAs data such as MINTbase that covering nuclear and mitochondrial tRNA-derived fragments (tRFs) in several human tissues have been developed. The original version of MINTbase included tRFs which discovered in 768 transcriptomic data (<https://doi.org/10.1093/nar/gkx1075>) (8).

Bioinformatics data analysis shows that the majority of these small RNAs were derived from tRNA under the stress condition. They play vital roles in various biological processes; such as cell proliferation, apoptosis, and angiogenesis in order to cancer progression. These regulatory functions can be performed by the intact tRNA and the fragments generated from tRNA (9, 10). The changes in the level of tRFs causes a transition phase in cell function through an effect on the DNA function, histone methylation, and chromatin structure (11).

Various studies show that tRFs have been linked to cancer development. The first report about *tRF-1001* in prostate cancer, which was derived from pre-tRNA Ser. Knocking down of *tRF-1001* led to a significant decrease in cell viability and proliferation(12). In addition, *tRF/miR-1280* has been studied in different cancers types, This tRF can suppress the thyroid carcinoma proliferation and invasion by targeting estrogen receptor α (13). Moreover, in bladder cancer cells, *tRF/miR-1280* can be inhibited invasion and metastasis by targeting Rock1 (14). the function of the *tRF/miR-1280* in CRC progression is so important and suppress Notch/Gata signaling by interaction with JAG2 3'UTR (7). *tRF/ miR- 1280* generated from cleave based in 3'fragment of a Lus tRNA. therefore, Schopman et al. suggested the annotated sequence named *tRF/miR-1280* then removed from the miRBase database ([http://www.mirbase.org/cgi bin/mirna_entry.placc¼MI0006437](http://www.mirbase.org/cgi-bin/mirna_entry.placc¼MI0006437)) (15).

tRF/miR -1308 is another non-coding RNA, that targets the apoptotic pathway through an NF KB inhibitor (NKIRA 1) (16). Nuclear factor-kappa B (NFkB) is playing a great role in regulating cell differentiation, proliferation, immune response and blocking apoptosis (17, 18). Recently the scientific are witness the reevaluation of the miRNA markers in the miRbase database. For instance, reported that miR-1308 is not a miRNA and it is a 5'-cleaved fragment of a Gly GCC tRNA,(tRNA fragment) (19). *miR-1280* and *miR-1308* classification turned to the set tRFs, which now are called as *tRFs/miR-1280* and *tRFs/miR-1308*, respectively (15, 19). Cellular validation research has proved that tRFs alteration patterns can be served as CRC metastatic predictors. We conducted a systematic review of tRNA-Derived small RNAs involved in

the pathogenesis of cancer types(20), Based on this, selected four tRFs that seem to be the role in cancer proliferation and metastasis. Moreover, we analyzed the MINTbase v2 database in order to check the profiling of small RNA transcripts (supplementary data). We next asked whether, these tRFs have the potential to the detective of CRC, by combining it with clinic pathological features that are routinely evaluated in these patients. In this study, we used Real-time PCR to demonstrate the four tRFs, in tissue samples of CRC, samples, In order to find new markers to diagnose or treat cancer.

Methods

Ethics statement

This study was conducted in accordance with the Ethics Committee of Iran medical university (number: IR.IUMS.REC.1397.1121) and all participants provided informed consent.

Specimen description

Sixty samples of paired colorectal cancer tissues (n = 30) and adjacent normal tissues (n= 30) resected surgically were collected from the Shariati and Firoozgar hospitals. None of these patients had chemotherapy before surgery. 30% of colorectal cancer patients had metastasized at the time of diagnosis of the original tumor. All of the fresh tissues transferred into "RNA later" and were stored frozen in liquid nitrogen in order to RNA extraction. The histopathological glass slides were microscopically reviewed by a pathologist to determine the cancer stage and clinical data are summarized in table 1.

RNA extraction from tissue samples

Total RNA from colorectal cancer tissues and adjacent non-tumor tissues was extracted by using miRNA easy Mini Kit (QIAGEN Cat No. ID/:217004). All steps of this protocol were performed according to the manufacturer's instruction kit. Briefly, Biological samples were lysed and homogenized in buffer containing the presence of a highly denaturing guanidine-thiocyanate, then inactivate RNases immediately and ensure the intact purification of RNA. Furthermore, ethanol was added to provide appropriate binding conditions. Then, the samples were put to an RNeasy Mini spin column, where the total RNA bind to the membrane, and contaminants are efficiently washed away. Lastly, High-quality RNA was eluted in 30–100 µl water and the RNAs quantity and purity were analyzed at 260/280 nm by NanoDrop (ND-1000 spectrophotometer).

Reverse transcription and cDNA synthesis

In the current study, we use total RNA, containing miRNA, for cDNA synthesis. Two µg of total RNA was used to prepare miRNA specific cDNA using the miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd.) all of these processes were done based on the manufacturer's instructions with the following temperature,1 h at 37°C and termination 5 min at 85°C in the Mini Thermal Cycler (QIAGEN).

Quantitative reverse transcription PCR (qRT-PCR) analysis

Q-PCR reactions were used for quantifying the expression of the RNAs on QIAGEN Rotor Gen Q system by using the SYBR green (Takara cat number: RR820Q). U6 RNA was used as an internal control for normalization and quantification. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ methods. The sequences of gene-specific primers were listed in Table 2.

- Statistical analysis

As noted, all quantified data has been replicated twice. Data distribution graphically was checked by Kolmogorov-Smirnov Test. Since data distribution was not normal, Mann-Whitney, Kruskal-Wallis, and Spearman correlation coefficient tests were used for analysis. All statistical analyses were performed in SPSS 20 software and the significance level was considered < 0.05 .

Results

Evaluation of candidate tRFs

The expression profiles of the four candidate tRFs were evaluated by using quantitative RT-qPCR method. As shown in Fig. 2, three tRFs, including *tRF / miR -1280*, *tRNA-derived fragment-Val and Asp* were down regulated in CRC patients compared with the healthy group ($p < 0.0001$); meanwhile, *tRF/miR 1308* did not show significant differential expression ($p < 0.394$).

Correlation tRFs in tissue sample

Based on past studies, we know that tRFs are derived from tRNAs and have three subclasses: tRF-5s, tRF-3s, and inter tRFs. The tRF-5s are derived from cleavage in the D-loop or arm region between the anticodon loop and the D loop of mature tRNA(21). The tRF-3s originate from cleavage in the T-loop and are ended with trinucleotides 'CCA'. The inter tRF is generated from the internal region of mature tRNA, which involves the anticodon loop and part of D-loop(22). It can be concluded that there is a co-relationship between tRF, probably has the same origin. Spearman's Correlations test was shown a positive correlation strongly between tRFs ($r_s > 0.66$, $p < 0.0001$) which is shown in Table 3.

Correlation tRFs with stage and size tumor of CRC

The primary stage CRC is called stage 0 (a very early cancer), and then classified from stages I through IV. As a principle, the lower the stage, the less tumor has spread. A higher number, such as stage IV, means cancer has expanse more. Tracing early-stage precancerous polyps before convert to cancerous in individuals at average risk for CRC, which leads to basic decreases in the incidence of cancer(23). Also, Tumor size is a reproducible predictive factor for the late stage in rectal cancers and helps to detect clinical staging and pre-operative management of patients(24). Kruskal-Wallis test shows that tRFs in stage IV have significantly lower expression levels than other stages ($p < 0.05$). There was no significant difference based on tumor size ($P > 0.05$).

Correlation tRFs with Differentiation degree

The studies proposed that three degrees of differentiation (poor, moderate, and well) are adequate for the grading of colorectal carcinomas and that grading based on the predominating degree of differentiation demonstrated in the primary tumor (25). Kruskal-Wallis test shows that tRF have relation with Cell differentiation. In Poorly-differentiated cell carcinoma, *tRF / miR 1308*, *tRF / miR -1280*, *tRNA-derived fragment-Val* and *Asp* are decreased. It is worth to mentioned that only *tRF / miR-1308* has a statistically significant relationship ($p=0.015$).

Correlation tRFs with metastases

Many colorectal cancers are probably to have diffuse from the site where they first formed to other parts of the body long before the original tumor can be detected by screening tests, new research results suggest. A Short time interval between CRC diagnosis and distant metastases indicates poor prognosis(26). Mostly colorectal tumors treated by radical surgery. Although 70%–80% of CRC are classified as high risk, less than 16% of these patients really have lymph node metastases. Biomarkers are needed to recognize patients with tumors with the highest risk of metastasis, to prohibit unnecessary radical surgeries(27). To evaluate the Correlation tRFs with metastases, the CRC samples are divided into two groups including metastasis to lymph node as well as distant metastasis groups. In the Mann-Whitney test, tRFs have a significant difference by metastases; it was found expression in distant metastasis is lower than lymph node metastasis. We observed decrease expression levels of *tRF / miR-1280* ($p=0.050$), *tRF / miR-1308* ($p=0.002$), *tRNA-derived fragment-Asp* ($p=0.018$) and *tRNA-derived fragment-Val* ($p=0.027$).

Discussion And Conclusions

Here, we identified the correlation between tRF and invasive stage CRC and reported that down-regulation of tRF/miR-1280, tRF Val, and tRF Asp relation with clinical outcomes, In addition, our study reveals a significant relationship between tRF and CRC invasive stage. Notably, this is first-time *tRNA-derived fragment-Val* and *Asp* investigated in CRC. The data presented here were identical with previous reports and indicate that tRFs play a key role in cancer progression and metastasis. Our outcomes could be valuable for designing a tRFs prognosis panel to determine invasive colorectal cancer.

Since cancer is extremely complex and heterogeneous, it needs too many studies research has been done to identify specific and practical biomarkers. The challenging field can be improved by the development of markers, which are allocated to only metastatic. Investigation of these biomarkers has great importance for personal medical care that leads to good prognosis and long median survival in neoplasm. .The expression of tRFs is deregulated in human cancer and caused many relevant changes at the molecular level for cancer progression. These days scientists suggested that tRFs can be used as novel molecular strategies in detection, monitoring, and treatment of cancer diseases (28).

It was shown that *tRF/miR-1280* plays a significant role in the migration/invasion by regulation of ROCK1 oncogene. a decrease of expressed *tRF/miR- 1280* in bladder cancer cell lines and tumor tissues, causes metastasis, therefore, targeting *tRF/miR-1280* might be useful for the treatment of bladder cancer (14).

Medulloblastoma (MB) is a malignant pediatric brain tumor, resistant to therapy and risk developing metastasis (29). Several studies have shown over-expression and over-activation of PDGF receptors (PDGFRs) and c-MYC in MB pathogenesis, malignant pediatric brain tumor (30). c-MYC is involved in PDGFR β signaling related to cell proliferation, cell death, and invasion(31). Moreover, previous studies showed delineating that *tRF/miR-1280* has an important role in the transcriptional regulation of JAG2 and *tRF/miR-1280* inhibitor can regulated expression of JAG2 in MB cells (32). *tRF/miR-1280* suppress cell proliferation and tumor growth by inhibiting the Notch signaling pathway by targeting JAG2. Also, transcriptional repression of the Gata1/3 and *miR-200b* genes, lead to decreased levels of *miR-200b* and elevated levels of JAG2, Gata1, Gata3, Zeb1, and Suz12 in colorectal cancer tissue specimens. Taken together, that *tRF/miR-1280* suppresses colorectal cancer growth and metastasis (7). Additionally, *tRF/miR-1280* is significantly suppressing Src proto-oncogene expression which frequently elevated in melanoma. *tRF/miR-1280* overexpression leads to suppress proliferation and invasive ability to melanoma cell. all of the above-mentioned data can be suggested that it represents a promising molecular target for anticancer therapy (33). The opposite results in the early stage of primary Non-small Cell Lung Cancer (NSCLC) patients reported that increasing rate of *tRF/miR-1280* can inhibit proliferation signaling pathways (34).

tRF/miR-1308 was considerably overexpression in non-small-cell lung cancer tissues (NSCLC). Concurrent *tRF/miR-1308* and *miR-124* are effects on *ADAM15* gene expression and up-regulated it(35). *ADAM15* is involved in the invasion and progression of tumors by up-regulating the matrix metalloproteinase 9 (MM9) expression in NSCLC patients, MM9 is a key regulator of cell invasion and promote lung cancer cell metastasis (36, 37). moreover in aggressive inflammatory breast cancer (IBC), and Hepatocellular carcinoma (HCC) analysis of microarray data in the cell line show a similar result for *tRF/miR1308* significantly upregulated (38) (39). *tRF/miR-1308 / miR-720* will be used as a diagnostic tool for screening healthy individuals in melanoma cancer patients. *tRF/miR1308* correlated with poor cell differentiation and increased invasiveness (18).

Although tRNA halves are commonly found in various mammalian cells, this tRNA cleavage can be induced under multiple stress conditions(40). There has been reported a decrease in *5'tRNA4-Val-AAC* levels in kidney and liver cancer. In clear cell renal cell carcinoma (ccRCC), tRNA halves have inversely correlated with the disease stage and grade (41). with comparing the exosomal tRNA-derived small RNA in liver cancer patients and healthy donors, the significantly different of tRNA-Val in the patient's plasma exosomes were identified (42). During cellular stress, tRF Val was related to Argonaute RISC Catalytic Component 2(AGO2), plays an inducing role in tumorigenesis and aggressiveness (43). In Bone marrow aspirate samples of patients with myelodysplasia syndromes (MDS), who were treated with DNA methyltransferase inhibitors (DNMTIs), tRF Val was identified. it shows that tRF Val can be used as a predictive biomarker for assessing response to DNMTIs treatment(40). *tRNA drive fragment Asp*, were up regulated in breast cancer cell lines under hypoxic conditions. These fragments by attaching to the RNA-binding protein YBX1 suppress the stability of multiple oncogenic transcripts. tRF Asp was significantly down-regulated in metastatic breast cancer rather than in non-metastatic ones. Thus, tRF Asp can play a role in cancer metastasis (44).

Evaluation of the optimal method for the screening of the sensitivity and specificity of these biomarkers is highly recommended. These novel biomarkers must be considered practically in the clinic, after following enough experiment validations optimized. Our analysis results indicated that tRFs can intervene in metastasis processes by genes regulating; therefore, they can be served to invasive stages prognosis or metastatic cancer treatment.

Abbreviations

AGO2: Argonaute RISC Catalytic Component 2

ANG: Angiogenin

CRC: colorectal cancer

CcRCC: clear cell renal cell carcinoma

DNMTIs: DNA methyl transferase inhibitors

HCC: Hepatocellular carcinoma

IBC: Inflammatory breast cancer

MDS: myelodysplastic syndromes

MB: Medulloblastoma

NGS: Next-generation sequencing

NSCLC: non-small-cell lung cancer

ncRNAs: non- coding RNA

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

qRT-PCR: Quantitative reverse transcription PCR

tRFs: tRNA- derived fragments

tiRNAs: tRNA-derived stress-induced RNAs

Declarations

-Ethics approval and consent to participate

This article was conducted in accordance with the **Ethics Committee of Iran medical university** (number: IR.IUMS.REC.1397.1121) and all of the cases and controls gave their informed consent prior to their inclusion in the study.

Consent for publication

The initial idea was given by J.Kiani and supervised the findings of this work. The responsibility of the authors is as follows: Maryam Sahlolbei: she did techniques, experiments and wrote the manuscript, Fahimeh Fattahi: provided technical help, Somayeh Vafaei: writing assistance, Rezvan Rajabzadeh: Data analysis and interpretation of data, Zahra Madjd: Critical revision of the article and discussed the results. All have consent to participate and publication

-Availability of data and materials

The data and materials that support the findings of this study are available from the corresponding author, upon reasonable request.

-Competing interests

Author Jafar Kiani had received research grants from IRAN Science Medical University. All of the authors declare that they have no conflict of interest.

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Tables

Table 1: Clinical and pathological data of CRC tissues.

Characteristics		Number of patients (n=30)
Gender	Female	12 (40%)
	Male	18 (60%)
Age	>60 years old	6 (20%)
	≤60 years old	13 (43%)
Pathological stage	Stage 1	2 (7%)
	Stage2	12 (40%)
	Stage 3	11 (36%)
	Stage 4	5 (16%)
Metastasis	Distant metastasis	11(36%)
	Lymph node metastasis	19(63%)
Differentiation degree(Grade)	Poor	11(36%)
	Moderate	3(10%)
	well	15(50%)

Table 2: list of primers

primers Name	Sequence
<i>tRF /miR -1280</i>	TCCCACCGCTGCCAC
<i>tRF /miR -1308</i>	GCATGGGTGGTTCAGT
<i>tRNA-derived fragment-Val CAC</i>	GTTTCCGTAGTGTAGTGG
<i>tRNA-derived fragment-Asp GTC</i>	GTCTCGATTCCCCGACGGG
<i>U6 RNA</i>	F: TTATGGGTCCTAGCCTGAC R: CACTATTGCGGGTCTGC

Table 3: Correlation between tRFs in tumor tissue (n=30).

Spearman's correlation coefficient	tRNA-derived fragment-Asp	tRNA- derived fragment-Val	tRNA- derived fragment-Leu /miR-1280	tRNA- derived fragment-Gly /miR-1308
<i>tRNA-derived fragment-Asp</i>	1	.748**	.853**	.766**
<i>tRNA- derived fragment-Val</i>		1	.746**	.815**
<i>tRNA- derived fragment-Leu/miR1280</i>			1	.662**
<i>tRNA- derived fragment-Gly/miR1308</i>				1

** . Correlation is significant at the 0.01 level (P<.0001).

Figures

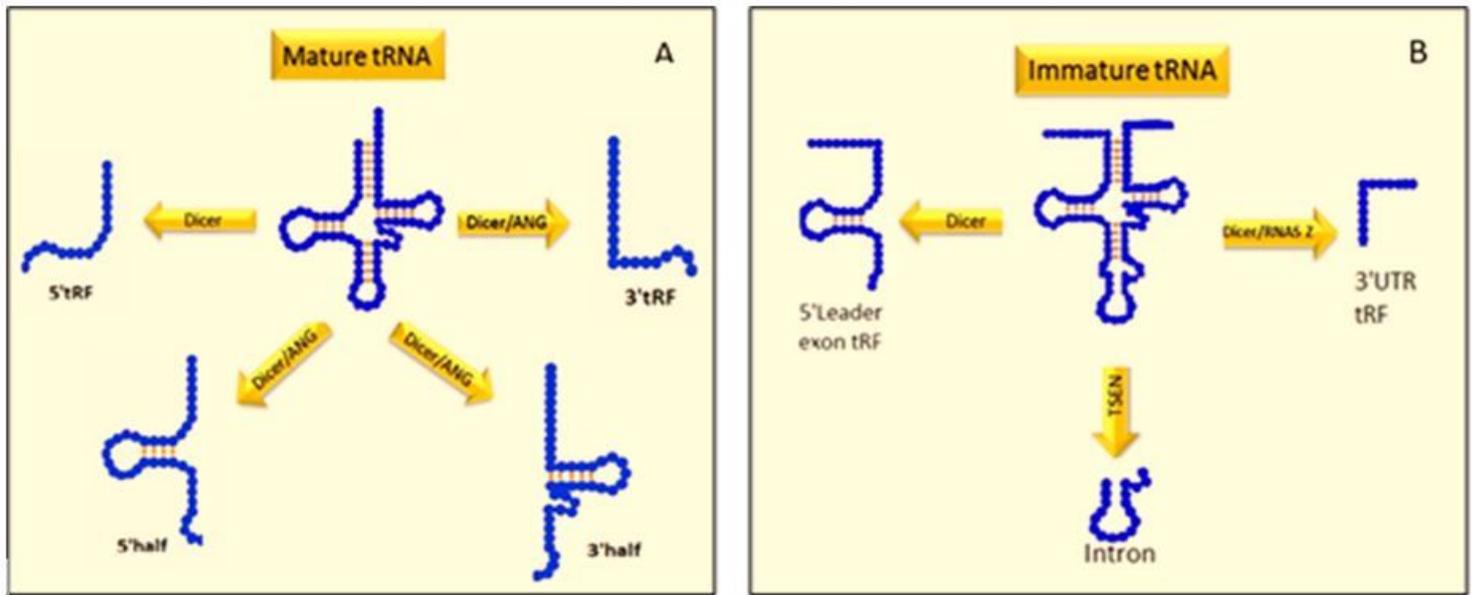


Figure 1

Processing of tRFs tRNAs drive fragment. The ribonucleases, RNase Z, Dicer, and ANG are involved in the biogenesis of tsRNAs. A) tRNA fragments derived from mature tRNAs and their processing. (B) tRNAs fragments derived from immature tRNAs and their processing.

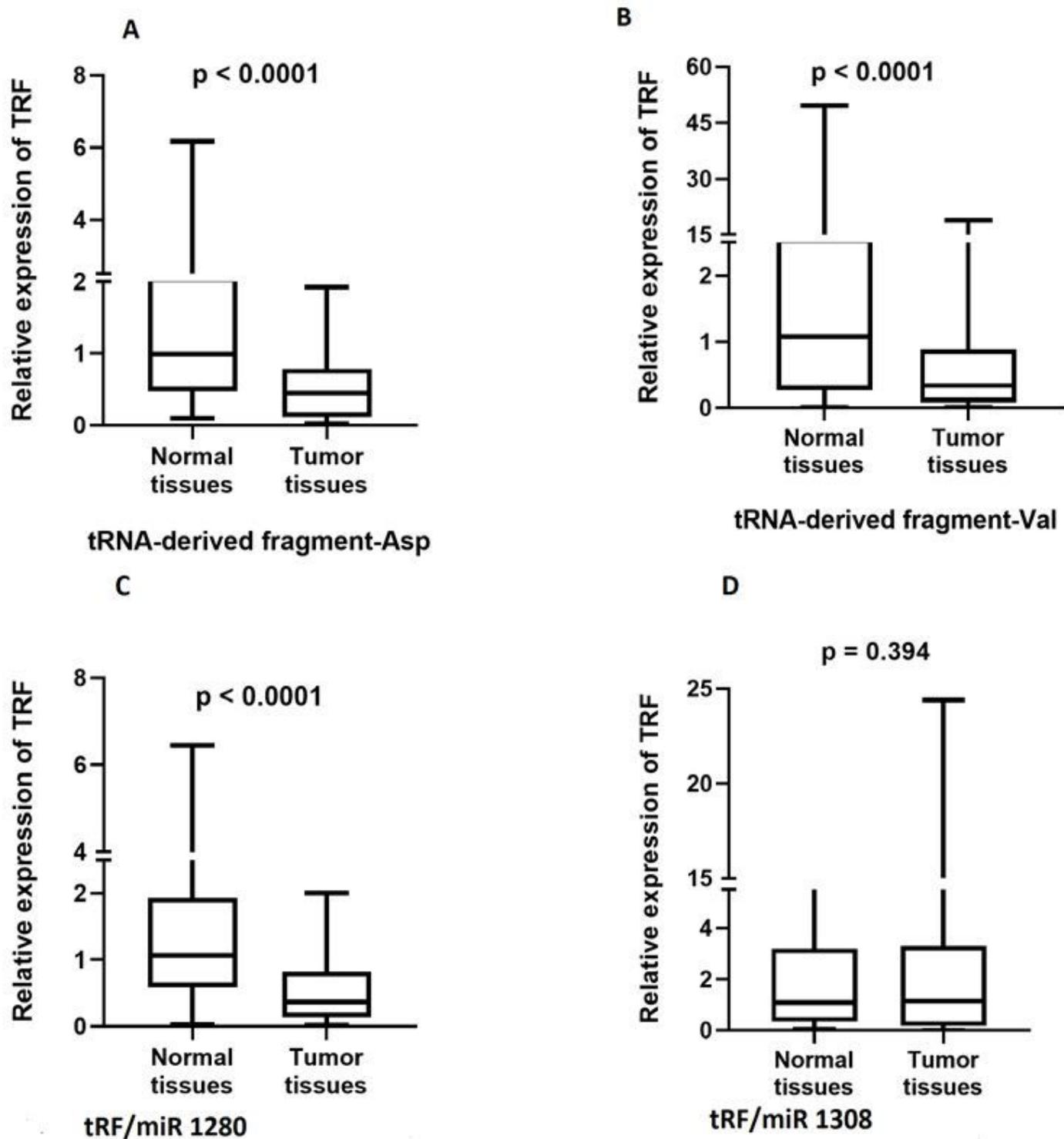


Figure 2

Expression profiles of candidate tRFs: A, B -The expression levels of tRNA-derived fragment- Asp and Val expression by qRT-PCR. C, D-The expression levels tRF/miR-1280 and tRF/miR-1308 in tumor tissue evaluated by qRT-PCR (n=30).

Supplementary Files

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