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Review

# Quantitative and qualitative detection of tRNAs, tRNA halves and tRFs in human cancer samples: Molecular grounds for biomarker development and clinical perspectives

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ABSTRACT

Transfer RNAs (tRNAs) are small non-coding RNAs playing a central role during protein synthesis. Besides translation, growing evidence suggests that in many contexts, precursor or mature tRNAs can also be processed into smaller fragments playing many non-canonical regulatory roles in different biological pathways with oncogenic relevance. Depending on the source, these molecules can be classified as tRNA halves (also known as tiRNAs) or tRNA-derived fragments (tRFs), and furtherly divided into 5'-tRNA and 3'-tRNA halves, or tRF-1, tRF-2, tRF-3, tRF-5, and i-tRF, respectively. Unlike DNA and mRNA, high-throughput sequencing of tRNAs is challenging, because of technical limitations of currently developed sequencing methods. In recent years, different sequencing approaches have been proposed allowing the quantification and identification of an increasing number of tRNA fragments with critical functions in distinct physiological and pathophysiological processes. In the present review, we discussed pros and cons of recent advances in different sequencing methods, also introducing the expanding repertoire of bioinformatics tool and resources specifically focused on tRNA research and discussing current issues in the study of these small RNA molecules. Furthermore, we discussed the potential value of tRNA fragments as diagnostic and prognostic biomarkers for different types of cancers.

## 1. Introduction

Despite recent advances in diagnostic techniques and therapeutic options, cancer is still a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020 (Cancer today, n.d.). Early detection is still one of the main challenges in the management of this pathology: many cancer types have long been called "silent killers" (Lõhmussaar et al., 2020), because signs of disease may arise very late, frequently after the illness has reached an advanced stage. To date, constant improvements of high-throughput genomic characterization technologies have allowed to measure nucleic acids at single-cell precision, leading to the discovery of many new molecular biomarkers of clinical utility, improving both early diagnosis and classification of several cancer types. Cancer biomarkers can be roughly classified into three main categories based on their clinical meaning: 1) diagnostic biomarkers, used to determine a specific disease to a patient; 2) prognostic biomarkers, informing clinicians about the expected outcomes or used for follow-up monitoring; and 3) predictive biomarkers, suggesting a patient's response to a given therapy (Goossens et al., 2015). Molecular profiling of tumor DNA to identify genomic variants (e.g. somatic or germline mutations) has been widely used to guide the clinical management of cancer patients, while the role of small non-coding RNAs as cancer biomarkers has been recently recognized and increasingly investigated (Zhang et al., 2021). Among non-coding RNAs, an emerging

*Abbreviations:* ALL, tRNAseq adapter-ligated libraries of tRNA-derived sequencing; ARM, seq AlkB-facilitated RNA methylation sequencing; DM, TGIRT-seq Demethylase-Thermostable Group II Intron RT tRNA sequencing; EMT, Epithelial to Mesenchymal Transition; HAC, seq Hydrazine-Aniline Cleavage sequencing; LC, liquid chromatography; mim-tRNAseq, modification-induced misincorporation tRNA sequencing; MS, mass spectrometry; OTTR, Ordered Two-Template Relay; PAR-CLIP, Photoactivatable crosslinking and immunoprecipitation; PHAM, Positive Hybridization in the Absence of Modification; QuantM-seq, Quantitative Mature tRNA sequencing; TLC, thin-layer chromatography; TRAC-seq, N7-methylguanosine ($m^7G$) reduction and cleavage sequencing; tRNA, Transfer RNA; tRNA, HydroSeq tRNA-enriched limited Hydrolysis Sequencing; tiRNA tRNA, derived stress-induced RNAs halves; tRF tRNA, derived fragments; YAMAT-seq, Y-shaped Adapter-ligated MAture TRNA sequencing.

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role for transfer RNAs (tRNAs) dysregulation and modification has been linked to cancer development (Gupta et al., 2022; Orioli, 2017).

tRNAs are small RNA molecules of approximatively 70–90 nucleotides in length, whose primary function lies in transferring amino acids to initialize and elongate peptide chains during mRNA translation, thus playing a regulatory role in both protein synthesis and the post-transcriptional regulation of gene expression. The different abundance of tRNAs matching synonymous codons has been shown to significantly change the efficiency of translation through a phenomenon called "codon usage bias" (Duret, 2002). Mature tRNAs fold into a characteristic "clover leaf" secondary and "L-shaped" tertiary structure (Root-Bernstein et al., 2016) (Fig. 1), whereas tRNA folding, stability, and function are influenced by different chemical modifications occurring during tRNAs biogenesis, one of which is tRNA methylation (Marchingo, 2022). Under various physiological conditions, including stress and the cellular response to different stimuli, mature tRNAs can be cleaved at the anticodon arm by the nuclease angiogenin and other ribonucleases (Su et al., 2019). This cleavage results in the formation of 5'-tRNA halves, which are fragments of approximately 34 nucleotides in length, and 3'-tRNA halves, representing the remaining portion of the mature tRNA molecule (Loher et al., 2017). Molecular functions of tRNA halves involve downregulating protein synthesis, producing stress granules, and interfering with siRNA-mediated silencing of stress-response genes (Kirchner and Ignatova, 2015; Su et al., 2019). While tRNA halves can be generated under a wide range of physiological circumstances and produced constitutively, some authors refer to tRNA-derived stress-induced RNAs halves (tiRNAs) as a subset of small RNAs that are specifically produced in response to cellular stress, such as exposure to oxidative stress, heat shock, or viral infection. This distinction may lead to potential confusion over the nature and role of these molecules (Fu et al., 2023; Magee and Rigoutsos, 2020; Shen et al., 2018). Shorter tRNA fragments are also produced by ribonuclease cleavage of mature or pre-tRNAs. These fragments, known as tRFs (also known as tRNA-derived small RNAs, or tsRNAs, and tRNA-derived RNAs, or tDRs), have been classified into 5 types (tRF-1, tRF-2, tRF-3, tRF-5, and i-tRF) according to the cleavage position of pre- and mature tRNAs (Magee and Rigoutsos, 2020; Sun et al., 2018; Zhang et al., 2020a) (Fig. 1).

It is becoming clear that tRNA halves and tRFs are an important class of regulatory small non-coding RNAs playing a role in several pathological contexts, including cancer, mitochondrial or metabolic diseases, viral infections, neurodevelopmental and neuropsychiatric disorders (Kirchner and Ignatova, 2015). Dysregulation of both tRNA processing and alterations of the tRNA pool have been described to play a role in cancer (Gupta et al., 2022; Kirchner and Ignatova, 2015; Wang and Lin, 2022): by regulating key processes like cell proliferation, apoptosis, epithelial to mesenchymal transition, drug resistance, and immunity, aberrant expression and dysregulated processing of tRNAs and tRFs have been shown to influence carcinogenesis, cancer progression, and the interplay between cancer cells and the tumor microenvironment (Chu et al., 2022; Jia et al., 2020; Kirchner and Ignatova, 2015), including the modulation of activity of T-cell-mediated immunity (Shan et al., 2020; Yue et al., 2021).

While the many aspects of tRNA biogenesis, signaling dynamics, and the emerging roles of both tiRNAand tRFs in cancer have been covered by several recent reviews (Chu et al., 2022; Fu et al., 2023; Gupta et al., 2022; Jiang and Yan, n.d.; Santos et al., 2019; Wang and Lin, 2022; Xie et al., 2020), the potential use of these molecules in cancer diagnosis, the possibility to detect alterations in tRNA halves and tRFs in body fluids or circulating tumor cells, and current methods and computational resources specifically designed to investigate small non-coding RNAs are

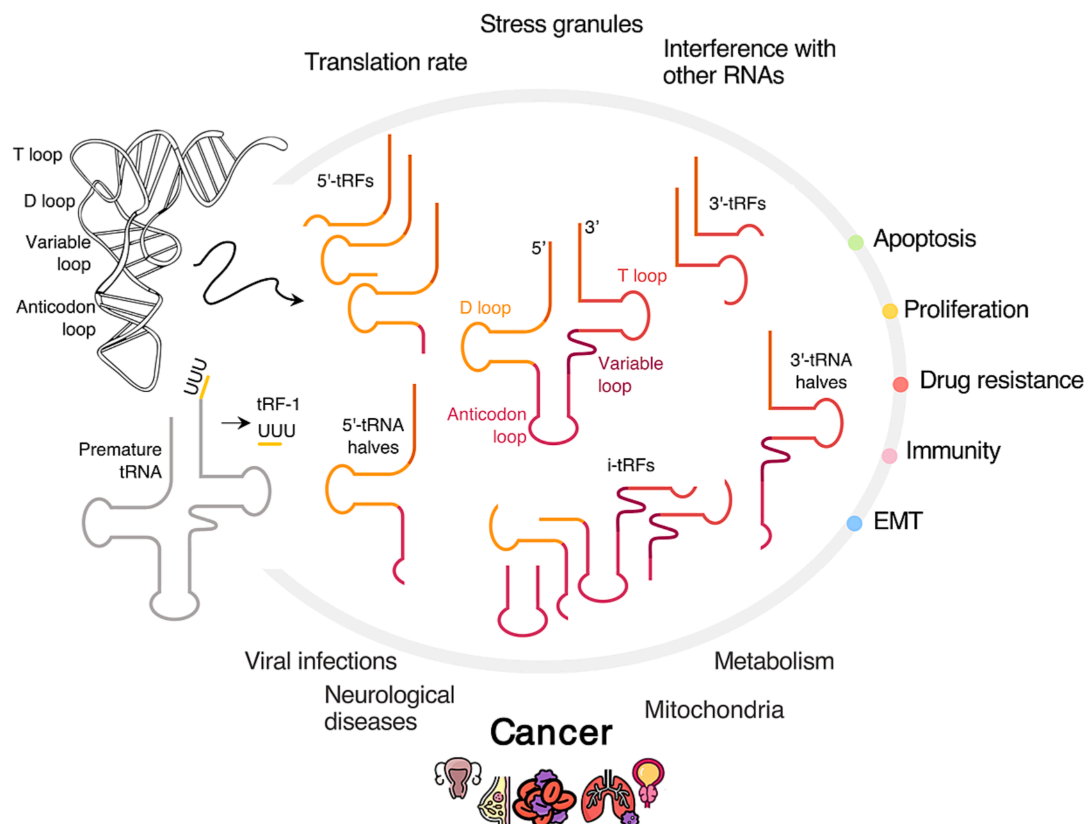


**Fig. 1. Classification of tRNA-derived fragments.** tRNAs regulates translation rates, gene expression, and the formation of stress granules. Precursor or mature tRNAs can also be processed into smaller fragments playing many non-canonical regulatory roles in different biological pathways with oncogenic relevance. Depending on their mapping positions on pre-tRNA or mature tRNA transcripts, tRNA fragments can be classified into 5′-tRNA and 3′-tRNA halves, or tRFs (tRF-1, tRF-2, tRF-3, tRF-5, and i-tRF).

still at a early stage. Therefore, the aim of the present review is to collect current knowledge about recently developed methods for the quantitative and qualitative detection of tRNAs and tRNA-derived fragments in human samples, providing a reasoned ground for their possible translation into clinical practice.

## 2. High-throughput quantification of tRNA expression and modifications

Unlike DNA and mRNA, high-throughput quantification of tRNA is still a challenging task (McArdle et al., 2020), mainly because tRNA has a highly stable secondary structure and it shows the highest density of post-transcriptional modifications among all RNAs (Lorenz et al., 2017). The presence of pervasive Watson-Crick face modifications makes tRNA-based cDNA synthesis very difficult because of the premature reverse transcription termination at modified sites, misincorporation of nucleotides (Liu and Pan, 2015), and challenges in adapter ligation, because the 5´-end of a mature tRNA binds tightly via base-pairs to the 3´-end as an acceptor stem, forming a rigid terminal structure that prevents adapter access (Shigematsu et al., 2017). Consequently, full-length tRNAs may appear as truncated species or fragments in sequencing data because of prematurely terminated cDNAs, leading to an underestimation of tRNA expression in biological samples. Furthermore, an extensive sequence similarity exists among tRNA genes, making reliable tRNA sequencing even more complicated. Taken together, these factors hampered the quantitative and qualitative analysis of the human tRNA landscape. To overcome the reverse transcription challenge, some authors applied array-based methods which do not require cDNA synthesis. For example, differential expression analysis of the tRNA pool in human cancer cell lines was made possible by the hybridization and quantification of tRNA-specific probes (Grelet et al., 2017). Essentially, tRNA microarrays involve a chemical ligation step to detect target tRNA isoacceptors, followed by the purification of chemical ligation products and then an hybridization step (Pavon-Eternod et al., 2009). A higher signal-to-noise ratio was achieved by the inclusion of the biotinylation of the tRNA population followed by streptavidin-mediated co-precipitation (Goodarzi et al., 2016). Another opportunity to analyze tRNA pools has been offered by northern blot. However, compared to sequencing-based methods, tRNA microarrays and northern blots can only achieve codon level resolution by recognizing the tRNA's anticodon loop, allowing to distinguish only tRNAs differing by at least eight nucleotide (Behrens et al., 2021). Moreover, arrays and northern blots do not provide information about potential tRNA modifications, losing a relevant portion of information about tRNA function. To overcome these limitations, some authors recently proposed a gel-based assay called positive hybridization in the absence of modification (PHAM) blot (Khalique et al., 2022). PHAM blot exploits the high sensitivity of DNA-oligo probe annealing to a tRNA sequence in the presence of nucleotides modification causing defective base-pairing. The use of modification-detection DNA-oligo allow to profile tRNA modifications occurring in different samples with reduced costs compared to sequencing techniques. Since this method relies on the differential annealing efficiency of a DNA-oligo to two or more sample tRNAs that vary in their levels of modification at a particular position of interest, it is strictly required to include in every experiment another DNA-oligo probe annealing to a different region of the same tRNA to be used as a control for normalization/calibration. Thus, experiments should be carefully planned to avoid misinterpretation of the results.

As discussed above, tRNA high-throughput sequencing is challenging because of difficulties in library generation. To date, many high-resolution approaches have been proposed with the aim to advance tRNA detection and analysis. Essentially, four main strategies have been followed by different authors: 1) enzymatic removal of known reverse transcriptase-blocking modifications (Cozen et al., 2015; Zheng et al., 2015); 2) use of improved reverse transcriptases (Behrens et al., 2021; Scheepbouwer et al., 2023; Zheng et al., 2015); 3) use of tRNA fragmentation strategies (Gogakos et al., 2017); 4) or improving the tRNA ligation step (Shigematsu et al., 2017). These strategies have been also coupled to enhance sequencing efficiency (Scheepbouwer et al., 2023).

Quantitative Mature tRNA sequencing (QuantM-seq) is a quick and simple method to monitor tRNA levels and to explore potential tRNA modifications (Pinkard et al., 2020). Although efficient, QuantM-seq does not take any action to overcome reverse transcription blocks, yielding mostly short reads due to premature stop at modified sites. An elegant solution to get a snapshot of the tRNA landscape of human cells has been offered by the protocol developed by Zheng et al. (Zheng et al., 2015), who developed a method named "DM-tRNA-seq" or DM-TGIRT-seq (demethylase-thermostable group II intron RT tRNA sequencing). This method involves the treatment with a demethylase mix before generating the cDNA by using a thermostable group II intron reverse transcriptase, which does not require adapter ligation, since it synthesizes cDNA by template-switching from the adapter to the 3′ end of the target RNA. The enzymatic removal of some base methylations is also the strategy adopted in AlkB-facilitated RNA methylation sequencing (ARM-seq) (Cozen et al., 2015), while library production by Ordered Two-Template Relay (OTTR) is a recently developed strategy to sequence both tRFs and mature tRNAs from the same sequencing pool by exploiting template jumping by nonretroviral reverse transcriptase (Upton et al., 2021). Other authors have included tRNA fragmentation in their protocols to overcome structure- and modification-induced reverse transcription barriers: it is the solution adopted by tRNA-HydroSeq (tRNA-enriched limited hydrolysis sequencing) (Arimbasseri et al., 2015; Karaca et al., 2014) also coupled with photoactivatable cross-linking and immunoprecipitation (PAR-CLIP) (Gogakos et al., 2017), and YAMAT-seq (Y-shaped Adapter-ligated MAture TRNA sequencing) (Shigematsu et al., 2017).

Despite having greatly improved our ability to investigate tRNA pools in human cells, all these protocols contain a potential source of bias in quantification of tRNAs due to a differential recovery toward tRNA subsets with few modified sites or those tRNAs showing sites that are more accessible to enzymes activity. The full-length cDNA library construction is the goal of modification-induced misincorporation tRNA sequencing (mim-tRNAseq), which is obtained by improving the efficiency of the thermostable template-switching reverse transcriptase used in DM-tRNA-seq. mim-tRNAseq integrates a primer extension assay to detect multiple tRNA modification types simultaneously. The overall objective is to reduce alignment ambiguity by increasing full-length cDNA reads and improving reads mapping (Behrens et al., 2021). While most of the current existing protocols apply reverse transcription before adapter ligation, Scheepbouwer et al. (Scheepbouwer et al., 2023) proposed the use of the ultraprocessive group II intron maturase MarathonRT combined with RNA demethylation to perform the reverse transcription step after adapter ligation to allow analysis of both full-length tRNA molecules and their derived fragments, overcoming the tRNA length bias. They named this method ALL-tRNAseq (adapter-ligated libraries of tRNA-derived sequencing).

The biological functions and the association of defects in post-transcriptional tRNA chemical modifications with several human diseases have been increasingly studied in the last few years (Hou et al., 2015). Direct detection of post-transcriptional tRNA modifications and structure can be achieved taking advantage of single-molecule nanopore sequencing by the Oxford Nanopore Technologies platform, which allows for the detection of modified nucleotides during the sequencing process without reverse transcription or amplification steps (Thomas et al., 2021). Some authors proposed modified sequencing protocols including chemical treatments to enhance the accuracy of specific tRNA modifications analysis: it is the case of Hydrazine-Aniline Cleavage sequencing (HAC-seq), specifically designed to profile 3-methylcytidine ($m^3C$) tRNA modifications (Cui et al., 2021), and N7-methylguanosine ($m^7G$) reduction and cleavage sequencing (TRAC-seq), designed to profile $m^7G$ tRNA modifications(Lin et al., 2019). Finally, a modified

version of RNA bisulfite sequencing /RBS-seq) has been used to profile 5-methylcytosine ($m^5C$) and 1-methyladenosine ($m^1A$) modifications, together with pseudouridine (Ψ) (Khoddami et al., 2019).

The analysis of tRNA fragments, like tRFs, has been accomplished by some authors using miRNA-sequencing protocols (miRNA-seq). In fact, during miRNA-seq library preparation, small RNAs ranging between 20 and 50 nucleotides in size are selected by electrophoresis. This range allows for the capture of many other species of small RNAs, in particular tRFs (Cole et al., 2009; Krishnan et al., 2016).

## 3. Quantification and analysis of tRNAs from biological samples

Although cheaper than RNA-sequencing, the quantification and characterization of tRFs using PCR suffers from several issues. The variability in tRF profiles stemming from different PCR methodologies and primer sets can lead to difficulties in comparing results across studies, and commercially available quantitative PCR (qPCR) methods are capable to detect variation in small RNAs (Magee et al., 2017). Sensitivity and specificity are paramount concerns, as PCR may struggle to detect low abundance tRFs or differentiate highly similar tRF species. Moreover, post-transcriptional modifications on tRFs can affect PCR efficiency, complicating accurate quantification, while proper normalization techniques to address variations in input RNA and reverse transcription efficiency are essential. The choice between qPCR and reverse transcription PCR (RT-PCR) methods can impact precision and accuracy (Olvedy et al., 2016). Reference gene selection, contamination prevention, and reproducibility considerations are additional factors that researchers must navigate in studying small RNA molecules. For these reasons, Honda et al. proposed a modified RT-PCR protocol, dumbbell-PCR (Db-PCR). Ensuring a single-nucleotide resolution at both the 5′- and 3′-terminal sequences, this method has been developed to specifically quantify unique variations to the 5′- and 3′-ends of individual small RNAs (Honda and Kirino, 2015).

Apart from sequencing-based techniques, quantification, characterization, and analysis of tRNA pools can be also performed via several biochemical techniques. In fact, quantitative analysis of tRNA modifications can be performed by thin-layer chromatography (TLC) (Grosjean et al., 2007) or liquid chromatography (LC) (Su et al., 2014) coupled with mass spectrometry (MS) (Wang and Lin, 2022). While these techniques may detect all modification types in purified tRNAs without requiring a reverse transcription step, they are unfortunately not suitable for the analysis of individual tRNAs' modification-specific sites and states (Wang and Lin, 2022), but they are still useful at measuring changes in relative levels of tRNAs and tRNAs modifications between different samples (Su et al., 2014). tRNA modifications have been traditionally investigated by primer extension assay, which recognizes reverse transcription termination or mismatched base insertion. Primer extension assay has the main drawback that it cannot identify the specific modification type, while returning a high false-positivity rate. Even if these techniques lack of the resolution power of high-throughput sequencing technologies, they are still useful to investigate the tRNA landscape of human cells, especially when the goal of the analysis is the detection of a given tRNA biomarker of interest for diagnostic/prognostic purposes. It is also to be considered that, despite sequencing platforms are currently widely spread also in clinical settings, not all the hospital facilities have direct access to these technologies, so biochemical/molecular biology-based assay may represent good alternatives to investigate tRNAs in biological samples.

### 3.1. Bioinformatics resources for tRNA analysis

In this section, we will review some bioinformatics tools to help researchers to investigate the tRNA landscape in human samples. A list of all bioinformatics resources to investigate tRNA biology discussed below has been included in Table 1.

**Table 1**
A collection of tRNA- focused bioinformatics resources.

| Tool | Description | Link |
|---|---|---|
| ARAGORN | Classic tool for the identification of novel tRNA genes and the prediction of tRNA secondary structure | ansikte.se/ARAGORN/ |
| GtRNAdb | A genomic database containing a collection of automated (not curated) tRNA gene predictions made by tRNAscan-SE on complete or nearly complete genomes. | gtrnadb.ucsc.edu/ |
| ENCORE | An upgraded version of RMBase that mainly focuses on the mechanism and function of 73 types of RNA modifications in 62 species. | rna.sysu.edu.cn/encore/ |
| MINTBase | A database collecting sequence information on mitochondrial and nuclear tRNA fragments. | cm.jefferson.edu/MINTbase/ |
| MINTmap | A software tool to map tRF profiles from short RNA-seq datasets. Source code is available from a dedicated GitHub repository. | cm.jefferson.edu/mintmap/ |
| MODOMICS | A database collecting information on sequence and structure of tRNA chemical modifications. Tables summarizing the role of tRNA modifications in human diseases and affected pathways are available for download in.csv and.pdf format. | genesilico.pl/modomics/ |
| OncotRF | A database of tRFs dysregulation in cancer. It integrates a survival analysis tool. | bioinformatics.zju.edu.cn/OncotRF/ |
| RMBase | A comprehensive database to integrate epitranscriptome sequencing data for exploring post-transcriptionally modifications of RNAs. It contains four web-apps: *Motif* allows to visualize position weight matrices (PWM) logos; *modRBP* allows the study of RNA-binding proteins and RNA modifications relationship; *modSNV* collects RNA modification sites and single nucleotide variants sites and their link with different disease; while *modMetagene* allows to plot metagenes of RNA modifications along a transcript model. | rna.sysu.edu.cn/rmbase/ |
| tatDB | A database collecting tRF and target genes information. A review of tRNA biology knowledges is also available in the Help tab of this tool. | grigoriev-lab.camden.rutgers.edu/tatdb |
| tDRMapper | A mapping tool specifically designed to map tRNA reads on Illumina small RNA-seq data (human samples) | github.com/sararselitsky/tDRmapper |
| tModBase | A database to investigate the relationships between tRNAs, their modifications, expression of tRNA-modifying enzymes and diseases | tmodbase.com |
| tncRNA Toolkit | A Python pipeline to detect and describe tRFs from sequence data. | nipgr.ac.in/tncRNA |
| tRAX | A software package for in-depth analyses of mature tRNAs, sRFs, and inference of RNA modifications from high-throughput small RNA-seq data | trna.ucsc.edu/tRAX/ |
| tReasure | An R-based tool to align, quantify, and perform tRNA differential expression analysis from small RNA-seq data | github.com/jinoklee/tReasure |
| tRFdb | A database collection of tRFs from H. sapiens and 4 model organisms (Mouse, Drosophila, S. pombe, C. elegans). | genome.bioch.virginia.edu/trfdb |
| tRFexplorer | Allows users to investigate expression profiles of tRNA fragments in TCGA tumor and NCI-60 cell samples | trfexplorer.cloud/ |
| tRic | A data portal to explore the expression landscape of tRNAs across 31 human cancer types | hanlab.uth.edu/tRic/ |
| tRFTAR | A tRF-Target database | rnanut.net/tRFTar/ |

(continued on next page)

Table 1 (*continued*)

| Tool | Description | Link |
|---|---|---|
| tRFTarget | A tRF-Target database and a pipeline to detect novel target genes from small RNA-seq datasets | trftarget.net/ |
| tRNAscan-SE | A web server to search for tRNA genes in genomic sequences | lowelab.ucsc.edu/tRNAscan-SE/ |
| tsRFun | A database to investigate tRNA-fragments expression in 32 human cancer types. It contains tsRFinder, a tool to identify tRNA fragments in small RNA-seq data. | rna.sysu.edu.cn/tsRFun/ |
| unitas | A Perl software to annotate and analyze small RNA-seq data | sourceforge.net/projects/unitas/ |
| YM500v3 | A cancer-focused human small RNA database | ngs.ym.edu.tw/ym500/ |

### 3.2. *tRNA sequencing reads mapping and annotation*

The unique features of tRNAs and tRNAs fragments make mapping and quantitation of sequencing reads complicated, especially in human samples. In fact, the human genome encodes more than 500 tRNA genes, with the majority of them having at least one identical copy elsewhere in the genome, indicating a high level of redundancy (Torres, 2019). Considering these challenges, some authors have developed specifically designed tools to characterize tRNAs and tRFs transcriptomes. The strategy adopted by the authors of tDRmapper, a tool designed for analyzing RNA transcripts originating from pre or mature tRNAs, is to map sequencing reads only to the tRNA space and not to the entire genome (Selitsky and Sethupathy, 2015). tDRmapper was specifically tested to work on human small RNA-seq data (single-end, 50x) generated on the Illumina sequencing platform using cDNA libraries prepared with the Illumina TruSeq protocol. Although effective, this strategy has been criticized because it could lead to the assignment of sequenced reads that can be unrelated to tRNAs, leading to the generation of many false positives (Telonis et al., 2016). The same issue is affecting also the tRAX (tRNA Analysis of eXpression) workflow, which uses a custom-built reference database including all tRNA loci in the reference genome (matching all pre-tRNAs), as well as fully processed mature tRNA transcripts to map sequencing reads. A prioritizing criterion is adopted, assigning reads firstly to mature tRNA transcripts, followed by reads aligning to genomic loci that cover all precursor tRNAs and then to other small non-coding RNAs. Multi-mapping is the default setting in tRAX, to account for the major contributions of identical or near-identical tRNA genes (Holmes et al., 2022). However, both tRAX and tDRmapper allow mismatches when mapping reads, thus leading to possible misassignment and incorrect quantification of tRFs (Loher et al., 2017).

Building an artificial tRNA genome to map tRNA sequencing reads is also the solution proposed by tReasure (Lee et al., 2022), a package to analyze tRNA expression profiles to be used in R (Giorgi et al., 2022). Similarly, MINTmap was designed to profile tRNA fragments from short reads RNA-seq (Loher et al., 2017), and tncRNA toolkit (Zahra et al., 2023), which uses tRNAscan-SE (Chan et al., 2021) to index the genome of interest predicting tRNAs sequences. Another option is provided by the unitas software (Gebert et al., 2017), which allows the complete annotation of small RNA sequence datasets, supporting all the species for which non-coding RNA reference sequences are available in the Ensembl databases (Cunningham et al., 2022).

### 3.3. *tRNA databases*

To date, several databases that collect information over tRNAs and their modifications are freely available online. One of the most known and most commonly cited web-based source of tRNA gene information is the Genomic tRNA Database (GtRNAdb) (Chan and Lowe, 2016). GtRNAdb collects and organize tRNA annotations from the tRNAscan-SE search tool (Chan et al., 2021) delivered as an interactive data portal. The current update (Data Release 20, Dec 2022) contains information over 584 eukaryotic genomes for a total of 168,849 (pseudogenes are not counted). The MODOMICS database is a manually curated resource providing comprehensive information about the chemical structures of modified ribonucleosides and their biosynthetic pathways that can be queried by SMILES codes or by drawing chemical molecules (Boccaletto et al., 2022). RMBase and tModBase are two resources developed by the same authors summarizing the landscape of tRNA modification profiles obtained from epitranscriptome sequencing data (Lei et al., 2023; Xuan et al., 2018). RMBase has evolved into ENCORE (The Encyclopedia of RNA Epitranscriptome), providing seven web-based tools to analyze 73 types of RNA modification among 9 clades and 62 species. This database currently maps 1,074,100 different RNA modification sites. Another very popular tool is MINTBase (Pliatsika et al., 2016), a repository collecting mitochondrial and nuclear tRFs. The authors encourage the submission of brand new tRF human datasets through the https://cm.jefferson.edu/MINTsubmit/ page. MINTBase was updated to version 2.0 in 2018. This new release includes nuclear and mitochondrial tRNA fragments identified through the analysis of all The Cancer Genome Atlas project datasets (Pliatsika et al., 2018). A collection of over 11,000 cancer-related small RNA sequencing datasets can be browsed at the YM500v3 database, offering also different sections to perform several analysis, like survival analysis (Chung et al., 2017), while tRFdb is another resource containing information over tRFs from publicly available sequencing dataset.

A collection of computationally predicted and experimentally validated tRF-target interactions is available at tRFtarget (Li et al., 2021), including information on putative binding sites. Predictions of tRF-target gene interactions via systemic re-analysis of Argonaute CLIP-seq datasets are collected in tRFTAR (Zhou et al., 2021), while tatDB (Guan and Grigoriev, 2023) offers a comprehensive collection of Ago1-mediated targets of transfer RNA fragment, allowing the user to search for gene targets of tRNA fragments (and vice-versa).

### 3.4. *Cancer-oriented tools*

Together with the above discussed database YM500v3, few web tools designed to sustain the biomedical research and discovery of the association between tRNA dysregulation and cancer have been released in recent years. tRF2Cancer (Zheng et al., 2016) (available from 2022 as a complete online suite to investigate tRFs expression, functions and prognostic value in cancer types, called tsRFun (Wang et al., 2022a) provides the user to browse the expression of tRFs among 32 different types of cancers from the TCGA cohort (Weinstein et al., 2013) and to visualize survival analysis searching for prognostic tRFs markers. Similar functions are also offered by OncotRF (Yao et al., 2022), integrating both data from TCGA samples and a literature review from PubMed searches, and the tRic data portal (Zhang et al., 2020a). Furthermore, the tRFexplorer database enable the user to browse the expression profile of each tRNA-derived fragment in every cell line included in the NCI-60 project as well as for each TCGA tumor type. Users can also perform their own differential expression analyses of tRNA fragments in all TCGA cancer subtypes (e.g. tumor vs. normal, metastatic vs. primary samples), also investigating the effect of variables such as gender, race, vital status, and tissue classification. Moreover, this database also allow to perform correlation analyses of tRNA fragments expression with gene and miRNA expression in TCGA or NCI-60 samples, in association with all omics and compound activities data available on CellMiner (La Ferlita et al., 2019).

### 3.5. *tRNA biomarkers for clinical applications in cancer*

Recent developments in liquid biopsies brought up the possibility to monitor circulating tumor nucleic acids released by tumor masses in free form or in circulating exosomes in oncology patients (Stejskal et al., 2023). Circulating tRNAs are usually found in the blood in fragmented

form, both tRNA halves and tRFs. It is thought that tRNAs may show higher turnover rates in cancers tissues compared to normal counterparts, and that expression levels may be higher in cancer under certain stress conditions, suggesting their potential use as cancer diagnostic biomarkers (S and A, 2022). In the last few years, an increasing number of studies linking specific tRFs profiles detected in plasma samples from oncology patients have been published (Vafaei et al., 2020; Yu et al., 2020). A comprehensive *meta*-analysis assessing the validity of tRNA fragments expression profiles from blood and solid tumor samples as biomarkers for cancer diagnosis/prognosis was released very recently (Gao et al., 2022a). The diverse roles of tRNA-derived fragments (tRFs) in cancer have been highlighted by a comprehensive pan-cancer analysis encompassing 32 different cancer types, as reported by Telonis et al. (Telonis et al., 2019). This analysis revealed distinct subtype-dependent associations between tRFs and mRNAs that regulate crucial cellular processes. These associations involve mRNAs related to developmental processes, receptor tyrosine kinase and MAPK signaling, regulation of protein degradation by proteasomes, and metabolic pathways that are frequently dysregulated in cancer. Intriguingly, gender differences have been reported in a few cancer subtypes, including bladder, lung, and kidney cancer (Telonis et al., 2019). Furthermore, it has been demonstrated that tRF expression is influenced by various factors such as gender, race, tissue type, and disease subtype. Notably, the fragmentation patterns of tRNA do not appear to correlate with the locations of tRNA modifications. Additionally, the length distribution of mitochondrially-encoded tRNAs differs from that of nuclearly-encoded tRNAs, underscoring the significance of tissue type in tRF characteristics. This distinction also highlights differences in the abundance of tRFs produced, with mitochondrial tRNAs exhibiting a higher propensity to generate tRFs (Telonis et al., 2019, 2015).

Although far from being a complete review of recent advances in tRNA biomarkers in cancer, a task that has been already accomplished elsewhere (Fu et al., 2023; Gupta et al., 2022; S and A, 2022; Santos et al., 2019; Sun et al., 2018; Vafaei et al., 2020; Yu et al., 2020; Zhang et al., 2020b; Zhang et al., 2021), our aim in the present work is to summarize main findings that we believe could be rapidly translated into clinical practice.

### 3.6. Breast cancer

In breast cancer samples, both nuclear- and mitochondrial-encoded tRNAs overexpression has been reported by several authors (Kwon et al., 2021; Mahlab et al., 2012; Pavon-Eternod et al., 2009), and linked to enhanced metastatic potential and cancer progression (Goodarzi et al., 2016). Frequently, breast cancer samples show altered post-transcriptional tRNA modifications, due to dysregulation of RNA methyltransferases activity, which has been shown to play a role in breast cancer pathogenesis (Delaunay et al., 2016; Liu et al., 2022). Mutations in mitochondrial tRNAs, such as mt-tRNA$^{Asp}$, also play a role in the carcinogenesis of breast cancer, possibly serving as diagnostic markers (Meng et al., 2016). Changes in the abundance of specific tRNA-derived fragments have been suggested to be a consequence of dysregulated oncogenes activity, and their characterization may be useful for cancer patient staging (Balatti et al., 2017). Recently, a panel of differentially expressed tRNA-derived fragments detected from plasma of breast cancer patients vs. healthy donors were described to be useful as diagnostic and prognostic biomarkers of breast cancer. In particular, the authors reported the diagnostic values of tRF-Arg-CCT-017, tRF-Gly-CCC-001, and tiRNA-Phe-GAA-003 in distinguishing patients with breast cancer from healthy controls, and the prognostic values of tRF-Arg-CCT-017 and tiRNA-Phe-GAA-003, being a higher expression associated with a worse disease-free or overall survival (Wang et al., 2021). Shan et al. reported five tRFs associated with prognosis emerging from the analysis of 1081 female patients with primary breast cancer. A decreased risk of death was reported for patients showing higher levels of tRF5024a, 5P_tRNA-Leu-CAA-4–1, and ts-49, while ts-34 and ts-58, were negatively associated with overall survival (Li et al., 2021; Shan et al., 2020). It has also been reported that the expression patterns of tRFs in breast cancer patients may vary depending on the patient's race (Telonis and Rigoutsos, 2018).

### 3.7. Gastrointestinal cancers

Tumors arising from the gastrointestinal tract are also affected by alteration in the tRNA pool (Balatti et al., 2017). Many tRFs and tRNA halves were found to be differentially expressed in gastric cancer samples compared to normal tissues. Among differentially expressed tRFs and tRNA halves, upregulation of tRF-3019a, tRF-31-U5YKFN8-DYDZDD, tRF-38-QB1MK8YUBS68BFD2, tRF-19-3L7L73JD, tRF-3017A, tRF-18-BS68BFD2, tRF-25-R9ODMJ6B26; and downregulation of tRF-24-V29K9UV3IU, tRF-5026a, hsa_tsr016141, tRF-33-P4R8YP9LON4VDP, tRF-Glu-TTC-027 was consistently reported in several independent studies (Kohansal et al., 2022). Plasma tRF-27 levels were found to be significantly increased in gastric cancer patients, and high levels of this tRF are suggestive of poor prognosis. Thus, detection of plasma tRF-27 levels has been suggested as a non-invasive assay for gastric cancer early diagnosis(Yu et al., 2023). Downregulation of tiRNA-5034-GluTTC-2 in tissues and plasmas from gastric cancer patients showed a correlation with increased tumor size and lower overall survival rate (Linwen Zhu et al., 2019).

Overexpression of tRF-phe-GAA-031 and tRF-VAL-TCA-002 was described in colorectal cancer, suggesting a correlation with metastatic potential (Chen et al., 2022). Detection of plasma levels of 5′-tRF-GlyGCC was suggested as a promising diagnostic biomarker for colorectal cancer diagnosis (Wu et al., 2021). A panel of dysregulated tRFs was obtained by matched analysis of tumor and normal peritumor tissues collected from surgical specimens obtained from 30 patients (Xiong et al., 2019). A diagnostic model based on 4 differentially expressed tRFs (tRF-22-WB86Q3P92, tRF-22-WE8SPOX52, tRF-22-WE8S68L52, and tRF-18-8R1546D2) and a prognostic model based on 6 tRFs (tRF-18-HSRVK7D2, tRF-33-PSQP4PW3FJI0W, tRF-33-PSQP4PW3FJIKW, tRF-18-H9Q867D2, tRF-32-O7M8LOMLQHWU3, and tRF-16-I3FJQSD) was proposed for colorectal cancer patients management (Zhu et al., 2021).

### 3.8. Gynecologic cancers

High-grade serous ovarian cancer cell proliferation was found to be promoted by tRF-03357 (M et al., 2019) and tRF5-Glu (K et al., 2017). Stratification of patients according to risk factors can be improved by detection of 3′U-tRF$^{ValCAC}$, while tRF$^{GlyGCC}$ may be used to assess ovarian cancer treatment outcome and progression (Panoutsopoulou et al., 2022).

In cervical cancer, tRF-Glu49 was showed to modulate cervical cell proliferation, migration, and invasion processes. Thus, it was suggested as a possible prognostic biological marker in patients with cervical carcinoma (Y et al., 2022).

### 3.9. Hematological malignancies

Rao et al. described a 6-tRFs signature for diffuse large B cell lymphoma early detection, drug response, and prognosis (Rao et al., 2022). The tRNA-derived CU1276 was reported to be consistently downregulated both in lymphoma cell lines and primary biopsies from diffuse large B cell lymphoma patients (Maute et al., 2013).

Downregulation of small tRNA fragments ts-46, ts-47, ts-53, ts-49, and ts-101, and upregulation of ts-4 in was reported in chronic lymphocytic leukemia, while a specific signature for this disease was described in from Balatti and co-workers (Balatti et al., 2017). More recently, a prognostic role was suggested for internal fragments i-tRF-GlyGCC and i-tRF-GlyCCC (Papageorgiou et al., 2019).

### 3.10. Lung cancer

In 2022, Gao et al. published a study to investigate the tRFs landscape of non-small cell lung cancer patients collecting 1,550 RNA-seq samples from publicly available datasets. They reported a panel of tRFs correlating with diagnosis, risk factors, and PD-L1 checkpoint signaling pathway, also useful for patients' subtyping (Gao et al., 2022b).

A signature of tRNA fragments detected from plasma samples was suggested as a predictive and prognostic biomarker in lung adenocarcinoma (Wang et al., 2022b). A prognostic score based on tRNAs was also proposed from Kuang et al. (Kuang et al., 2019).

### 3.11. Prostate cancer

Olvedy et al. reported the ratio of tRFs derived from tRNALysCTT and tRNAPheGAA (tRF-315/tRF-544) as a good indicator of progression-free survival and a candidate prognostic marker in prostate cancer patients. An excess of tRNA halves was also reported in seminal fluids and prostate tissue of cancer patients compared to noncancer patients (Dhahbi et al., 2018). Dysregulation of several tRFs was also reported by Liu et al. (Liu et al., 2023), suggesting that measuring tRFs levels may provide clinicians with a source of diagnostic and prognostic information independent of the Gleason score. Magee et al. investigated the association between tRFs expression profiles and Gleason score. They reported that samples classified as Gleason score 7 exhibited different tRFs profiles, which were associated with varying prognosis. They also reported that 3′-tRFs from mitochondrially-derived tRNAs differes between normal prostate and tumor samples by length: in fact, short (19 nt) tRFs were usually found in normal samples, whereas longer (23 nt) tRFs characterized tumor samples. (Magee et al., 2018).

### 3.12. Other cancers

Together with the above discussed cancer subtypes, functional aberrations of tRNAs and dysregulation of the expression of tRNA fragments have been reported for many cancer types (Vafaei et al., 2020; Yu et al., 2020), including renal (Zhao et al., 2018), head and neck (Diez-Fraile et al., 2020), liver (Lei Zhu et al., 2019), bladder (Papadimitriou et al., 2020), bone sarcomas (Tang et al., 2021), and uveal melanoma (Londin et al., 2020). These findings suggest that these classes of molecules deserve a more in-depth investigation, considering their cancer-wide role in tumor initiation and progression.

## 4. Conclusion and perspectives

tRNA and tRNA-derived fragments constitute a vast universe of molecular species which has been vastly ignored by the scientific community until the last few years. As our capability to reliably detect them has improved, so has the evidence of their role in cellular pathways, both in terms of ribonucleoproteic complexes and as players in signal transduction. Their role in these processes has revealed them to be pivotal biomarkers for pathological progress, specifically in the field of cancer diagnosis and prognosis; more importantly, experiments have shown that tRNA, tRNA halves and tRFs could be used as predictors for pharmacological strategies and for personalized treatments, with a huge number of clinical investigations which appeared in scientific literature only in the last few years.

In our review, we discussed recent discoveries related to tRNA biology in the context of cancer and the latest advances in methods for detecting tRNA expression and modifications from biological samples. We provided a collection of currently available bioinformatics tools for investigating these molecules. To the best of our knowledge, this work represents the first attempt to gather all computational resources for characterizing tRFs alongside biochemical strategies for quantifying them.

Early detection, diagnosis, new treatment strategies, and methods to accurately evaluate or predict surgical or chemotherapy outcomes are considered key factors towards an improved management of cancer patients. To date, the toolset of clinical biomarkers to be used for the early diagnosis and accurate prognosis of cancer remains limited. Together with recent advances in non-invasive methods to accurately assess and evaluate tRNAs from biological samples, we are still at the beginning of the exploration of these kind of molecules in the context of cancer, and as many studies are progressing, tRNAs, tRNA halves, and tRFs are emerging as such biomarkers with high potentials in several cancer types, such as lung, prostate, breast, and gastrointestinal tumors.

A frequently overlooked aspect of studying tRNA derived fragments is the potential ambiguity associated with labeling these molecules. This ambiguity arises when the same nucleotide sequence can be assigned to multiple labels, leading to challenges in accurate classification and analysis (Magee and Rigoutsos, 2020). The complexity of accurately annotating and classifying tRNA halves, which are alternatively referred to as 'tRNA halves' or 'tiRNA' by different research groups, and tRFs was emphasized by several researchers, including the work of Telonis et al. (Telonis et al., 2015). Their analysis emphasized the necessity of rigorous bioinformatics approaches to disambiguate tRFs with identical sequences but different structural or functional properties. Furthermore, due to the multiple tRNA isodecoders sharing identical sequences, a single tRF can originate from various tRNA genes. This cross-gene sequence identity adds an additional layer of complexity when attempting to pinpoint the exact source of a particular tRF. The importance of addressing this labeling ambiguity becomes especially significant when researchers aim to understand the distinct functions, origins, and regulatory roles of tRFs in different cellular processes. Profiling the expression of tRNA fragments from short-read sequencing poses several challenges. Many tRNA fragment sequences exhibit extended regions of similarity with numerous sequences in the human genome, complicating efforts to identify the fragment's source. This challenge is exacerbated by the fact that tRNAs are repeated elements. Furthermore, when mapping a tRNA fragment, the absence of the rest of the tRNA sequence makes it difficult to unequivocally establish its tRNA nature (Telonis et al., 2016). Many authors have addressed these challenges by mapping sequence reads exclusively to databases of tRNA sequences, known as a tRNA space, rather than the entire human genome. However, this approach may result in numerous false positives, potentially leading to a misrepresentation of tRF abundancy. In this context, the strategy employed by MINTmap appears to be a promising way to navigate this complexity. MINTmap endeavors to assign each fragment to both the complete genome and a tRNA space. This dual approach provides researchers with the flexibility to concentrate on fragments originating exclusively from the tRNA space or to relax this constraint for improved sensitivity, considering the entire genome. However, this flexibility comes at the cost of an increased level of noise (Loher et al., 2017).

To tackle all these challenges, advancements in high-throughput sequencing and bioinformatics tools are being continually developed to improve the accuracy of tRFs annotation and to provide clearer insights into the functions and implications of these enigmatic RNA fragments. Researchers are encouraged to remain vigilant about these issues and incorporate robust bioinformatics strategies in their studies to ensure precise profiling and unambiguous labeling of tRFs.


## CRediT authorship contribution statement

**Chiara Cabrelle:** . **Federico Manuel Giorgi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Daniele Mercatelli:** .

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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