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RESEARCH ARTICLE

tRNA fragment biomarkers of Neurological Disease: Challenges and Opportunities

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ABSTRACT

Transfer RNAs play a crucial role in protein translation where they bring amino acids to the ribosome to be incorporated into nascent polypeptide chains. During stress conditions tRNAs can be cleaved to generate tRNA-derived fragments. Several ribonucleases have been identified that cleave tRNA, however mutations in the stress-induced ribonuclease Angiogenin have been identified in a range of neurological disorders including Amyotrophic Lateral Sclerosis, Parkinson's Disease, and Alzheimer's Disease, suggesting that tRNA cleavage may be dysregulated in neurological disease. tRNA fragments have been detected in biofluids indicating they may be of use as biomarkers for neurological diseases. There is considerable variability in the methods used to quantify tRFs from size selection, adapter ligation, removal of RNA modifications, and sequence analysis approaches which can make it difficult to reconcile multiple studies. Here we review the biology of transfer RNAs and the biogenesis of tRNA-derived fragments, with a focus on the methods used to identify and quantify tRNA fragments and how different methodological approaches can influence tRNA fragment detection. We provide an overview of current literature on the identification of tRNA fragments in neurological disease models and patient samples, with a focus on circulating tRNA fragments as potential biomarkers of neurological diseases.

Introduction

Recent advances in sequencing technologies have highlighted the abundance of non-coding RNA species that were previously considered to be non-specific degradation products. In this review we discuss one group of these small non-coding RNAs that are derived from transfer RNAs. We discuss the biogenesis of tRNA and tRNA-derived fragments and approaches used to identify and quantify their abundance in tissues and biofluids. The review concludes with a summary of recent literature identifying tRNA-derived fragments in tissues and biofluids from neurological disease models and patient samples, where they may be of use as circulating biomarkers.

Transfer RNA

Transfer RNA (tRNA) are short, non-coding RNAs with a distinctive cloverleaf secondary structure and an "L" shaped tertiary structure[1,2]. In the classic cloverleaf structure, the tRNA contains 4 stem loop regions which are named the acceptor stem, the TψC-loop, the anticodon loop, and the dihydrouridine or D-loop. In the tertiary structure the anticodon loop is located at one end of the "L" shape which has complementary base pairing to the codon within messenger RNA (mRNA). The cognate amino acid is attached to the acceptor stem at the other end of the "L" via an ester bond between the 3' hydroxyl group and the amino acid carboxyl group. The elbow region of the "L" shape comprises the TψC-loop and the D-loops stabilised by multiple hydrogen bonds formed between highly conserved residues[3]. Magnesium ions also help to stabilise the tertiary structure of tRNAs by forming contacts with the phosphate backbone[4]. These features stabilise the tertiary structure of tRNAs, which is highly conserved, and enable protein translation to occur within the ribosome.

Genes encoding tRNAs occur in clusters throughout the human genome and are transcribed by RNA polymerase III in the nucleolus, suggesting production and processing of rRNA and tRNAs is coordinated[5]. There are 428 nuclear tRNA genes in the human genome of which 28 include introns, and these decode 20 standard amino acids[6]. There is considerable variation in the number of genes for each isoacceptor type (tRNAs with different anticodons that carry the same amino acid), with up to 38 genes encoding Alanine tRNAs. These can be further subdivided into isodecoder types, which have the same anticodon and amino acid, but show variation in the primary sequence. Originally, the abundance of tRNA isodecoders were thought to be redundant, however recent advances in tRNA sequencing have revealed different expression patterns for isoacceptors and isodecoders. In the first example demonstrating clearly that isodecoders show different expression and function, a mutation in a CNS-specific Arginine isodecoder influenced penetrance of a neurodegenerative phenotype caused by a mutation in the ribosome recycling factor GTPBP2[7]. There are also many genes in the human genome that closely resemble tRNAs but contain insertions, deletions, point mutations, or contain Polymerase III promoter elements but do not resemble tRNA sequences, these are collectively termed tRNA pseudogenes and predicted tRNA pseudogenes comprise almost 10-fold more genes than predicted *bona fide* tRNA genes[6].

Transcribed tRNAs are in an immature form with a 5' leader and 3' trailer sequence which are cleaved by RNase P and RNase Z respectively. Human nuclear RNase P is a ribonucleoprotein complex with a weakly catalytic RNA component, H1 RNA, and 10 protein subunits, and removal of the 5' leader sequence usually occurs first[8]. Removal of the 3' trailer sequence follows and is performed by RNase Z, which recognises the discriminator nucleotide, the first non-paired nucleotide after the acceptor stem, and cleaves to the 3' side of this, releasing the trailer sequence[9]. tRNA intronic sequences and RNase P also show nucleolar organisation indicating tRNAs are both transcribed and processed within the nucleolus[10]. The non-templated CCA-tail is added by tRNA nucleotidyltransferase enzyme, which simultaneously checks the integrity of the tRNA backbone and will not add the CCA-tail to defective tRNAs that are subsequently degraded, thereby performing a crucial quality control step, reviewed in[11].

There are four isodecoder types (ArgTCT, LeuCAA, IleTAT, and TyrGTA) that contain introns within the anticodon loop region. tRNA splicing is performed in two steps, firstly intron removal by the tRNA Splicing Endonuclease Complex (TSEN), which is comprised of 4 subunits: 2 endoribonuclease subunits (TSEN2 &TSEN34) and 2 scaffolding subunits (TSEN15 & TSEN54), reviewed in[12]. TSEN2 is responsible for cleaving the 5' splice site leaving the 5' exon with a 2'3'cyclic phosphate end, and TSEN34 cleaves the 3' splice site and leaves the 3'exon with a 5'hydroxyl group. Secondly, the 5' and 3' exons are directly ligated by the RTCB ligase complex[13]. Mutations in the four subunits that form the TSEN complex and Cleavage Factor Polyribonucleotide Kinase Subunit 1 (CLP1) result in Pontocerebellar Hypoplasia (PCH), a clinically heterogeneous neurodevelopmental disorder which is associated with mutations in 12 distinct genes that play diverse roles in RNA processing[14,15]. Symptoms range from motor neuron disease features like

hypotonia and respiratory distress, similar to the phenotype of Spinal Muscular Atrophy (SMA), to intellectual disability and seizures, reviewed in [16]. CLP1 has also been found to physically associate with the TSEN complex and can phosphorylate 5' hydroxyl ends of single or double stranded RNA[17], however this abrogates direct ligation of 5' and 3' ends generated when tRNA introns are removed, so it remains unclear what role CLP1 plays in tRNA processing. A mouse model expressing a catalytically-inactive CLP1 kinase develops motor neuron disease and recapitulates features of PCH, and also shows accumulation of Tyrosine-derived tRFs due to defects in tRNA splicing indicating CLP1 is required for efficient tRNA splicing[18].

The final stage in tRNA maturation is the extensive modification of specific nucleotides within the tRNA to aid stability and structure, reviewed in [19]. Over 150 distinct modifications have been identified in tRNA, yet these are dynamic and can vary according to cell type or condition. Modifications regulate many aspects of tRNA function, from recognition by the aminoacyl tRNA synthetases (AARS) which add amino acids, to recognition by the ribosome and maintenance of structure and stability. Recent reports have shown that RNA modifications can influence cleavage of tRNAs[20–22], indeed, rapid degradation of incorrectly modified tRNAs occurs indicating modifications are essential for the longevity of tRNA molecules[23]. As such, defects in RNA modification enzymes can have severe consequences and give rise to a range of disorders collectively termed "RNA Modopathies", many of which impact neuronal development or function, reviewed in [24]. The MODOMICS site provides a comprehensive database of tRNA modifications[25].

Once these steps have been completed the mature tRNA be exported into the cytoplasm to perform its role in protein translation. In the cytoplasm tRNAs are charged with their specific amino acid by 20 individual AARS which catalyse the addition of the amino acid to the adenosine residue of the CCA-tail. Mutations in Glycine AARS which prevent the release of the charged tRNA Glycine lead to ribosome stalling which triggers the Integrated Stress Response (ISR) and causes the peripheral neuropathy Charcot-Marie-Tooth disease[26,27].

Estimates vary depending on cell type but it has been proposed that tRNAs constitute approximately 10-15 % of total cellular RNA[28]. The most abundant RNA type is ribosomal RNA, but due to the small size of tRNA molecules (70-90 nucleotides), this equates to approximately 10 tRNA molecules per ribosome[29]. It has been shown that mature tRNA levels are selectively altered during cellular stress to regulate protein synthesis[30,31]. One method which may contribute to the change in abundance of mature tRNAs is the enzymatic cleavage of tRNAs to generate tRNA-derived fragments (tRF).

Ribonucleases that cleave tRNA

As previously mentioned, tRNAs require processing to remove a 5' leader sequence which is performed by RNase P and the 3' trailer sequence which is removed by RNase Z. However, recent advances in sequencing technologies and bioinformatic analysis have led to the identification of multiple fragments derived from tRNAs. The nomenclature used to define tRFs is not formalised which leads to confusion when comparing different studies. The general convention is that tRFs derived from the 5' end of the mature tRNA are termed 5'tRFs, those cleaved within the anticodon loop are termed 5' or 3' tRH (halves) or tiRNAs (tRNA-derived stress-induced RNAs), and those originating from the 3' end are 3'tRFs, see **Figure 1**. Internal (i-tRFs) are generated from cleavage sites within the T and D loops and contain the full anticodon loop. 1-tRFs refers to tRFs generated from pre-tRNAs which include the 3' trailer sequence. However, the start and end sites of fragments can vary considerably and it is unclear if this influences function. Convention in the cancer field is to assign individual numbers to each tRF, and some neurological studies have assigned "barcodes", but as these are study-specific their use makes comparisons between studies difficult. The minimum information required when discussing tRfs is the tRNA of origin and the location of the fragment (5' or 3'). Several ribonucleases that cleave tRNA have been identified along with their preferred cleavage sites (**Figure 1**), but it is likely there are others that can perform these reactions. They can be divided into stress-induced tRNA ribonucleases exemplified by Angiogenin, and basal ribonucleases such as Dicer.

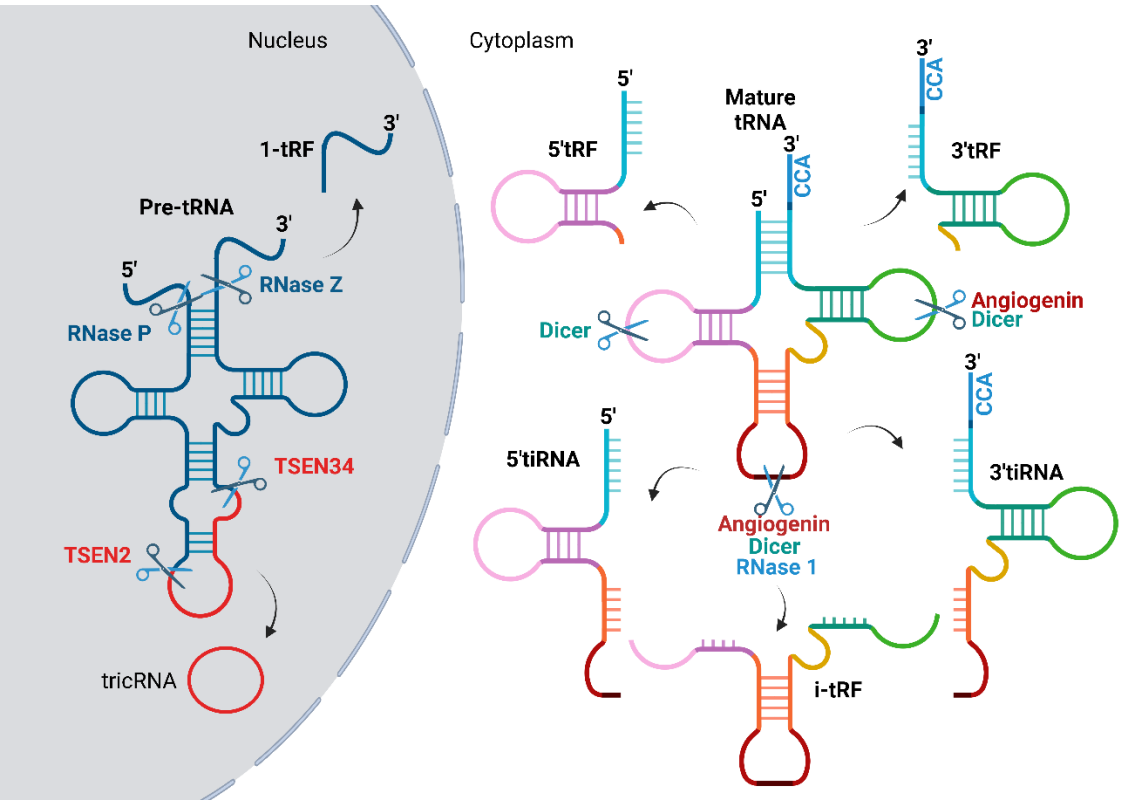

Figure 1: tRNA and tRF biogenesis pathways. Pre-tRNAs are transcribed in the nucleus and processed by RNaseP and RNase Z to remove the 5' leader and 3' trailer sequences. 1-tRFs are generated by cleavage by RNase Z and contain the 3'trailer sequence. Introns are removed by TSEN2 and TSEN34 and the 5' and 3' halves are ligated. The intron can be ligated to form a tRNA intronic circular RNA (tricRNA). In the cytoplasm mature tRNAs can be cleaved at multiple sites to generate 5'tRFs, 3'tRFs, 5'tiRNAs, 3'tiRNAs, or i-tRFs (internal), by ribonucleases including Angiogenin, Dicer, and RNase 1. Figure created in BioRender.

Angiogenin is a 14 kDa member of the vertebrate secreted ribonuclease family which is expressed in motor neurons and epithelial cells lining blood vessels[32], and expression is upregulated in response to a range of stresses including hypoxia[33]. Angiogenin is transcribed from an unusual locus where the 5'UTR and two promoter regions are shared between Angiogenin (RNase5) and the neighbouring gene RNase4, directing ubiquitous and liver-specific expression[34]. RNA cleavage by members of the vertebrate specific ribonuclease family generates 5' fragments with a 2'3' cyclic phosphate at the 3' end, and 3' fragments with a 5' hydroxyl group. However, Angiogenin shows significantly reduced ribonuclease activity and higher substrate specificity than other members of the family[35], using ribosomal RNA Angiogenin generated specific cleavage products, unlike other family members which digest RNA non-specifically resulting in single nucleotides[35]. Angiogenin shows preference for cleaving after a pyrimidine base located within a single-stranded region of RNA, and it has been shown to cleave tRNAs within the anticodon loop to generate 5' and 3' tRNA halves, sometimes referred to as tRNA-derived stress-induced RNA fragments (tiRNAs)[36,37]. Angiogenin has also been found to cleave within the TψC-loop, however this site is less frequently used.

Ribonuclease (RNase) 1 is also a member of the vertebrate secreted ribonuclease family which shows high activity against double-stranded RNA (dsRNA)[38], where it digests RNAs to single nucleotide products. RNase 1 is ubiquitously expressed but has been found to be upregulated during oxidative stress, and has been reported to cleave tRNA indicating it may also function to generate stress-induced tRFs[39,40]. However, it has also been suggested that these tRFs may be generated extracellularly by secreted RNase 1, and the CCA tail was also found to be cleaved[40].

tRNA fragments have also been identified with 5'phosphate and 3'hydroxyl groups indicating they were not generated by vertebrate secreted ribonuclease family members[41]. The majority of these tRF's were shown to be generated by Dicer. These fragments are shorter than Angiogenin-derived tRFs, with a size range of 20-25 nt, which is consistent with cleavage occurring at the base of the D or TψC-loop. 5'tiRNAs were also detected indicating Dicer can also cleave within the anticodon

loop[42]. Human Dicer is 218 kDa protein which binds to dsRNA and functions within the microRNA biogenesis pathway to cleave precursor microRNAs to generate ~21-23 nt fragments[43]. It is not clear how Dicer may interact with tRNA, which have short stretches of dsRNA and extensive tertiary structure, but Dicer enzymes contain a helicase domain which may assist with resolving local structure[43].
One tRF which was generated in a Dicer-independent manner, was found to be processed by RNase Z[41] from the pre-tRNA, however this does not appear to be an abundant class of tRFs.

tRNA fragments in circulation

Fragments from 5' region of tRNAs were originally detected in serum where they were found to be present in large macromolecular complexes, that were depleted in plasma samples prepared with EDTA[44]. Subsequent large-scale sequencing projects have demonstrated that tRFs are present in many biofluids including serum, plasma, and CSF[45–47]. However, vast differences in abundance have been reported, with one study reporting similar levels of tRF reads in serum and CSF (~40 %), but negligible levels of tRF reads from plasma[45], and another indicating plasma tRF reads of approximately 30 % and CSF tRF reads of 45 %[47]. Different purification approaches also influenced RNA type read distributions, but this study reported similar read counts from plasma and serum[46]. tRFs have also been detected in cell culture media[39,46,48], one study which profiled intra- and extra- cellular tRFs generated in response to stress found that intracellular tRF species overlapped in multiple stress conditions, whereas extracellular tRFs provided distinct profiles depending on the stress type[39]. Interestingly, extracellular microRNA profiles did not vary between stress types, indicating secreted tRFs may provide more informative biomarkers than secreted microRNAs. Of note, we found no correlation between tRF levels and haemolysis (as quantified by UV absorbance at 414 nm[49]) indicating lysed red blood cells are not a major source of tRFs[48], whereas this can be a problem for certain microRNAs.

Detection methods

Modified nucleotides have been identified in many RNA species, but tRNAs are amongst the most heavily modified RNAs. However, bulky modifications including methyl groups can prevent enzymes including Reverse Transcriptase or Polymerase from travelling along the RNA molecule. This has led to the development of treatments to remove or accommodate modifications and secondary structure prior to sequencing. One method involves pre-treatment of RNAs with an *E.coli* demethylating enzyme (AlkB) to remove bulky methyl groups, and has been called AlkB-facilitated RNA methylation sequencing (ARM-seq)[50]. Another group proposed to combine demethylation of RNA with use of a thermostable group II intron reverse transcriptase to combat issues with highly structured tRNAs (DM-tRNA-seq)[51]. However, when these techniques are used on total cellular RNA, they can lead to very high reads originating from ribosomal RNAs and tRNAs, as they more accurately reflect the original RNA type distribution (rRNA: ~70 %, tRNA: ~10-15 %). It has recently been shown that tRFs in neural cells are hypomodified indicating demethylation may have little impact on the abundance of tRFs originating from neurons[52]. This is understandable given numerous studies that demonstrate modifications can block generation of tRFs by preventing ribonucleases from accessing or acting on their preferred site in the tRNA[20–22,53]. This also suggests that tRFs generated in neural cells do not appear to be substrates for *de novo* methylation, although this was a short experimental time frame[52].
Cleavage by vertebrate secreted ribonucleases generates 2'3' cyclic phosphates at the 3' end of the 5'tRF, and a 5' hydroxyl group on the 3'tRF[38]. To assist with adapter ligation some library preparation protocols include pre-treatment steps to remove these modifications.
Library preparation kit choice and preparation methods vary widely across publications on tRFs. The classical small non-coding RNA library preparation includes size selection, usually after adapter ligation, which restricts the insert to under 40 nt. However, some are restricted to under 30 nt. As tRNA halves (or tiRNAs) are approximately 30-34 and 40-45 nt this may exclude the larger 3' tRFs from the outset. Some methods involve gel purification and size selection of input RNA prior to library generation. A recent paper compared several well-known library preparation kits for their ability to detect small non-coding RNAs from high and low input samples, along with plasma. They concluded that the Illumina TruSeq kit resulted in detection of almost twice as many tRFs than the other kits tested (NEBNext and BiooScientific NEXTFlex) across three tissue types tested (brain, liver, and placenta)[54]. The Illumina TruSeq kit contains modified adapters designed to capture small RNA with 3'OH and 5'P modifications, such as those generated by Dicer processing. Therefore, this result may indicate that Dicer, or Dicer-like ribonucleases, are contributing significantly to the pool of tRNA fragments in the tissues analysed. Alternatively, the 5' & 3' ends generated following cleavage may be further trimmed or modified within the cell following cleavage.

However, plasma samples did not show the same pattern and tRF levels were similar between 2 kits analysed (Illumina TruSeq and BiooScientific NEXTFlex). Here tRFs constituted a minor contribution to the total reads which were mostly comprised of miRNA and Y RNA derivatives[54]. This result is consistent with the profiling results presented by Godoy et al, which suggest tRFs constitute a minor component of plasma but a much higher proportion of reads from serum and CSF[45]. As there was minimal difference between the kits tested, this finding could indicate that the tRFs in circulation are not generated by Dicer (or Dicer-like ribonucleases), which correlates with our observation that one Angiogenin-generated tRF, was robustly secreted from neural cells and detected in animal and human serum[48]. Indeed, this has also been observed in conditioned media from cells exposed to a range of physiological stresses, where the majority of extracellular tRFs were cleaved within the anticodon loop, whereas intracellular tRFs showed more varied cleavage site use[39]. Therefore, it appears that stress-induced tRFs cleaved within the anticodon loop may be the predominant species of secreted tRFs.

The presence of an amino acid at the 3'end of tRNA and tRFs can inhibit amplification-based methods and lead to loss of species from libraries or lack of validation of results. In our screen for angiogenin substrates we identified a number of 3'tRNA halves, primarily derived from Arginine isoacceptors, which were validated robustly by northern blotting but showed substantially weaker fold-change when quantified using qPCR methods[48]. Subsequently it has been found that 3'tRFs derived from Arginine tRNAs retain their aminoacyl moiety following cleavage and can act as arginine donors in protein arginylation reactions mediated by the enzyme Arginyltransferase (ATE1)[55]. Interestingly, Arg-tRF levels were modified in response to demand for substrate by ATE1, such that when ATE1 was deleted levels of Arg-tRFs fell resulting in a significant change in the ratio of tRNA:tRF and indicating a regulatory loop involving tRNA cleavage enzymes exists. Additional examples of 3'tRF aminoacylation indicate this may be common[56], and some studies perform deacylation prior to adapter ligation when preparing sequencing libraries to capture tRFs.

Therefore, size selection, RNA sample preparation, and choice of library preparation kit can greatly influence the abundance and species of tRFs detected in small RNA sequencing data. Unfortunately, comprehensive details of methods employed during sample preparation are not always included in publications or noted in the information with publicly available datasets.

tRNA Sequence analysis

Genes encoding tRNAs are present in multiple copies within the genome, hence they are classified as repeats and reads will not be aligned using classical RNA sequencing analysis workflows. In addition, tRNAs are post-transcriptionally modified by cleavage of the 5' leader and 3' trailer sequences, and addition of a CCA-tail, which means that reads from tRNAs do not align to the genome. Due to the high level of sequence similarity between tRNA genes, reads derived from tRFs can align to multiple copies of a gene, and therefore alignments must be limited to 1 hit per read to avoid unequal amplification of read counts. One way to avoid these issues is to create a custom tRNA genome using the GtRNAdb (gtrnadb.ucsc.edu)[6], and manually add the CCA-tails with a string of N's at the 5' and 3' end to allow detection of fragments with the leader or trailer sequences attached[48]. However, there are many analysis pipelines now available which perform similar functions including MINTmap[57], tRNAstudio[58], and tDRmapper[59]. Interestingly, one study compared in-house analysis performed by the RNA sequencing company against a custom analysis performed using the Galaxy server and found dramatically different read counts for most RNA types including tRNAs, which accounted for 6 % reads with Qiagen analysis and 39 % reads (making tRNA the predominant RNA type detected) with the Galaxy analysis pipeline[60]. Similarly, analysis of differentially expressed RNAs varied according to the analysis pipeline used with Galaxy analysis indicating 26 tRNAs were differentially expressed and Qiagen analysis indicating none were. Therefore, choice of analysis method can also dramatically influence tRNA read counts.

Neurological disorders

Mutations in Angiogenin have been identified in patients with Amyotrophic Lateral Sclerosis (ALS)[32] and Alzheimer's disease (AD)[61], and variants within angiogenin are associated with Parkinson's disease (PD)[62,63]. Several studies have shown that tRNAs can be cleaved within the anticodon loop in the absence of angiogenin, suggesting that there are other ribonucleases that can perform this reaction[41,42,64], however the association between Angiogenin variants and neurological diseases suggests that Angiogenin may be the predominant ribonuclease performing this reaction in neurons, and that this function may be required for neuronal health. As tRFs can be detected in biofluids, many studies have sought to investigate whether tRFs could be of use as biomarkers of neurological disease, and here we review the current information. Of note, tRFs have also be investigated as biomarkers in a range of

cancers, which has recently been reviewed elsewhere[65].

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is the leading cause of dementia worldwide, but shows considerable clinical heterogeneity[66]. Neuronal degeneration leads to widespread atrophy affecting multiple brain regions which causes a range of cognitive and behavioural changes that can result in a loss of independent living. Pathologically, AD is characterised by the presence of intraneuronal neurofibrillary tangles comprised of hyperphosphorylated microtubule-associated protein Tau, and neuritic plaques comprised of beta-amyloid peptides of 40-42 amino acids (βA40-42) derived from the Amyloid Precursor Protein (APP). Mutations within the *APP* gene, or genes encoding the enzyme Presenilin (*PSEN1 & 2*) which forms the catalytic component of the gamma secretase complex responsible for generating the βA40-42 peptide, cause familial AD which typically has a younger age of onset than sporadic forms of the disease[67]. Recent publications investigating small non-coding RNAs in AD mouse models and AD patient tissues provide evidence of tRF dysregulation in AD.

Fragment levels were profiled in the Senescence-accelerated mouse prone 8 (SAMP8) strain of mice that show a rapid aging phenotype, with learning and memory deficits and evidence of βA40-42 deposition, indicating they can be used to model aspects of AD[68]. A set of 8 tRFs were identified that were differentially expressed in SAMP8 mice compared to a senescence-accelerated mouse resistant (SAMR) strain, with five of these were upregulated in the resistant strain. These included a variety of tRF types from different tRNAs, but interestingly some tRFs were only detected in one strain and were absent from the other[68]. Investigations using transgenic mice overexpressing a mutant chimeric mouse/human APP (APPswe) and a mutant human Presenilin 1 (PSEN1dE9) that develop age-dependent cognitive impairment and show abundant pathological βA40-42 inclusions also identified dysregulated tRFs[69]. Lu et al, performed small RNA sequencing on hippocampal tissue from ~12-month-old male mice and identified dysregulated tRFs, validation confirmed that a tRF derived from ThrCGT was upregulated in AD mice compared to controls, whereas a tRF from LeuCAA was significantly downregulated[70].

Wu et al, found that microRNAs and tRFs were elevated in a publicly available sequencing dataset from 6 late onset AD patients and 6 age-matched healthy controls[71]. To validate these findings hippocampus tissue from 10-15 AD patients and healthy controls was examined, revealing that Angiogenin levels were significantly higher in AD patients, whilst Dicer levels were unchanged. 5'tRFs derived from GlyGCC, GluCTC, and ProAGG were found to be elevated in AD tissue compared to control, with 5'ProAGG showing a positive correlation with Braak staging. Additional samples that were analysed according to disease onset revealed that all 3 tRFs were significantly upregulated in early onset AD patients compared to age-matched controls, with only 5'ProAGG showing a significant difference in late onset AD patients compared to age-matched controls. Interestingly, they also identified a significant downregulation of NSun2 tRNA methyltransferase enzyme in early onset AD patients which may explain why tRFs are abundant in this patient group[71]. It remains to be seen whether blood-based tRFs can be of use as biomarkers in AD.

Parkinson's Disease

Parkinson's disease (PD) is the second most prevalent neurological disorder in the world, which is characterised by classical motor signs of resting tremor and rigidity, but can also present with a wide range of non-motor symptoms including cognitive decline, depression, pain, constipation, and sleep behaviour abnormalities[72]. Pathological hallmarks of PD include formation of Lewy bodies comprised of aggregated alpha synuclein (αS) protein, which leads to degeneration of the dopaminergic neurons within the substantia nigra pars compacta. The first investigation into tRF biomarkers in PD used several existing sequencing datasets from PD patients and healthy controls to examine small RNAs from prefrontal cortex, serum, and CSF. Magee et al, identified specific tRF expression patterns dependent on sex, in distinct tissue and biofluid compartments, between PD patients and healthy controls, and identified differentially expressed tRFs based on whether PD patients exhibited dementia or not[73]. Their sequence analysis study concluded a specific tRF signature may be of diagnostic use in PD patients, but further work to validate these findings is required.

A subsequent study using a two large sample cohorts from the Parkinson's Progression Markers Initiative (PPMI) and the Luxembourg Parkinson's Study (NCER-PD)) found that microRNAs were the most abundant species present in whole blood, and hence mainly focussed on this species[74]. tRFs were detected in this dataset but contributed only 0.5 % total reads whereas microRNAs accounted for over

90 % total reads, but as whole blood was analysed, both cellular and extracellular RNAs are contributing to these read counts. It has recently been shown that Shlafen2 specifically protects tRNAs from cleavage within the anticodon loop in T cells to allow rapid proliferation to occur despite high levels of metabolic stress, providing an explanation for why tRFs may not be abundant in whole blood analysis[75].

More recently, Paldor et al, performed a more targeted analysis of the PPMI whole blood samples previously described but restricted to idiopathic PD patients only, along with an existing dataset of post mortem CSF samples collected from PD patients and healthy controls[76]. Using a global analysis of tRF types regardless of origin tRNA this study again highlighted a sex difference, which was more pronounced in CSF than in whole blood, however an age-dependent decline in CNS 3'tRFs was also identified. Distinct tRFs could discriminate PD patients from controls in CSF and whole blood, indicating tRFs may be of use as a diagnostic aid in PD patients. However, further validation is required.

Progressive Supranuclear Palsy

Progressive Supranuclear Palsy (PSP) is an age-related neurodegenerative disease caused by mutations in the microtubule-associated protein Tau, hence PSP belongs to a group of dementias termed "Tauopathies". Tau is also implicated in AD pathology as previously discussed, and patients with PSP develop neurofibrillary tangles containing hyperphosphorylated Tau similarly to AD patients, however they also demonstrate glial Tau pathology in affected brain regions including the subthalamic nucleus, the brainstem, and the substantia nigra[77]. Patients with PSP can exhibit a range of cognitive and motor symptoms including supranuclear gaze palsy, which gave the disorder its name, but can also exhibit Parkinsonism features including posturing and rigidity which can lead to difficulty in diagnosis. Simoes et al, performed small non-coding RNA sequencing on matched serum and CSF samples from patients diagnosed with possible or probable PSP and healthy controls[78]. They identified 16 dysregulated tRFs in serum including tRFs derived from GlyGCC and GluCTC but read counts for tRFs were low in serum and only ValCAC tRF downregulation was confirmed by qPCR. Nine non-coding RNAs were dysregulated in both serum and CSF, but when comparing PSP patients and controls only four changed in the same direction, and no tRFs were amongst those validated by qPCR.

Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a CAG triplet repeat expansion which occurs within exon 1 of the Huntingtin gene. HD is one of a number of CAG-repeat associated disorders which lead to neuronal degeneration, although the specific neuronal population affected is different in each disorder[79]. CAG repeats are translated resulting in tracks of polyglutamine amino acids within the Huntingtin protein which are prone to aggregation. Pathological aggregates of polyglutamine proteins are found within neurons in the striatum and cortical degeneration can also occur[79]. A study by Banez-Coronel et al, found that short RNAs of approximately 21 nt were generated from the CAG triplet expansion in a Dicer-dependent manner[80]. To determine the pathogenicity of this species small RNA populations were purified from post-mortem brain tissue from patients with HD or healthy aged controls, which were then infused into the brains of mice via intracerebral injections, and motor function and histological analysis performed[81]. The authors were examining the ability of short RNAs derived from the CAG-repeat expansion to induce neurological deficits in the mice, but found these CAG-RNAs only recapitulated part of the phenotype. In addition to the CAG-RNA they had purified tRFs from the HD brains, which likely reflects cellular stress pathway activation had occurred prior to death, although the level of ribonucleases in the post-mortem tissue was not investigated. Abundant "clusters" aligning to Glycine, Alanine, Valine, and Glutamic acid tRNAs were detected in putamen from HD patients which were not detected in other brain regions or in healthy controls. *In vitro* experiments indicated exogenously applied 5'AlaAGC tRF caused striatal neuron death within 1 hour of administration, whereas other tRFs tested had no effect, indicating this species was partially responsible for the observed phenotype.

Stroke

Stroke is a neurological disorder caused by the blockage of blood vessels in the brain and it can result in severe disabilities, dementia, or death. Blockages within blood vessels can lead to neurons being starved of oxygen (ischaemic stroke, approx. 85 % cases), or it can lead them to rupture and bleed (haemorrhagic stroke approx. 10-15 % cases). Treatment focusses on removing the blockage, often a blood clot targeted by thrombolytics, and timing of treatment is imperative with improved outcomes associated with rapid intervention[82]. Interestingly, serum angiogenin levels have been shown to be elevated in patients within 48 hours of suffering a ischaemic stroke, and these levels remained significantly higher than control subjects for 1 week following the event[83]. Serum

angiogenin levels correlated with the size of the infarct as measured by MRI or CT scan with larger infarcts (> 4 cm) associated with higher serum angiogenin levels. In a rat model of Middle Cerebral Artery Occlusion (MCAO) small RNA sequencing (< 35 nt) identified numerous reads aligning to ValCAC and GlyGCC tRNAs that were upregulated post injury, with the highest levels detected at 48 hours[84]. The angiogenin-generated tRFs inhibited proliferation of endothelial cells *in vitro*, suggesting they may inhibit angiogenesis in the local area.

More recently, tRFs in whole blood have been interrogated for their potential role in modulating immune responses following ischaemic stroke. In samples collected 2 days after ischemic stroke Winek et al, found that microRNAs were significantly downregulated while tRFs were significantly upregulated compared to healthy controls, particularly fragments derived from Alanine and Glycine tRNAs and predominantly 3'tRFs[85]. Assuming the tRFs function in the same way as microRNAs, the authors then identified an abundance of tRFs targeting cholinergic-associated transcripts which they termed "Cholino-tRFs" and suggested that these were responsible for modulating post-stroke peripheral immunosuppression that is mediated via cholinergic signalling pathways. *In vitro* experiments showed that tRFs were upregulated in macrophages in response to lipopolysaccharide exposure, indicating tRFs could directly modulate the immune response[85]. The tRFs described in this study were assigned individual barcodes, so it remains to be seen which tRNA they originated from.

In a clinical study to distinguish between haemorrhagic stroke, ischemic stroke, and stroke mimics using blood-based non-coding RNAs, Nguyen et al identified a tRF signature comprised of 3 tRFs (ValCAC, TyrGTA, and ThrCGT) that could accurately discriminate ischaemic from haemorrhagic stroke, and from mimics[86]. In a validation cohort of stroke and healthy controls the tRF signature could identify the stroke patients with an Area Under the Curve (AUC) value of 0.875. Interestingly, ValCAC and GlyGCC were again highlighted here as elevated following ischaemic stroke whereas a different tRF profile was found in haemorrhagic stroke patients[86]. Multiple studies have now indicated that angiogenin and tRFs derived from ValCAC and GlyGCC are elevated following ischaemic stroke and may provide diagnostic and prognostic value.

Traumatic Brain Injury

Traumatic Brain Injury (TBI) results from trauma to the head which leads to confusion, loss of consciousness, amnesia, or neurological abnormalities and is the most common cause of disability in adults[87]. The primary insult causes a secondary inflammatory reaction which can exacerbate the damage from the initial insult. In addition, there is a great need to identify biomarkers of TBI that could help in assessing the severity of the insult and therefore influence the immediate response and recovery plan. Puhakka and colleagues postulated that tRFs may be implicated in the neuroinflammatory reaction which occurs in the months following TBI and can lead to the development of epilepsy[88]. They investigated small non-coding RNAs in the thalamus and cortex from rats 3 months post TBI and sham treated control rats. They identified two 3'tRFs derived from LysCTT and IleAAT that were significantly elevated in both cortex and thalamus; however, these were generated by cleavage within the variable region of the tRNA and Angiogenin levels were not upregulated, suggesting they were generated by a different ribonuclease. Interestingly, they found that higher levels of tRFs correlated with a worse behavioural outcome[88]. It would be interesting to see if these tRFs can be detected in blood following TBI.

A study investigating non-coding RNA biomarkers of concussion in rugby players identified dysregulated tRFs in saliva samples collected from players immediately following a confirmed head injury assessment (HIA+) compared to baseline samples collected pre-season, samples from HIA negative players, or uninjured players[89]. Nine tRFs were part of a panel of non-coding RNAs investigated in a validation study using qPCR analysis, and four of these could discriminate between HIA+ and HIA- players. Only one tRF, derived from 3'ArgCCT lacking the CCA tail, was included in the final panel of 14 non-coding RNAs which could identify concussed players from uninjured, or those injured but receiving a negative HIA, when saliva was collected immediately following the game or 24-36 hours later. However, it remains to be seen if saliva tRFs can be influenced by other factors such as local tissue damage within the mouth sustained during a collision which causes head injury, but which may be absent from other injuries.

Epilepsy

Epilepsy is a complex neurological condition characterised by uncontrolled neuronal hyperactivity, termed seizures. Epilepsy can be present from birth as a result of a genetic abnormality or it can be acquired as a result of trauma or injury to the brain, which can take time to develop into spontaneous seizures. Approximately

one third of people with epilepsy do not respond to the currently available medications leaving them with uncontrolled seizures which can occur at any time. The ability to predict the onset of seizures could revolutionise the way epilepsies are treated and give people living with epilepsy greater control over their lives. Many studies have sought to identify blood-based biomarkers to aid with diagnosis, mainly focussing on microRNAs[90]. Studies into seizure prediction have mainly focussed on detecting patterns of electrical activity in the brain that could indicate seizure imminence[91].

We performed the first tRF study in plasma samples collected from people with epilepsy before and after a seizure occurred and identified 3 tRFs that are elevated in advance of seizures and return to baseline post-seizure[92]. At a cellular level we found that tRFs were detected in primary mouse hippocampal neurons, the culture media they were incubated in, and that tRF levels in the media fell following sustained epileptiform activity induced by magnesium withdrawal[92]. Investigations using differentiated SH-SY5Y cells demonstrated that intracellular tRF levels change rapidly upon depolarisation with potassium, and returned to normal after a 2 hour recovery window[93]. The study by Kiltschewskij & Cairns also investigated mRNA levels and ribosome occupancy and showed that immediately following depolarisation, gene expression was either regulated by mRNA abundance or by translational activity, and that neuronal genes were more often associated with translational regulation. tRFs have been identified in synaptic vesicles which may indicate how they are released into the extracellular fluid and explain how extracellular tRF levels are regulated by neuronal activity[94]. A range of neurological insults are thought to result in epileptogenesis, the latent period before spontaneous seizures occur, including hypoxia, viral infection, and TBI. Interestingly tRFs are reportedly upregulated in response to hypoxia[84], viral infection[95], and TBI[88], raising the possibility that tRFs or variation within mature tRNA levels may be implicated in the epileptogenic process.

Interestingly, a recent paper demonstrated that expression of a brain specific ArgTCT tRNA modulated seizure thresholds in mice[96]. A single point mutation within the T stem loop region inhibited tRNA processing such that mature tRNA ArgTCT levels were reduced by 60 %, but this increased the threshold for seizures induced pharmacologically or due to mutations within a GABA Receptor gene linked to childhood epilepsy disorders in humans. The authors demonstrated that the decrease in available ArgTCT resulted in an increase in inhibitory signalling in the hippocampus that influenced seizure thresholds, and also led to ribosome pausing which activated the integrated stress response (ISR)[96]. This study demonstrates again that tRNA abundance may modulate penetrance of disease-associated mutations.

Spinal Cord Injury

Primary spinal cord injury is often caused by trauma and is largely unavoidable, however following on from insult a secondary pathophysiology occurs including oedema, ischaemia, apoptosis and oxidative stress which can exacerbate the direct damage and prolong the recovery significantly. Qin et al, investigated differentially expressed tRFs 24 hours after surgical spinal cord contusion in rats and identified 155 differentially expressed tRFs[97]. 5'GlyGCC was found to be upregulated while Brain Derived Neurotrophic Factor (BDNF) was downregulated, and 5'GlyGCC could negatively regulate BDNF 3'UTR in a luciferase assay, raising the possibility that knocking down 5'GlyGCC may improve outcomes in Spinal Cord injury.

Amyotrophic Lateral Sclerosis

The motor neuron disease Amyotrophic Lateral Sclerosis (ALS) is a progressive, debilitating neuromuscular disorder characterised by loss of upper and lower motor neurons which leads to degeneration of respiratory muscles resulting in death[98]. Patients with ALS can face delays with diagnosis, and disease onset and progression shows considerable clinical heterogeneity. In an effort to identify non-coding RNA diagnostic and prognostic biomarkers for ALS, Joilin et al, performed small non-coding RNA sequencing on pooled serum samples collected from slow and fast progressing ALS patients, disease mimics, and healthy controls[60]. Custom analysis using the Galaxy server indicated 26 tRNAs were differentially expressed, and qPCR validation confirmed a 5'tRF from ValAAC was upregulated in ALS disease mimics compared to healthy controls, and 5'AlaAGC was downregulated in slow progressing ALS patients compared to healthy controls and disease mimics. Examining these tRF's in a second patient cohort revealed 5'ValAAC and 5'AlaAGC were upregulated in slow and fast progressing ALS patients compared to controls or disease mimics.

We approached this in a different way, using cellular models to identify the substrates of the ALS-associated ribonuclease Angiogenin and then exploring whether these could provide prognostic value to people with ALS[48]. We identified that the 5'ValCAC tRF which was robustly generated by Angiogenin in cellular models, was also upregulated in spinal cord from transgenic ALS mouse models at disease onset, when Angiogenin expression is

upregulated in response to stress. Interestingly, we also found protein translation initiation factors eIF4e and eIF4eBP1 were dysregulated in spinal cord motor neurons, where increased 5'ValCAC correlated with increased eIF4EBP1 expression and slower disease progression. 5'ValCAC tRF was secreted from neural cells and elevated at disease onset in serum from ALS mouse models. Finally we investigated serum collected from a cohort of ALS patients at diagnosis and found that higher levels of 5'ValCAC indicated a slower disease progression and longer survival leading us to conclude that serum 5'ValCAC levels provide prognostic value[48]. Notably, both biomarker studies conclude 5'Val tRF's are elevated in serum from ALS patients.

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating neurodegenerative disease which is classified according to disease progression into either Relapse-Remitting (RR), primary progressive (PP), or secondary progressive (SP) MS. Initial stages of the disease are characterised by episodes of immune-mediated neurological dysfunction which can result in a range of symptoms depending on the site of the lesion including problems with vision, movement, balance, and sensation[99]. Over time, recurrence of inflammatory episodes can lead to permanent dysfunction or disability. MS has a younger onset than most neurodegenerative diseases, with onset typically occurring between 20-40 years of age. Several risk factors are known to contribute to development of MS including female sex, vitamin D levels, and race, and recently a large case study has confirmed that previous infection by Epstein-Barr Virus predisposes to developing MS[100]. Current disease-modifying treatments focus on reducing inflammation during episodes (relapses) to limit neurological damage, or therapies designed to reduce symptoms such as muscle relaxants or anti-fatigue medicines. Early treatment can prevent permanent disability therefore diagnostic biomarkers may be of use.

Zheleznyakova et al, 2021 profiled small non-coding RNAs in plasma and peripheral blood monocytes (PBMCs), and CSF and CSF cells, from patients with RR MS and non-inflammatory neurological disease control (NINDC) patients[47]. In PBMCs and CSF cells they identified opposing expression profiles for tRFs; whereby tRFs were significantly downregulated in RR PBMCs compared to NINDCs and significantly upregulated in RR CSF cells compared to NINDCs. This group included Lysine, Glutamine, and Proline derived tRFs. A distinct group of tRFs were only significantly altered in the CSF compartment which included Valine, Glycine, and Methionine-derived tRFs. These patterns were reflected in plasma and CSF, whereby tRF levels were higher in CSF but no tRFs were significantly altered in the cell-free CSF sample. The authors postulate that the upregulation of tRFs in CSF cells may reflect alterations in protein translation that occur in response to inflammatory conditions. In a follow up study, Needhamsen et al, performed unsupervised Multiple Co-Inertia Analysis (MCIA) to integrate multiple datasets, including small RNA sequencing data from CSF cells, cell-free CSF, PBMCs, and plasma, and methylome data collected from CSF cells, to distinguish between RR MS and controls[101]. This analysis indicated that small non-coding RNAs from CSF cells, cell-free CSF, and plasma could accurately distinguish RR MS from controls, and interestingly, this was mostly due to tRFs which contributed 12 out of 15 features included in this discriminatory panel[101].

Validation of tRF changes

Once tRFs have been identified by small RNA sequencing, validation of altered expression for a selection of tRFs is often required. Quantitative PCR (qPCR) can be affected by RNA modifications in the same way as sequencing, however it remains a well-used method for high-throughput quantification of tRFs in a research setting. The gold standard for tRNA quantification remains northern blotting, and this is often required for validation of tRF changes as this method is not influenced by RNA modifications. Northern blotting is a laborious method and detection limits may not be as sensitive as qPCR, nevertheless, it is possible to detect tRFs in plasma samples using the northern blotting technique[102]. However accurate these approaches may be, they are not routinely used in hospital labs and therefore may not be of use in a clinical environment where quick and accurate results are required. This has led researchers to investigate alternative methods for quantifying tRFs in a clinical setting. In a novel approach Ishida et al, focussed on the RNA modification m1A located within the tRFs rather than quantifying the fragments themselves. Using an m1A modification-specific ELISA they showed that patients with acute large vessel occlusion that had lower levels of m1A modification in plasma upon arrival to hospital had better clinical outcomes[103]. However, survival outcomes also correlated with a smaller initial infarction volume indicating tRFs, quantified via m1A modification, may be a useful biomarker of infarction size and clinical outcome. This supports results discussed earlier showing that serum Angiogenin levels correlate with infarct size[83].

Instead of measuring a proxy for tRFs, we utilised a direct detection method capable of quantifying tRFs in small volume biofluids[102]. Here complementary sequences to 3 epilepsy-associated tRFs were immobilised on a gold electrode to capture tRFs in the applied biofluid. In a second step, sequences complementary to the other end of the tRF were immobilised on platinum-coated nanoparticles, so that in the presence of the correct tRF, the platinum-coated nanoparticles are brought to the gold electrode surface. When hydrogen peroxide is added the platinum nanoparticles electrocatalyse the reduction of hydrogen peroxide, which generates a current that is directly proportional to the number of nanoparticles present. The nanoparticle number is dependent on the concentration of the tRF present in the biofluid, therefore the electric current generated is also proportional to the concentration of tRF present allowing direct quantification of tRFs in small volume biofluids. This method may be adaptable to a portable hand-held device allowing users to quantify tRFs in a pin-prick of blood.

Conclusion

Transfer RNA derived fragments are under investigation in a range of tissues and conditions as fluid-based biomarkers, and to uncover their functional contributions to cellular processes. The vast array of methodologies used to identify and quantify tRFs in tissues and biofluids creates a challenge when seeking to compare studies. Pre-treating samples to remove modifications from nucleotides or modify ends prior to adapter ligation can dramatically alter the species and abundance of tRFs detected. As yet, no unified approach has been adopted by the field. Size selection of input RNA, libraries, and read lengths can bias towards shorter fragments, which predisposes to certain tRF species over others. Variation in sequence analysis methods is not widely discussed but appears to have dramatic influence over the tRFs detected. Validation methods that do not rely on amplification-based detection can demonstrate robust, disease-associated alteration in tRF levels, but these are often absent from large-scale profiling studies. Nomenclature used to discuss tRFs varies widely across field and a standardised format should be adopted to enable comparisons and ensure that basic information on the parental tRNA and site of the fragment is included in all publications. In conclusion, the field would benefit from a unified approach to tRF detection, quantification, and nomenclature, to increase evaluation of findings across neurological diseases.

Conflict of Interest

The authors report no conflict of interest.

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