5′ValCAC tRNA fragment generated as part of a protective angiogenin response provides prognostic value in amyotrophic lateral sclerosis

Running title: Angiogenin tRNA Amyotrophic Lateral Sclerosis paper

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Abstract

Loss-of-function mutations in the ribonuclease angiogenin are associated with amyotrophic lateral sclerosis. Angiogenin has been shown to cleave transfer RNAs during stress to produce ‘transfer-derived stress-induced RNAs’ (‘tiRNAs’). Stress-induced tRNA cleavage is preserved from single-celled organisms to humans indicating it represents part of a highly conserved stress response. However, to date the role of tRNA cleavage in amyotrophic lateral sclerosis remains to be fully elucidated. To this end, we performed small RNA sequencing on a human astrocytoma cell line to identify the complete repertoire of tRNA fragments generated by angiogenin. We found that only a specific subset of tRNAs are cleaved by angiogenin and identified 5’ValCAC tiRNA to be secreted from neural cells. 5’ValCAC was quantified in spinal cord and serum from SOD1\(^{G93A}\) amyotrophic lateral sclerosis mouse models where we found it to be significantly elevated at symptom onset correlating with increased angiogenin expression, imbalanced protein translation initiation factors, and slower disease progression. In amyotrophic lateral sclerosis patient serum samples, we found 5’ValCAC to be significantly higher in patients with slow disease progression and interestingly we find 5’ValCAC to hold prognostic value for amyotrophic lateral sclerosis patients. Here we report that angiogenin cleaves a specific subset of tRNAs and provide evidence for 5’ValCAC as a prognostic biomarker in amyotrophic lateral sclerosis. We propose that increased serum 5’ValCAC levels indicate an enhanced angiogenin-mediated stress response within motor neurons that correlates with increased survival. These data suggest that the previously reported beneficial effects of angiogenin in SOD1\(^{G93A}\) mice may result from elevated levels of 5’ValCAC tRNA fragment.

Key words: amyotrophic lateral sclerosis, angiogenin, tRNA fragment, tiRNA, biomarker

Abbreviations: ALS = Amyotrophic Lateral Sclerosis, Ang = Angiogenin, Arg = Arginine, EV = Extracellular Vesicle, FUS = Fused in Sarcoma, Gly = Glycine, miRNA = microRNA, RNase = Ribonuclease, rRNA = ribosomal RNA, snoRNA = small nucleolar RNA, snRNA = small nuclear RNA, SOD1 = Superoxide Dismutase 1, TG = transgenic, tiRNA = transfer-derived stress-induced RNA, tRNA = transfer RNA, Val = Valine, WT = Wild type.
Introduction

Amyotrophic Lateral Sclerosis (ALS) is a debilitating disease characterised by progressive paralysis, which ultimately leads to death within an average of 3 years from diagnosis (van Es et al., 2017). However, considerable heterogeneity is observed with some patients showing rapid decline and death within 2 years of onset whilst others show a much slower disease progression with survival times extending over 5 years from symptom onset (Pupillo et al., 2014). Therefore, there is a great need to identify prognostic biomarkers for use in ALS.

Angiogenin is a ribonuclease from the RNase A family however, it shows significantly lower RNase activity and increased substrate specificity compared to other members of the family (Shapiro et al., 1986). Loss-of-function mutations in angiogenin were identified in sporadic and familial ALS patients of Irish and Scottish heritage in 2006 (Greenway et al., 2006) and since then mutations have been identified in ALS patients around the world (Aparicio-Erriu et al., 2012). Angiogenin variants have also been identified as risk factors for Parkinson’s disease (van Es et al., 2011; van Es et al., 2014), and more recently a nonsense mutation was identified in a patient with Alzheimer’s disease (Gagliardi et al., 2018), indicating Angiogenin may have a more widespread role in neuronal protection. Angiogenin is expressed in many cell types, including motor neurons and endothelial cells (Greenway et al., 2006; Wu et al., 2007), and production and secretion of angiogenin is upregulated in response to stresses such as hypoxia or trophic factor withdrawal (Sebastia et al., 2009; Skorupa et al., 2012). Our group and others have shown that angiogenin protects primary neurons against metabolic and toxic challenges in vitro (Fu et al., 2009; Ivanov et al., 2014; Kieran et al., 2008; Skorupa et al., 2012; Steidinger et al., 2011). Furthermore, systemic administration of recombinant human angiogenin protein prevented motor neuron death and increased lifespan in the SOD1G93A mouse model of ALS (Crivello et al., 2018; Kieran et al., 2008). Interestingly, transcriptomic analysis of fast and slow progressing mice expressing the same SOD1G93A transgene on different genetic backgrounds revealed that angiogenin expression in motor neurons was higher in slow progressing compared to fast progressing mice (Nardo et al., 2013), again suggesting that angiogenin elicits protective stress responses. Subsequent studies indicated that secreted or systemically administered angiogenin is endocytosed predominantly by astrocytes in vitro and in vivo (Crivello et al., 2018; Skorupa et al., 2012).
Angiogenin has been shown to cleave transfer RNAs during stress to produce ‘transfer-derived stress-induced RNAs’ (‘tiRNAs’) (Honda et al., 2016; Saikia et al., 2012; Yamasaki et al., 2009). Stress-induced tRNA cleavage is preserved from single-celled organisms to humans indicating it represents part of a highly conserved stress response (Lee et al., 2005). Using small RNA sequencing and custom bioinformatic analysis, we found that a specific subset of tRNAs were robustly cleaved by angiogenin within the anticodon loop, from which only one half was retained. We found that 5’ValCAC was elevated at disease onset in spinal cord from slow progressing SOD1<sup>G93A</sup> transgenic mice, which correlated with increased angiogenin levels and differential expression of protein translation initiation factors. We validated these results in a second preclinical ALS mouse, the FUS (1-359) model. We describe serum 5’ValCAC tRNA fragment as a novel prognostic biomarker in ALS, which results from a beneficial angiogenin-mediated neuroprotective stress response in motor neurons.
Materials and Methods

Cell culture

MZ-294 cells (RRID:CVCL_M410) were originally isolated from a primary glioblastoma (Hetschko et al., 2008) and maintained at 37°C/5 % CO₂ in DMEM media (Lonza) supplemented with 10% FBS (Sigma), 2mM L-Glutamine (Sigma), 100 U/mL Penicillin and 0.1 mg/ml Streptomycin (P/S: Sigma). Astrocyte lineage was confirmed by S100β expression (Raponi et al., 2007). Cells were treated with recombinant human angiogenin (rhAng: R&D Systems, #265-AN/CF) in serum-free Neurobasal media (ThermoFisher Scientific).

SH-SY5Y human neuroblastoma cells (RRID:CVCL_0019) were obtained from the ATCC and maintained in DMEM/F12 media (ThermoFisher Scientific) with additions as above. SH-SY5Y cells stably overexpressing angiogenin were generated by transfection of pcDNA3.1-Ang WT or mutant plasmids with Metafectene (Biontex Laboratories GmbH), and selection performed with neomycin (G418, Sigma).

Cell lines were confirmed as free from mycoplasma infection.

Nuclear and cytoplasmic fractionation

Nuclear and cytoplasmic fractionation was performed as described (Suzuki et al., 2010). Briefly, cells were washed in ice-cold PBS, scraped into 1 ml PBS and pelleted by centrifugation for 10 seconds at 4°C with 16,000 x g. Supernatant was discarded and cell pellet resuspended in 100 µl PBS with 0.1% NP-40 with protease inhibitors (Sigma). The cell pellet was triturated five times with a P1000 pipette tip and the sample centrifuged for 10 seconds at 4°C with 16,000 x g. The supernatant was collected as a cytoplasmic fraction and the pellet was washed twice by resuspending in PBS with 0.1% NP-40. Supernatant was discarded and the pellet (Nuclear fraction) resuspended in 30 µl RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1 % Triton X-100, 0.25 % sodium deoxycholate) with protease inhibitors (Sigma). Nuclear and cytoplasmic fractions for protein analysis were triturated and incubated on ice for 15 minutes before a final centrifugation step for 1 minute at 4°C and 10,000 x g. Nuclear and cytoplasmic fractions for RNA analysis were extracted with Trizol reagent as described.
Extracellular Vesicle purification

Extracellular vesicles (EVs) were purified from conditioned media (CM: 24 hours, serum-free Neurobasal media (Sigma)) following a described protocol (Chakrabortty et al., 2015). Briefly, CM was collected and centrifuged at 2000 x g for 10 minutes, then passed through a 0.22 um syringe filter. EVs were precipitated from 10 ml CM by addition of 2 ml ExoQuick TC solution (System Biosciences). Samples were incubated overnight at 4°C. EVs were pelleted by centrifugation at 1500 x g for 30 minutes at 4°C, supernatant was discarded. EVs were resuspended in 110 μl PBS with a 10 μl sample used for NanoSight NTA particle analysis and 100 μl used for RNA extraction. Expression of exosomal markers was confirmed by western blotting, where EVs from 10 ml CM were resuspended in 25 μl RIPA buffer, and analysed as described. Antibodies used were Rabbit anti-Alix (Bethyl Laboratories, A302-938A, RRID: AB_10681518), and Rabbit anti-Flotillin 1 (Abcam, ab RRID: AB_941621).

RNA extraction and analysis

Total RNA was extracted from cells using Trizol reagent and protocol (ThermoFisher Scientific). Briefly, a 10 cm dish was collected in 1 ml Trizol reagent and incubated for 5 minutes at room temperature. 200 μl of chloroform was added and samples were shaken for 15 seconds. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was removed to a new tube and an equal volume of isopropanol was added. Samples were incubated overnight at -20 °C. Samples were centrifuged at 12,000 x g for 15 minutes at 4 °C. The pellet was washed with 75% ethanol and air dried before resuspension in water supplemented with RNaseOUT ribonuclease inhibitor (ThermoFisher Scientific). RNA was extracted from mouse tissue using the Qiagen miRNeasy kit (Qiagen) and quantified by Nanodrop. RNA was purified from serum or conditioned media using the Qiagen serum/plasma miRNeasy kit (Qiagen). 3.5 ul of C.elegans miRNA-39 spike-in (at 1.6x10^8 copies/ul) was added during purification according to the manufacturer’s instructions (Qiagen). RNA was eluted in 20 ul water containing 1 ul RNaseOUT ribonuclease inhibitor. Small RNA levels were quantified by Fragment Analyser (Advanced Analytical).
Small RNA sequencing & analysis

Small RNA sequencing was performed on total RNA <100 nt extracted from 3 biological replicates of MZ-294 cells treated with angiogenin or vehicle by Eurofins MWG (error ~25%; Supplementary Figure 1). Adaptor sequences were trimmed using Trim Galore with 10 bp adaptor overlap and 10% errors (using cutadapt -O 10 -e 0.1 parameters). Reads with quality score <20 or length <15 bp were filtered out. A tRNA database was built from the tRNA genome downloaded from GtRNAdb (gtrnadb.ucsc.edu). Intron locations were added for 32 tRNAs. Tophat (v 2.0.14) and Bowtie (v 2.2.5.0) were used for alignments. Alignments were done in three stages:

1) Filtered reads were aligned to custom tRNA database with 1 hit allowed per read (-x 1 parameter) and mapped to tRNAs.
2) Unmapped reads from step 1 were aligned to the human genome (GRCh37/hg19) with 1 hit allowed per read (-x 1 parameter) and mapped to the sno/miRNA transcriptome.
3) Unmapped reads from step 2 were aligned to the human genome with 60 hits allowed per read, and reads were mapped to RefSeq transcriptome.

Aligned reads were counted and differential expression analysis was performed using DESeq2, including adjustment of p-values to correct for multiple testing (Love et al., 2014). RNA secondary structures were predicted using the Vienna RNAfold program (Lorenz et al., 2011).

RNA gel and Northern blotting

RNA gels were performed as described (Skorupa et al., 2012). Briefly, 15 μg total RNA isolated with Trizol reagent and protocol was diluted in formamide loading buffer and denatured at 90°C for 5 minutes before loading onto a 15% TBE-Urea PAGE gel. Gels were electrophoresed at 200 V for 95 minutes at 4 °C, stained with SYBR Gold (ThermoFisher Scientific), and imaged on an LAS 4000 Reader (Fujifilm). Northern blotting was performed as described (S. W. Kim et al., 2010). Briefly, following RNA gel electrophoresis, RNA was transferred to a Hybond+
nitrocellulose membrane (GE Lifesciences) at 10 V for 60 minutes at 4 °C. RNA was UV-cross-linked in a Stratalinker (1200 mJ/cm2). Membranes were blocked in ultraHyb Oligo (ThermoFisher Scientific) for 30 minutes at 37°C. Dual digoxigenin labelled DNA probes were added to blocking solution (final concentration 1 nM), and incubated overnight at 37°C. Membranes were washed twice with low-stringency wash buffer (2x SSC, 0.1 % SDS) and twice with high-stringency wash buffer (0.1x SSC, 0.1 % SDS) at 37°C, then 2xSSC at room temperature. Membranes were processed using the DIG wash and block set according to instructions (Roche). CPD-Star Development Reagent (Roche) was added and images were acquired using an LAS 4000 Reader (Fujifilm). Dual digoxigenin-labelled probes are listed in Supplementary Table 2.

qPCR

Custom small RNA Taqman assays (ThermoFisher Scientific) were designed to 5‘ValCAC tiRNA fragment (5’-GUUUCCGUAGUGUGGUUAUCACGUUCGCCUC-3’) and quantification performed on StepOnePlus or Quantstudio 5 PCR machines (ThermoFisher Scientific). Assay I.D. available on request. Mouse tiRNA levels were normalised to U6 snRNA, and human tiRNA levels were normalised to C.elegans miRNA-39 spike-in, using the 2^-DDCt method (Livak et al., 2001). 2 ul RNA per reverse transcription performed according to Taqman small RNA Assay protocol (ThermoFisher). 1 ul reverse transcription per qPCR reaction, and qPCRs were performed in triplicate. Angiogenin expression analysis was performed using Quantitect SYBR green PCR kit (Qiagen) using primers from (Fu et al., 2009).

Immunocytochemistry & immunohistochemistry

Immunocytochemistry was performed on MZ-294 cells treated with rhANG or vehicle using goat anti-human angiogenin (1/100, PC317L, Calbiochem, RRID:AB_213593) and mouse anti-tubulin (1/500, T6199, Sigma, RRID:AB_477583). Cells grown on coverslips were treated with angiogenin or vehicle and fixed in 4 % paraformaldehyde for 15 minutes. Cells were permeabilised by 10 minutes incubation in 95 % ethanol: 5 % glacial acetic acid. Coverslips were blocked in 5 % BSA in PBS with 0.1% triton X-100. Primary antibodies were incubated
overnight at 4°C. Fluorescent Alexa-conjugated secondary antibodies were used (1/500, Molecular Probes) for 2 hours and coverslips mounted in Prolong Gold (Invitrogen). Images were captured on a Zeiss LSM 710 confocal microscope.

Anaesthetized mice were perfused transcardially with 4% paraformaldehyde and spinal cord dissected, post-fixed in 4% paraformaldehyde overnight at 4°C, preserved in 30% sucrose and then included in OCT compound. Immunohistochemical analyses were done on free-floating spinal cord cryosections (30 μm), after mounting on glass slides (Waldeirmar Knittle) with 1:1 0.1M PBS: glycerol, samples were analysed under an Olympus Fluoview Laser scanning confocal microscope (Olympus BX61 light microscope). Z-stacked composites were generated and used for image analysis. The primary antibodies used: Mouse anti eIF4e (1/200, sc-9976, Santa Cruz Biotechnology, RRID:AB_627502) and Rabbit anti eIF4eBP1 (1/500, SAB4300475, Sigma-Aldrich, RRID:AB_10634621). Alexa- 488 and 647 secondary antibodies (Invitrogen) were used at 1:500. The intensity of fluorescence was calculated with Fiji (Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA) on single cell by making a ROI on the cytosol of each MN with a diameter > 400 μm² and measuring the Integrated Density following background subtraction. ~3-5 MNs > 400 μm² from at least three serial L3-L4 slices were evaluated.

**Western blotting**

Lysates were collected in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.25 % sodium deoxycholate) with protease inhibitor (Sigma). Total protein was quantified using a macro BCA assay (ThermoFisher Scientific) and denatured for 10 minutes at 90°C in 1x laemmli buffer. Proteins were separated on a 12 % SDS-PAGE gel and transferred to nitrocellulose membrane. Membranes were incubated overnight in antibodies: goat anti-human angiogenin (1/500, AB-265-AN, R & D Systems, RRID: AB_354325) and mouse anti-actin (1/1000, A5441, Sigma, RRID: AB_476744). Membranes incubated for 2 hours in HRP-conjugated secondary antibodies. ECL reagent was added (Immobilon, Merck) and images taken on an LAS 4000 Reader (Fujifilm).
ALS mouse models

FUS (1-359) mice (Shelkovnikova et al., 2013) were kindly donated by Professor Vladimir Buchman (School of Biosciences, Cardiff University, Cardiff, CF10 3AX, UK), embryos were re-derived in The Institute of Molecular Genetics ASCR, Prague, Czech Republic, and the colony maintained at RCSI. Ethical approval for the study was granted by the RCSI Research Ethics Committee (REC1122) and a licence was obtained from the HPRA (AE19127/P004). After weaning on post-natal day (PND) 21, pups from litters of the same generation and gender were housed in groups (3-5 per cage). N=6-8 mice of both sexes were used in this study.

Transgenic SOD1<sup>G93A</sup> mice of C57BL/6JOlahsd (C57-TG) or 129SvHsd (129Sv-TG) genetic background and non-transgenic littermates (C57-WT and 129Sv-WT) derived from the B6SJLtgNSOD-1-SOD1G93A-1Gur line (Jackson Laboratories) expressing 20 copies of human SOD1<sup>G93A</sup> transgene were maintained at the Mario Negri Institute animal facility. Procedures involving animals were conducted according to the Mario Negri institutional guidelines. The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01). Symptom onset was determined by the first sign of impaired paw grip strength and decline in body weight. N=8 female mice at symptom onset were used in this study. Mice were maintained at 22 ±2°C with a relative humidity 55 ±10% and a 12h light/dark cycle with food and water supplied <em>ad libitum</em>.

Tissue and serum collection

Animals were terminally anaesthetized with Dolethal, and after absence of reflexes was confirmed, blood was collected. Serum was separated by centrifugation at 1,000 x g for 10 minutes at 4°C. Mice were transcardially perfused with PBS and the spinal cord was dissected and frozen.

Patient samples

Serum samples were from the Prospective ALS study Nederland (PAN), which is a large ongoing epidemiological study on environmental and genetic risk factors for ALS (Huisman et al., 2011). Male and female ALS patients fulfilled the criteria for suspected, possible, probable
or definite ALS according to the 1994 El Escorial criteria (Brooks, 1994). In the PAN study, practitioners are asked to recruit a matched for age, sex, and ethnicity control individual for each ALS case. Samples included in this study came from participants enrolled in the PAN study in 2013 and 2014. DNA samples from the individuals in the discovery cohort were screened for mutations in \textit{C9orf72}, \textit{TARDBP}, \textit{SOD1}, \textit{FUS}, and \textit{ANG} by whole genome sequencing (Dolzhenko \textit{et al.}, 2017; van Rheenen \textit{et al.}, 2016). All participants provided informed consent according to the Declaration of Helsinki. Ethical approval for this study was obtained from the Institutional Review Board of UMC Utrecht. Blood was centrifuged at 1,000 x g and serum collected and stored at -80°C. Serum was checked for haemolysis on the Nanodrop using a protocol adapted from (Appierto \textit{et al.}, 2014).

\textbf{Statistical analysis}

Statistical analysis was performed in Graphpad Prism, SPSS, or R version 3.4.3. Data are fold change tiRNA levels in transgenic (TG) compared to wild type (WT) mice, or ALS patients compared to controls. Student’s t-tests and ANOVA were used to determine whether mean tiRNA levels differed between WT and TG groups and groups of more than two, respectively. Tukey’s honest significant difference test was used to assess differences between specific group means. In patient samples, tiRNA levels were tested using a Kruskal-Wallis test with post-hoc Dunn’s test. As exploratory analyses, Poisson regression with robust standard errors was used to compute relative risk (RR) estimates and 95% confidence intervals (CI) to examine whether each continuous linear tiRNA was independently associated with the risk of slow vs. fast ALS progression. Analyses were adjusted for continuous linear age at sample collection and binary gender. Statistical significance was determined from Wald p-values computed using robust standard errors. Receiver operating characteristics (ROC) curve analysis was performed in SPSS and Youdens J statistic was used to identify the maximal potential effectiveness of the biomarker.

\textbf{Data Availability}

https://mc.manuscriptcentral.com/braincom
Raw RNA sequencing reads are deposited in the NCBI Sequence Read Archive (BioProject PRJNA507979).

Results

Small RNA sequencing to identify substrates of angiogenin

Motor neurons express angiogenin and during stress conditions expression is increased and angiogenin is secreted (Greenway et al., 2006; Wu et al., 2007). We have shown angiogenin is endocytosed by astrocytes where it cleaves RNA and mediates neuroprotection in paracrine (Skorupa et al., 2012). The human glial-derived cell line MZ-294 was used to characterise angiogenin substrates, as these cells do not express detectable levels of angiogenin and efficiently endocytose angiogenin from media (Fig 1A). Exogenous recombinant human angiogenin (rhAng) added for 3 hours is predominantly located in the cytoplasm (Fig 1B) and induces RNA cleavage in a dose-dependent manner (Fig 1C). To determine the composition of RNA fragments we isolated total RNA from angiogenin-treated (500 ng/ml in PBS with 1% BSA; 3 hrs; R&D Systems) and vehicle-treated cells (PBS with 1% BSA; 3 hrs) and performed small RNA sequencing (<100 nt, Supplementary Fig 1). We developed a custom analysis pipeline using a tRNA library generated from the human tRNA genome at GtRNAdb (gtrnadb.ucsc.edu; (Chan et al., 2009)). The GtRNAdb is a publicly available catalogue of predicted tRNA gene sequences from over 700 different species with direct links to the UCSC genome browser to allow users to view tRNAs in their genomic context. To account for post-transcriptional addition of CCA-tails to tRNAs, “CCA” was added to the 3’ end of each tRNA. Reads aligning to tRNAs were pooled by isoacceptor type, differential gene expression was performed, and read coverages plotted to determine the angiogenin cleavage profile. Read distribution (listed in Supplementary Table 1) indicated a similar proportion of reads aligned in angiogenin and vehicle-treated cells, including tRNA aligned reads (4% angiogenin, 3.6% vehicle).

Specific tRNAs are cleaved by angiogenin
Read profiles indicated a small but highly significantly altered subset of tRNAs were robustly cleaved by angiogenin (Table 1). Full-length tRNAs were not detected in this analysis due to the high frequency of modified bases, which inhibits progression of polymerases. When we limited analysis to significantly altered tRNAs with an adjusted p-value < 0.05 we identified 10 tRNAs, 9 of which showed profiles indicative of cleavage by angiogenin, suggesting tRNA cleavage is a highly specific process. Specific aminoacyl groups were favoured by angiogenin (e.g. Arginine and Valine) whilst others remained intact. In most cases, reads were detected aligning to either the 5’ or 3’ end of the tRNA, which appeared to be determined by aminoacyl group as 3’ fragments were only identified from Arginine tRNAs. Aligned reads for Valine CAC genes are shown (Fig 1D) with chromosomal location and gene sequence below (Fig 1E). A dramatically different profile is seen in angiogenin-treated cells where many short RNA fragments aligned to the 5’ end of Valine CAC which are absent in vehicle-treated cells. Mapping the cleavage site onto the secondary structure indicated angiogenin cleavage predominantly occurred within the anticodon loop (Fig 1F, Mature full-length Valine CAC secondary structure; Fig 1G predicted 5’Valine CAC tRNA secondary structure). Read coverage plots and predicted secondary structures for tRNAs listed in Table 1 are provided in Supplementary Figs 2-10. The tRNA fragments are predicted to form stable hairpin structures (Supplementary Fig 11 & 12) and interestingly, the preserved fragments have higher predicted minimum free energy than the undetected fragments (Supplementary Fig 13).

Validation of tRNA fragment accumulation in response to angiogenin.

Northern blotting was used to validate tRNA cleavage by angiogenin (Fig 2A) with probes that recognise 5’ValCAC, 3’ArgTCG, and 5’GlyGCC. Probes recognising 3’ValCAC and 5’ArgTCG fragments showed these are barely detectable indicating they undergo rapid degradation, and validating the RNA seq results. The 5S rRNA band from the RNA gel is included to show equal loading of total RNA. Nuclear and cytoplasmic fractionation revealed cleavage occurs within the cytoplasm (Fig 2B), a western blot confirming fractionation is shown (Fig 2D). The proportion of tRNA that is cleaved by angiogenin relative to full-length tRNA Valine CAC is low (conservatively estimated at 5-fold more full-length compared to fragment, see supplementary Fig 14), suggesting there would be no loss-of-function of full-length tRNAs.
associated with tRNA cleavage. Custom small RNA Taqman assays were designed to recognise specific tiRNA fragments 5’ValCAC, 3’ArgTCG, and 5’GlyGCC. Custom synthetic tiRNA mimics were synthesised for 5’ValCAC, 3’ArgTCG, and 5’GlyGCC (IDT, USA), and used as template for qPCR. Synthetic tiRNAs amplified linearly over a range of four orders of magnitude (Fig 2C) indicating the Taqman assays were robust. The Taqman assays displayed a dose response to increasing angiogenin levels in both human astrocytoma and mouse primary astrocytes (Fig 2E & F), although the response to angiogenin was muted in primary mouse astrocytes.

tiRNA secretion from neuronal cells is altered by ALS-associated Angiogenin mutations

As endogenous angiogenin is expressed in motor neurons (Greenway et al., 2006), we explored whether tRNA cleavage also occurs in neuronal cells. Further as miRNAs are actively secreted from cells in Argonaute-bound complexes or protected within exosomes (Chen et al., 2012), and tiRNAs have been found within synaptic vesicles (H. Li et al., 2015) we sought to determine whether tiRNAs are secreted from neuronal cells, and could be detected in exosomes. We used human neuroblastoma SH-SY5Y cell lines that stably overexpress wild type (WT) angiogenin, or ALS-associated mutants K40I and R31K. The K40I mutation renders Angiogenin catalytically inactive while the R31K mutation is reported to inhibit nuclear translocation (Thiyagarajan et al., 2012). qPCR and western blotting confirmed angiogenin over-expression compared to the control, which stably expresses the pcDNA3.1 plasmid (Fig 3A & B, pcDNA). The K40I mutant was consistently expressed at lower levels (in multiple clones generated) than WT Ang or R31K mutant indicating this mutation may affect stability of Ang mRNA (data not shown). Cells were incubated for 24 hours in serum-free media and conditioned media (CM) collected. Quantification of 5’ValCAC indicated 5’ValCAC was secreted at elevated levels from cells overexpressing WT Ang and R31K mutant Ang compared to the pcDNA control and the K40I mutant (Fig 3C). The R31K mutation is located within the nuclear localisation signal (NLS) of angiogenin, and we see increased 5’ValCAC secreted indicating the R31K mutation may interact with cytoplasmic factors involved in the secretion pathway. We explored the mechanism of tiRNA secretion by precipitating extracellular vesicles (EVs) from CM. There was no significant difference in EV numbers purified from cells stably overexpressing WT or mutant Ang (Fig 3D). Exosomal markers were present on purified
EVs as shown by western blotting with Alix and Flotillin 1 antibodies (Fig 3E). We found that the fraction of 5′ValCAC in EVs was not significantly different between the cell lines, with approximately 2-4% total secreted 5′ValCAC found in EVs (Fig 3F). This suggests that the un-encapsulated fraction of 5′ValCAC is dynamically regulated while basal levels of 5′ValCAC are secreted in EVs.

5′ValCAC levels indicate disease progression in mouse models of ALS

As tiRNA production is linked to protective stress signalling (Emara et al., 2010; Fu et al., 2009; Saikia et al., 2014; Saikia et al., 2012), we investigated 5′ValCAC levels in slow vs. fast progressing SOD1\(^{G93A}\) mice bred on two distinct genetic backgrounds (C57Bl/6JOLA/Hsd and 129Sv), which display differences in disease progression and lifespan (Nardo et al., 2013). SOD1\(^{G93A}\) transgenic (TG) mice on the 129Sv background show symptom onset at 14 weeks whereas TG mice on the C57/Ola background show delayed symptom onset at 18 weeks (Fig 4A). This difference could not be explained by differences in SOD1\(^{G93A}\) transgene copy number or levels of mutant SOD1 protein (Nardo et al., 2013). Interestingly, Nardo et al., found significantly increased angiogenin expression in the C57/Ola strain at symptom onset (Nardo et al., 2013). Therefore, we examined angiogenin expression and 5′ValCAC levels in lumbar spinal cord RNA collected at symptom onset in these SOD1\(^{G93A}\) mice. Initially we confirmed \(mAng1\) levels were significantly elevated in the slow progressing TG mice compared to wild type (WT) mice on the C57/Ola background, and also compared to fast progressing 129Sv WT and TG mice (Fig 4B). We then found that 5′ValCAC levels were significantly higher in spinal cord from the slow TG mice compared to the fast TG and WT mice (Fig 4C), suggesting that 5′ValCAC may serve as a biomarker of disease progression in ALS.

tiRNAs have been shown to inhibit protein translation in several recent studies (Fricker et al., 2019; Goncalves et al., 2016; Ivanov et al., 2011). The protein translation initiation factor elf4e plays a crucial role in recruiting elf4G, and subsequently the ribosomal subunits to the 5′m7G mRNA cap structure. elf4e is the rate-limiting factor in the protein translation process and levels are tightly regulated via interaction with the elf4e-BP inhibitor proteins, where elf4e and elf4e-BP1 are normally present in a 1:1 ratio (Rau et al., 1996). Interestingly, 5′Alanine tiRNA has been shown to interact directly with elf4e, and can displace elf4e from
the 5’m7G cap (Ivanov et al., 2011). To determine whether increased 5’ValCAC levels were associated with dysregulation of protein translation initiation factors we examined expression of elF4e and elF4eBP1 in motor neurons by immunohistochemistry. This revealed that slow progressing TG mice had significantly lower levels of elF4e and higher levels of eiF4eBP1 compared to WT mice on the same background (Fig 4D & E, and Supplementary Fig 15 & 16). The opposite was observed in the fast progressing mice where eiF4e was significantly elevated in TG compared to WT mice, while eiF4eBP1 showed no significant difference but levels were lower in TG mice. Together, we observe an upregulation of angiogenin in motor neurons, along with increased 5’ValCAC levels in spinal cord homogenate, and altered levels of protein translation factors within motor neurons. It remains to be seen whether these changes occur as part of a synchronised stress response.

To determine whether 5’ValCAC levels were altered in a second ALS model, we employed the FUS (1-359) mouse model (Shelkovnikova et al., 2013) from which spinal cord tissue and serum samples were available. FUS (1-359) transgenic mice overexpress a truncated human FUS gene lacking a nuclear localisation signal, which is prone to cytoplasmic aggregation, and show an early vasculature defect similar to the SOD1$^{G93A}$ model (Crivello et al., 2019; Crivello et al., 2018). We analysed mice collected at PND 90 which equates to around symptom onset as the average lifespan of TG mice from the FUS (1-359) colony is 120 days (+/-22 days)(Hogg et al., 2017). Immunohistochemistry of angiogenin protein (ANG) levels in lumbar motor neurons from 6 WT and 6 TG mice showed that ANG levels were significantly higher in TG mice than WT littermates (Fig 4F (i) and supplementary Fig 17). Quantification of 5’ValCAC levels in lumbar spinal cord homogenate revealed that 5’ValCAC was significantly elevated in TG mice compared to WT littermates (Fig 4F(ii)). As we have demonstrated that 5’ValCAC is secreted from cells, we sought to determine whether it could be detected in serum. We found that 5’ValCAC was significantly elevated in serum from TG FUS (1-359) mice compared to WT littermates at disease onset (Fig 4G) indicating 5’ValCAC may be of use as a blood-based biomarker in ALS. Analysis of 5’ValCAC levels at a pre-symptomatic time point (PND 50) revealed there was no significant difference in 5’ValCAC levels between WT and TG littermates in spinal cord or serum (Supplementary Fig 18).
Serum 5′ValCAC levels as a prognostic biomarker for ALS patients

The preclinical data in the slow and fast progressing SOD1<sup>G93A</sup> transgenic mouse models suggested that 5′ValCAC levels are related to activation of protective stress responses during disease progression and may hold prognostic potential. To further explore the role of serum 5′ValCAC as a biomarker for ALS progression, we next investigated serum collected at diagnosis from a cohort of slow and fast-progressing ALS patients (n = 114, consisting of n = 70 fast and n = 16 slow progressing ALS patient) and 91 healthy controls in The Netherlands as part of the PAN study (Huisman et al., 2011). Patients were stratified according to disease progression determined by survival time: fast progressors <24 months and slow progressors >60 months (Fig 5A). 5′ValCAC levels were significantly elevated in slow progressing patients when compared to healthy controls or to fast progressing patients indicating 5′ValCAC levels in serum may be of use as a prognostic biomarker in ALS (Fig 5B: Kruskal-Wallis test p = 0.012, Dunn’s post-hoc p < 0.05 for Fast Vs Slow and Control Vs Slow).

No significant correlation was found between 5′ValCAC levels and RNA concentration (Supplementary Fig 19) and no significant difference was detected in 5′ValCAC levels from haemolysed and non-haemolysed samples (data not shown). ALS patients were screened for repeat expansions (RE) at the C9orf72 locus and whole exome sequencing was performed to screen for mutations at the SOD1, TARDBP, FUS, and ANG loci (Supplementary Table 3). No significant difference was detected in 5′ValCAC levels in ALS patients with C9orf72 RE compared to those without (wt), and no differences were detected in patients with FUS, TARDBP, or SOD1 mutations, (Supplementary Fig 20; n=4, 2, and 3 respectively, labelled wt(+mut)). Unfortunately, no patients in the discovery cohort carried mutations in ANG. These results suggest serum 5′ValCAC levels may provide prognostic information for sporadic and familial ALS patients, irrespective of whether an underlying genetic cause has been identified.

Next, findings were validated in a second independent cohort of serum samples from the PAN study (Huisman et al., 2011). Serum samples collected at diagnosis from slow (n = 92) and fast (n = 93) progressing ALS patients, and healthy controls (n = 128) were included to replicate the initial study and optimise contrast and power. Validation cohort demographics are shown (Fig 5C). Analysis of serum 5′ValCAC levels indicated a significant difference across survival
groups (Fig 5D; Kruskal-Wallis test p = 0.0005, Dunn’s post-hoc p < 0.01 for Control Vs Fast and Fast Vs Slow). In unadjusted analyses, 5’ValCAC was significantly associated with disease progression when comparing controls, fast, and slow ALS progressors (p = 0.008). For every unit increase in 5’ValCAC, the risk of slow vs. fast ALS progression increased by 38% (RR 1.38, 95% CI 1.01-1.89) and the risk of fast ALS progression vs. controls decreased by 34% (RR 0.66, 95% CI 0.49-0.89). Relative risk estimates and 95% CIs were similar after adjusting for age and sex. Results were statistically significant after correcting for multiple comparisons (Bonferroni p = 0.02).

To determine whether serum 5’ValCAC levels could be prognostic for a continuous clinical spectrum of ALS patients, we combined cohorts 1 and 2 and additionally included an intermediate survival group from cohort 1 (n = 16) and cohort 2 (n = 91). To determine a cut-off value, we performed Receiver Operating Characteristic (ROC) Curve analysis on fast and slow progressing patients from cohorts 1 and 2. This analysis gave an area under the curve of 0.66, with p = 0.000003 (Supplementary Fig 21). Youden’s J statistic indicated a cut-off value of 0.735 provided the best discrimination between fast and slow progressing patients, with a sensitivity of 82.9 % and a specificity 47.4 %. We used this cut-off to stratify patients into low 5’ValCAC (<0.735) and high 5’ValCAC (>0.735) groups. Kaplan-Meier analysis of survival for high and low 5’ValCAC groups indicated that patients with high serum 5’ValCAC at diagnosis survived significantly longer than those with low 5’ValCAC levels (Fig 6A; Log Rank test, χ² = 12.29, p = 0.0005). The number of patients surviving at each 12 month time point are indicated below the x-axis (Fig 6B). Of note, the prognostic value of 5’ValCAC levels remain when patients with spinal-onset disease are analysed alone (Supplementary Figure 22, Log-Rank test, p = 0.008), indicating that although spinal onset indicates a better prognosis, 5’ValCAC levels provide additional prognostic value. These data suggest 5’ValCAC levels in serum, collected at diagnosis, may provide prognostic information for ALS patients.
Discussion

Angiogenin shows neuroprotective properties \textit{in vitro} and loss of functional angiogenin due to mutation or decreased expression has been implicated in a range of neurodegenerative disorders (Gagliardi \textit{et al.}, 2018; Greenway \textit{et al.}, 2006; Kieran \textit{et al.}, 2008; Skorupa \textit{et al.}, 2012; van Es \textit{et al.}, 2011). We sought to characterise angiogenin-generated tRNA fragments and investigated their prevalence throughout disease progression in ALS. We found angiogenin cleaves a subset of tRNAs, and only one half is preserved. The tRNA fragments are predicted to form hairpin structures, which appear to be protected from degradation. Interestingly we find that the 5’ValCAC fragment is significantly elevated in ALS mouse models and serum from ALS patients with a slow disease progression. The clinical findings were validated in a second cohort of ALS patients, suggesting that 5’ValCAC could serve as a novel prognostic biomarker.

Cleavage of tRNAs by angiogenin has been widely reported yet we were surprised as only a small subset of tRNAs were cleaved robustly (Fu \textit{et al.}, 2009; Saikia \textit{et al.}, 2012; Yamasaki \textit{et al.}, 2009). We identified both 5’tiRNA and 3’tiRNAs in our screen yet never both from the same tRNA. Recent work has shown that tRNA cleavage by angiogenin can be inhibited by 5-methylcytosine indicating some tRNAs may be protected by the presence of RNA modifications (Blanco \textit{et al.}, 2014; Schaefer \textit{et al.}, 2010). Many reports describe the presence of tRNA fragments in a range of cell types, animal models and human patients but little is known about their function. They were originally described to inhibit protein translation (Ivanov \textit{et al.}, 2011; Yamasaki \textit{et al.}, 2009), promote stress granule assembly (Emara \textit{et al.}, 2010), and were found to be neuroprotective in motor neurons (Ivanov \textit{et al.}, 2014). More recently, several groups have reported that tRNA fragments can inhibit protein translation and regulate ribosome biogenesis (Fricker \textit{et al.}, 2019; Guzzi \textit{et al.}, 2018; H. K. Kim \textit{et al.}, 2017). Translation initiation factor eIF4e plays a crucial role in recruiting the ribosome to the mRNA to initiate protein translation via binding to the 5’m7G cap structure. eIF4e interacts with scaffolding protein eIF4G which recruits the RNA helicase eIF4A to form the eIF4F complex. The eIF4F complex then engages with other initiation factors and ultimately recruits the large and small ribosomal subunits to allow protein translation to occur. eIF4e is the rate-limiting factor in the translation initiation process, and as such levels are tightly regulated under normal physiological conditions via interaction with eIF4eBP inhibitor proteins. Indeed,
eIF4e and eIF4eBP1 are normally present in a 1:1 ratio (Rau et al., 1996). Lower levels of eIF4e coupled with increased levels of eIF4eBP1 that we observed in TG mice from the slow progressing SOD1G93A colony would likely result in decreased protein translation; however, translation initiation factors are also regulated via phosphorylation. Ivanov et al., demonstrated that 5’Alanine tRNA can displace eIF4G from the m7G cap, and to a lesser extent eIF4e (Ivanov et al., 2011). Interestingly, when they analysed the proteins bound to 5’Alanine tRNA they found that eIF4e precipitated at much higher levels than eIF4G. This data and our results suggest that tiRNAs may interact directly with eIF4e and influence cap-dependent protein translation, however further work is required to determine whether these observations are linked. Of note, pharmacological intervention targeting protein translation with Guanabenz, which blocks dephosphorylation of translation initiation factor eIF2α, have been shown to improve motor performance, protect against motor neuron loss, and extend lifespan in the SOD1G93A ALS mouse model (Jiang et al., 2014; L. Wang et al., 2014). However, a subsequent study found that Guanabenz exacerbated disease in SOD1G93A mice (Vieira et al., 2015). Recently angiogenin has been shown to preserve stemness in haematopoietic stem cells via generation of tiRNAs that inhibit protein translation, whilst promoting proliferation of myeloid progenitor cells via stimulation of ribosomal RNA (Goncalves et al., 2016).

To date little research has focused on the extracellular function of tRNA fragments, yet here we find 5’ValCAC to be secreted from neuronally-derived cells. We found basal levels of 5’ValCAC within EVs secreted from neural cells overexpressing WT or mutant angiogenin, but higher levels of 5’ValCAC were found in the total CM fraction, indicating the majority of 5’ValCAC is secreted via a different pathway. Further, the un-encapsulated fraction of secreted 5’ValCAC was greatly influenced by mutations in angiogenin suggesting angiogenin may interact with the (as yet unidentified) factors involved in this pathway, within the cytoplasm. This is supported by the increased level of 5’Val CAC secretion we observed from cells overexpressing the R31K angiogenin mutant. Interestingly, sorting of RNAs, including tRNAs, prior to secretion in EVs can be dependent on the RNA sequence and the cell or tissue of origin, and YBX-1 protein has been implicated (Shurtleff et al., 2017; Temoche-Diaz et al., 2019). Un-encapsulated extracellular miRNAs have been found in complexes with High Density Lipoproteins (HDLs) (Vickers et al., 2011), nucleophosmins (K. Wang et al., 2010), and argonaute protein complexes (Kumar et al., 2014), indicating there are many potential
candidates that may be involved in transporting un-encapsulated tiRNAs. Research on miRNAs has shown cell-type specific secretion and uptake occurs (Chivet et al., 2014). This raises the possibility that extracellular tiRNAs may function similarly as specific signaling molecules, particularly within the CNS (Hogg et al., 2019). tiRNAs have been shown to be generated in endothelial cells in response to hypoxia (Q. Li et al., 2016), and in airway epithelial cells in response to respiratory syncytial viral infection (Q. Wang et al., 2013), indicating that tiRNAs from sources other than spinal cord motor neurons may also contribute to tiRNAs quantified in serum in this study. Further investigations are required to determine how tiRNAs are secreted from neural cells and protected from degradation in circulation.

Serum Angiogenin protein levels have been investigated as a biomarker of ALS and found to be elevated in ALS patients compared to controls (Cronin et al., 2006; van Es et al., 2014). Interestingly, in both studies serum Ang levels were higher in patients with spinal onset ALS than those with bulbar onset, highlighting the role of angiogenin in spinal motor neurons under stress conditions. However there was no association between Ang levels and survival. This apparent discrepancy with our results can be explained as the levels of Ang protein were quantified in serum, whereas we postulate tRNA cleavage occurs within cells in the spinal cord, and the resulting tRNA fragments are secreted. Therefore we posit that we are quantifying tRNA fragments as a measure of angiogenin activity within cells, as a “read-out” of underlying stress response. As tiRNAs are widely reported to inhibit protein translation (Emara et al., 2010; Goncalves et al., 2016; Ivanov et al., 2011; Yamasaki et al., 2009), and have been shown to specifically inhibit cap-dependent translation (Ivanov et al., 2011), they may alleviate neuronal stress by reducing cap-dependent translation while allowing vital stress-response factors to be generated via IRES-mediated translation.

There is a great need to develop prognostic biomarkers for ALS to monitor disease progression and treatment response and enable stratification of patients entering clinical trials. Here we found that 5’ValCAC tiRNA levels are associated with a slower disease progression in both SOD1<sup>G93A</sup> ALS mouse model and ALS patient serum collected at diagnosis. tiRNAs generated by angiogenin occur as part of a highly conserved stress response, it is interesting to speculate that patients with a more robust stress response survive longer, although it cannot be excluded that these patients simply have a higher number of motor neurons surviving that are experiencing stress and exhibiting a stress response. Serum tiRNAs
are highly suited for development as biomarkers as they are stable, serum is easily accessible, and the custom Taqman assays described here are reliable and easy to perform. Further, we have recently developed an electrochemical direct detection method for quantification of tRNA fragments in small volume biofluids which could be utilised for prognostic profiling of patients upon entry into clinical trials (McArdle et al., 2020). Therefore, we present a novel class of non-coding RNA, which may be of use as a prognostic biomarker indicating a protective neuronal stress response in ALS patients. Further, our data suggests that the previously reported beneficial effects of angiogenin administered as a therapeutic in SOD1G93A mice may result from the 5'ValCAC tRNA fragment. 5'ValCAC warrants further investigation in ALS to determine whether beneficial effects of angiogenin are mediated by this tRNA fragment.
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Supplementary data

Supporting data figures and RNA structures are provided as a word document and a summary of the RNA sequencing results are provided in an excel spreadsheet.

Competing Interests

The authors declare no competing interests.
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FIGURE LEGENDS

Figure 1: Small RNA sequencing to identify substrates of angiogenin. A) Western blot showing rhAng in serum-free media is endocytosed by glial-derived MZ-294 cells in a dose dependent manner (doses are 100, 250, and 500 ng/ml). Untreated (NT) or vehicle-treated (Veh) MZ-294 cells do not contain detectable levels of angiogenin. Uncropped blots are available in a Supplementary document. B) Immunocytochemistry of angiogenin and vehicle-treated MZ-294 showing that Ang (Green) is clearly detectable in the cytoplasm highlighted here with tubulin (Red) following 3 hours treatment (500 ng/ml). Nuclei are stained with DAPI, scale bar = 25 μm. C) SYBR Gold stained TBE-Urea RNA gel showing RNA fragmentation induced by Ang in a dose dependent manner. tiRNAs of approximately 34 and 43 nt are indicated, a ribosomal RNA band is shown as a loading control. D) Pooled read coverage (counts per million, CPM) of reads aligned to Valine CAC genes, with genomic location and gene sequences shown in (E). A clear cleavage profile is present in angiogenin-treated samples where the 5' ValCAC fragment is retained while the 3’ fragment is absent. Full-length tRNA reads were not detected due to the highly modified nature of tRNAs, which inhibits the progression of polymerases. F) Mature tRNA Valine CAC secondary structure with the Ang cleavage site in the anticodon loop indicated (red triangle). G) Predicted secondary structure of the 5’ValCAC tiRNA fragment.

Figure 2: Specific tRNA fragments accumulate in Angiogenin-treated astrocytes. A) tiRNA production was validated using northern blotting with dual DIG labelled probes specific for either the 5’ or 3’ end of the tRNA, which shows the 5’Val CAC fragment increases with higher doses of Ang whereas the 3’ Val CAC fragment is barely detectable in all samples. Similarly, the 5’GlyGCC and 3’ArgTCG tiRNAs increase in a dose-dependent manner whereas the 5’ ArgTCG cannot be detected at all. 5S rRNA on the RNA gel is shown to demonstrate similar levels of RNA were loaded in each lane. B) Nuclear & Cytoplasmic fractionation was performed to determine where Ang cleavage occurs which revealed the tiRNA fragments are generated in the cytoplasm. Uncropped blots are available in a Supplementary document. C) Custom Taqman assays were designed to recognise 5’ ValCAC, 3’ ArgTCG and 5’ GlyGCC tiRNAs and standard curves were generated using synthetic tiRNAs (Integrated DNA technologies) which showed the Custom Taqman assays are linear log over a 4-fold dilution series (R2 values: 5’ValCAC = 0.99, 3’ArgTCG = 0.99, 5’GlyGCC = 0.97), n=4 independent experiments, individual data points are shown. D) Nuclear and cytoplasmic fractionation was confirmed by western blotting with nuclear (Fibrillarin) and cytoplasmic (Tubulin) antibodies. Taqman assays were validated in E) MZ-294 human astrocytoma cells and F) Primary mouse astrocytes treated with increasing amounts of rhAng. N=4 independent experiments, with individual data points and mean +/- SEM shown. * indicates P< 0.05, 1-way ANOVA post-hoc Dunnett’s compared to 0 Ang. In E) all 3 assays show significant difference between 0 and 500 ng/ml Ang, and 5’ValCAC assay shows significant difference between 0 and 250 ng/ml Ang. In F) 5’ValCAC assay shows a significant difference between 0 and 500 ng/ml Ang.
Figure 3: tiRNAs are secreted from neuronally-derived cells. SH-SY5Y human neuroblastoma cells stably over-expressing wild type human Ang (Ang) or ALS associated mutants (K40I, R31K), or the empty vector alone (pcDNA) were generated and Ang levels were quantified by A) qPCR and B) western blot. Recombinant human angiogenin (5 ng) was loaded as a control. The K40I mutant consistently displayed reduced expression indicating this mutation may alter stability of the Ang mRNA. Uncropped blots are available in a Supplementary document. C) Absolute levels of 5'ValCAC tiRNA were examined in conditioned media (CM) using a custom Taqman assay revealing levels were increased in WT and R31K expressing cells, whereas no increase was detected in CM from cells expressing the catalytically inactive K40I mutant, or the empty vector. N=3 independent experiments each, data represents mean +/- SEM with individual data points shown. E) Precipitation of extracellular vesicles (EVs) from all cell lines showed no significant difference in the number of particles purified from stable cell lines expressing WT or mutant Ang. F) EVs expressed Alix and Flotillin 1 indicating exosomes were present. Uncropped blots are available in a Supplementary document. G) 5'ValCAC was present in EVs but levels were low across all cell lines analysed, compared to the amount in total CM (indicated as percent of total below the graph). The majority of the extracellular 5'ValCAC is un-encapsulated, and this appear to be dynamically regulated by angiogenin as significantly higher levels were found in WT and R31K mutant overexpressing cell lines compared to K40I or pcDNA control.

Figure 4: 5'ValCAC tiRNA is elevated in a slow compared to a fast-progressing SOD1G93A mouse model and in spinal cord and serum from the FUS (1-359) mouse model. A) Cartoon showing differences in disease progression between SLOW (C57Bl/6JOlaHsd) and FAST (Sv129) mice carrying the SOD1G93A transgene. B) mAng1 levels were analysed by qPCR in lumbar spinal cord from wild type (WT) and transgenic (TG), SLOW and FAST mice carrying the SOD1G93A transgene, confirming significantly elevated mAng1 levels at symptom onset in spinal cord from slow progressing TG ALS mice. C) 5'ValCAC tiRNA levels were significantly higher in TG mice on the slow progressing background. N = 8 mice per group, ANOVA with post-hoc Tukey’s for 5'ValCAC p = 0.0079. * indicates p < 0.05. Levels of protein translation initiation factors D) elf4e and E) elf4eBP1 were profiled in motor neurons by immunohistochemistry and quantified in relation to WT. Data are shown as mean +/- SEM of the number of individual motor neurons analysed from three/four mice per group (animal numbers indicated below). Two-way ANOVA with post-hoc Sidak was performed on the average Int_Den value for each mouse. **p <0.01; ***p <0.01; ****p <0.0001. D) elf4e was significantly decreased in TG mice from the SLOW colony and significantly increased in TG mice from the FAST colony indicating lower levels of elf4e are beneficial to ALS disease progression. Representative images are shown in supplementary Figure 15. E) Conversely elf4eBP1 was significantly elevated in TG mice from the SLOW colony and slightly lowered in TG mice from the FAST colony. Representative images are shown in supplementary Figure 15. F) In a second preclinical ALS mouse model the FUS (1-359) colony both i) ANG and ii) 5'ValCAC were significantly elevated in lumbar spinal cord from TG mice compared to WT littermates around symptom onset. ANG levels were quantified by immunohistochemistry in 6 WT and 6 TG mice per group, with representative images provided in supplementary Figure 17. G)
5’ValCAC was significantly elevated in serum collected at symptom onset from TG mice compared to WT littermates. For FUS (1-359) tissue samples n = 7 mice per group, for serum samples n = 6-8. * indicates significance of p < 0.05, two-tailed t-test. Individual data points are plotted with mean +/- S.E.M, and significance indicated.

**Figure 5: Serum 5’ValCAC levels are significantly elevated in ALS patients with slow progressing disease.** Serum from a cohort of 114 ALS patients and 91 age and sex matched healthy controls from the Netherlands was investigated for tiRNA levels. A) Demographics of human samples used and patient stratification based on survival to death or tracheostomy. B) When patients were stratified by survival serum 5’ValCAC levels were significantly elevated in patients with slow progressing disease, both when compared to healthy controls and when compared to patients with fast progressing disease (Kruskal-Wallis test p = 0.012, Dunn’s post-hoc test, p < 0.05 for Fast Vs slow and Control Vs Slow. C) Demographics of the validation cohort of ALS patients and healthy controls from the Netherlands. D) A significant difference in serum 5’ValCAC levels between fast and slow progressing patients was confirmed in a second independent cohort of patients (Kruskal-Wallis test p = 0.0005, Dunn’s post-hoc test indicates p < 0.01 for Fast Vs Slow and Control Vs Fast). Individual data points are plotted with median and interquartile range indicated.
Figure 6: Kaplan-Meier survival analysis indicates high 5’ValCAC at diagnosis holds prognostic value for a continuous spectrum of ALS patients. A) ALS patients from cohorts 1 and 2 combined including intermediate survival group from cohort 2 (n = 378) were stratified into high and low 5’ValCAC groups using the cut-off 0.735. Kaplan-Meier survival analysis indicates patients with high 5’ValCAC survive for significantly longer than this with low 5’ValCAC levels, Log Rank test $\chi^2 = 12.29$, p = 0.0005. The median survival for the Low 5’ValCAC group is 22 months and for the High 5’ValCAC is 35 months. B) Patient survival numbers for each 12-month time point are indicated.

Table 1: Table displaying the significantly altered tRNAs. tRNAs ranked by adjusted p-values, with raw p-values also listed. Mean of the normalised read counts for all samples are shown indicating the abundance of the tRNA fragments (Pseudo CAC reads were negligible), and a summary of whether 5’ or 3’ tRNA was detected, * fragments from both 5’ & 3’ ends of SerAGA were detected, see supplementary data for the cleavage profiles of all listed tRNAs.
Figure 1

148x215mm (300 x 300 DPI)
Figure 2

143x164mm (300 x 300 DPI)
A SH-SY5Y cells overexpressing Ang

B SH-SY5Y cells overexpressing Ang

C CM 5'ValCAC

D EV particles

E EV protein

F EV 5'ValCAC

Figure 3

152x149mm (300 x 300 DPI)
Figure 4

117x170mm (300 x 300 DPI)
Figure 5

158x124mm (300 x 300 DPI)
Figure 6

A

Low 5'ValCAC (n=143)
High 5'ValCAC (n=235)

Log-Rank test:
χ² = 12.29
p = 0.0005

B

| Months | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
|--------|----|----|----|----|----|----|----|----|
| High   | 229| 159| 114| 92 | 79 | 59 | 42 | 26 |
| Low    | 132| 53 | 41 | 36 | 31 | 24 | 17 | 13 |

130x105mm (300 x 300 DPI)
Motor neuron

Motor neurons express angiogenin which cleaves RNA as a protective stress response

Stress

Angiogenin is secreted by motor neurons under stress

Angiogenin is endocytosed by astrocytes

Astrocytes mediate neuroprotection

Astrocyte

Angiogenin cleaves RNA in astrocytes

135x53mm (300 x 300 DPI)
Summary (50 words)

Angiogenin is a stress-induced ribonuclease linked to amyotrophic lateral sclerosis via pathogenic loss-of-function mutations. Here we investigate substrates of Angiogenin and identify 5'Valine CAC tRNA fragment as a prognostic biomarker in mouse models and in serum samples from patient with Amyotrophic Lateral Sclerosis, where higher levels are associated with longer survival.