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Protein Functional Effector (pfe) Noncoding RNAS are Identical to Fragments from Various Noncoding RNAs

Roberto Patarca * **and William A. Haseltine**

ACCESS Health International, 384 West Lane, Ridgefield, CT 06877, USA; roberto.patarca@accessh.org; william.haseltine@accessh.org

Feinstein Institutes for Medical Research, 350 Community Dr, Manhasset, NY 11030 USA;

* Correspondence: roberto.patarca@accessh.org

Abstract: Protein functional effector (pfe)RNAs were introduced in 2015 as PIWI-interacting-like small noncoding (nc)RNAs and later categorized as a novel group. Here, we document that human 3′-end 2′-O-methylated, protein-binding pfeRNAs match fragments of GenBank-database-annotated human ncRNAs. PDLpfeRNAa, matching the 3′-half fragment of a mitochondrial transfer (t)RNA, and PDLpfeRNAb, a 28S ribosomal (r)RNA fragment, bind tumor programmed death ligand (PD-L)1, respectively enhancing or inhibiting its interaction with lymphocyte PD-1, favoring or inhibiting tumor immune escape. In an 8-pfeRNA-set, validated as a pulmonary nodule presence and nature classifier, seven pfeRNAs match one or more transfer, micro, Y, PIWI, long (lnc)RNAs, and a PDLpfeRNAa fragment, with partially overlapping chromosomal locations. In a 2-pfeRNA-set distinguishing among controls, pulmonary tuberculosis, and lung cancer patients, a p60-DMAD-binding-pfeRNA affecting apoptosis complements small nucleolar RNA SNORD45C, matching smaller 18S rRNA and lncRNA segments. These sequence-identical, diversely-sourced, not-of-degradation-or-precursor-maturation-origin, multifunctional ncRNA fragments enrich the regulatory transcriptome and targetome. Differential ncRNA fragment modification may contribute to multifunctionality. For tRNA fragments, stabilizing 3′-end-2′-O-methylation, 3′-aminoacylation, and glycosylation may respectively regulate protein function, translation, and extracellular effects. One ncRNA gene can encode multiple fragments, multiple ncRNA genes can encode the same fragment, and ncRNA fragments can synergize or antagonize each other, further fostering medical applications.

Keywords: protein functional effector RNA; noncoding RNA; RNA-protein interactions; transfer RNA fragment; ribosomal RNA fragment; microRNA fragments; Y RNA fragments; PD-1/PD-L1 interaction; cancer; RNA modifications

Introduction

The eukaryotic transcriptome consists mainly of noncoding (nc)RNAs, predominantly underlying biological diversity and regulation [1,2]. Due to their abundance, structural flexibility, and varied conformations, RNAs can regulate nearly every aspect of cellular physiology, influencing chromatin organization, gene expression regulation, biochemical reaction catalysis, cellular signaling pathways, embryogenesis, and immune responses, among other processes [1,3,4].

ncRNAs can be broken down into fragments that are not simply byproducts of nonspecific degradation or remnants of RNA precursor maturation but result from specific enzymatic pathways and have distinctive regulatory functions [5]. Even transfer (t)RNAs and ribosomal (r)RNAs, considered housekeeping RNAs, and their fragments have regulatory functions [5-11].

Among ncRNAs, protein functional effector (pfe)RNAs were characterized as novel ones that play a critical role in tumorigenesis and differentiation of non-small cell lung cancer (NSCLC) [12-

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15]. These 26-60 nucleotide-long pfeRNAs display differentiating features, including 2′-O-methylation at the 3′ end, and direct interactions with target proteins rather than transcripts, regulating protein function without changing their levels [16].

Two pfeRNAs, termed PDLpfeRNAs, affect the interaction between the programmed death (PD)-1 surface protein on T lymphocytes and the PD-1 ligand (PD-1L) on tumor cells. By binding to PD-L1, PDLpfeRNAa enhances the PD-1/PD-L1 interaction, while PDLpfRNAb inhibits it, favoring or inhibiting, respectively, tumor immune escape [16,17]. Whether PDLpfeRNAs' structural features or differential binding affinities to the PD-L1/PD-1 complex mediate their effects on PD-1/PD-L1 interactions remains undetermined.

Normal tissues show a balanced expression of PDLpfeRNA a and b, while PDLpfeRNAa expression is significantly higher during tumorigenesis [17]. In patients with unresectable malignant pleural mesothelioma, the plasma relative expression levels of these pfeRNAs predicted response to first-line treatment [17]. Said treatment included the PD-L1 inhibitor dorvalumab, cisplatin, which synergizes with PD-1/PD-L1 inhibition, and pemetrexed, which induces transcriptional activation of PD-L1 and secretion of cytokines that further increase PD-L1 levels. PDLpfeRNAa/PDLRNAb plasma relative expression levels had significant prognostic value for overall and progression-free survival, regardless of histological subtype and age [17].

Another eight pfeRNAs, termed pfeRNA a-h, were validated as a set in a multicenter study in China and the United States as cost-effective, non-invasive, liquid-biopsy biomarkers for pulmonary nodule presence and nature [18]. A classifier based on these pfeRNAs differentiated between the presence and absence of pulmonary nodules (mean 96.2% specificity, 97.35% specificity), and malignant vs. benign pulmonary nodules (mean 77.1% sensitivity, 74.25% specificity) [18].

Lastly, two additional pfeRNAs, termed PIWI-interacting-like small RNAs (piR-L)-163 [12] and piR-L-138 [15] were characterized. piR-L-163 directly binds phosphorylated ERM (p-ERM) proteins, playing a critical role in ERM activation and signal transduction regulation. Cell cortex ERM (ezrin, radixin, and moesin) proteins bridge transmembrane and cytoskeleton proteins. Phosphorylation of ERM proteins exposes binding sites for transmembrane proteins, such as EBP50, and the cytoskeleton, including filamentous actin. piR-163 is expressed in immortalized human bronchial epithelial cells, with distinctive expression downregulation in lung cancer cells. The second pfeRNA, piR-L-138, is upregulated in association with apoptosis inhibition in chemoresistance to cisplatin-based chemotherapy in lung squamous cell carcinoma (LSCC) cells and patient-derived xenograft LSCC models [15]. piR-L-138, directly interacts with the phosphorylated mouse double minute 2 homolog (p60-MDM2) in response to cisplatin-based therapy of LSCC, inducing chemoresistance by inhibiting apoptosis [15].

Here, comparisons of pfeRNA primary sequences against sequences in the GenBank database revealed that the pfeRNAs mentioned above match fragments derived from various annotated ncRNAs, including mitochondrial and nuclear transfer, ribosomal, micro, Y, PIWI-interacting, and long ncRNAs. Two pfeRNAs are segments of others; sometimes, pfeRNAs match more than one ncRNA type; and one pfeRNA had no matches in the GenBank database. piR-L-163 matched an intronic region in the human laminin subunit gamma 2 oncogene. piR-L138 matched complementary sequences in small nucleolar and long ncRNAs, and shorter sense regions in 18S rRNA and long ncRNA fragments. These findings further exemplify that one ncRNA-type gene can express more than one fragment, and more than one ncRNA-type gene can give rise to the same fragment. Furthermore, differential modifications to each RNA fragment, determining their stability level and specific function, may increase the versatility of this regulatory network.

2. . Results

2.1. Analysis of pfeRNAs related to PD-1/PD-L1 interactions, termed PDLpfeRNAs

PDLpfeRNAa, induced during non-small cell lung cancer tumorigenesis, binds to PD-L1 and enhances PD-1/PD-L1 interaction, favoring tumor immune escape [16,17]. A search of the GenBank

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database using the 39-nt-long human PDLpfeRNAa as query sequence revealed that it is identical to the 3'-half fragment of the human mitochondrial tRNA for glutamic acid (Glu) (Fig. 1A) [19]. This 3'-half fragment could be generated via the action of angiogenin at the level of the tRNA's anticodon loop [6,20] (Fig.1B). The visualized secondary structure (Fig. 1B) has an estimated free minimal energy (ΔG) of -6.8 Kcal/mol.

A similar analysis revealed that the human 41-nucleotide-long PDLpfeRNAb, which is also induced during tumorigenesis, is identical to a fragment towards the 3' end in human 28S ribosomal (rRNA), part of its domain 5 (Fig. 2A,B). The visualized secondary structure of this rRNA fragment has an estimated ΔG of -7.4 Kcal/mol (Fig. 2B). Like PDLpfeRNAa, PDLpfeRNAb binds to PD-L1 [6,17]; however, in contrast to PDLpfeRNAa, it inhibits the functional interaction between PD-1 and PD-L1, consequently inhibiting tumor immune escape, as do the PD-1 inhibitors in cancer immunotherapy (Fig. 2B).

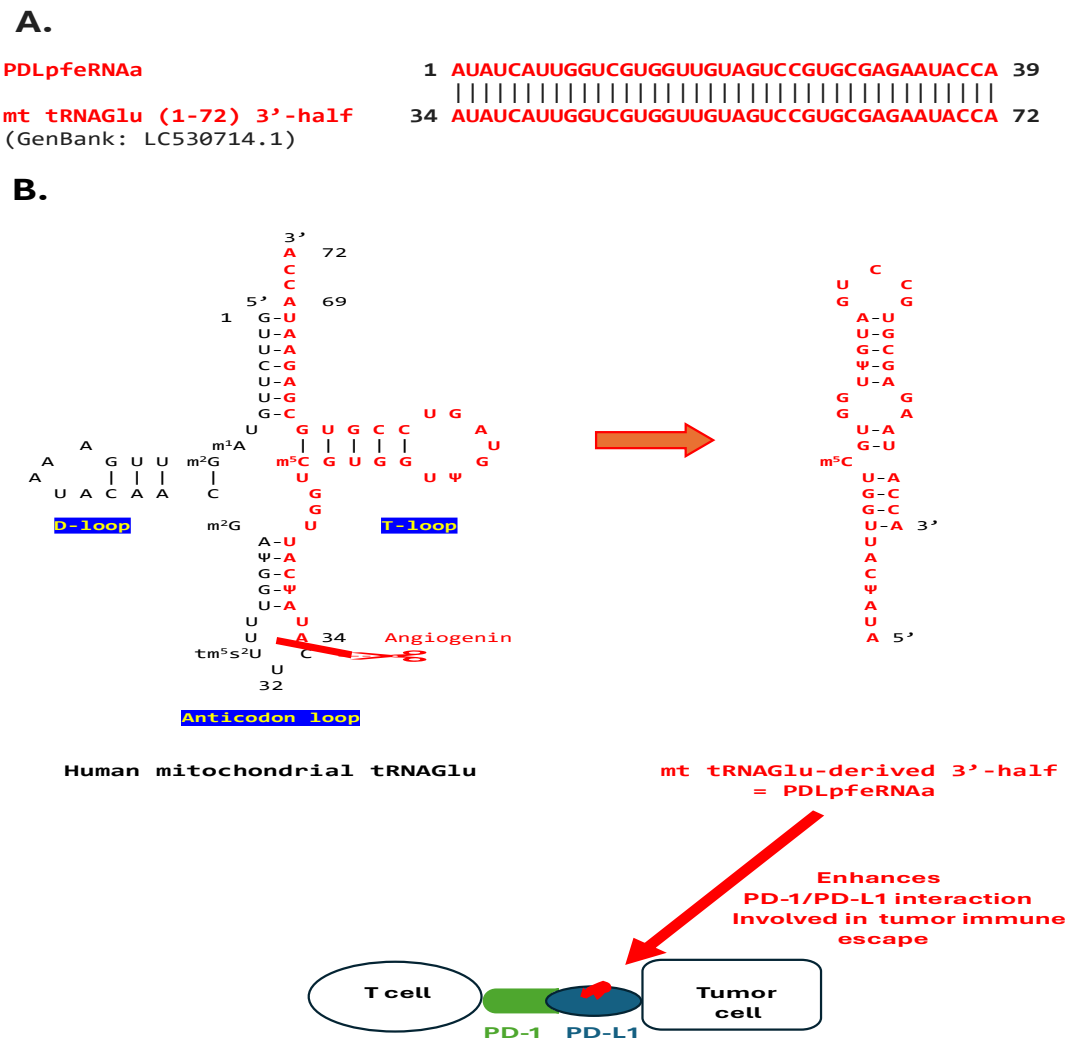

Figure 1. Sequence analysis of PDLpfeRNAa. A. PDLpfeRNAa matches the 3' half of human mt tRNAGlu. **B.** Angiogenin can cut the mt tRNAGlu at the site depicted by a pair of scissors, and the 3'-half whose secondary structure is visualized might, as PDLpfeRNAa, bind to PD-L1.

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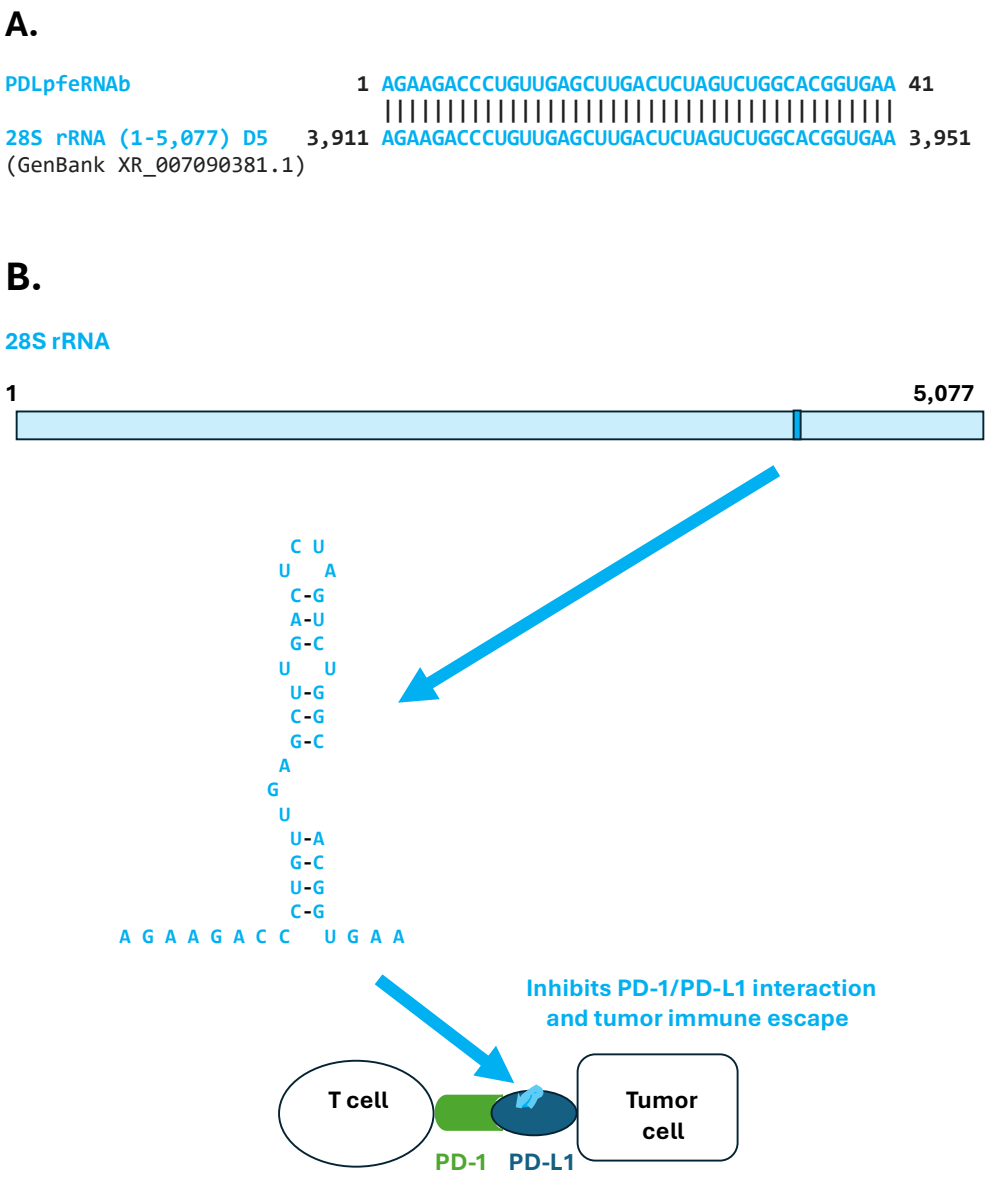

Figure 2. Sequence analysis of PDLpfeRNAb. A. PDLpfeRNAb matches a fragment located towards the 3' end of human 28S rRNA. **B.** The 28S rRNA fragment whose secondary structure is visualized might, as PDLpfeRNAb, bind to PD-L1.

2.2. *Analysis of plasma pfeRNAs related to pulmonary nodules: pfeRNAa-h*

A set of 8 pfeRNAs was characterized and validated as a low-cost, noninvasive liquid-biopsy classifier for pulmonary nodule presence vs. absence, and nature, i.e., benign vs. malignant [18]. A search of the GenBank database revealed identical sequences in fragments of Y RNA, microRNA-21, Piwi-interacting and long ncRNAs, and mitochondrial and nuclear tRNAs, including one, pfeRNAb, that is a fragment of PDLfeRNAa and therefore also matching the mitochondrial tRNA Glu 3'-half (Fig. 3). One of the 8 pfeRNAs, pfeRNAh, did not have any highly similar matches in the GenBank database (Fig. 3).

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Figure 3. Sequence analysis of the eight plasma pfeRNAs (a-h) related to pulmonary nodule presence and nature. pfeRNAb overlaps with PDLpfeRNAa (in red as in Fig. 1) except for five nucleotides. pfeRNAs d and g also overlap, differing by three nucleotides.

GenBank accession numbers for the matches are: Ro60 Y4 RNA (NR_004393.1) [21], microRNA21 (NR_029493.1) [22], Hy40 Ro RNA (L32608.1) [23], tRNA-Lys (HG983908.1, HG983910.1, HG983915.1, HG983916.1) [24-26]; piRs: piR-31104 (DQ57099.2), piR-3452 (DQ597346.1), piR-35410 (DQ597344.1), piR-35411(DQ597345.1), piR-35412 (DQ597346.1), piR-35413 (DQ597347.1), piR-35413 (DQ597347.1), PiR-143604 (DQ575492.1) [27]; lncRNA 1128 (MN298243.1); and mt tRNA Val (LC530724.1) [19].

Chromosomal locations of pfeRNAa-h were predicted in the publication that characterized pfeRNAs a-h using the QIAGEN CLC Genomics Workbench 10.11 software package [18]. Chromosomal locations of the matched sequences in the annotated GenBank database, as determined here, overlap with those of 5 of the 7 pfeRNAs (Fig. 4). For the two pfeRNAs without chromosomal location overlaps, one, pfeRNAb, matches PDLpfeRNa. We infer it has a mitochondrial genome localization based on its identical sequence with mitochondrial tRNA Glu. The other, pfeRNAc, is identical to a fragment spanning 48 nucleotides in microRNA-21 whose gene has been mapped to human chromosome 17q3.2 in the 11th intron of the gene encoding the TMEM49 transmembrane protein, which is a precursor of VMP1 (vacuole membrane protein (VMP)1 (Fig. 4) [28,29]. This contrasts with the location on chromosome 5 in the reference characterizing pfeRNAc and using a software package for location assignment [18].

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	Chromosome
pfeRNAa	1,7,8,9q21.2-22.1,12,17
Ro60 Y4 RNA (1-96)	7q36.1,8,9q21.2-22.1,11,13,14,17,Xq13.1-21.1
pfeRNAb	13,X
PDLpfeRNAa, mt tRNA Glu 3'-half	mitochondrial
pfeRNAc	5
MicroRNA21 (1-72)	17q3.2
pfeRNAd	2 (reverse),6,7
pfeRNAg	6,7
Hy4 Ro RNA (1-72)	1,2,3, 6,7q36.1,9,10,11,13,15,17
pfeRNAe	1,5,6,14,16
tRNA-Lys (1-73) 5'-half	1,5,6,14,16,19
PiR-31104,3452,35410,35413	
lncRNA 1128	
pfeRNAf	7, mitochondrial
mt tRNA Val (1-72)	mitochondrial
pfeRNAh	1, mitochondrial
No match	-

Figure 4. Chromosomal location of pulmonary nodule-related pfeRNAs and matching GenBank sequences shown in Fig. 3. Overlapping locations are highlighted in green. .

2.3. *Analysis of plasma pfeRNAs related to p-ERM proteins and p60-MDM2*

The pfeRNA, piR-L-163, that binds to p-ERM proteins (ERM-pfeRNA) affecting signal transduction matched a sequence in the intron after exon 10 of the human laminin subunit gamma 2 oncogene (Fig. 5A). The pfeRNA, piR-L-138, that binds to p60-MDM2 (p60-MDM2-pfeRNA) influencing apoptosis matched the complementary sequences of a fragment of the small nucleolar RNA SNORD45C and a smaller segment of a long noncoding RNA (in green in Fig. 5B). p60-MDM2-pfeRNA also matches a similarly smaller segment of 18S rRNA and a lncRNA (Fig. 5B).

GenBank accession numbers for matches to p60-MDM2-pfeRNA are (Fig. 5): small nucleolar RNA C/D box 45 C (1-79: LN848108.1, 1-78: NR_003042.1, 1-83: LN848102.1, 1-84: NR_002749.1); LNC_000006 lncRNA (MN308643.1); 18S rRNA, chain 2 (8UKB_52, 8ZDC-2, 6ZOJ_2, 6ZON_2,5A2Q_2, 6G4S_2), 45S pre-ribosomal N2 rRNA (NR_146144.1, NR_146117.1), 18S rRNA chain L1 (1-1,872: 7MQ9_L1), 18S rRNA chain B1 (1-1,1869: 5AJO_B1), 18S pre-ribosomal 4 (NR_146119.1); and LNC_00012 (MN308649.1).

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A.

```
ERM-pfeRNA (piR-L-163)                1  AUAUCAUGAUGUUACUUUGAUUCUCUGACC  30
                                         ||||||||||||||||||||||||||||||
Intron in human laminin          47,446  ATATCATGATGTTACTTTGATTCTCTGACC  47,475
subunit gamma 2 gene after
exon 10 on chromosome 1
(NG_007079.3)
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B.

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p60-MDM2-pfeRNA (PiR-L-138)           1  AGGUCUCAGAGUAAUUCUAGAGCUAAAGU  29
                                         ||||||||||||||||||||||||||||||
Small nucleolar RNA SNORD45C (1-79)  78  AGGUCUCAGAGUAAUUCUAGAGCUAAAGU  50
                                                 ||||||||||||||||
LNC_000006 lncRNA (1-632)                    19  GUAAUUCUAGAGCUAA  4
                                                 ||||||||||||||||
18S rRNA, chain 2 (1-1,868)                 156  GUAAUUCUAGAGCUAA  171
                                                 ||||||||||||||||
LNC_00012 lncRNA (1-629)                    424  GUAAUUCUAGAGCUAA  439
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Figure 5. Sequence analysis of pfeRNAs that bind to **A.** ERM proteins and **B.** p60-MDM2. Complementary sequence matches are shown in green and reverse numbering corresponding to sense-strand positions.

Discussion

The present study identified matches between characterized pfeRNAs and segments of annotated ncRNA sequences in the GenBank database, further exemplifying the known ability of one ncRNA to encode multiple fragments and highlighting that the same fragment can originate from multiple ncRNAs. The relevance of these matches is further underscored by the overlap between the published chromosomal locations for 5 of 7 pulmonary nodule-related pfeRNAs determined using a genomic software program and those annotated for the GenBank database matches. Moreover, matching sequences may have functional overlaps based on studies on similar fragments. Only one of the 12 characterized pfeRNAs had no matches in the GenBank database. A pfeRNA that interacts with the epidermal growth factor receptor (EGFR), affecting cell growth and colony formation of lung cancer H226 cells, was mentioned in a publication [16]. However, its sequence was not provided and therefore not included in this analysis.

3.1 PD-L1-binding pfeRNAs (PDLpfeRNAs a and b)

PDLpfeRNAa, which binds to the PD-L1 protein and enhances PD-1/PD-L1 interaction, favoring tumor immune escape [16,17], is identical in sequence to the 3′-half fragment of human mitochondrial tRNA Glu. tRNA fragments of different types and lengths regulate cell viability, differentiation, and homeostasis in health and disease, including cancer, participating via diverse mechanisms, in various processes, including ribosome biogenesis and gene silencing, by binding to complementary nucleic acids and various proteins [30-33]. tRNA halves bind to specific proteins. For instance, a 5′-tRNA half binds to the YBX1 (YB-1) protein, promoting stress granule formation and inhibiting global protein translation during stress conditions [6]. Ribosomes with a vacant A site, whose abundance increases during cellular stress, activate angiogenin [34], which cleaves at the anticodon loop, generating tRNA halves. The match described here makes it possible that the 3′-half fragment of human mitochondrial tRNA Glu is the origin of PDLpfeRNA or, like it, binds to PD-L1.

PDLpfeRNAb, which also binds to PD-L1 but inhibits PD-1/PD-L1 interaction and tumor immune escape [16,17], is identical to a sequence towards the 3′-end of human 28S rRNA. rRNA

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fragments can associate with specific proteins, including those binding small RNAs, such as the P19 protein [35]. Consistent with the match here between PDLpfeRNAb and a 28S rRNA fragment, the generation of 28S rRNA fragments in tumor cells has been associated with cell death and cytotoxicity, indicating that these fragments may be markers or mediators of cancer cell stress responses [36].

28S rRNA fragments dominate the rRNA fragment pool [37]. The primary precursor, 45S pre-rRNA, undergoes sequential cleavage to generate intermediate transcripts (32S or 36S pre-rRNAs) before maturing into 28S rRNA. During this process, parallel pathways involving endonucleases and exonucleases generate rRNA fragments [38]. From ticks to humans, rRNA fragments, whose length progresses with one nucleotide difference, align to the 5'- and 3'-ends of the 5.8S and 28S rRNA genes and are predominantly expressed over those in the body of the rRNA genes [5].

3.2. Pulmonary nodule-related pfeRNAs

A set of eight pfeRNAs, pfeRNA a-h, classifies presence vs. absence and benign vs. malignant pulmonary nodules.

pfeRNAa matches a fragment spanning almost the entire 3′-half of Ro60 Y4 RNA. The ring-shaped Ro60 protein forms a ribonucleoprotein complex by binding RNAs such as Y RNAs, which regulate the subcellular localization of Ro60, tether it to effector proteins, and regulate other RNAs' access to its central cavity [21,23].

Because mammalian cells and bacteria lacking Ro60 are sensitized to ultraviolet irradiation, Ro60 function in RNA quality control may be particularly relevant during exposure to some environmental stressors, where Y RNA and its expression increase. Y RNA fragments are dysregulated in tumors and associated with cancer progression [39,40]. Ro60 RNP is a target of autoantibodies in patients with some rheumatic diseases, potentially contributing to their initiation and progression (reviewed in [21]).

pfeRNAb matches, except for five nucleotides, PDLpfeRNAa, which, in turn, matches the 3′-half fragment of mitochondrial tRNA Glu. The predicted chromosomal location for pfeRNAb was on chromosomes 13 and X in the publication that characterized pfeRNAb [18]. Although a study identified nuclear genomic sequences resembling mitochondrial tRNAs, with Glu tRNAs being among the most represented [41], the discrepancy in predicted chromosomal location between PDLpfeRNAa, based on its similarity to mitochondrial tRNA Glu, and that for pfeRNAb, based on the QIAGEN CLC Genomics Workbench 10.1.1 [18], remains to be elucidated.

pfeRNAc matches a 48-nucleotide segment of the 72-nucleotide-long human microRNA (miR)-21. miRs silence gene expression by binding partially complementary sequences within target messenger RNAs. miRNAs recognize canonical target sites by base-pairing in their 5′ region. However, there are non-canonical target sites [42].

First discovered in 2008 in human stem cells [43], isomicroRNAs (isomiRs) are miR sequence variants that differ from the canonical miR sequence by changes, such as trimming at the 5' end (5′-isomiRs), often due to alternative cleavage during mRNA processing; trimming at the 3' end; nucleotide additions at the 3′ end, often through post-transcriptional modifications; internal substitutions; or combinations of the above including trimming at both 5′ and 3′ ends (mixed-type) [44,45].

5′- and 3′-isomiRs are widespread and represent approximately half of miR copies in cells and tissues [46]. IsomiRs are generated through regulated processes during miRNA biogenesis and are often conserved across species [47,48]. IsomiR expression changes in diseases such as cancer, making them potential biomarkers [46,47,49], as was shown for PNpfeRNAc [18]. pfeRNAc lacks the first 7 nucleotides at the 5′ end (most of the seed sequence from nucleotides 2 to 8 that binds to the target messenger RNA) and 17 nucleotides at the 3′ end, rendering it a mixed-type isomiR. pfeRNAc would be a templated isomiR because its sequence matches the parental miR21 gene [50].

miR-21 is classified as an "oncomiR," a bona fide oncogene consistently upregulated in nearly all types of cancers. It promotes cell proliferation, migration, invasion, and survival by targeting tumor

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suppressor genes such as PTEN and PDCD4 [51-53]. Other associations of mR-21 include cardiac and pulmonary fibrosis [51], immunity, inflammation [52], and osteogenesis [54].

miR-21 acts as an oncogene by targeting tumor suppressor genes and promoting cell proliferation, migration, and invasion in lung cancer cells. In non-small cell lung cancer (NSCLC), miR-21 is consistently overexpressed and detectable in both serum and sputum. Elevated miR-21 levels are associated with poor prognosis, advanced clinical stage, lymph node metastasis, and lower survival rates [55].

Cells have multiple isomiRs of miR-21 [56]. Overall, for 5' isomiRs, changes in the miR 5′ seed region (nucleotides 2–8), as present in PNpfeRNAc, can alter the set of mRNA targets (targetome shifting), potentially leading to different regulatory outcomes. IsomiRs may also act with canonical miRNAs to regulate the same pathways or have distinct, even opposing, effects (cooperative or divergent function). For instance, a 3′ isomiR of miR-21 suppresses hepatoma cell growth [57]. Moreover, consistent with the association of pfeRNAc with pulmonary nodules and their presence vs. absence and benign vs. malignant nature, miR-21 fragments drive tumor progression by silencing tumor suppressors like PTEN, promoting uncontrolled cell proliferation in lung and hepatocellular carcinomas [58].

pfeRNAd overlaps except for 3 nucleotides with **pfeRNAg**, which matches almost the entire 5′-half of Y4 RNA, whose 3′-half matches pfeRNAa. Therefore, the items discussed for Y4 RNA apply to pfeRNAs d and g.

pfeRNAe matches, except for its first five nucleotides, a 5′ fragment of tRNA Lys, and minus an additional nucleotide, several piRs and a lncRNA. piRs participate in transposon silencing, heterochromatin modification, germ cell maintenance, and tumorigenesis [15,59-61]. These matches illustrate how fragments can differ by sometimes one nucleotide, as has been described for rRNA fragment series [5].

pfeRNAf matches mitochondrial tRNA Val except for the latter's first 19 nucleotides. Mitochondrial-derived tRNA fragments are biomarkers for chronic lymphocytic leukemia and Mycobacterium tuberculosis infection [58].

pfeRNAh had no highly similar matches with GenBank sequences.

3.3. ERM-pfeRNA and p-60-MDM2-pfeRNA

A set of two pfeRNAs, one binding p-ERM proteins (ERM-pfeRNA) influencing signal transduction [12], and the other p60-MDM2 (p-60-MDM2 pfeRNA) affecting cancer cell apoptosis and chemoresistance to cisplatin-based therapy [15], distinguishes among controls and pulmonary tuberculosis and lung cancer patients. Likewise, Gu et al. [62] later developed a TRY-RNA signature composed of tRNA fragments, rRNA-derived small RNAs, and YRNA-derived small RNAs from human peripheral blood mononuclear cells, which exhibited diagnostic potential for precise discrimination between healthy controls, lung cancer and pulmonary tuberculosis [62]. In the latter study, tRNALys-derived small RNAs (in this report one matched with pulmonary nodule-related pfeRNAe), along with fragments from tRNA-Ala, tRNA-Asn, tRNA-Leu, and tRNA-Tyr, were the only five tRNA-derived small RNA groups that were upregulated in the lung cancer patients relative to controls and pulmonary tuberculosis patients [62].

As Gu et al. [62] concluded, changes in the composition of tRNA-derived, rRNA-derived, and Y RNA-derived small RNA may result in altered ribosome heterogeneity that directs the cell to a specific functional state [63]. Permutations of various small noncoding RNAs may provide specificity to distinguish complex diseases and represent a "disease RNA code" in lung cancer screening [62].

ERM-pfeRNA (piR-L-163) matches an intron segment after exon 10 in the laminin subunit gamma 2 (LAMC2) oncogene. Laminins, a family of extracellular matrix glycoproteins, are the principal noncollagenous constituent of basement membranes. In certain cancers, notably intrahepatic cholangiocarcinoma, LAMC2 facilitates tumor growth and metastasis through molecular pathways including epidermal growth factor receptor (EGFR) signaling. LAMC2 constitutes a diagnostic and prognostic biomarker in neoplasias [64-71]. There is no published evidence that

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introns in said gene encode ncRNAs. However, through further processing after splicing, introns in other genes encode small regulatory RNAs, such as miRs, small nucleolar RNAs, long intronic RNAs, and circular RNAs [72-74].

p-60-MDM2-pfe RNA (piR-L-138) is complementary to a segment of the small nucleolar RNA SNORD45C and a smaller segment of an lncRNA, which may antagonize it. P-60-MDM2-pfeRNA matches a similar segment of 18S rRNA and another lncRNA.

Some fragments originating from small nucleolar RNAs function like miRNAs [75-77]. Dysregulation of small nucleolar RNAs plays a vital role in lung tumorigenesis, and sputum small nucleolar RNA biomarkers might improve lung cancer diagnosis [78]. The rates of nucleolar ribosome production and ribosomal protein biosynthesis are tightly correlated with cell growth and proliferation rates. Deregulation of factors, including oncogenes, controlling these processes, especially ribosome biosynthesis, can lead to cell transformation [79].

A study characterized 21 antisense small nucleolar RNAs from human cells required for site-specific 2'-O-methylation of preribosomal RNA through direct base pairing interactions [80]. The antisense element and the small nucleolar RNA's D or D' box provide the information necessary to select the target nucleotide for the methyltransfer reaction [80-82]. Most small nucleolar RNAs modify rRNA [83]. This is consistent with the matching here of p-60-MDM2 pfeRNA with an antisense small nucleolar RNA fragment and a sense 18S rRNA fragment, and the effects of this pfeRNA on cancer cell apoptosis and chemoresistance to cisplatin-based therapy [15]. Small nucleolar RNAs regulate alternative splicing through sequence-specific RNA interactions and protein complex formation [83].

3.4. pfeRNAs, glycoRNAs, and nicked tRNA halves among RNAs affecting cell surface and extracellular protein functions and PD-1/PD-L1 interactions

pfeRNAs are not the only small ncRNAs that have an extracellular effect. For instance, as with proteins and lipids, glycosylation modifies RNAs, giving rise to glycoRNAs primarily present at the cell surface. GlycoRNAs consist of small nuclear RNAs modified with secretory N-glycans rich in sialic acid and fucose via their attachment to the modified base 3-(3-amino-3-carboxypropyl)uridine (acp3U) [84,85]. GlycoRNAs in mammals and other eukaryotes interact with antibodies and cellular receptors, influencing neutrophil recruitment, immunity, and pathogenesis [84-89]. GlycoRNAs, including glycosylated transfer, ribosomal, small nuclear, small nucleolar, and Y noncoding RNAs, could explain why RNAs, traditionally considered intracellular molecules, act as autoantigens [86]. This is beyond the attachment of RNAs to autoantigenic proteins, such as Ro, as described above.

Here, we showed that pfeRNAs thus far characterized and with an available sequence in the literature are identical or highly similar to fragments of transfer, ribosomal, Y, and small nucleolar noncoding RNAs, which might become extracellular after being glycosylated. For instance, Y RNAs are classically mainly cytoplasmic with a minor fraction in the nucleus [90], and tRNAs localize to the soluble cytosol and nucleus. The biogenesis of sialylated glycans occurs across many subcellular compartments, including the cytosol (processing of ManNAc to Neu5Ac), the nucleus (charging of Neu5Ac with CMP), and the secretory pathway (where sialyltransferases add sialic acid to the termini of glycans) [84].

As mentioned above, antibodies targeting RNA have been associated with systemic lupus erythematosus [91]. Besides the described RNA role in PD-1/PD-L1 interactions, another example of RNA-mediated improvement of immune checkpoint blockade treatment of glioblastomas in mice is the lupus-derived 4H2 anti-guanosine autoantibody, which enters cells through a membrane transit nucleoside salvage-linked pathway after systemic administration. It then binds endogenous RNA, stimulating the cytoplasmic pattern recognition receptor cyclic GMP-AMP synthase (cGAS), immune signaling, and cytotoxicity [92].

Fragments of specific long-coding and noncoding RNAs are present on the surface of cells, indicating an expanded role for RNA in cell-cell and cell-environment interactions [93]. tRNA and rRNA fragments, Y and microRNAs, are present in extracellular vesicles [94], and there is non-vesicle-associated extracellular RNA [95]. Among the latter, full-length tRNAs containing broken

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phosphodiester bonds (i.e., nicked tRNAs) are stable reservoirs of tRNA halves in cells and biofluids [96]. A recently developed protocol distinguishes between structurally distinct but sequence-identical tRNA-derived fragments and nicked tRNAs, disentangling their biological functions [97].

3.5. 3′-end 2′-O-methylation is also present in ncRNAs other than the characterized pfeRNAs

Methylation of the 2′-hydroxyl group of the ribose sugar of a nucleotide, or 2′-O-methylation, is among the most common RNA modifications across kingdoms of life, increasing RNA stability. It is a characteristic feature of the 3′-end of Piwi-interacting RNAs in animals and miRNAs in plants carried out by the S-adenosylmethionine-dependent methyltransferase (MTase) Hen1 [98-101]. In the ciliated protozoan Tetrahymena, 3'-end 2'-O-methylation on a selected class of small RNAs regulates the function of a specific RNA interference pathway [102].

2′-O-methylation is also a highly abundant posttranscriptional modification at internal sites in ncRNAs, such as ribosomal, transfer, small nuclear RNAs [103-107]. Each methylation site in tRNA, rRNA, or other RNAs is typically modified by a distinct methyltransferase, highlighting the specificity of these enzymes for their RNA substrates and target sites [108]. 2′-O-methylation is an essential feature of the 5′ cap of eukaryotic mRNAs [109].

In plants, specific 5′-tRNA fragments (e.g., tRF-5a Ala and tRF-5b Gly) bear 3′-end-2′-O-methylation, which protects them from degradation and may guide Argonaute proteins for gene regulation. This modification is independent of HEN1, the enzyme responsible for miRNA methylation, and is conserved across species [110]. It remains to be determined if the 3′-ends of fragments from human transfer (mitochondrial and nuclear), ribosomal, Y, and micro RNAs undergo 2′-O methylation as characterized for the pfeRNAs as a distinguishing feature, along with binding to proteins to influence their function directly.

Other chemical groups might be present in some of the ncRNAs instead of the 2′-O-methyl group and might be removed and substituted for it. For instance, tRNA 3′-halves are aminoacylated, as are the full tRNAs from which they originated [111]. However, 2′-O-methylation is not universally required for RNA-protein binding, and tRNA and rRNA fragments can retain 2′-O-methylated nucleotides depending on the cleavage site [108,112].

4. . Conclusion

Beyond the function of messenger RNA as a template for protein synthesis, characterizing pfeRNAs enriches the central molecular biology dogma by highlighting how noncoding RNAs directly bind to and regulate the function of intracellular and surface proteins. The findings of the current analysis underscore the concept of a gene capable of producing not only one product, in this case an RNA, but multiple ones, including the most abundant transfer and ribosomal RNAs and their fragments. This is exemplified by the various noncoding RNA fragments generated, often in response to cellular stress, with diverse functions, enriching the targetome, and biogenesis tracking to gene expression, not degradation pathways. Conversely, the same RNA fragment can have a multigene origin. The chromosomal location of these genes is diverse, including mitochondrial and nuclear genomes, introns, gene clusters, genes encoding messenger, noncoding, or multifunctional messenger and noncoding RNAs.

Other known levels of versatility in RNA genes and the functions of their transcripts include bi- or dual-function RNAs [113,114], including mRNAs that encode noncoding RNAs and, vice versa, noncoding RNAs that encode proteins [115]. The bifunctional transfer-messenger RNA (tmRNA) has properties of a tRNA and an mRNA for trans-translation by releasing ribosomes stalled during translation and targeting the nascent polypeptides for degradation. This concerted reaction contributes to translational quality control and regulation of bacterial gene expression. Underscoring its importance for bacterial fitness, tmRNA is conserved and one of the most abundant RNAs among bacteria [116].

Additional examples of bifunctional RNAs are the steroid receptor activator/SRA [117], VegT RNA [118,119], Oskar RNA [120], ENOD40 [121], p53 RNA [122], SR1 RNA [123], and Spot 42 RNA

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[124,125]. As further exemplified here by the matches between pfeRNAS and other ncRNAs, many ncRNAs encompass different ncRNA categories, for instance, H/ACA box small nucleolar (sno)RNA and microRNA [126,127].

Insights into gene expression versatility, posttranscriptional modifications, and the complex regulatory network of noncoding RNAs, accounting for most of the transcriptome, will continue to enrich the diagnostic, prognostic, and therapeutic armamentarium against human diseases, with applications in all kingdoms of life. It remains to be determined if pfeRNAs originate from their genes, differentially modified fragments of various noncoding RNAs, or both.

5. . Methods

5.1. Detection of genomic sequences identical or highly similar to pfeRNAs, and their chromosomal location for pulmonary nodule-related pfeRNAs

We searched for genomic sequences identical or highly similar to published human pfeRNA sequences using the GenBank database (National Library of Medicine) and the BLASTN program (nucleotide collection [nr/nt]; expect threshold: 0.5; mismatch scores: 2, -3; gap costs: linear; up to 5,000 sequences) [128], which evaluates sense and antisense strands. For the matches shown, the Expect values, describing the number of hits one can expect by chance when searching a database of a particular size, are less than e^{-4}. A first search was done without organism restriction and a subsequent one specifying "Homo sapiens" as organism.

Chromosomal locations and other details of the detected genomic sequences were obtained from the GenBank database annotations. Chromosomal locations for the pulmonary nodule-related pfeRNAs had been predicted using the QIAGEN CLC Genomics Workbench 10.11 software package [18].

5.2. Visualization of RNA secondary structures and estimation of their minimum free energies

RNA secondary structures were visualized using forna, a force directed graph layout (ViennaRNA Web services) [129]. Optimal secondary structures were also visualized using the RNAfold webserver, which was used to estimate the minimum free energy reflecting the robustness of the pairings [130,131].

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