

Aberrantly Expressed tRNA-Val Fragments Can Distinguish Canine Hepatocellular Carcinoma from Canine Hepatocellular Adenoma

Saki Hashimoto , [MD Nazmul Hasan](#) ^{*} , [Mohammad Arif](#) , Nobuhiro Nozaki , Al Asmaul Husna , [Yu Furusawa](#) , Takeshi Sogawa , Kaori Takahashi , Tomohide Kuramoto , Aki Noguchi , Masashi Takahashi , [Osamu Yamato](#) , [Md Mahfuzur Rahman](#) , [Naoki Miura](#) ^{*}

Posted Date: 11 July 2024

doi: 10.20944/preprints2024070990.v1

Keywords: tRNA-Val; canine hepatocellular carcinoma; canine hepatocellular adenoma



Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Article

Aberrantly Expressed tRNA-Val Fragments Can Distinguish Canine Hepatocellular Carcinoma from Canine Hepatocellular Adenoma

Saki Hashimoto ¹, MD Nazmul Hasan ^{1,*}, Mohammad Arif ¹, Nobuhiro Nozaki ¹, Al Asmaul Husna ², Yu Furusawa ², Takeshi Sogawa ², Kaori Takahashi ², Tomohide Kuramoto ², Aki Noguchi ², Masashi Takahashi ^{1,2}, Osamu Yamato ^{1,2}, Md Mahfuzur Rahman ^{2,†} and Naoki Miura ^{1,2,*}

¹ Joint Graduate School of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

² Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

[†] Current address: Department of Human Oncology, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA.

* Correspondence: nazmulriaj.bau.vet@gmail.com (MD Nazmul Hasan); k9236024@kadai.jp (Naoki Miura)

Abstract: Hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC) can be difficult to differentiate but must be diagnosed correctly as treatment and prognosis for these tumors differ markedly. Relevant diagnostic biomarkers are thus needed, and those identified in dogs may have utility in human medicine because of the similarities between human and canine HCA and HCC. A tRNA-derived fragment (tRF), tRNA-Val, is a promising potential biomarker for canine mammary gland tumors but has not previously been investigated in hepatic tumors. Accordingly, we aimed to elucidate the potential utility of tRNA-Val as a biomarker for canine HCA and HCC using clinical samples (tumor tissue and plasma extracellular vesicles [EVs]) and tumor cell lines with qRT-PCR assays. We also investigated relevant functions and signaling pathways with bioinformatic analyses (Gene Ontology and Kyoto Encyclopedia of Genes and Genomes). tRNA-Val was markedly downregulated in HCC tumor tissue versus HCA tumor tissue and normal liver tissue, and a similar trend was shown in plasma EVs and HCC cell lines versus healthy controls. Based on areas under the receiver operating characteristic curves (AUCs), tRNA-Val significantly distinguished HCC (AUC=1.00, P=0.001) from healthy controls in plasma EVs and HCC from HCA (AUC=0.950, P=0.01). Bioinformatics analysis revealed that tRNA-Val may be primarily involved in DNA repair, mRNA processing, and splicing and may be linked to the N-glycan and ubiquitin-mediated proteasome pathways. This is the first report on the expression of tRNA-Val in canine HCC and HCA, and its possible functions and signaling pathways. We suggest that tRNA-Val could be a promising novel biomarker to distinguish canine HCC from HCA. This study provides evidence for a greater understanding of the role played by tRNA-Val in the development of canine HCC.

Keywords: tRNA-Val; canine hepatocellular carcinoma; canine hepatocellular adenoma

1. Introduction

Hepatocellular adenomas (HCAs) are uncommon benign liver tumors that arise from the proliferation of hepatocytes and may transform into hepatocellular carcinoma (HCC), a common and malignant form of liver cancer in humans and dogs [1]. HCCs are the most common hepatic tumors in dogs, accounting for 50-70% of the total, and also represent the sixth most widespread cancer in humans globally [2–5]. Differentiating between HCA and HCC is a complex task, and as their prognosis and treatments vary, a molecular identification of the tumor type is vital for the patient

[6,7]. Accordingly, further research on molecular diagnostics to facilitate the differentiation between these two tumors would be potentially beneficial for oncologists.

Transfer RNA (tRNA)-derived fragments (tRFs) are recently discovered noncoding RNAs (ncRNAs) generated through precise cleavage of pre-mature and mature tRNA molecules [8]. tRFs may contain seed sequences that can target mRNA, resulting in gene silencing. They enter argonaute (AGO) complexes in a similar way to miRNAs [9,10]. tRFs are crucial in various cellular processes, such as cell proliferation, invasion, migration, and drug resistance. These molecules also regulate RNA stability and significantly impact target gene expression [11,12]. tRFs have diagnostic utility in identifying human HCC, ovarian, gastric, and breast cancer [13–20]. Research has also focused on tRFs in different canine cancers, including oral melanoma and MGTs [21,22]. The role of tRFs has not previously been investigated in canine HCC or HCA.

Extracellular vesicles (EVs) are small membrane-bound structures that cells release. These structures are crucial for intercellular communication as they transport vital molecules like DNA, RNA, and proteins between cells [23]. EV-associated tRFs are aberrantly expressed in gastric carcinoma, hypopharyngeal carcinoma, and canine oral melanoma [24–27]. However, to date, there is a total paucity of research on EV-associated tRFs in canine and human HCC and HCA.

Studying cancer in dogs can improve our understanding of its development in humans. There are marked similarities in biology and appearance between canine and human cancers, which underlines the need for cross-species research that could enhance therapeutic options for both species [28,29]. Canine HCC has many clinical and histological similarities to human HCC, suggesting that treatments for canine HCC could also be utilized for human HCC [2]. Investigating the involvement of tRFs in dogs with HCC offers a valuable opportunity to uncover important information about the development of HCC in humans.

In this study, we determined the expression levels of tRNA-Val in dogs with HCC and dogs with HCA using qRT-PCR analysis. We also validated the expression level of tRNA-Val in clinical canine HCC and HCA tissues, plasma EVs, and HCC cell lines for the first time. Our findings provide useful insight into the diagnosis of canine HCC and HCA.

2. Materials and Methods

2.1. Clinical Samples

We targeted 38 dogs (20 males and 19 females) aged between 8- and 14-years undergoing surgery at Kagoshima University Veterinary Teaching Hospital or an affiliated clinic in or around Kagoshima City, Japan, as potential tissue/blood donors for the study. Based on liver tissue samples, thirteen of these cases were diagnosed as HCC, and fifteen as HCA histopathologically by the hospital's certified pathologists (Diplomates of the Japanese College of Veterinary Pathologists). These 28 HCC or HCA samples were evaluated in this study. Normal liver tissue samples were obtained from healthy adult beagle dogs (n=9), purpose-bred for research, at the time of their scheduled necropsy as control samples.

Blood samples were collected from a subset of the tissue donors (n=20), consisting of healthy controls (n=6) and dogs with HCA (n=5) or HCC (n=9). The patients' (HCC and HCA donors') information is presented in Table S1.

The tissue samples were placed in RNeasy lysis buffer immediately after collection. Blood samples were centrifuged after collection to obtain plasma as the supernatant, which was then transferred to new tubes. All samples were stored at -80°C until downstream analysis.

This study was approved by the Ethics Committee of Kagoshima University (KVH210001). All procedures were accorded with Kagoshima University by laws and regulations. All owners gave informed consent for samples from their dogs to be evaluated in this study.

2.2. Cell Lines and Cell Culture

We used two canine HCC cell lines in this study, a fast-proliferating HCC cell line, 95-1044, and an intermediate-proliferating HCC cell line, AZACH [30,31]. Cell lines were cultured using D-MEM

medium (Sigma-Aldrich, St. Louis, Missouri), fetal bovine serum (Thermo Fisher Scientific, Waltham, Massachusetts), L-glutamine (Sigma-Aldrich), and spectinomycin (Sigma-Aldrich). All cells were cultured in a humidified incubator with 5% CO₂ at 37°C. Cells were counted using an automated cell counter instrument (Luna cell counter II, Logos Biosystems Inc., USA).

2.3. Isolation of EVs

EVs were isolated from plasma using the Total Exosome RNA and Protein Isolation Kit (Invitrogen, Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Briefly, an equal volume of phosphate-buffered saline (PBS) was added to a 300 µl aliquot of each plasma sample. The exosome precipitation reagent was added, the test sample was then vortexed well and centrifuged, and the supernatant was discarded. The pellet was reconstituted in 200 µl 1X PBS and kept at -80°C until analysis.

2.4. RNA Extraction

To extract total RNA from tissues and cells, a mirVanaTM RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used. Total RNA was isolated from EVs using the mirVana PARIS Kit (Thermo Fisher Scientific). To normalize variations, each plasma EV sample was mixed with 5 µl of synthetic cel-miR-39 prior to extraction. To elute total RNA, an elution solution was used. The total RNA concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to assess RNA quality and integrity. Cells and tissues had RNA Integrity Numbers (RINs) ranging from 8.5 to 9.5.

2.5. qRT-PCR Analysis for tRNA-Val

tRNA-Val was selected based on a previously reported NGS dataset (SRA: PRJNA716131) for canine mammary gland tumors [22]. The qRT-PCR protocol has been described previously [21,32,33]. Briefly, the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) was used with a T100 thermal cycler to reverse transcribe 2 ng (for tissues and cell lines) or 1.25 µl (for plasma EVs) of total RNA into cDNA in accordance with the manufacturer's instructions. Synthetic cel-miR-39 was spiked into the plasma samples to normalize RNA isolation. The Quant Studio 3 real-time PCR system (Thermo Fisher Scientific) and TaqMan First Advanced Master Mix Kit were used for qRT-PCR. All experiments were carried out twice. The 2^{-ΔΔCT} method was used to assess relative tRNA-Val expression. The internal controls were RNU6B and miR-186 for tissues and EVs, respectively [27]. The following custom-made Taqman primer sequences have been designed specifically for qRT-PCR: 5'-GTTTCCGTAGTGTAGTGGTTATCACGTTTCGCTGCC-3' tRNA-Val (URS000044730B_9606).

2.6. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment and Protein-Protein Interaction (PPI) Analysis

The tRNA-Val sequence was submitted to the miRDB database (<https://mirdb.org/custom.html>) for a custom gene prediction. Next, the targeted genes were submitted to the DAVID bioinformatics database (<https://david.ncifcrf.gov/>) for GO and KEGG enrichment analyses. The results of GO and KEGG enrichment analyses were also verified using SRPlot bioinformatics (<https://www.bioinformatics.com.cn/en>). A string database (<https://string-db.org/>) was used to identify the PPI interactions among the genes. A confidence score >0.400 was set for the prediction.

2.7. Statistical Analysis

Statistical analysis and graph visualization were performed using GraphPad Prism 9 (<https://www.graphpad.com/>). The qRT-PCR data were evaluated using a Mann-Whitney U test and one-way analysis of variance (ANOVA) with the Kruskal-Wallis test. The Wilson/Brown method was used to calculate receiver operating characteristic (ROC) curves and areas under the curve (AUCs). P-values <0.05 were regarded as statistically significant.

3. Results

3.1. Expression of tRNA-Val Using qRT-PCR

3.1.1. Relative Expression in Clinical Tissue Samples

The relative expression of tRNA-Val was determined for HCA and HCC tissue samples. tRNA-Val expression was significantly downregulated in HCC versus both normal liver (fold change [FC]=0.22, $P=0.003$) and HCA (FC=0.10, $P=0.004$), but did not significantly differ between HCA and normal liver (Figure 1A). Overall, tRNA-Val in liver tissue significantly differentiated HCC from HCA and normal liver.

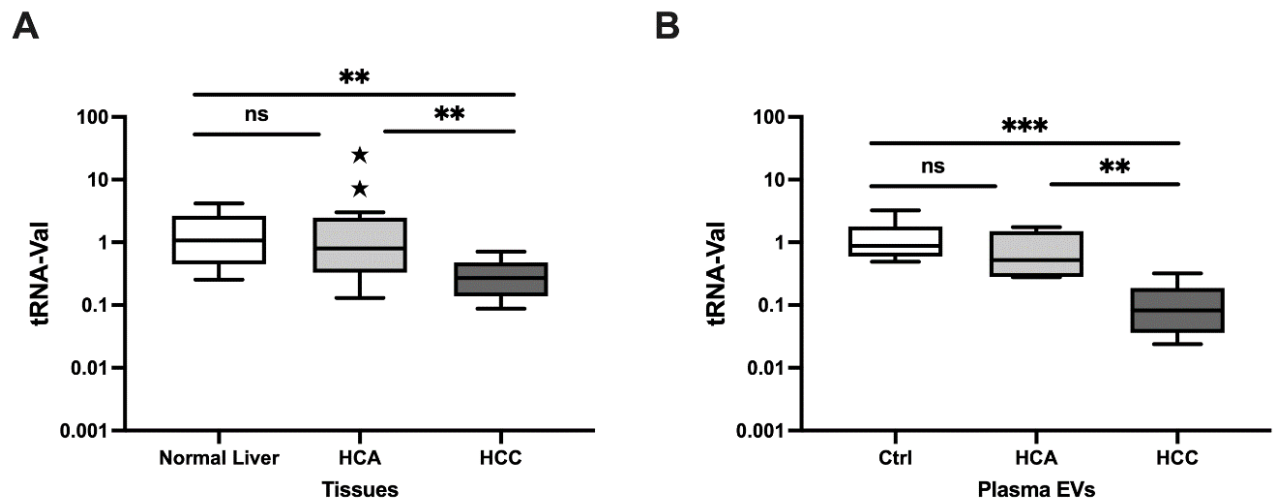


Figure 1. Relative expression in HCA and HCC tissue samples and plasma EVs using qRT-PCR. A. Relative expression levels of tRNA-Val in HCA (n=15) and HCC (n=13) versus normal liver (n=9) tissue. B. Relative expression levels of tRNA-Val HCA (n=5) and HCC (n=9) versus healthy control (n=6) plasma EVs. The Y-axis represents relative noncoding RNA expression levels in log₁₀ units. Data were analyzed with one-way ANOVA (nonparametric), followed by the Kruskal-Wallis and Mann-Whitney U tests. $p < 0.05$ (** $p < 0.01$, *** $p < 0.001$) was considered significant. HCA: hepatocellular adenoma, HCC: Hepatocellular carcinoma, ns; not significant, Ctrl: control, EVs: extracellular vesicles.

3.1.2. Relative Expression in Plasma EVs

Relative tRNA-Val expression was further investigated in plasma EVs, and found to be significantly downregulated in HCC cases versus healthy controls (FC=0.10, $P=0.0002$) and HCA cases (FC=0.19, $P=0.008$); however, it did not significantly differ between HCA cases and healthy controls (Figure 1B). Overall, tRNA-Val in plasma EVs could distinguish HCC from HCA and healthy controls.

3.1.3. Relative Expression in Canine HCC Cell Lines

We also investigated tRNA-Val expression in two canine HCC cell lines, the fast proliferating 95-1044 and the intermediate-proliferating AZACH cell lines. We found that tRNA-Val expression was decreased significantly in 95-1044 (FC=0.03, $P=0.0002$) and AZACH (FC=0.15, $P=0.004$) cells versus normal liver tissue, and in 95-1044 cells versus AZACH cells (FC=0.08, $P=0.004$) (Figure 2). Overall, these findings were in line with those for clinical samples and show that tRNA-Val is able to distinguish between fast- and intermediate-proliferating cell lines.

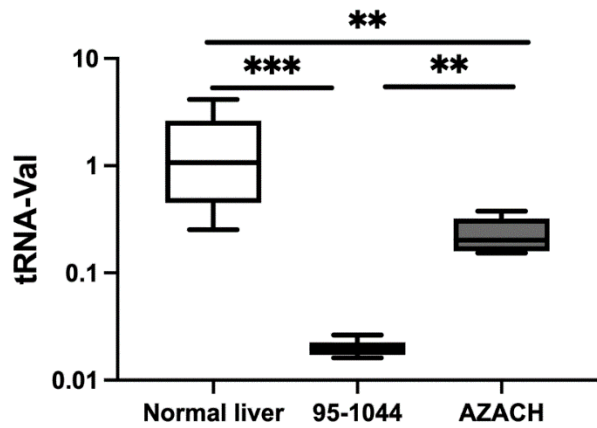


Figure 2. Relative expression in HCC cell lines using qRT-PCR. Relative expression levels of tRNA-Val in HCC-1044 (n=6) and AZACH (n=6) versus normal liver tissue (n=9). The Y-axis represents relative noncoding RNA expression levels in log10 units. Data were analyzed with one-way ANOVA (nonparametric) followed by the Kruskal-Wallis and Mann-Whitney tests. The differences were considered significant when the p-value was <0.05 (**p<0.01, ***p<0.001).

3.2. Diagnostic Value of tRNA-Val

ROC curves were plotted and AUCs were calculated to determine the diagnostic efficacy of tRNA-Val. In plasma EVs, tRNA-Val showed strong diagnostic efficacy for distinguishing HCC from healthy controls (AUC=1.00, P=0.001; Figure 3A), and from HCA (AUC=0.950, P=0.01; Figure 3B). However, tRNA-Val was unable to distinguish between HCA and healthy controls (AUC=0.708, P=0.286; Supplementary Figure 1). In summary, tRNA-Val could serve as a diagnostic biomarker to differentiate HCC from both HCA and healthy controls.

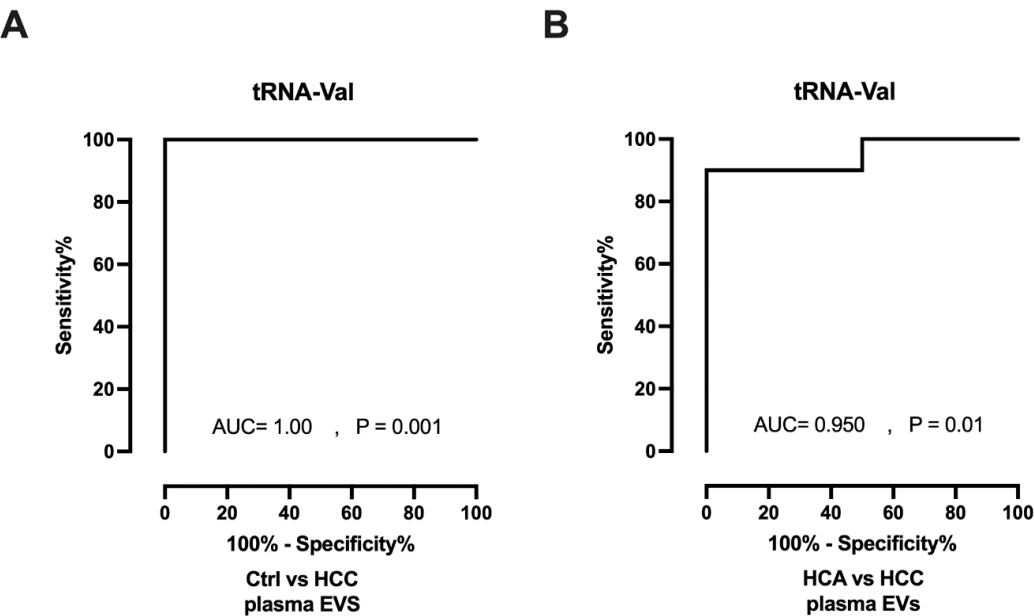


Figure 3. Potential of tRNA-Val as a diagnostic biomarker. ROC curves for tRNA-Val as a differentiator between HCC (n=9) and Ctrl (n=6), A, and HCA (n=5), B. Ctrl: control, HCA: hepatocellular adenoma, HCC: hepatocellular carcinoma, EVs: extracellular vesicles.

3.3. Predicted Targets for tRNA-Val and Enrichment Analyses

tRFs are known to target genes by binding to AGO complexes, based on complementarity to their seed sequence, in a fashion resembling the seed-sequence-mediated targeting by microRNAs [9]. tRNA-Val target genes were predicted using the miRDB custom gene prediction program. GO

enrichment and KEGG enrichment analyses were performed using the targeted gene list to identify the functional roles and pathways in which tRNA-Val is involved. GO enrichment analysis revealed a potential association between tRNA-Val and regulation of mRNA processing and splicing (Figure 4A). KEGG enrichment analysis revealed potential involvement of tRNA-Val in various types of N-glycan biosynthesis and ubiquitin-mediated proteolysis. To further elucidate the intrinsic mechanism, a PPI network was established based on a gene-to-gene confidence score using the STRING database (<https://string-db.org/>) (Figure 4B). Target genes involved in the PPI network are summarized in Table S2. The tRNA-Val target genes included those such as APLF, which are primarily involved in DNA repair; GTF2H1, which regulates transcription by RNA polymerase II; CDC 27, which acts on cell cycle division; and SMAD11, which acts as a negative regulator of transcription by RNA polymerase II.

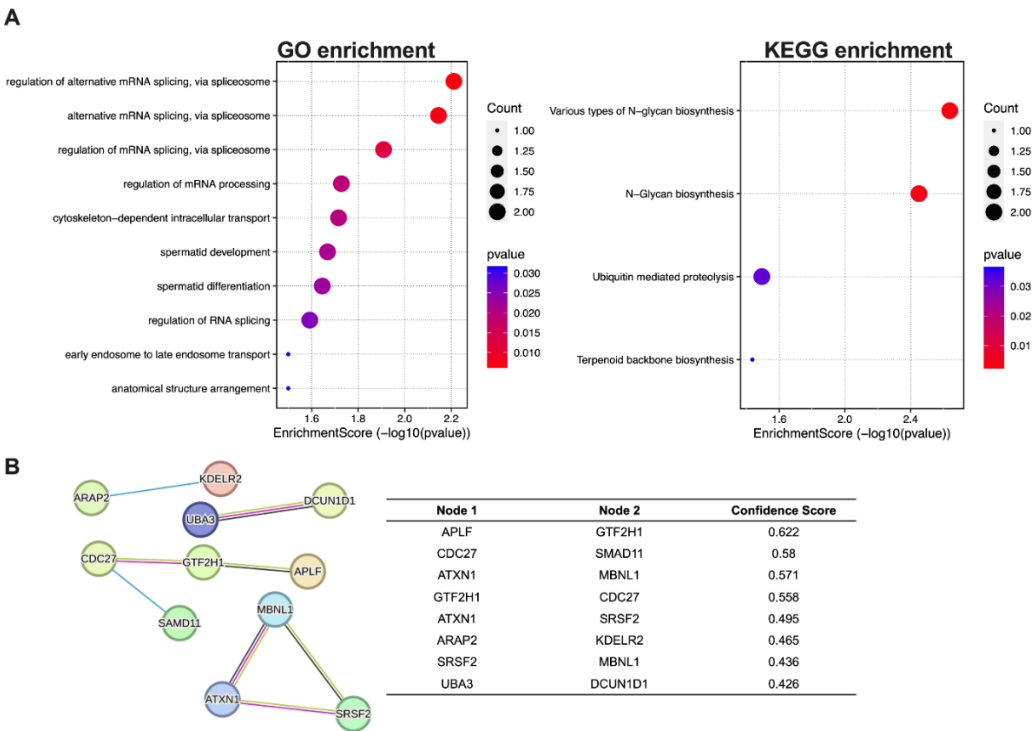


Figure 4. Enrichment and protein-protein interaction (PPI) analysis of target genes of tRNA-Val. A. GO and KEGG enrichment analyses and B. PPI of tRNA-Val. Results were considered significant when $p < 0.05$. The lower panel table shows the interaction confidence score between nodes 1 and 2. GO: Gene Ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes.

4. Discussion

We aimed to elucidate the diagnostic efficacy of tRNA-Val as a tRF biomarker for distinguishing canine HCA from canine HCC by evaluating its expression in liver tissue, plasma EVs, and HCC cell lines. To the best of our knowledge, this is the first report on tRF expression in canine liver tumors. As key findings in this study, we demonstrated significant downregulation of tRNA-Val in liver tissue and plasma EVs from dogs with HCC versus both healthy controls and dogs with HCA. By contrast, we found no difference in tRNA-Val expression between dogs with HCA and healthy controls (in either tissue or plasma EVs). Our findings are consistent with reports that tRFs can act as diagnostic and prognostic biomarkers for human cancers, even at an early stage [11,12], and that this tRF has specific diagnostic utility for differentiating between benign and malignant canine MGTs [22]. Furthermore, various tRFs reportedly show diagnostic utility for a range of human cancers, interestingly including 5'-tRNA-Glnm, which is significantly downregulated in HCC tissue versus tumor-adjacent tissue and in metastatic versus non-metastatic HCC [13]. In other examples, hsa-tsrf 016141, tRNA-Val-CAC-001, tRF-Glu-TTC-027, and tRF-5026a are preferentially downregulated in

gastric cancer and inhibit the proliferation of this carcinoma [16–19]. tRF-Gly-CCC-046, tRF-Tyr-GTA-010, and tRF-Pro-TGG-001 are reportedly downregulated in breast cancer and have potential as diagnostic biomarkers for early-stage detection of this cancer [20].

To validate our findings in clinical samples, we investigated tRNA-Val expression in fast-proliferating (95-1044) and intermediate-proliferating (AZACH) canine HCC cell lines. tRNA-Val was preferentially decreased in 95-1044 and AZACH cells versus healthy control tissue, reflecting the trend in clinical tissue and normal liver samples. These findings are consistent with those for tRF expression in human cell lines: 3'U-tRFValCAC promotes cell growth and migration in human ovarian cancer [14] and, as a novel tRF, tRF-Val is upregulated in gastric cancer tissues and cell lines, promoting cell proliferation and invasion and inhibiting cell apoptosis [15]. mt-tRFs are also reportedly significantly downregulated in canine oral melanoma tissues and cell lines versus controls [21], and tRNA-Val is downregulated in metastatic-site versus primary-site canine MGT cell lines [22].

Another noteworthy feature of this study was the particularly strong downregulation of tRNA-Val in plasma EVs from dogs with HCC versus healthy controls and dogs with HCA. EV-associated tRFs are evidently involved in cancer progression and have potential diagnostic utility to become biomarkers for various cancers [34–36]. EV cargos are important in intracellular communication, and EVs have been implicated in cancer cell communication and proliferation and tumor and metastasis [23]. Our findings in plasma EVs, taken together with our overall expression findings in the fast-proliferating cell line, suggest that EVs play a similar role in canine HCC development and progression. Interestingly, plasma EV-associated tRNA-Ala-TGC-5-1 can distinguish canine oral melanoma patients from tumor-free dogs [27]. In human medicine, EV-associated tRFs are aberrantly expressed in non-small cell lung cancer, gastric carcinoma, and hypopharyngeal carcinoma [24–26]. Taken together with our findings, these reports indicate the utility of plasma EVs as a sample matrix for oncological molecular diagnostics.

Through bioinformatics analysis, we predicted genes that tRNA-Val may target, considering that tRFs have a gene-regulating ability similar to that of miRNAs [37]. The predicted target genes include APLF, GTF2H1, CDC27, and SMAD11, which are involved in DNA repair, and mRNA splicing and processing. tRNA-Val may be involved in the N-glycan biosynthesis and ubiquitin-mediated proteasome pathways, which play pivotal roles in protein trafficking and enzyme complex formation [38], and the regulation of many biological processes such as cell cycle control, proliferation, signal transduction, and transcription regulation [39,40], respectively.

This study has some limitations. We did not evaluate plasma tRF-Val as a biomarker, which means further investigations are necessary to fully elucidate the intrinsic mechanism of this tRF. The numbers of tissue and blood donors in this study (n=28/20) were relatively small, and further investigation is needed in a larger study population.

5. Conclusions

In conclusion, we suggest that tRF-Val has potential diagnostic efficacy as a tRF biomarker for differentiating canine HCA from canine HCC. Our findings are of interest for research on new molecular diagnostic techniques and, ultimately, new therapeutic targets in canine and human oncology.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: ROC curves of tRNA-Val; Table S1: HCC and HCA patient information; Table S2: Targeted genes of tRNA-Val with their seed sequence and binding locations.

Author Contributions: Conceptualization, Saki Hashimoto and Naoki Miura; Methodology, Saki Hashimoto, Al Asmaul Husna, Osamu Yamato and Naoki Miura; Validation, Saki Hashimoto, MD Nazmul Hasan, Mohammad Arif, Nobuhiro Nozaki, Al Asmaul Husna, Osamu Yamato and Md Mahfuzur Rahman; Formal analysis, Saki Hashimoto, MD Nazmul Hasan, Mohammad Arif, Nobuhiro Nozaki, Yu Furusawa, Takeshi Sogawa, Kaori Takahashi, Tomohide Kuramoto, Aki Noguchi, Masashi Takahashi and Md Mahfuzur Rahman; Investigation, Saki Hashimoto, MD Nazmul Hasan, Mohammad Arif, Nobuhiro Nozaki, Al Asmaul Husna and Md Mahfuzur Rahman; Resources, Saki Hashimoto, Yu Furusawa, Takeshi Sogawa, Kaori Takahashi, Tomohide

Kuramoto, Aki Noguchi, Masashi Takahashi, Osamu Yamato and Naoki Miura; Writing – original draft, Saki Hashimoto and MD Nazmul Hasan; Writing – review & editing, Saki Hashimoto, MD Nazmul Hasan and Naoki Miura; Visualization, Saki Hashimoto, MD Nazmul Hasan, Mohammad Arif, Nobuhiro Nozaki, Al Asmaul Husna, Yu Furusawa, Takeshi Sogawa, Kaori Takahashi, Tomohide Kuramoto, Aki Noguchi, Masashi Takahashi and Md Mahfuzur Rahman; Supervision, Naoki Miura; Project administration, Naoki Miura; Funding acquisition, Naoki Miura.

Funding: This work was supported by JSPS KAKENHI (Grant nos. 21H02366, 20K21375).

Institutional Review Board Statement: This study was approved by the Ethics Committee of Kagoshima University (KVH210001).

Informed Consent Statement: All owners gave written consent for samples from their dogs to be evaluated in this study.

Data Availability Statement: All data of this study are included within this article and supplementary materials.

Acknowledgments: The authors express their gratitude to Professor Dr. Henry Smith for editing a draft of this manuscript.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Zheng, J.; Sadot, E.; Vigidal, J.A.; Klimstra, D.S.; Balachandran, V.P.; Kingham, T.P.; Allen, P.J.; D'Angelica, M.I.; DeMatteo, R.P.; Jarnagin, W.R.; et al. Characterization of hepatocellular adenoma and carcinoma using microRNA profiling and targeted gene sequencing. *PLoS One* **2018**, *13*, e0200776.
2. Patnaik, A.K.; Hurvitz, A.I.; Lieberman, P.H.; Johnson, G.F. Canine hepatocellular carcinoma. *Vet. Pathol.* **1981**, *18*, 427-438.
3. Van Sprundel, R.G.; van den Ingh, T.S.; Guscetti, F.; Kershaw, O.; Kanemoto, H.; van Gils, H.M.; Rothuizen, J.; Roskams, T.; Spee, B. Classification of primary hepatic tumours in the dog. *Vet. J.* **2013**, *197*, 596-606.
4. Forner, A.; Reig, M.; Bruix, J. Hepatocellular carcinoma. *Lancet* **2018**, *391*, 1301-1314.
5. Patnaik, A.K.; Hurvitz, A.I.; Lieberman, P.H. Canine hepatic neoplasms: a clinicopathologic study. *Vet. Pathol.* **1980**, *17*, 553-564.
6. Zucman-Rossi, J.; Jeannot, E.; Nhieu, J.T.; Scoazec, J.Y.; Guettier, C.; Rebouissou, S.; Bacq, Y.; Leteurtre, E.; Paradis, V.; Michalak, S.; et al. Genotype-phenotype correlation in hepatocellular adenoma: new classification and relationship with HCC. *Hepatology* **2006**, *43*, 515-524.
7. Bioulac-Sage, P.; Laumonier, H.; Couchy, G.; Le Bail, B.; Sa Cunha, A.; Rullier, A.; Laurent, C.; Blanc, J.F.; Cubel, G.; Trillaud, H.; et al. Hepatocellular adenoma management and phenotypic classification: the Bordeaux experience. *Hepatology* **2009**, *50*, 481-489.
8. Zhu, L.; Ge, J.; Li, T.; Shen, Y.; Guo, J. tRNA-derived fragments and tRNA halves: The new players in cancers. *Cancer Lett.* **2019**, *452*, 31-37.
9. Kescu, C.; Kumar, P.; Kiran, M.; Su, Z.; Malik, A.; Dutta, A. tRNA fragments (tRFs) guide Ago to regulate gene expression post-transcriptionally in a Dicer-independent manner. *Rna* **2018**, *24*, 1093-1105.
10. Kumar, P.; Anaya, J.; Mudunuri, S.B.; Dutta, A. Meta-analysis of tRNA derived RNA fragments reveals that they are evolutionarily conserved and associate with AGO proteins to recognize specific RNA targets. *BMC Biol.* **2014**, *12*, 78.
11. Gong, M.; Deng, Y.; Xiang, Y.; Ye, D. The role and mechanism of action of tRNA-derived fragments in the diagnosis and treatment of malignant tumors. *Cell Commun. Signal* **2023**, *21*, 62.
12. Fu, M.; Gu, J.; Wang, M.; Zhang, J.; Chen, Y.; Jiang, P.; Zhu, T.; Zhang, X. Emerging roles of tRNA-derived fragments in cancer. *Mol. Cancer* **2023**, *22*, 30.
13. Wu, C.; Liu, D.; Zhang, L.; Wang, J.; Ding, Y.; Sun, Z.; Wang, W. 5'-tiRNA-Gln inhibits hepatocellular carcinoma progression by repressing translation through the interaction with eukaryotic initiation factor 4A-I. *Front. Med.* **2023**, *17*, 476-492.
14. Panoutsopoulou, K.; Magkou, P.; Dreyer, T.; Dorn, J.; Obermayr, E.; Mahner, S.; van Gorp, T.; Braicu, I.; Magdolen, V.; Zeillinger, R.; et al. tRNA-derived small RNA 3'U-tRF(ValCAC) promotes tumour migration and early progression in ovarian cancer. *Eur. J. Cancer* **2023**, *180*, 134-145.
15. Cui, H.; Li, H.; Wu, H.; Du, F.; Xie, X.; Zeng, S.; Zhang, Z.; Dong, K.; Shang, L.; Jing, C.; et al. A novel 3'tRNA-derived fragment tRF-Val promotes proliferation and inhibits apoptosis by targeting EE1A1 in gastric cancer. *Cell Death Dis.* **2022**, *13*, 471.

16. Gu, X.; Ma, S.; Liang, B.; Ju, S. Serum hsa_tsr016141 as a Kind of tRNA-Derived Fragments Is a Novel Biomarker in Gastric Cancer. *Front. Oncol.* **2021**, *11*, 679366.
17. Zheng, J.; Li, C.; Zhu, Z.; Yang, F.; Wang, X.; Jiang, P.; Yan, F. A 5'-tRNA Derived Fragment Named tRNA-Val-CAC-001 Works as a Suppressor in Gastric Cancer. *Cancer Manag. Res.* **2022**, *14*, 2323-2337.
18. Xu, W.; Zhou, B.; Wang, J.; Tang, L.; Hu, Q.; Wang, J.; Chen, H.; Zheng, J.; Yan, F.; Chen, H. tRNA-Derived Fragment tRF-Glu-TTC-027 Regulates the Progression of Gastric Carcinoma via MAPK Signaling Pathway. *Front. Oncol.* **2021**, *11*, 733763.
19. Zhu, L.; Li, Z.; Yu, X.; Ruan, Y.; Shen, Y.; Shao, Y.; Zhang, X.; Ye, G.; Guo, J. The tRNA-derived fragment 5026a inhibits the proliferation of gastric cancer cells by regulating the PTEN/PI3K/AKT signaling pathway. *Stem Cell Res. Ther.* **2021**, *12*, 418.
20. Zhang, Y.; Bi, Z.; Dong, X.; Yu, M.; Wang, K.; Song, X.; Xie, L.; Song, X. tRNA-derived fragments: tRF-Gly-CCC-046, tRF-Tyr-GTA-010 and tRF-Pro-TGG-001 as novel diagnostic biomarkers for breast cancer. *Thorac. Cancer* **2021**, *12*, 2314-2323.
21. Rahman, M.M.; Lai, Y.C.; Husna, A.A.; Chen, H.W.; Tanaka, Y.; Kawaguchi, H.; Hatai, H.; Miyoshi, N.; Nakagawa, T.; Fukushima, R.; et al. Aberrantly expressed snoRNA, snRNA, piRNA and tRFs in canine melanoma. *Vet. Comp. Oncol.* **2020**, *18*, 353-361.
22. Hasan, M.N.; Rahman, M.M.; Husna, A.A.; Nozaki, N.; Yamato, O.; Miura, N. YRNA and tRNA fragments can differentiate benign from malignant canine mammary gland tumors. *Biochem. Biophys. Res. Commun.* **2024**, *691*, 149336.
23. Zaborowski, M.P.; Balaj, L.; Breakefield, X.O.; Lai, C.P. Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Bioscience* **2015**, *65*, 783-797.
24. Zheng, B.; Song, X.; Wang, L.; Zhang, Y.; Tang, Y.; Wang, S.; Li, L.; Wu, Y.; Song, X.; Xie, L. Plasma exosomal tRNA-derived fragments as diagnostic biomarkers in non-small cell lung cancer. *Front. Oncol.* **2022**, *12*, 1037523.
25. Xi, J.; Zeng, Z.; Li, X.; Zhang, X.; Xu, J. Expression and Diagnostic Value of tRNA-Derived Fragments Secreted by Extracellular Vesicles in Hypopharyngeal Carcinoma. *Onco Targets Ther.* **2021**, *14*, 4189-4199.
26. Lin, C.; Zheng, L.; Huang, R.; Yang, G.; Chen, J.; Li, H. tRFs as Potential Exosome tRNA-Derived Fragment Biomarkers for Gastric Carcinoma. *Clin. Lab.* **2020**, *66*.
27. Husna, A.A.; Rahman, M.M.; Lai, Y.C.; Chen, H.W.; Hasan, M.N.; Nakagawa, T.; Miura, N. Identification of melanoma-specific exosomal miRNAs as the potential biomarker for canine oral melanoma. *Pigment Cell Melanoma Res.* **2021**, *34*, 1062-1073.
28. Gustafson, D.L.; Duval, D.L.; Regan, D.P.; Thamm, D.H. Canine sarcomas as a surrogate for the human disease. *Pharmacol. Ther.* **2018**, *188*, 80-96.
29. Gardner, H.L.; Fenger, J.M.; London, C.A. Dogs as a Model for Cancer. *Annu. Rev. Anim. Biosci.* **2016**, *4*, 199-222.
30. Lai, Y.C.; Ushio, N.; Rahman, M.M.; Katanoda, Y.; Ogihara, K.; Naya, Y.; Moriyama, A.; Iwanaga, T.; Saitoh, Y.; Sogawa, T.; et al. Aberrant expression of microRNAs and the miR-1/MET pathway in canine hepatocellular carcinoma. *Vet. Comp. Oncol.* **2018**, *16*, 288-296.
31. Fujimoto, A.; Neo, S.; Ishizuka, C.; Kato, T.; Segawa, K.; Kawarai, S.; Ogihara, K.; Hisasue, M.; Tsuchiya, R. Identification of cell surface antigen expression in canine hepatocellular carcinoma cell lines. *J. Vet. Med. Sci.* **2013**, *75*, 831-835.
32. Hino, Y.; Rahman, M.M.; Lai, Y.C.; Husna, A.A.; Chen, H.W.; Hasan, M.N.; Nakagawa, T.; Miura, N. Hypoxic miRNAs expression are different between primary and metastatic melanoma cells. *Gene* **2021**, *782*, 145552.
33. Husna, A.A.; Rahman, M.M.; Chen, H.W.; Hasan, M.N.; Nakagawa, T.; Miura, N. Long non-coding RNA and transfer RNA-derived small fragments in exosomes are potential biomarkers for canine oral melanoma. *Vet. Comp. Oncol.* **2022**, *20*, 653-663.
34. Zhu, L.; Li, J.; Gong, Y.; Wu, Q.; Tan, S.; Sun, D.; Xu, X.; Zuo, Y.; Zhao, Y.; Wei, Y.Q.; et al. Exosomal tRNA-derived small RNA as a promising biomarker for cancer diagnosis. *Mol. Cancer* **2019**, *18*, 74.
35. Weng, Q.; Wang, Y.; Xie, Y.; Yu, X.; Zhang, S.; Ge, J.; Li, Z.; Ye, G.; Guo, J. Extracellular vesicles-associated tRNA-derived fragments (tRFs): biogenesis, biological functions, and their role as potential biomarkers in human diseases. *J. Mol. Med.* **2022**, *100*, 679-695.
36. Suresh, P.S.; Thankachan, S.; Venkatesh, T. Landscape of Clinically Relevant Exosomal tRNA-Derived Non-coding RNAs. *Mol. Biotechnol.* **2023**, *65*, 300-310.

37. Wang, J.; Ma, G.; Li, M.; Han, X.; Xu, J.; Liang, M.; Mao, X.; Chen, X.; Xia, T.; Liu, X.; et al. Plasma tRNA Fragments Derived from 5' Ends as Novel Diagnostic Biomarkers for Early-Stage Breast Cancer. *Mol. Ther. Nucleic Acids* **2020**, *21*, 954-964.
38. Bieberich, E. Synthesis, Processing, and Function of N-glycans in N-glycoproteins. *Adv. Neurobiol.* **2014**, *9*, 47-70.
39. Tu, Y.; Chen, C.; Pan, J.; Xu, J.; Zhou, Z.G.; Wang, C.Y. The Ubiquitin Proteasome Pathway (UPP) in the regulation of cell cycle control and DNA damage repair and its implication in tumorigenesis. *Int. J. Clin. Exp. Pathol.* **2012**, *5*, 726-738.
40. Shang, F.; Taylor, A. Roles for the ubiquitin-proteasome pathway in protein quality control and signaling in the retina: implications in the pathogenesis of age-related macular degeneration. *Mol. Asp. Med.* **2012**, *33*, 446-466.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.