Serum levels of vault RNA significantly varied in patients with haematological malignancies

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Abstract. Among extracellular non-coding RNAs, serum levels of microRNAs have been extensively investigated in cancers. In contrast, the serum levels of vault RNAs (vtRNAs) in relation to various disease conditions remain poorly understood. The present study evaluated the clinical significance of serum vtRNA1-1 levels in patients with blood diseases. The stability and sub-localisation of serum vtRNA1-1 was assessed and a reverse transcription-quantitative PCR method using spiked RNA to quantify serum vtRNA1-1 was developed. Serum vtRNA1-1 levels were assessed in 102 individuals with blood diseases. Serum vtRNA1-1 was demonstrated to be stable for three weeks at 4˚C and was not confined to the exosome fractions. Spiking RNA was used to correct for the inconsistency in RNA extraction. The serum vtRNA1-1 levels ranged between 7.28 and 8.76 $\log_{10}$ cps/ml (median 8.05) in control individuals (n=46). Serum vtRNA1-1 levels correlated with leukocyte counts and increased to a maximum of 10.01 $\log_{10}$ cps/ml in patients with bulky leukaemia and lymphoma and decreased to 6.52 $\log_{10}$ cps/ml during intensive chemotherapy. The serum vtRNA1-1 levels varied significantly in patients with haematological malignancies. Serum vtRNA1-1 may originate from haematological cells and are a potential biomarker of normal and malignant haematological activities.

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Abbreviations: AD, active disease; CTx, chemotherapy; ICTx, intensive chemotherapy; ID, indolent disease; R_S, Spearman correlation coefficient; RT-qPCR, reverse transcription-quantitative PCR; SD, standard deviation

Key words: vault RNA, exosome, extracellular vesicle, haematological malignancy, biomarker

Introduction

Vault RNAs (vtRNAs) have been reported as components of vault, the largest ribonucleoprotein particles found in eukaryotic cells, from amoebas to mammals (1,2). Four vault RNA genes; *VTRNA1-1* (98 bp), *VTRNA1-2* (88 bp), *VTRNA1-3* (88 bp) and *VTRNA2-1* (108 bp), are expressed in humans and these non-coding RNAs are transcribed by polymerase III (3). *VTRNA1-1*, *VTRNA1-2* and *VTRNA1-3* are highly homologous and are clustered on chromosome 5q31.3. The less homologous paralogue, *VTRNA2-1*, located on chromosome 5q31.1, encodes vtRNA2-1, previously named as precursor microRNA-886 (2,4,5). Among these genes, the gene product of *VTRNA1-1*, vtRNA1-1, has been reported to be strongly up-regulated during influenza A virus and Epstein-Barr virus infections, and to serve a role in viral establishment and apoptosis suppression (6-8). These previous studies have suggested the involvement of vtRNA1-1 in viral evasion from the innate immune system. It has also been reported that expression of vtRNA1-1 suppressed apoptosis in cancer cells and its overexpression was related to drug resistance in cancer cells (9-12). As a mechanism of drug resistance, vtRNA1-1 can recognise and bind chemotherapeutic compounds, such as mitoxantrone, in human glioblastoma-, leukaemia- and osteocarcinoma-derived cell lines (9). VtRNA1-1 also associates with an RNA/DNA-binding protein, PSF, in MCF-7 cells, inducing the oncogene, *GAGE6* (10). Knock-out of vtRNA1-1 in HeLa cells has been reported to cause mis-regulation of the PI3K/Akt signalling pathway and the ERK1/2 MAPK cascade, leading to increased apoptosis (11). Another study with liver cancer cells suggested the role of vtRNA1-1 in lysosome-mediated chemoresistance (12). Recently, Horos *et al* (13) reported that vtRNA1-1 binds to sequestosoma-1/p62, repressing autophagy as a riboregulator (14). Therefore, vtRNA1-1 is one of the multifaceted modulators of pro-survival characteristics and tumorigenesis (15). However, the function of extracellular vtRNA1-1 remains unknown.

Vault RNAs are expressed in numerous cell types, are localised in the cytoplasm and are secreted from cells into the extracellular space. Extracellular vtRNA1-1 is found in exosomes, small extracellular vesicles, which serve roles in inter-cellular communication, and in the promotion and metastasis of cancer cells (16). Transport of vtRNA1-1 into exosomes

is regulated by YBX1 proteins (17). Ramayanti *et al* (18) used vtRNA1-1 as an internal control to measure serum microRNAs in patients with malignancy considering its consistent detection, small variation in its levels and its PCR amplification with efficiencies similar to those for microRNAs (18). However, the relation between serum vtRNA1-1 levels and certain diseases is unknown.

In the present study, the clinical significance of serum vtRNA1-1 levels in patients with blood diseases was assessed. First, a method to measure the serum levels of vtRNA1-1 using reverse transcription-quantitative PCR (RT-qPCR), with spiked RNA to correct for the inconsistencies in RNA extraction and measurement, was proposed and optimised. The stability and sub-localisation of extracellular vtRNA1-1 in serum was then determined. The serum levels of vtRNA1-1 in patients with blood diseases in different disease states were assessed to determine the clinical significance of serum vtRNA1-1.

Materials and methods

Clinical samples. Archived serum samples from patients requiring laboratory tests and transplant donors visiting or hospitalised in the Department of Haematology, Tottori University Hospital (Yonago, Japan) between September 2020 and February 2022 were used in the present study. Serum was obtained from blood collected without anticoagulants by centrifugation at 1,900-2,200 x g for 7 min at room temperature. The serum samples were used for the subsequent clinical tests. The residual samples were stored at 4˚C until use in this study. The present study was approved by the Ethics Committee at Tottori University Faculty of Medicine (approval no. 18A115). Informed consent was obtained using an opt-out approach.

Fractionation of exosomes and the other extracellular vesicles. Exosomes were isolated from serum using the affinity purification method with Tim4-bound beads (MagCapture™ Exosome Isolation Kit PS; FUJIFILM Wako Pure Chemical Corporation) as per the manufacturer's instructions (19). Briefly, serum samples were incubated with Tim4-bound beads at 4˚C for ≥3 h. After incubation and magnetic selection, the supernatant was transferred and centrifuged at 10,000 x g for 30 min at 4˚C. Large extracellular vesicles were concentrated in the pellet (P10 fraction) and the residual supernatant (S10 fraction) contained serum proteins, ribonucleoproteins and phosphatidylserine-negative small extracellular vesicles. The beads were washed three times to obtain the Tim4 fraction. The three fractions were subjected to lysis for protein and RNA extraction.

RNA extraction and RT-qPCR with spiked MS2 RNA. RNA was extracted from 200 μl serum (stored for ≤21 days at 4˚C) using the spin column method (miRNeasy Serum/Plasma Advanced Kit; Qiagen KK) by centrifugation at 12,000 x g for 1 min at room temperature, and from serum fractions (Tim4-bound beads, pellets and supernatants) using the organic method (RNAzol RT; Molecular Research Center, Inc.) according to the manufacturers' instructions. Before RNA extraction, lysis buffer was mixed with bacteriophage MS2 RNA (0.8 ng/sample, Roche Diagnostics) as a spiked control to correct for inconsistent RNA extraction rates and transfer RNA from *Saccharomyces cerevisiae* (spin column method, 1 μg; organic method 5.5 μg; Sigma-Aldrich; Merck KGaA) as a carrier. One-step RT-PCR was performed using a 50 μl reaction mixture containing 900 nM forward and reverse primers, 250 nM probe, 1 μl ROX Reference Dye (50 X), One Step PrimeScript III RT-qPCR Mix (both from Takara Bio, Inc.) and 2 μl extracted RNA (equivalent to 20 μl serum). The mixture was incubated at 52˚C for 5 min for reverse transcription, followed by the inactivation of transcriptase at 95˚C for 10 sec and subsequent PCR amplification consisted of 50 cycles of denaturation at 95˚C for 15 sec and annealing and extension at 60˚C for 1 min using an ABI7300 Real Time PCR System (Thermo Fisher Scientific, Inc.). Primers and probes were purchased from Takara Bio, Inc. (Table I).

PCR products of vtRNA1-1 were examined using 5% polyacrylamide gel electrophoresis and ethidium bromide staining after 50 more cycles of PCR amplification, which was performed using 2 μl qPCR product and TaKaRa Ex Taq DNA polymerase (Takara Bio, inc.) with the aforementioned conditions, with the exception of denaturation at 95˚C for 10 min. The amplified band (Fig. 1) was purified after gel electrophoresis and amplified again with the same conditions. The product obtained was purified using the spin column method (QIAamp DNA Mini Kit; Qiagen KK) by centrifugation at 6,000 x g for 1 min at room temperature and used to generate calibration curves for the measurement of vtRNA1-1 expression levels. The correction for the measured values of vtRNA1-1 levels was made using the C_t values for MS2 RNA spiked in lysis buffer according to the following equation: corrected value (cps/ml)=measured value (cps/ml)/$2^{(\text{Ct value of MS2 RNA spiked in RNA extraction lysis buffer-Ct value of MS2 RNA in each sample})}$.

DNA contamination was assessed using PCR without RT. RNA samples were subjected to semi-qPCR amplification using GeneAce Probe qPCR Mix II (Nippon Gene Co., Ltd.), which consisted of denaturation at 95˚C for 10 min followed by 50 cycles of denaturation at 95˚C for 15 sec, and annealing and extension at 60˚C for 1 min using an ABI7300 Real Time PCR System (Thermo Fisher Scientific, Inc.). C_t values for vtRNA1-1 were compared with the measured C_t values for vtRNA1-1 aforementioned.

The amount of vtRNA1-1 in each serum fraction was determined using RT-qPCR, as aforementioned. The corrected values were normalised to that in the Tim4 fraction.

Gel electrophoresis and western blotting. The three fractions were lysed using SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% w/v glycerol, 0.005% w/v BPB), separated using SDS-PAGE on 10% gels and transferred onto a membrane (ClearTrans SP PVDF Membrane, Fujifilm, Japan). Western blot signals were detected using Amersham ECL Prime Western Blotting Detection Reagents (Cytiva) and images were captured using a MultiImager II (MISVS II, BioTools, Inc.). Membranes were incubated at 4˚C overnight with anti-CD63 (cat. no. 3-13; FUJIFILM Wako Pure Chemical Corporation; 1:1,000), anti-CD9 (cat. no. 1K; FUJIFILM Wako Pure Chemical Corporation; 1:1,000) and anti-CD81 (cat. no. 17B1; FUJIFILM Wako Pure Chemical Corporation; 1:2,000) primary monoclonal antibodies. Membranes were

Table I. Sequence of primers and probes used for reverse transcription-quantitative PCR.

Target	Sequence (5'-3')
vtRNA1-1	F: GGCTGGCTTTAGCTCAGCG
	R: AAGGACTGGAGAGCGCCC
	P: ^{FAM}CAAGCAACCTGTCTGGGTTGTTCGAGACC^{TAM}
MS2 RNA	F: GGGTTTCCGTCTTGCTCGTA
	R: GGACTTCATGCTGTCGGTGA
	P: ^{FAM}CCACTGTCGTGCTTTTCGCTGAAGAACTTG^{TAM}

F, forward; R, reverse; P, probe; FAM, fluorescein amidites; TAM, tetramethyl-rhodamine.

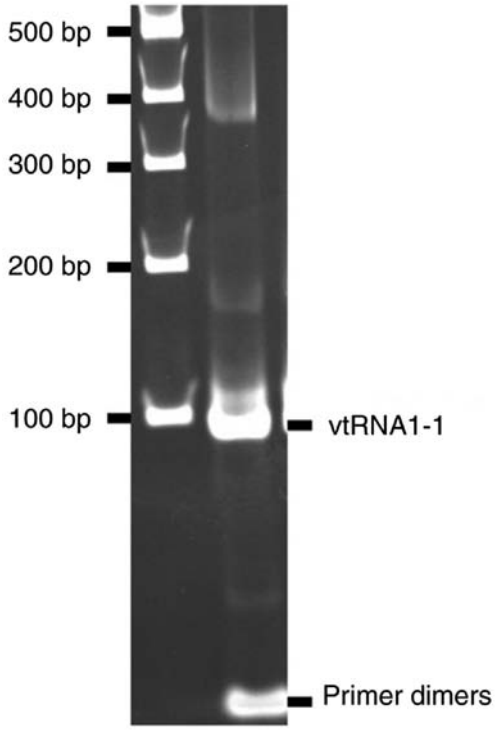

Figure 1. Specific amplification of vtRNA1-1. PCR products separated using polyacrylamide gel electrophoresis. DNA marker (100 bp ladder) was loaded in the left lane. A single band of the expected size (98 bp) corresponded to vtRNA1-1. Smaller bands (88 bp) corresponding to vtRNA1-2 and vtRNA1-3 were not detected, demonstrating the specific amplification of vtRNA1-1. The small band seen at the bottom consisted of primer dimers. Representative results of four similar experiments are presented. vtRNA, vault RNA.

washed with PBS and 0.05% Tween-20 and then incubated with peroxidase-labelled goat polyclonal anti-murine IgG and IgM (H+L) (cat. no. 5220-0343; SeraCare Life Sciences, Inc.; 1: 6,000 for negative control, 1:12,500 for CD63, and 1:25,000 for CD9 and CD81).

Statistical analysis. The enrolled individuals were classified into four groups, namely patients with malignancy in active disease (AD), patients with malignancy receiving chemotherapy (CTx), patients with malignancy receiving intensive chemotherapy (ICTx) and patients with malignancy in inactive disease (ID). The ID group included patients with malignancies in partial or complete remission after chemotherapy or indolent diseases without symptoms. Patients with non-malignant diseases, regardless of treatment, and transplant donors were included in the ID group. The ICTx group included patients receiving intensive chemotherapy requiring hospitalisation. Patients receiving other chemotherapies, which included cytotoxic drugs, targeted drugs and/or antibody drugs, were classified in the CTx group.

Data were analysed using EZR (version 1.35, Jichi Medical University Saitama Medical Center), which is a Japanese user interface for R (version 2.3-0) (20). The correlation coefficient between two variables was calculated using the Spearman's rank correlation test and, pairwise comparisons were performed using the Student's t-test or Mann-Whitney *U* test, and multiple comparisons were performed using one-way ANOVA with Bonferroni correction or Kruskal-Wallis test followed by pairwise Mann-Whitney U comparisons with the Bonferroni correction.

Results

Stability of serum vtRNA1-1. vtRNA1-1 levels in the serum, stored overnight at 4, -20, or -80˚C, were quantified. The vtRNA1-1 levels in frozen samples were significantly reduced in the -20˚C and markedly reduced in the -80˚C compared with those in the 4˚C sample, which indicated the degradation of vtRNA1-1 during the freezing and thawing process (Fig. 2A). The serum was then stored for three weeks and vtRNA1-1 was quantified. The vtRNA1-1 levels were significantly decreased after a single day of storage at -20˚C and remained stable thereafter whereas serum vtRNA1-1 stored at 4˚C was stable for three weeks (Fig. 2B). Freezing and thawing resulted in a marked reduction in serum vtRNA1-1 levels. Therefore, serum was stored at 4˚C for use in subsequent assays. RNA extraction was performed within 21 days of storage.

Sub-localisation of serum vtRNA1-1. The affinity method using Tim4-bound beads allowed the collection of phosphatidylserine-positive vesicles and highly purified exosomes (19). Centrifugation at 10,000 x g following the affinity method was used to separate the serum into the Tim4, S10 and P10 fraction. Tetraspanins, which are exosomal markers, were analysed using western blotting (Fig. 3). CD63, the marker that was used to isolate exosomes (17), was found in the Tim4 fraction, which indicated efficient purification of exosomes. CD9 and CD81 were identified in all fractions, which suggested that

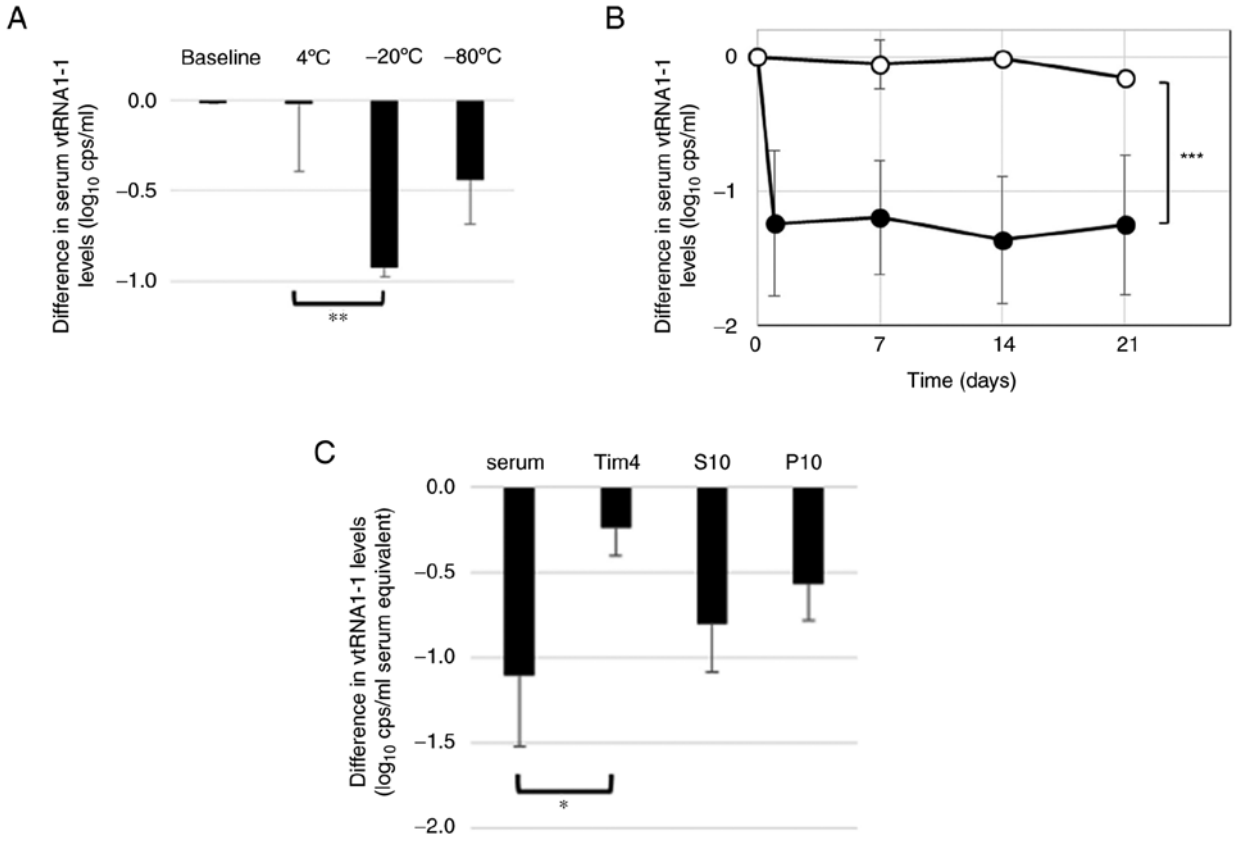

Figure 2. Stability of serum vtRNA1-1. RT-qPCR was used to quantify serum vtRNA1-1 levels at baseline and in serum stored at 4, -20, or 80˚C (A) overnight or (B) 4˚C (open circles, n=4) or -20˚C (closed circles, n=4) for 1, 7, 14 and 21 days. (C) The affinity purification method with Tim4-bound beads followed by centrifugation was used to fractionate serum samples (fractions stored at -20˚C). RT-qPCR was used to quantify vtRNA1-1 in each fraction before and after storage. The values were normalised to the baseline values obtained before storage in the log scale; the differences from the baseline values are shown. Bars indicated the standard deviation. Pairwise comparison was performed using the (B) Student's t-test and (A and C) ANOVA with the Bonferroni correction as required. *P<0.05, **P<0.01 and ***P<0.001. RT-qPCR, reverse transcription-quantitative PCR; S10, supernatant fraction; P10, pellet fraction; vtRNA, vault RNA; cps, copies.

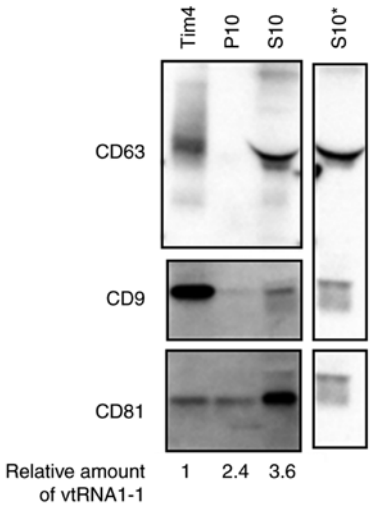

Figure 3. Sub-localisation of serum vtRNA1-1. Western blotting was used to assess the expression of CD63, CD9 and CD81 in each fraction (Tim4, S10 and P10). The representative results from four similar experiments are presented, images were cropped from different blots and the original full-length blots were presented in Fig. S1. The loaded Tim4, P10 and S10 fractions were equivalent to 5, 5 and 1 μl serum, respectively. The amounts of vtRNA1-1 in the corresponding fraction were determined using RT-qPCR and the relative amounts normalised to the amount in the Tim4 fraction are indicated at the bottom (mean values, n=7). *Primary antibody was excluded from comparison because of the presence of non-specific signals. S10, supernatant fraction; P10, pellet fraction; vtRNA, vault RNA.

these proteins were also present in other vesicles, including the large extracellular vesicles (separated in the P10 fractions) and phosphatidylserine-negative small extracellular vesicles (separated in the S10 fractions). Furthermore, vtRNA1-1 was quantified using RT-qPCR in all fractions and a markedly larger amount was present in the S10 fraction (Fig. 3). Thus, serum vtRNA1-1 was not confined to the exosome fractions. Freezing and thawing degraded serum vtRNA1-1, as aforementioned. The stability of vtRNA1-1 in each fraction was tested by freezing and thawing. The amount of vtRNA1-1 in any fraction reduced after freezing and thawing. vtRNA1-1 in the Tim4 fractions was demonstrated to be the most resistant to freezing and thawing whereas that in P10 and S10 fractions was more susceptible (Fig. 2C). These results suggested that serum vtRNA1-1 existed in different modalities in different fractions.

Serum levels of vtRNA1-1 in patients with blood diseases. To investigate the clinical significance of serum vtRNA1-1, serum vtRNA1-1 levels in patients with blood diseases were quantified. Bacteriophage MS2 RNA was added as a spiked RNA to the lysis buffer in advance and its C_t value was used for correction of inconsistent extraction rate and PCR efficiency. DNA contamination rates were evaluated using PCR without RT and were negligible [mean ± standard deviation (SD) 0.52±1.08%;

Table II. Diagnosis and disease status.

A, Patients with malignant disease (n=76)

Diagnosis	n	Disease status, n			
		AD	CTx	ICTx	ID
Total	76	22	28	5	21
Myeloid neoplasm	29	11	10	4	4
Acute myeloid leukemia	8	1	2	4	1
Myelodysplastic syndrome	4	2	1	0	1
Myeloproliferative neoplasms	16	7	7	0	2
Chronic myelomonocytic leukemia	1	1	0	0	0
Lymphoid neoplasm	47	11	18	1	17
Non-Hodgkin lymphoma	26	8	7	0	11
Hodgkin lymphoma	2	0	1	0	1
Acute lymphoid leukemia	2	1	1	0	0
Chronic lymphocytic leukemia	1	1	0	0	0
Multiple myeloma	15	1	9	1	4
Langerhans cell histiocytosis	1	0	0	0	1

B, Patients with nonmalignant disease (n=26)

Diagnosis	n	Disease status, n			
		AD	CTx	ICTx	AD
Total	26	0	1	0	25
Iron deficiency anemia	3	0	0	0	3
Hemophilia A	2	0	0	0	2
Factor XIII deficiency	4	0	0	0	4
Pernicious anemia	1	0	0	0	1
Immune thrombocytopenia[a]	6	0	1	0	5
Aplastic anemia	4	0	0	0	4
Castleman disease	1	0	0	0	1
Stress erythrocytosis	1	0	0	0	1
Heart disease	1	0	0	0	1
Transplant donor	3	0	0	0	3

[a]A patient with immune thrombocytopenia had prostate cancer receiving chemotherapy. AD, patients with active blood malignancy; CTx, patients receiving chemotherapy with cytotoxic drugs, targeted drugs and/or antibody drugs; ICTx, patients receiving intensive chemotherapy requiring hospitalization; ID, patients with blood malignancies in partial or complete remission after chemotherapy, those with indolent disease without symptoms and those with nonmalignant disease.

n=7]. Thereafter, the serum levels of vtRNA1-1 in 102 individuals with various blood malignancies and non-malignant diseases, in different disease status, including transplant donors were assessed. Diagnoses and disease status were presented in Table II. The mean and median serum vtRNA1-1 levels in all individuals were 8.10±0.54 $\log_{10}$ cps/ml and 8.06 $\log_{10}$ cps/ml (range, 6.52-10.01), respectively (Table III). Mean and median of RNA extraction rates for 102 serum samples determined using C_t values of spiked MS2 RNA were 49.7% (SD 27.1%) and 51.9% (range, 1.1-127.5%), respectively. The inconsistent RNA extraction rates were apparent. Without correction using the spiked controls, the mean value decreased to 7.72 $\log_{10}$ cps/ml and the SD increased to 0.69; the variance increased from 0.29 to 0.47. These findings indicated that correction using spiked RNA was necessary to determine accurate values for serum vtRNA1-1 levels.

The ID group included patients with malignancy in complete remission for >1 year and patients with non-malignant diseases which were under control. Therefore, among the four groups, this group was the closest to a group of healthy individuals; therefore, the ID group was taken as a control. The mean and median serum vtRNA1-1 levels in the ID group (n=46) were 8.04 $\log_{10}$ cps/ml (SD 0.34) and 8.05 $\log_{10}$ cps/ml (range, 7.28-8.76), respectively (Table III). Comparison of demographic values demonstrated that vtRNA1-1 levels in males were significantly higher compared with those in females. No correlation was observed between age and vtRNA1-1 levels (R_S -0.0203, P=0.840, n=102; Tables III and IV). There was a significant

Table III. Serum vtRNA1-1 levels in relation to age, sex, diagnosis and disease status.

		vtRNA1-1, $\log_{10}$ cps/ml		
Group	n	Mean (SD)	Median (range)	P-value[d]
Total	102	8.10 (0.54)	8.06 (6.52-10.01)	
Age				NS
<65 years	45	8.11 (0.52)	8.16 (6.79-9.38)	
≥65 years	57	8.10 (0.55)	8.04 (6.52-10.01)	
Sex				
Male	52	8.21 (0.56)	8.10 (6.52-9.38)	P<0.05
Female	50	7.99 (0.49)	8.00 (6.79-10.01)	
Diagnosis				NS
Myeloid malignancy	29	8.05 (0.75)	8.04 (6.52-10.01)	
Lymphoid malignancy	47	8.16 (0.47)	8.04 (7.37-9.38)	
Non-malignant disease	26	8.06 (0.33)	8.09 (7.28-8.76)	
Disease status[d]				
AD	22	8.55 (0.65)	8.53 (7.61-10.01)	P<0.05[a], P<0.01[b], P<0.05[c]
CTx	29	8.06 (0.32)	8.04 (7.59-8.80)	P<0.05[b]
ICTx	5	6.98 (0.53)	6.83 (6.52-7.90)	P<0.01[c]
ID	46	8.04 (0.34)	8.05 (7.28-8.76)	

[a]vs. CTx, [b]vs. ICTx, [c]vs. ID using Kruskal-Wallis test, [d]Bonferroni correction was made for diagnosis and disease status. SD, standard deviation; AD, patients with active blood malignancy; CTx, patients receiving chemotherapy with cytotoxic drugs, targeted drugs and/or antibody drugs; ICTx, patients receiving intensive chemotherapy requiring hospitalization; ID, patients with blood malignancies in partial or complete remission after chemotherapy, those with indolent disease without symptoms, and those without malignant disease; NS, not significant.

correlation between serum vtRNA1-1 levels and physical constitution except body mass index (R_S 0.275, P<0.01, n=101; Fig. 4A, Table III). Even in the ID group, body height remained significantly correlated with vtRNA1-1 levels (R_S 0.304, P<0.05, n=45; Fig. 4B), which suggested that physique, but not obesity, had certain correlation with serum vtRNA1-1 levels. Comparison of laboratory data with vtRNA1-1 levels demonstrated that blood counts, particularly leukocyte counts, regardless of leukocyte components, had a strong significant correlation with serum vtRNA1-1 levels (R_S 0.596, P<0.001, n=102; Fig. 4C, Table IV). Furthermore, liver enzymes and C-reactive protein levels were significantly correlated with serum vtRNA1-1 levels (R_S 0.419, P<0.001, n=102 and R_S 0.220, P<0.05, n=96, respectively; Fig. 4D, Table IV). However, creatinine levels demonstrated no correlation with serum vtRNA1-1 levels (R_S 0.117, P=0.243, n=102; Fig. 4E, Table IV).

The serum levels of vtRNA1-1 were not significantly different between myeloid and lymphoid malignancies. However, serum levels of vtRNA1-1 varied significantly in different disease status (median vtRNA1-1 levels of AD, CTx, ICTx, and ID groups were 8.53, 8.04, 6.83 and 8.05 $\log_{10}$ cps/ml, respectively; Table III; Fig. 5). The serum vtRNA1-1 levels increased to a maximum of 10.01 $\log_{10}$ cps/ml in patients with active blood malignancies, levels which were demonstrated in a patient with advanced myeloid leukaemia. Furthermore, markedly increased levels of vtRNA1-1, >9.0 $\log_{10}$ cps/ml, were demonstrated in patients with primary myelofibrosis, chronic lymphocytic leukaemia, hairy cell leukaemia, large granular lymphocytic leukaemia and anaplastic large cell lymphoma. In patients with lymphoid malignancy, levels of soluble interleukin-2 receptor, a biomarker of lymphoma (21), were significantly correlated with serum vtRNA1-1 levels (R_S 0.368, P<0.05, n=30; Fig. 4F; Table IV). However, patients who received intensive chemotherapy (particularly those with myeloid malignancy) and required hospitalisation, had significantly lower serum levels of vtRNA1-1 (n=29; ICTx vs. AD, P<0.01; ICTx vs. CTx, P<0.05; ICTx vs. ID, P<0.01).

Discussion

The present study developed a method of quantifying the serum levels of vtRNA1-1 using RT-qPCR with MS2 RNA as a spiked control. Due to inconsistencies in RNA extraction from clinical samples, correction with internal controls was preferable for measuring serum RNA levels. However, the use of internal controls for measuring the levels of non-coding RNAs remains controversial (18). Instead of internal controls, MS2 RNA was spiked in the lysis buffer and used to correct vtRNA1-1 values, which demonstrated that the variance of corrected vtRNA1-1 values was decreased compared with that of the uncorrected, measured values. These results supported the use of spiked RNA as a control.

Using the affinity purification method, with Tim4-bound beads, exosomes were purified as demonstrated by the presence of CD63, an exosome-specific marker. The relative amounts of vtRNA1-1 appeared to have no association with the intensity of any band. Serum vtRNA1-1 was not confined to exosomes, but appeared to be distributed widely in the

Table IV. Age, physical constitution, and laboratory data and their correlations with serum vtRNA1-1 levels.

Characteristic	n	Mean (SD)	Median (range)	R_S	P-value
Age, years	102	62.4 (16.8)	66 (13-94)	-0.0203	
Physical constitution					
Body height, cm	101	162 (9.1)	162 (136-185)	0.275	P<0.01
Body weight, kg	102	57.2 (11.9)	55.1 (29.1-100)	0.303	P<0.01
Body mass index, kg/m^2	101	21.6 (3.4)	21.5 (13.2-34.3)	0.151	
Body surface area, m^2	101	1.60 (0.19)	1.58 (1.11-2.24)	0.296	P<0.01
Blood counts					
White blood cells, x10^3/μl	102	11.0 (32.2)	4.8 (0.1-256)	0.596	P<0.001
Granulocytes, x10^3/μl	100	4.4 (8.8)	2.9 (0-80.9)	0.416	P<0.001
Monocytes, x10^3/μl	100	0.75 (4.27)	0.26 (0-42.9)	0.407	P<0.001
Lymphoid cells, x10^3/μl	100	3.1 (12.9)	1.4 (0.056-126)	0.441	P<0.001
Red blood cells, x10^4/μl	102	381 (98)	404 (153-609)	0.267	P<0.01
Reticulocytes, x10^4/μl	98	7.2 (4.8)	6.7 (0.22-27.6)	0.145	
Platelets, x10^3/μl	102	188 (226)	171 (8-2173)	0.127	
Chemistry					
Total protein, g/dl	94	6.6 (0.8)	6.7 (4.2-8.7)	0.109	
Albumin, g/dl	93	3.9 (0.7)	4.0 (1.5-5.5)	0.037	
Total bilirubin, mg/dl	101	0.85 (0.57)	0.7 (0.2-4.6)	-0.060	
Aspartate aminotransferase, U/l	102	27.1 (17.0)	22 (9-111)	0.374	P<0.001
Alanine aminotransferase, U/l	102	27.4 (25.8)	19 (7-189)	0.419	P<0.001
Alkaline phosphatase, U/l	99	92.3 (72.2)	76 (33-695)	0.308	P<0.01
γ-glutamyl transpeptidase, U/l	93	48.8 (64.0)	27 (8-417)	0.245	P<0.05
Lactate dehydrogenase, U/l	101	262 (208)	201 (75-1339)	0.318	P<0.01
Creatine kinase, U/l	44	113 (110)	90.5 (7-617)	-0.094	
C-Reactive protein, mg/dl	96	1.77 (5.88)	0.09 (0.01-33.2)	0.220	P<0.05
Creatinine, mg/dl	102	1.06 (1.24)	0.78 (0.4-10.6)	0.117	
Soluble interleukin-2 receptor, U/ml	30	3389 (7201)	589.8 (218.1-26004)	0.368	P<0.05

Body mass index was body weight divided by body height^2. Body surface area was calculated using the DuBois formula (27). P-values were calculated using Spearman rank correlation test. SD, standard deviation; R_S, Spearman correlation coefficient.

extracellular space. This finding was consistent with a recent report that vtRNAs were enriched in non-vesicular fractions of cell lines in culture medium (22). Because of their resistance to detergent-mediated disruption, vtRNA1-1 may not be contained in vesicles but may be present as ribonucleoproteins (22). In the present study, serum vtRNA1-1 in the Tim4 fractions were more resistant to the freeze-thaw process than those in the other fractions. The lipid bilayer membranes of exosomes may partly protect vtRNA1-1 from destruction through freezing and thawing whereas vtRNA1-1 included in ribonucleoproteins are more susceptible as they lack such protection. In either case, vtRNA1-1 was protected from RNase in the serum as it was stable for 3 weeks at 4˚C.

To the best of our knowledge, the present was the first study to measure serum vtRNA1-1 levels in patients with blood diseases to evaluate the clinical significance of this vtRNA. Ramayanti *et al* (18) previously reported the measurement of relative levels of serum and plasma vtRNA1-1 in patients with head and neck cancer and used them as controls for normalising microRNA levels considering their small variation. In the present study, certain patients received different intensities of chemotherapy and others were not subjected to any intervention. Among all these patients, increased vtRNA1-1 levels were demonstrated in patients with bulky leukaemia and lymphoma. Myeloid and lymphoid malignancies correlated with increased levels of vtRNA1-1. Lactate dehydrogenase levels have frequently been used as a tumour marker of haematological malignancy in clinical practice (23,24) and were significantly correlated with vtRNA1-1 levels. Although the increase in serum vtRNA1-1 levels may depend on specific disease types, requiring further study of more cases, the present study indicated that serum vtRNA1-1 levels could be used as a biomarker to monitor tumour mass in response to treatment. Serum vtRNA1-1 levels were significantly decreased only in the ICTx group, which underwent intensive chemotherapy, which not only reduces tumour mass but also extensively eliminates normal haematological cells (25,26). Furthermore, significant correlation was demonstrated between serum vtRNA1-1 levels and leukocyte counts. Therefore, serum vtRNA1-1 appeared to originate largely from haematological cells regardless of whether they were malignant or normal. Moreover, the correlation of serum vtRNA1-1 with body height, but not with body

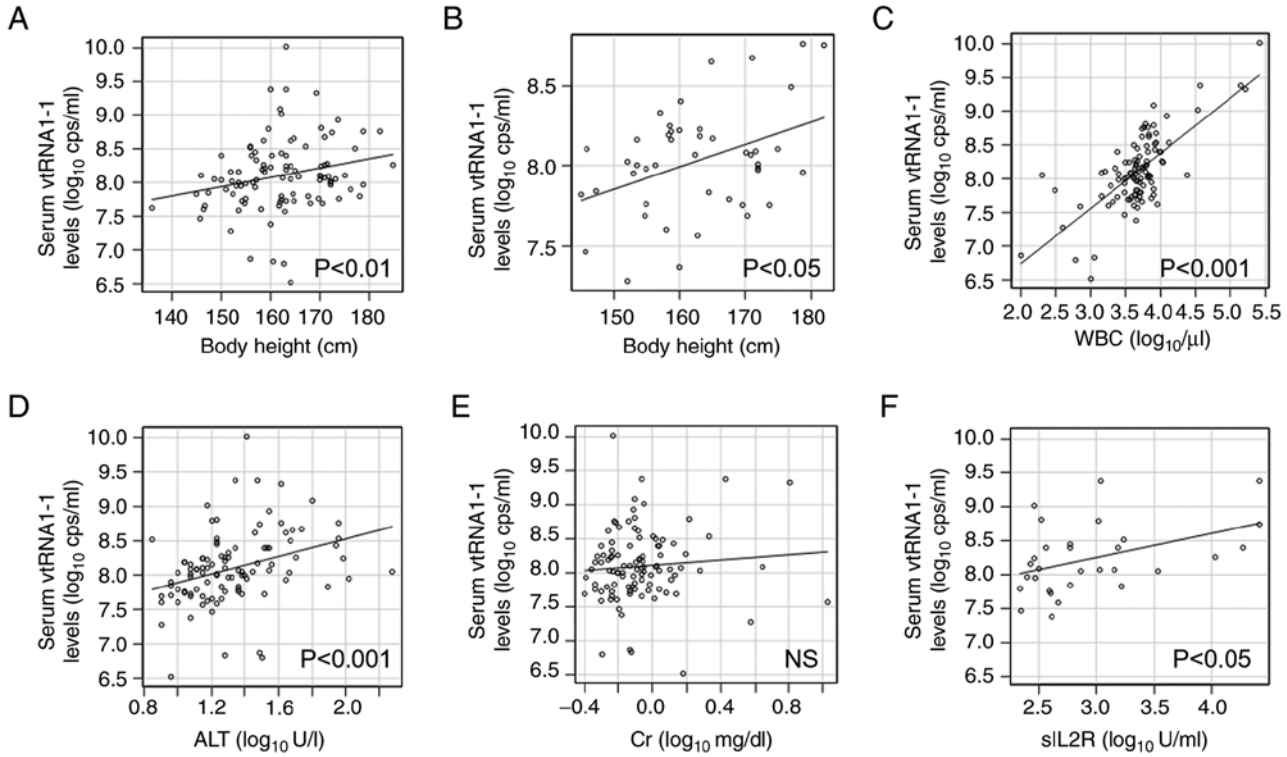

Figure 4. Correlation of serum vtRNA1-1 levels with height and blood components. Scatter dot plots for the correlation of serum vtRNA1-1 with (A) height (n=101), (C) WBC count (n=102), (D) ALT (n=102), (E) Cr (n=44) and (F) sIL2R (n=30). (B) Correlation between serum vtRNA1-1 levels and body height was significant even in the control group (n=46). P-values were calculated using the Spearman rank correlation test. WBC, white blood cells; ALT, alanine aminotransferase; Cr, creatinine; sIL2R, soluble interleukin-2 receptor; NS, not significant.

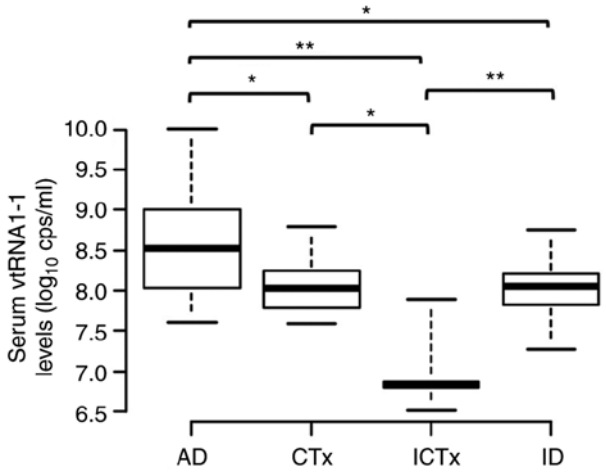

Figure 5. Serum vtRNA1-1 levels in different disease states. Serum vtRNA1-1 levels assessed in each group. Error bars denote minimum and maximum values in the four patient groups. Bold lines denote median values, boxes represent the 25th and 75th percentiles. *P<0.05 and **P<0.01 using Kruskal-Wallis test with the Bonferroni correction. AD, patients with active blood malignancy (n=22); CTx, patients receiving chemotherapy (n=29); ICTx, patients receiving intensive chemotherapy requiring hospitalisation (n=5); ID, patients with indolent blood malignancy without symptoms and blood malignancy in partial or complete remission after chemotherapy and those without blood malignancy (n=46).

mass index, led to a hypothesis that the serum vtRNA1-1 levels partially reflect the bone marrow capacity. Therefore, correlation between bone marrow cellularity and serum vtRNA1-1 levels should be further evaluated.

Metabolism of extracellular RNAs is largely unknown. No correlation of renal function, represented by creatinine levels, with serum vtRNA1-1 levels was demonstrated, which indicated that renal excretion of vtRNA1-1 was negligible. Conversely, significant correlation was demonstrated between serum vtRNA1-1 levels and liver enzyme levels, and leakage of vtRNA1-1 from damaged liver cells or decreased hepatic clearance of serum vtRNA1-1 may be the mechanism underlying this correlation. However, patients with severe liver dysfunction were not included in the cohort used in this study. It was recently reported that vtRNA1-1 was not detected in normal hepatic cells but its expression was increased in metastatic tumour cells (15). Therefore, infiltrating inflammatory cells were a possible origin of increased serum vtRNA1-1 levels. The time course of the serum vtRNA1-1 levels during severe liver damage due to different causes would be informative and should be considered in future studies.

The results of the present study suggested that serum vtRNA1-1 level was a potential biomarker of haematological activities. However, advanced solid tumours and inflammatory or infectious diseases, which induce immune cell response, remain to be evaluated. Because serum levels of vtRNA1-1 vary significantly, depending upon haematological activities, which can be detected using routine clinical examination, serum vtRNA1-1 may be used as an internal control for measuring the levels of other non-coding RNAs, such as microRNAs.

In conclusion, serum vtRNA1-1 remained stable at 4˚C and was not confined to exosomes. Serum vtRNA1-1 levels varied significantly in patients with haematological malignancies,

with varying disease status, which suggested serum vtRNA1-1 as a potential biomarker of haematological cells, either malignant or normal. These findings supported the need for further investigation of serum vtRNA1-1 as a potential biomarker of haematological activities.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HK, YHas, HS and TM designed the study. HK, YHat and TM performed the study. HK wrote the first draft of the manuscript. HS and TM supervised the study and reviewed and edited the manuscript. YHat and TM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee at Tottori University Faculty of Medicine (approval no. 18A115). Informed consent was obtained through an opt-out approach. All methods were performed in accordance with Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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