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RNU2-1 gene copy number variations do not affect serum levels of miR-1246 as a biomarker for lung adenocarcinoma

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Abstract. Circulating microRNA (miR)-1246, a fragment of U2 small nuclear RNA, represents a promising biomarker for certain cancers, including lung adenocarcinoma. The gene that encodes miR-1246, *RNU2-1*, is located on human chromosome 17q21.31 and is organized as a tandem array that exhibits copy number variations (CNVs). The range of copy numbers has been reported to be quite wide, ranging from 5 to 82 per haploid genome. The present study analyzed the correlation between *RNU2-1* copy numbers and serum miR-1246 levels in healthy controls and patients with stage IV lung adenocarcinoma. Serum miR-1246 levels were quantified using reverse transcription-quantitative polymerase chain reaction (PCR), and *RNU2-1* copy numbers per diploid genome were measured via digital PCR using blood-derived DNA. The median copy numbers were 50 and 49 in the control and lung adenocarcinoma groups, respectively, with no significant difference observed between the groups. In addition, no correlation between *RNU2-1* copy number and serum miR-1246 level was identified in either group. These results suggest that CNVs of *RNU2-1* do not affect serum levels of the lung adenocarcinoma biomarker miR-1246.

Introduction

The novel microRNA miR-1246, first identified in 2008 (1), has been investigated as a biomarker for pancreatic adenocarcinoma, hepatocellular carcinoma, and esophageal, breast, and lung cancers (2-6). A fragment of U2 small nuclear RNA (U2 snRNA) that carries an identical sequence to miR-1246, referred to as RNU2-1f (7) or miR-U2-1 (8), has also been evaluated as a potential biomarker for pancreatic and colorectal adenocarcinoma, melanoma, central nervous system lymphoma, and ovarian and lung cancers (7-11). The area under the receiver-operator characteristics curve (AUC) of miR-1246 was 0.878 (CI: 0.818-0.925) for discriminating healthy controls (HC) (n=96) from patients with all stages of non-small cell lung cancer (NSCLC) (n=62) (8). The AUC of miR-1246 was 0.891 (CI: 0.819-0.962) for distinguishing lung cancer patients of all histological types (n=211) from HC (n=58) and 0.873 (CI: 0.761-0.985) for distinguishing stage 0-II patients (n=54) from controls (12).

Various isoforms of miR-1246 have been detected in sera and tumor samples from patients with cancer, some of which were longer than its archetypical sequence as described in the micro-RNA database, miRBase (7-8,13). Sequences at both ends of these isoforms correspond to the U2 snRNA sequence rather than the predicted pre-miR-1246 sequence (7-8,13). These results revealed that miR-1246 is not derived from pre-miR-1246, but from U2 snRNA. Genomic deletion of the predicted region of the miR-1246 gene (chromosome 2: 176,600,980-176,601,052) was shown to reduce neither miR-1246 expression nor exosomal miR-1246 levels in Panc-1 cells (14). These findings strongly suggest that miR-1246 is not encoded by the miR-1246 gene, but rather by the U2 snRNA gene *RNU2-1* (14).

RNU2-1 is located on human chromosome 17q21.31, and is organized as a tandem array (15,16). A 6.1 kb repeat unit includes a188 bp U2 snRNA coding region, and the repeat number of the unit exhibits certain polymorphisms [i.e., copy number variations (CNVs)] (17) (Fig. 1A). The identified copy number range was 6-82 per haploid genome, as determined through direct visualization of the polymorphic *RNU2-1* gene copy number using fluorescence *in situ* hybridization (FISH) on 46 unrelated chromosomes (18). In addition, 53 alleles were identified via Southern blot analysis (19).

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Abbreviations: CNVs, copy number variations; U2 snRNA, U2 small nuclear RNA; PCR, polymerase chain reaction; RT-qPCR, reverse transcription-quantitative PCR; FISH, fluorescence *in situ* hybridization; dPCR, digital PCR; CEP17, centromere of chromosome 17; SD, standard deviation; PSE, proximal sequence element; DSE, distal sequence element; NSCLC, non-small cell lung cancer; CI, confidence interval

Key words: miR-1246, circulating microRNA, *RNU2-1*, copy number variation, digital polymerase chain reaction, non-small cell lung cancer

The present study aimed to clarify whether the polymorphic copy number of *RNU2-1* affects serum miR-1246 levels in both HC and patients with lung adenocarcinoma. *RNU2-1* copy numbers have traditionally been measured using Southern blot analysis (19) or FISH (18). Recently, the depth of coverage values of sequences from over 1,000 individuals from various genome projects were used to determine the *RNU2-1* copy number per diploid genome (20). In this study, we used digital PCR (dPCR) as a relatively simple method to determine the extremely wide-ranging *RNU2-1* copy number per diploid genome, and analyzed the correlation between *RNU2-1* copy number and serum miR-1246 levels.

Materials and methods

Patients and clinical specimens. This study was approved by the Ethics Committee of the Faculty of Health Sciences, Kyorin University for the HC (approval number: 2022-30) and the patients with stage IV lung adenocarcinoma whose samples were obtained from Kanagawa Cancer Center (approval number: 2023-22). The study protocol for the patients was also approved by the Ethics Committee of Kanagawa Cancer Center (approval number: 2023 epidemiology-84). Samples from the HC were collected at Kyorin University in October 2014 and October 2020. The HC consented to the use of their samples in the present study, and they provided written informed consent. Samples from patients were collected from the biobank of the Kanagawa Cancer Center between April 2021 and March 2023 and provided to us in November 2023. The patients consented to the use of their samples in comprehensive cancer research, including genetic analysis. The exclusion criteria for patients with lung adenocarcinoma were as follows: participants who were pregnant, had a history of other malignancies, or had received prior treatment.

Extraction of serum RNAs. All serum samples were centrifuged at 20,000 x g for 10 min at 4°C to remove cell debris, divided into 200 μl aliquots, and stored at -80°C until further use. An miRNeasy Serum/Plasma kit (Qiagen GmbH, cat. no. 217184) was used for small RNA extraction. Briefly, 3.5 μl of 0.16 fmol/μl 5'-phosphorylated cel-miR-39-3p was added to 200 μl of serum sample as a spike-in control for RT-qPCR. RNA was extracted according to the manufacturer's instructions, with the only minor modification being that the volume of RNase-free H_2O used to elute the RNA was changed to 28 μl (21).

RT-qPCR. The experimental protocol was essentially identical to the one described in our previous study (21). MiR-X miRNA First-Strand Synthesis and TB Green qRT-PCR systems (Takara Bio Inc., cat. no. 638313) were used to quantify miR-1246. The cDNA was synthesized according to the manufacturer's instructions. Briefly, 5 μl of 2x mRQ buffer, 3.75 μl of RNA sample, and 1.25 μl of mRQ Enzyme Mix were added to 0.2 ml tubes, incubated at 37°C for 1 h, then inactivated at 85°C for 5 min. Thereafter, 90 μl of DNase-RNase-free H_2O was added to the solution. The qPCR reaction mixture consisted of 7.8 μl of DNase/RNase-free H_2O, 10 Ml of 2x TB Green Advantage Premix (Takara Bio Inc., cat. no. 638314), 0.4 Ml of 50x ROX Reference Dye LMP, 0.4 μl of the primers (10 μM), and 1 μl of cDNA. A two-step qPCR was performed in duplicate on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.), using the cycling protocol: 95°C for 10 sec, 40 cycles of 95°C for 4 sec, and 60°C for 32 sec. The forward primer sequences are presented in Table I. A reverse transcription primer and a reverse primer were provided as part of the MiR-X miRNA First-Strand Synthesis and TB Green qRT-PCR systems. The $2^{-\Delta Cq}$ method was used to perform relative quantification of the miRNAs as follows: ΔCq=(Cq of miR-1246)-(Cq of spike-in control cel-miR-39-3p).

Quantification of gene copy number via dPCR. Genomic DNA was extracted from peripheral venous blood using a QIAamp DNA Mini kit (Qiagen GmbH, cat. no. 51304), according to the manufacturer's instructions. The restriction enzyme HindIII was used to divide *RNU2-1* tandem repeats into repeat units (17) (Fig. 1A). A total of 35-75 ng of DNA was digested using 10.5 U of HindIII (Takara Bio Inc., cat. no. 1060A) in a 20 μl reaction mixture at 37°C for 60 min, after which HindIII was inactivated at 65°C for 20 min. The HindIII-digested DNA was then centrifuged at 20,630 x g for 3 min before being added to the dPCR reaction mixture. A 10 μl aliquot of reaction mixture, containing 1 μl of HindIII-digested DNA, 0.8 μl of 2.5 μM TaqMan probe, 0.45 μl each of 10 μM primers, 0.5 μl of TaqMan™ Copy Number Reference Assay RNase P (Thermo Fisher Scientific Inc., cat. no. 4403328), 2 μl of 5x Absolute Q DNA Digital PCR Master Mix (Thermo Fisher Scientific Inc., cat. no. A52490), and 4.8 μl of ultra-pure H_2O was prepared. After centrifuging the reaction mixture at 20,630 x g for 1 min, 9 μl of the mixture and 15 μl of Absolute Q Isolation Buffer were applied to each well of the Absolute Q MAP16 Plate (Thermo Fisher Scientific Inc., cat. No. A52865), according to the manufacturer's instructions. The plate was centrifuged at 160 x g for 1 min, after which PCR was performed in duplicate according to the cycling protocol: 96°C for 10 min, 40 cycles of 96°C for 5 sec, and 61°C for 30 sec, on a QuantStudio Absolute Q Digital PCR system (Thermo Fisher Scientific Inc.). The sequences of all TaqMan probes and primers are listed in Table I. The primers and TaqMan probe for *RNU2-1* were designed using Primer Express software 3.0.1 (Thermo Fisher Scientific Inc.) based on the human *RNU2-1* sequence (NCBI Gene ID: 6066). Similarly, the copy number of *CEP-17*, a marker for the centromere of chromosome 17, was quantified using the *RPPH1* gene as a reference to evaluate any chromosome 17 polysomy. The CEP17-R19 primer was modified from CEP17-R (22) by adding three nucleotides (AGC) at the 5' end.

Statistical analysis. All statistical analyses were conducted using JMP 13.2.1 software (SAS Institute Inc.). The ages of the study participants were expressed as means ± standard deviations (SDs). The Shapiro-Wilk test indicated that each experimental dataset was non-normally distributed ($P<0.005$). Therefore, the Mann-Whitney U test was used to compare miR-1246 serum levels or *RNU2-1* copy numbers between the patient and control groups. Fisher's exact test was used to assess the significance of any differences in age, and the chi-squared test was used to assess differences in sex. Spearman's rank correlation coefficient test was used to perform a correlation

Table I. Oligonucleotides used in the present study.

A, Primers for digital PCR

Name	Sequence (5'-3')	(Refs.)
RNU2-1F97	GGATTTTGGAGCAGGGAGAT	-
RNU2-1R172	GAGGTACTGCAATACCAGGTCGAT	-
CEP17-F	GCTGATGATCATAAAGCCACAGGTA	(22)
CEP17-R19	AGCTGGTGCTCAGGCAGTG	(22)

B, Primers for real-time PCR

Name	Sequence (5'-3')	(Refs.)
cel-39 miR-XF	CACCGGGTGTAAATCAGCTTG	(21)
U2 miR-XF	CCAATGGATTTTTGGAGCAGG	(21)

C, TaqMan probes for digital PCR

Name	Sequence (5'-3')	(Refs.)
RNU2-1TM132	FAM-CTCCGTCCACTCCAC	-
CEP17-*p* FAM	FAM-TGCTGCAATAGGCGG	(22)

-, primers/probes were designed in the present study. CEP17-R19 primer was modified from CEP17-R (22) by adding three nucleotides (AGC) at the 5' end.

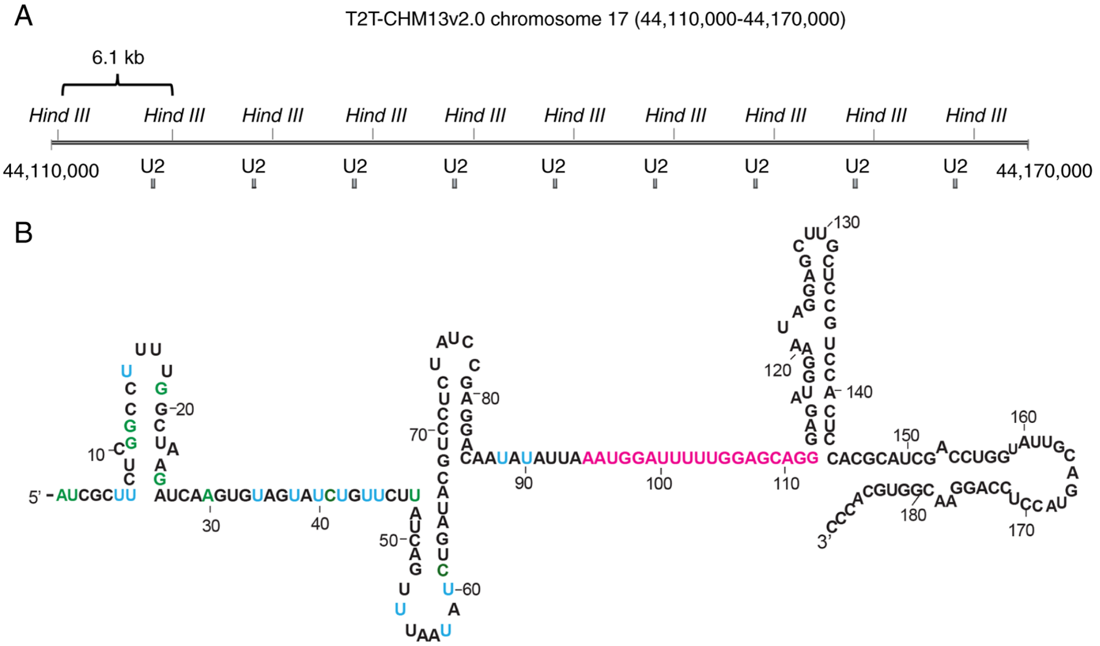

Figure 1. Schematic overview of the *RNU2-1* gene and its products. (A) Arrangement of 6.1 kb *RNU2-1* repeat units and HindIII restriction sites in the T2T-CHM13v2.0 human reference genome. (B) Secondary structure of U2 snRNA adapted from a report by Mazières *et al* (8), with minor modifications. Magenta, miR-1246; Blue, pseudouridine; Green, methylation.

analysis. Stepwise linear regression analysis was used to identify factors influencing serum miR-1246 levels. Differences were considered statistically significant when their P-values were <0.05.

Results

RNU2-1 copy numbers of healthy individuals and patients with lung adenocarcinoma. We first quantified serum miR-1246

Table II. Characteristics of patients with lung adenocarcinoma and control subjects.

Characteristics	No. of patients with lung adenocarcinoma	No. of control subjects	P-value
Total	45	51	
Age			<0.0001
≥50	45	17	
<50	0	34	
Sex			<0.001
Male	27	12	
Female	18	39	
Stage			
IV	45		

Fisher's exact and Chi-squared tests were used to evaluate age and sex, respectively. Staging of the malignant tumors was performed according to the eighth edition of the Tumor-Node-Metastasis classification (32).

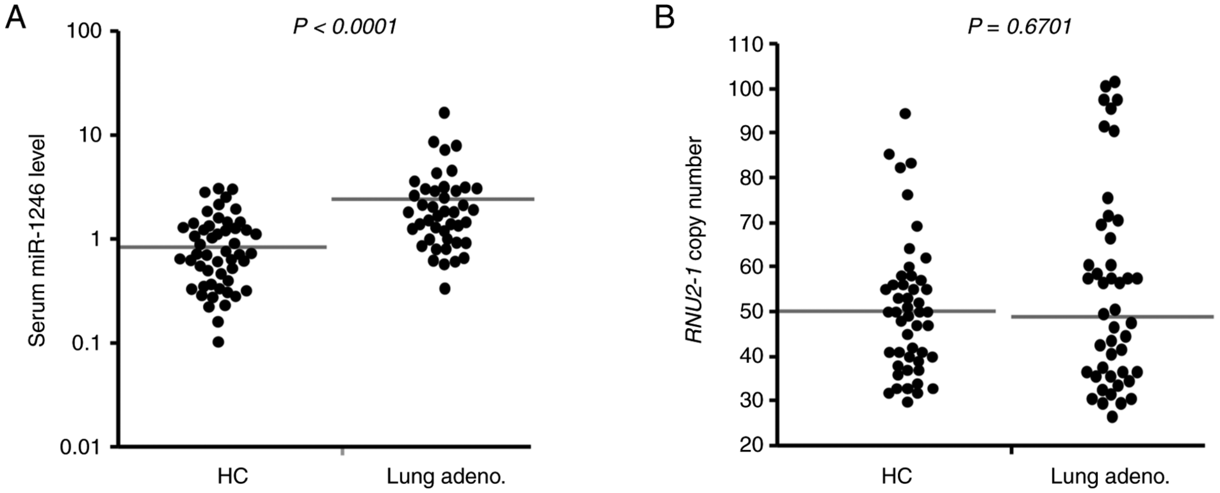

Figure 2. Quantification of serum miR-1246 levels and *RNU2-1* copy number. (A) Serum miR-1246 levels quantified by reverse transcription-quantitative polymerase chain reaction (PCR). The $2^{-\Delta\Delta Cq}$ method was used for relative quantification. (B) *RNU2-1* copy number, measured via digital PCR, showing copy number per diploid. Statistical significance of differences between two groups was evaluated using the Mann-Whitney U test. Horizontal lines represent the median. Lung adeno., lung adenocarcinoma; HC, healthy controls; miR, microRNA.

levels in 51 healthy individuals and 45 patients with stage IV lung adenocarcinoma using RT-qPCR. The characteristics of the participants are described in Table II. Serum miR-1246 levels were significantly higher in patients with stage IV lung adenocarcinoma than in the HC (P<0.0001; Fig. 2A). These results obtained from patients with stage IV adenocarcinoma were consistent with the results in our previous report on patients with stage III or IV adenocarcinoma (21). There was a significant difference in the age distribution between the patients and HC (P<0.0001; Table II). The age (mean ± standard deviation) was 68.56±7.31 years in the patient group and 36.19±14.74 years in the HC group. Therefore, we examined whether age affected serum miR-1246 levels. We first performed a stepwise multiple regression analysis, with age as a covariate. Age was the only retained variable (P<0.0001) in the current cohort, because the ages of the HC were significantly lower than those of patients with lung adenocarcinoma. To investigate the potential confounding effect of age, we performed the same analysis using data from our previous study (21). Results showed that disease status was the strongest factor associated with serum miR-1246 levels (P<0.05). In the current cohort, a correlation between age and serum miR-1246 level was not observed in patients with lung adenocarcinoma (Fig. S1A). Although a moderate age-related correlation was observed (R=0.569) in the HCs (Fig. S2B), this correlation was largely driven by a subset of HC. This subset was a young healthy cohort (20-22 years old;) whose samples were collected on the same day. The correlation was no longer significant after excluding this young healthy subset (Fig. S1C).

Next, the *RNU2-1* copy numbers per diploid were measured via dPCR using genomic DNA derived from peripheral blood samples (Fig. 2B). Each *RNU2-1* gene in the tandem array was digested using HindIII, as described in the Materials and Methods section (Fig. 1A). The range of the *RNU2-1* copy numbers was 30-94 (median: 50) in the HC group, and 26-101 (median: 49) in the stage IV lung adenocarcinoma group, with

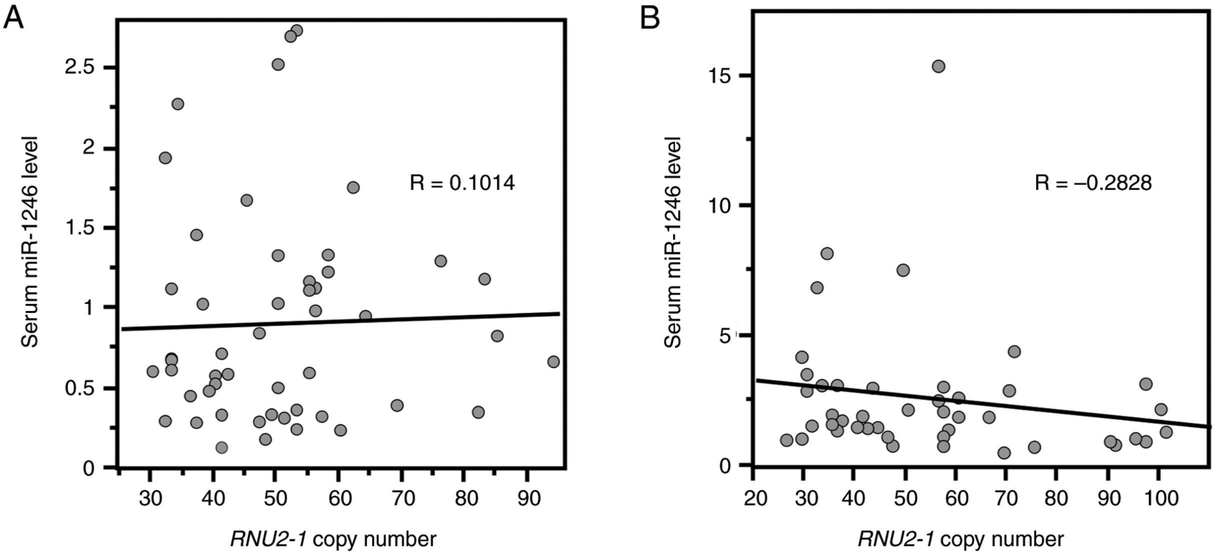

Figure 3. Correlation between serum miR-1246 levels and *RNU2-1* copy number. The significance of the correlation was evaluated using Spearman's rank correlation coefficient test. (A) Correlation in HCs (R=0.1014; P=0.4790). (B) Correlation in patients with lung adenocarcinoma (R=-0.282; P=0.0598). Lung adeno., lung adenocarcinoma; HC, healthy controls; miR, microRNA.

no significant differences observed between the two groups (P=0.4349; Fig. 2B). We also investigated the possibility of chromosome 17 polysomy in seven patients with stage IV lung adenocarcinoma who had particularly high *RNU2-1* copy numbers (Table III), using a TaqMan probe for CEP17 (Table I). The CEP17 copy numbers per diploid genome ranged between 1.97-2.37, revealing that there was no chromosome 17 polysomy (Table III).

Correlation analysis between serum miR-1246 levels and RNU2-1 copy number. Finally, we analyzed the correlation between serum miR-1246 levels and *RNU2-1* copy numbers in the control and stage IV lung adenocarcinoma groups. Spearman's rank correlation coefficients between serum miR-1246 levels and *RNU2-1* copy numbers were 0.0692 in the control group (P=0.6594) and -0.2828 in the stage IV lung adenocarcinoma one (P=0.0598), indicating no significant correlation (Fig. 3).

Table III. Number of *CEP17* signals in seven patients with lung adenocarcinoma found to have particularly high *RNU2-1* copy numbers.

Case ID	*RNU2-1* copy number	*CEP17* copy number
LC no.1	91.91±2.08	2.27±0.05
LC no.13	98.68±0.91	2.27±0.03
LC no.27	110.00±7.19	1.99±0.04
LC no.32	84.26±2.51	2.26±0.05
LC no.38	85.25±2.40	2.28±0.07
LC no.39	87.56±1.65	1.97±0.04
LC no.43	99.66±1.11	2.37±0.04

Data are shown as means ± SEs (n=3). SE, standard error.

Discussion

In this study, we investigated whether polymorphisms in *RNU2-1* copy number affect serum miR-1246 levels in patients with stage IV lung adenocarcinoma and healthy individuals. First, we confirmed that serum miR-1246 levels were significantly higher in patients with stage IV lung adenocarcinoma than in healthy individuals, as has been reported previously (8,12,21). We then analyzed the *RNU2-1* copy number per diploid genome using dPCR with DNA derived from peripheral blood samples. The median *RNU2-1* copy numbers in HC and patients with stage IV lung adenocarcinoma were 50 and 49, respectively, with no significant difference being observed between them. We also did not identify any correlation between *RNU2-1* copy number and serum miR-1246 level in either group. These results suggest that *RNU2-1* CNVs have no effect on serum levels of the lung adenocarcinoma biomarker miR-1246. Other studies have previously investigated correlations between gene copy numbers and serum levels of the corresponding gene products, for some other genes. Serum amylase levels, for example, were not found to correlate with *AMY2* or *AMY2B* copy numbers (range: 1-4), but did correlate with that of *AMY1A* (range: 2-27) (23). A correlation was also demonstrated between the copy numbers of the *FCGR3B* gene (range: 0-3) and serum levels of Fc gamma receptor III-B (FCGR3B) (24). The *RNU2-1* copy numbers, which range from 26 to 101, had no effect on serum miR-1246 levels in both healthy individuals and patients with lung adenocarcinoma.

U2 snRNA is transcribed by RNA polymerase II. Each repeat unit in the *RNU2-1* tandem repeat contains a proximal sequence element (PSE) and an enhancer-like distal sequence element (DSE), located ~55 and ~220 bp, respectively, upstream of the U2 snRNA coding region. *RNU2-1* transcription is regulated by Oct-1 and snRNA-activating protein complex, which bind to DSE and PSE, respectively (25,26).

Intracellular U2 snRNA levels were not changed by either the overexpression or knockdown of *RNU2-1* in Panc-1 cells, in a previous study. In both cases, exosomal miR-1246 levels (i.e., RNU2-1f), an intermediate degradation product of U2 snRNA, were found to be elevated (14). These results suggest that the transcription and degradation of U2 snRNA are tightly regulated under such circumstances. The miR-1246 sequence within the U2 snRNA sequence contains a binding sequence for the RNA-binding protein, SmB/B'. Exosomal miR-1246 levels were found to be reduced in SmB/B' knockdown cells, suggesting that SmB/B' binds to miR-1246 to protect it from degradation (14,27). Exosomal miR-1246 was also found to be reduced by knockdown of the RNA-binding protein, SRSF1 (28); therefore, SRSF1 may be involved in the sorting of miR-1246 into exosomes. In the present study, no correlation was observed between *RNU2-1* copy number and serum miR-1246 levels in our HC group (Fig. 3A). These results imply that a significant difference in *RNU2-1* copy number does not affect circulating miR-1246 level, which may instead be regulated by other mechanisms such as transcription of *RNU2-1*, or the stability or releasing efficiency of miR-1246.

MiR-1246 expression was elevated in lung cancer (6) and colorectal cancer (13) tissues, and its serum levels decreased after surgery (29). U2 snRNAs are upregulated in certain subtypes of breast cancer, suggesting that U2 snRNA expression may vary under specific cancer types or physiological conditions (30). Cancer tissues may represent one of the major sources of circulating miR-1246. Although this study focused on germline *RNU2-1* CNV, circulating miR-1246 levels may be influenced by other tumor-derived regulatory factors (e.g., *RNU2-1* transcriptional activity and regulation of miR-1246 release via exosomal packaging), and these mechanisms should be investigated in future studies.

The estimated total length of the *RNU2-1* tandem repeat ranges 30-492 kb when the copy number of *RNU2-1* ranges 5-82. It is impossible to amplify the entire region of the tandem repeat even when using enzymes for long PCR. In this study, we used dPCR to analyze *RNU2-1* copy numbers. This approach is a relatively simple method that facilitates the quantification of gene copy number per diploid genome. The *RNU2-1* copy number per diploid genome ranged from 30 to 94 (median, 50) in the 51 healthy individuals we analyzed. An estimated *RNU2-1* copy number per diploid, based on depth of coverage value from the 1000 Genomes Project, ranged from 2.5 to 160 (mean, 40.6) (20). The copy number of each allele, as revealed through Southern blot analysis and a fiber FISH approach, ranged from 5 to 63 (19) and 6 to 82 (18), respectively.

The major limitation of this study is relatively small sample size. At least 53 alleles on *RNU2-1* copy numbers have been identified thus far (19); therefore, our sample size was too small to analyze all possible copy numbers. Tessereau *et al* estimated *RNU2-1* copy numbers based on data from public genomics databases (20). Schaap *et al* analyzed *RNU2-1* copy numbers using DNA from 270 individuals by pulsed-field gel electrophoresis and Southern blot (19). We drew histograms of *RNU2-1* copy numbers using their data supplied as the additional file 3 in reference (19) or our dataset and then reconfirmed whether the sample size impacted on distribution range of copy number. The distribution range of copy number in this study was consistent with that in these two reports, although our sample size was quite smaller than that in these two reports.

The second major limitation of this study is that age was significantly different between the patient and HC group. We performed stepwise regression analysis and correlation analysis to examine whether age affected serum miR-1246 levels. No significant correlation was observed between age and serum miR-1246 levels in both HC and patients with lung adenocarcinoma; thus, we concluded that the significant difference in age distribution between the patients and HC did not affect the results of this study. However, several age-dependent serum microRNAs were reported in large cohort studies (31), and further studies with increased sample size will be needed.

In this study, we analyzed serum levels of miR-1246 and copy number of *RNU2-1* and found that copy number polymorphism of *RNU2-1* has no effect on serum levels of miR-1246 as a biomarker for lung adenocarcinoma.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MU and TA conducted the experiments and wrote the manuscript. MU and TA designed the study and interpreted the experimental results. SS performed the pathological diagnoses of the patients. SS and YM prepared the specimens. MU and TA confirmed the authenticity of the raw data. All of the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study's protocol was approved by the Ethics Committees of the Faculty of Health Sciences, Kyorin University (approval nos. 2022-30 and 2023-22) and Kanagawa Cancer Center (approval no. 2023epidemiology-84). Signed informed consent was obtained from all of the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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