5'-isomiR is the most abundant sequence of miR-1246, a candidate biomarker of lung cancer, in serum

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Abstract. MicroRNA (miRNA/miR) 5'-isoforms (5'-isomiRs) differ from canonical sequences registered in the microRNA database in the length of their 5' ends. The 'seed sequence' of miRNAs that bind to target mRNAs is 2-8 nucleotides from the 5' end; thus, shifts at the 5' end can cause a 'seed shift'. Accumulating data from miRNA deep sequencing have revealed that, in a substantial number of miRNAs, sequences corresponding to specific isomiRs, not the canonical form, are the most abundant. Studies have so far focused on circulating miRNAs as either markers or intercellular communication factors. miR-1246 is abundant in the serum and is a candidate diagnostic and prognostic marker for esophageal squamous cell carcinoma, pancreatic cancer, hepatocellular carcinoma, colorectal adenocarcinoma and non-small cell lung cancer (NSCLC). The present study analyzed the 5'-end of serum miR-1246 by fragment analysis and found that a 5'-isomiR, which is two bases shorter than the canonical sequence, was the most abundant sequence in patients with NSCLC as well as healthy donors. To quantify the 5'-isomiR, 5'-isomiR-specific primers based on primers for allele specific-PCR were used, primarily because commercially available methods for miRNA Reverse transcription-quantitative PCR cannot discriminate among sequences, especially those located at the 5' end of miRNA. The total miR-1246 levels were significantly increased in patients with NSCLC; by contrast, the level of the canonical sequence was significantly decreased. Significant positive correlations were observed between the total miR-1246 levels and the 5'-isomiR levels, but not that of the canonical sequence. These results imply that the increase in levels of serum miR-1246 in patients with NSCLC depends on increase of the 5'-isomiR.

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Introduction

MicroRNA (miRNA/miR) isoforms (isomiRs) with 5' or 3' end lengths differ from those of the canonical sequences registered in the microRNA database and can be detected using next-generation sequencing of small RNAs (1,2). Systematic analyses have revealed that these isoforms are not solely degradation intermediates but also enable recruitment to the RNA-induced silencing complex to suppress target mRNAs (3-5). IsomiRs are generated from a unique miRNA precursor (pre-miRNA) during RNA processing and have been detected in blood, urine and milk (6-10). Accumulating data have revealed that, in a substantial number of miRNAs, sequences corresponding to specific isomiRs, not the canonical form, are the most abundant (3,11-15). In next-generation sequencing, sequences of 5'-isomiRs are observed at a lower frequency (5-15%) than that of 3' isomiRs (40-50%) (16). A canonical type and relevant isomiRs derived from a pre-miRNA can function cooperatively on common target mRNAs based on different affinities (17,18).

The 'seed sequence', used by miRNAs to bind to the 3'-untranslated regions of target mRNAs, is located 2-8 nucleotides from the 5' end of the miRNAs; consequently, shifts in the 5' end can cause seed shifts (19,20). In fact, some naturally co-expressed 5'-isomiRs can act on individual target mRNAs that are distinct from those of the canonical type (5,18,21). In such cases, it is necessary to discriminate and quantify the specific 5'-isomiR. However, in deep sequencing, reads of relevant isomiRs are generally included in the total number of reads for the miRNA. In addition, the individuality of such 5'-isomiRs is currently ignored in reverse transcription-quantitative (RT-q) PCR as commercially available protocols for miRNA are unable to discriminate among sequences based on the 5' end, although these can identify a single nucleotide difference in the center of the miRNA (22-25).

miR-1246 is abundant in serum exosomes and could serve as a candidate marker for various cancers (26-30). Using RNA deep sequencing, the authors previously identified a 5'-isomiR of miR-1246, two bases shorter than the canonical sequence, which was extremely abundant in the serum of a patient with non-small cell lung cancer (NSCLC) and increased with recurrence (31). The present study analyzed the 5' end-sequence of miR-1246 in the sera of 20 patients with NSCLC using fragment analysis and 5' end-specific RT-qPCR.

Materials and methods

Patients and clinical specimens. The protocol of the present study was approved by the Ethics Committee of the Faculty of Medicine (approval no. H26-010) and the Ethics Committee of the Faculty of Health Sciences (approval no. 2019-45), Kyorin University. All Japanese participants provided signed informed consent. Serum samples were collected between October 2014 and May 2016 at the Kyorin University Hospital, Japan, from 20 patients with non-small cell lung cancer (NSCLC) and 20 cancer-free control participants (Table I). Histological typing and staging of the tumors were performed according to the World Health Organization criteria (32) and the seventh edition of TNM classification of malignant tumors (33), respectively. Blood samples were collected prior to all therapeutic procedures. Peripheral blood was collected in VP-AS109K Vacutainer tubes (Terumo Corporation), kept at room temperature (20-25˚C) for 30 min and then centrifuged at 1,500 x g for 10 min at 4˚C to separate the serum. Thereafter, the serum was 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. Pooled serum was prepared by mixing equal volumes of serum from 55 healthy individuals [male: n=13, female: n=42; age (mean): 40.1 years].

Target prediction for isomiRs. Target prediction for miR-1246 isomiRs was performed using miRDB-Custom Prediction (https://mirdb.org/custom.html).

Oligonucleotides. The oligonucleotides used in the present study are listed in Table II. Primers were synthesized by Life Technologies Japan Ltd. and RNA and chimera adaptors were synthesized by Sigma-Aldrich (Merck KGaA). The notation of isomiRs was performed according to the method reported by Telonis *et al* (34). The canonical type (miR-1246 |0|0|) and 5'-isomiRs (miR-1246 |-1|0| and miR-1246 |-2|0|) of *Homo sapiens* miR-1246 and *Caenorhabditis elegans* cel-miR-39-3p, were 5'-phosphorylated. The chimera 5' adaptors, Adp1 (protocol I) and Adp2 (protocol II), were 5'-biotinylated and two ribonucleotides were added at the 3' ends. The PCR reverse primers, used in the allele-specific PCR (AS-PCR) for 5' isomiR quantification, contained one mismatch nucleotide at the 3' region to prevent cross reactions (35).

Serum RNA extraction. The miRNeasy Serum/Plasma kit (Qiagen GmbH) 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 serum sample in RT-qPCR as a spike-in control. RNA was extracted according to the manufacturer's instructions with minor modifications: The volume of RNase-free H_2O used to elute the RNA was changed to 28 μl.

Fragment analysis of relevant 5'-isomiRs. A total of 2 μl of 10 X T4 RNA ligation buffer, 10 μl of 50% PEG 6000 (Nacalai Tesque, Inc.), 1.2 μl of 0.1% BSA, 1 μl of 5 μM 5' adaptor Adp1 (protocol I) or Adp2 (protocol II), 0.4 μl of 40 U/μl recombinant RNase inhibitor (Takara Bio, Inc.), 0.4 μl of 40 U/μl T4 RNA ligase (Takara Bio, Inc.) and 5 μl of serum RNA were mixed in a 1.5 ml tube and incubated at 15˚C for 1 h. The 5'-adaptor-ligated RNAs were purified using Magnosphere MS300/Streptavidin magnetic beads (JSR Life Sciences) and resuspended in 20 μl of RNase-free H_2O in protocol I. Then 10 μl of adaptor-ligated RNA was mixed with 3 μl of RNase-free H_2O, 1 μl of 5 μM RT primer (1246 FRG-RT or cel-39 FRG-RT) and 1 μl of 10 μM dNTPs, incubated at 65˚C for 5 min and then placed on ice for 2 min. Thereafter, 4 μl of 5X PrimeScript II buffer, 0.5 μl of 40 U/μl recombinant RNase inhibitor and 0.5 μl of 200 U/μl PrimeScript II reverse transcriptase (Takara Bio, Inc.) were added to the mixture and incubated at 48˚C for 45 min. In protocol I, magnetic bead-bound DNA-RNA double-strands were captured and single-stranded cDNA was obtained via denaturation at 95˚C for 5 min. In protocol II, all of the purification steps using magnetic beads were omitted to simplify the procedure. PCR was performed using Takara Taq Hot Start Version (Takara Bio, Inc.), with 30 reaction cycles as follows: 98˚C for 10 sec, 55˚C for 30 sec and 72˚C for 1 min. The primer pairs Adp1-F/1246 FRG-R or Adp1-F/Cel-39 FRG-R and Adp2-F/U2 FRG-R were used in protocols I and II, respectively (Table II). Following verification by 4% agarose gel electrophoresis and ethidium bromide staining, DNA fragment analysis was performed using the Capillary Sequencer ABI3730XL (Thermo Fisher Scientific, Inc.) at Macrogen Japan Corporation. The Peak Scanner (Thermo Fisher Scientific, Inc.) was used to analyze the size and relative fluorescent units (RFUs) of the fragments.

RT-qPCR. The MiR-X miRNA First-Strand Synthesis and TB Green qRT-PCR systems (Takara Bio, Inc.) were used to quantify the total amount of miR-1246. cDNA was synthesized according to the manufacturer's instructions. In brief, 5 μl of 2X mRQ buffer, 3.75 μl of RNA sample and 1.25 μl of the mRQ Enzyme Mix were added to a 0.2 ml tube, incubated at 37˚C for 1 h and then inactivated at 85˚C for 5 min. Thereafter, 90 μl of DNase-RNase-free H_2O was added to the solution. The primers, U2 miR-XF or cel-39 miR-XF, were used as forward primers (Table II). A two-step qPCR (Takara Bio, Inc. protocol) was performed in duplicate on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.) as follows: 95˚C for 10 sec, followed by 40 cycles of 95˚C for 4 sec and 60˚C for 32 sec. The $2^{-\Delta\Delta Cq}$ method was used for the relative quantification of miRNAs (36) as follows: ΔCq=(Cq of miR-1246)-(Cq of spike-in control cel-miR-39-3p).

For the 5'-end-specific quantification of miR-1246, cDNAs synthesized using protocol II of fragment analysis were diluted and used as follows: 7.8 μl of DNase-RNase-free H_2O, 10 μl of 2X TB Green Advantage Premix (Takara Bio, Inc.), 0.4 μl of 50x Rox Reference Dye LMP, 0.4 μl of Adp2-F, 0.4 μl of 5'-isomiR-specific primers (c1-R, c2-R, i1-R, i2-R, or Cel-junc-R; Table II) and 1 μl of the cDNA were added to each well. Then two-step qPCR was performed, as described in the previous section. The melting curves were verified after completion of the reaction. The $2^{-\Delta\Delta Cq}$ method was used for the relative quantification of miRNAs (36).

Statistical analyses. The data were presented as the mean ± SEM or SD. Fisher's exact probability test was performed to compare the demographic features between patients with NSCLC and the control group. Mann-Whitney

Table I. Characteristics of patients with NSCLC and control subjects.

Characteristics	No. of patients with NSCLC (%)	No. of control subjects (%)	P-value
Total	20	20	
Age, years			
≤60	9 (45.0)	7 (35.0)	0.374
>60	11 (55.0)	13 (65.0)	
Sex			0.257
Male	14 (70.0)	11 (55.0)	
Female	6 (30.0)	9 (45.0)	
Smoking status			0.048
Former/never	10 (50.0)	16 (80.0)	
Current	10 (50.0)	4 (20.0)	
Lung cancer stage			
III	3 (15.0)		
IV	17 (85.0)		
Type of carcinoma			
AC	20 (100.0)		

Fisher's exact probability test was used. Age (mean ± SD); NSCLC, 60.8±15.2; control, 62.7±13.6. AC, adenocarcinoma; NSCLC, non-small cell lung cancer.

U-test was performed to compare the miRNA levels between the patient and control groups. Spearman's rank correlation coefficient test was used for correlation analysis. Statcel3 software (Ohmsha, Ltd.) was used for all statistical analyses. All P-values were two-sided and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Simultaneous detection of relevant 5'-isomiRs in fragment analysis. A description of this procedure is presented in Fig. 1. Chemically synthesized miR-1246 was used to demonstrate this procedure. miR-1246|-1|0| and miR-1246|-2|0| are one and two bases shorter than the canonical sequence at the 5'-end, respectively (Table II). The cDNAs synthesized according to the procedure, shown in the top panel of Fig. 1A, were amplified and subjected to fragment analysis (Fig. 1B). The expected lengths of the PCR fragments derived from miR-1246|-2|0|, miR-1246|-1|0|, miR-1246|0|0| (canonical) and cel-miR-39-3p, using protocol I were 78, 79, 80 and 82 bp, respectively. Specific peaks of the three 5'-isomiRs were detected in lanes 1 to 3. In lanes 4 and 5, a mixture of miR-1246|-2|0|, miR-1246|-1|0|, miR-1246|0|0| and cel-miR-39-3p, was observed. Peaks of miR-1246|-2|0|, miR-1246|-1|0| and miR-1246|0|0| (lane 4) and cel-miR-39-3p peak (lane 5) were detected using primers specific for miR-1246 (lane 4) and cel-miR-39-3p (lane 5), respectively. In addition, the protocol was confirmed using a chemically synthesized cDNA (Adp1-miR1246|0|0|-cDNA), which has the same sequence as the cDNA synthesized using the 5'-adaptor-ligated miR-1246|0|0| (Table II and Fig. S1). The relevant 5'-isomiRs were detected as unique peaks specifically and simultaneously during fragment analysis using this protocol.

Fragment analysis of serum miR-1246. The present study examined the 5'-isomiRs of miR-1246 in pooled serum samples obtained from healthy volunteers using fragment analysis (Figs. 1C and S2). The peaks detected in fragment analysis of the pooled sera were the same as those for the chemically synthesized miR-1246|0|0|, miR-1246|-1|0| and miR-1246|-2|0| (Fig. S2). It was assumed that various isomiRs of miR-1246, each with a unique pairing of the 5' and 3' ends, existed in the sera. During fragment analysis of serum miRNAs, peaks were derived from various types of isomiRs that had the same 5' ends as the marker, but variable 3' ends. Therefore, the 5'-isomiR groups were referred to as 5' isomiR (0), 5' isomiR (-1), or 5' isomiR (-2). Semi-quantification of the 5'-isomiR-groups in the pooled sera was attempted by fragment analysis using chemically synthesized miR-1246|0|0| after verifying an optimal detection range (Fig. S3). RFUs of 5' isomiR (-2), 5' isomiR (-1) and 5' (0) were 23963.3±1453.1, 9503.3±423.7 and 1648.7±114.7, respectively (Fig. 1C). Substantial levels of 5'-isomiR (-1) and 5'-isomiR (-2) were detected in the pooled sera.

Next, the ratio of each 5'-isomiR of miR-1246 in the serum samples of patients with NSCLC as well as those of healthy controls was analyzed (Fig. 1D). The 5' isomiR (-2) was the most abundant in the control and NSCLC patient groups, with ratios of 0.525±0.083 and 0.581±0.052, respectively, followed by that of 5' isomiR (-1) (0.312±0.102 and 0.260±0.032, respectively). By contrast, 5' (0) was low in both groups, with ratios of 0.069±0.040 and 0.062±0.039 in the control and patients with NSCLC groups, respectively.

Evaluation of qPCR primers in specific quantification of miR-1246 5'-isomiRs. The specificity of the qPCR primers to quantify each 5'-isomiR of miR-1246 was verified as shown

Table II. Oligonucleotides used in the present study.

A, RNAs (5'-Phospholilated, 3'-OH)	
Name	Sequence (5'-3')[a,b]
miR1246 |0|0|	AAUGGAUUUUUGGAGCAGG
miR1246 |-1|0|	AUGGAUUUUUGGAGCAGG
miR1246 |-2|0|	UGGAUUUUUGGAGCAGG
cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG
B, 5' adaptors (5'-Biotinylated, 3'-OH)	
Name	Sequence (5'-3')[a,b]
Adp1	CTACGACTACATCCCAGGCTCCATAACTCCCACACACC-AGAACGAACCrArC
Adp2	CTACGACTACAACGATAACTCGCACACACCAGAACGAArCrC
C, RT primers	
Name	Sequence (5'-3')[a,b]
1246 FRG-RT	CCTGCTCCAAAAATCC
cel-39 FRG-RT	CAAGCTGATTTACACCC
D, PCR primers	
Name	Sequence (5'-3')[a,b]
Adp1-F	cccacCTACGACTACATCCCAG
1246 FRG-R	FAM-cgcataCCTGCTCCAAAAATCCA
Cel-39 FRG-R	FAM-gcataCAAGCTGATTTACACCCG
Adp2-F	cacCTACGACTACAACGATAACTCG
U2 FRG-R	FAM-gcataCCTGCTCCAAAAATCCA
c1-R	ACCCTGCTCCAAAAATCCATTGG
c2-R	CCTGCTCCAAAAATCCGTTGG
i1-R	ACCCTGCTCCAAAAATCGATGG
i2-R	ACCCTGCTCCAAAAATCGAGG
Cel-junc-R	AAGCTGATTTACACCCGATGAGG
U2 miR-X F	CCAATGGATTTTTGGAGCAGG
cel-39 miR-X F	CACCGGGTGTAAATCAGCTTG
E, DNA	
Name	Sequence (5'-3')[a,b]
Adp1-miR1246|0|0|-cDNA	CCTGCTCCAAAAATCCATTGTGGTTCGTTCTGGTGTGT-GGGAGTTATGGAGCCTGGGATGTAGTCGTAG

[a]rN, ribonucleotides; [b]underline, mismatch nucleotide.

in Table III and Fig. 2. These primers were designed based on AS-PCR (Fig. 1A and Table II). A single mismatch nucleotide was introduced at the 3' region of these primers to reduce the cross reaction. Reverse primers c1-R, i1-R and i2-R were designed to specifically amplify the chemically synthesized miR-1246|0|0|, miR-1246|-1|0| and miR-1246|-2|0|, respectively. Amplification of these targets by the c1-R, i1-R and i2-R reverse primers generated Cq values of 23.36, 23.65 and

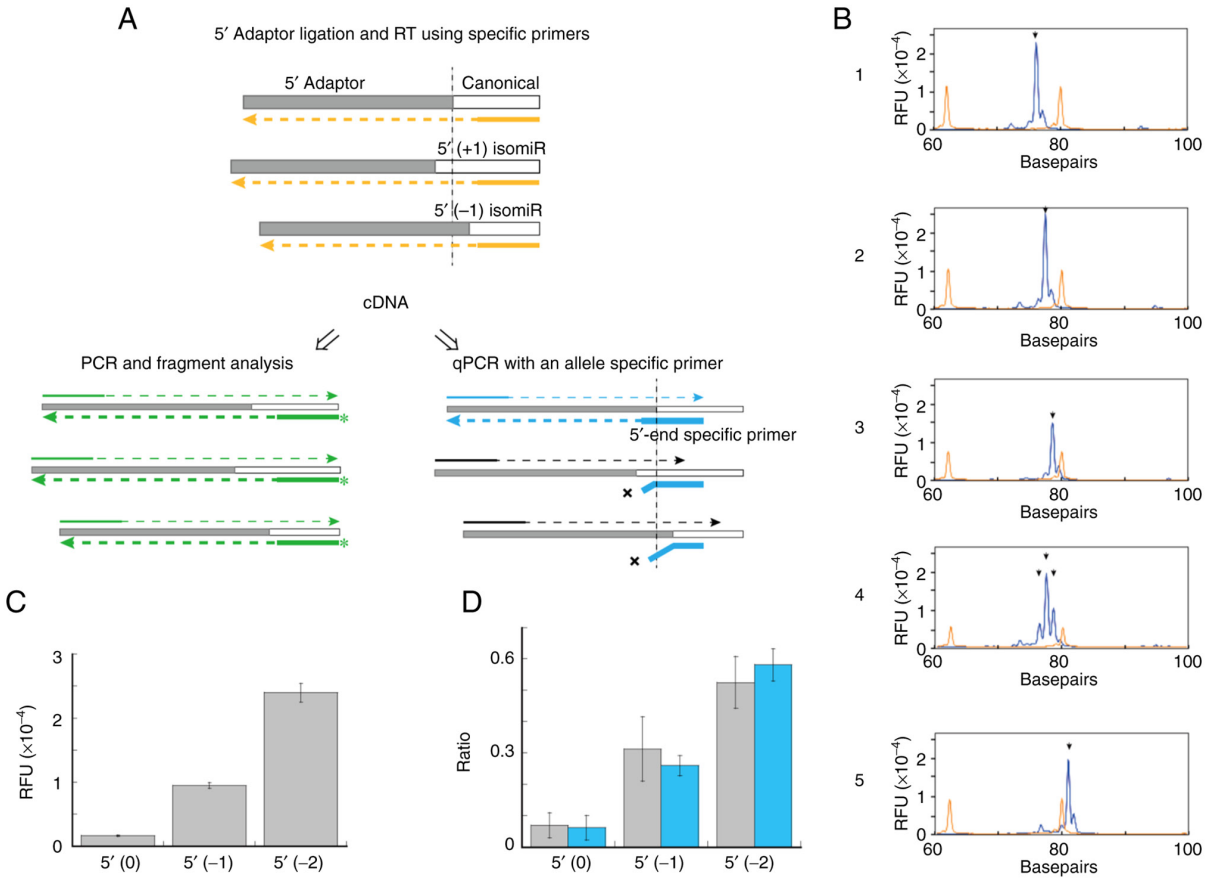

Figure 1. Depiction of procedure used to analyze 5'-isomiRs of miR-1246. (A) Schematic representation of fragment analysis of relevant 5'-isomiRs and 5'-end-specific quantification of miR-1246. miRNAs were ligated to a 5'-adaptor and cDNAs were synthesized using miR-1246-specific RT primers (orange broken lines). (Left) PCR fragments of specific lengths derived from individual 5'-isomiRs (green broken lines) during fragment analysis. (Right) The cDNAs used in 5'-isomiR-specific miRNA quantification. The 3' end of the PCR reverse primer (thick blue line) was specific to the sequence of a junction of the 5'-adaptor and a 5'-isomiR. (B) Peaks corresponding to chemically synthesized miR-1246 5'-isomiRs observed during fragment analysis. Arrowheads indicate expected peaks. Lane 1, miR-1246|-2|0|; lane 2, miR-1246|-1|0|; lane 3, miR-1246|0|0|; lanes 4 and 5, a mixture of miR-1246|-2|0|, miR-1246|-1|0|, miR-1246|0|0| and cel-miR-39-3p. RT primers: lanes 1-4, 1246 FRG-RT; lane 5, cel-39 FRG-RT. PCR primers: lanes 1-4, Adp1-F/1246 FRG-R; lane 5, Adp1-F/Cel-39 FRG-R. Protocol I was used. (C) Fragment analysis of a pooled serum sample. RFUs corresponding to specific peaks of miR-1246 isomiRs are shown. For descriptions of 5' isomiR (0), 5' isomiR (-1) and 5' isomiR (-2), please refer to the text. Three independent experiments were performed. Data are presented as mean ± SE. (D) Fragment analysis of serum samples from healthy controls and patients with NSCLC. Ratio of (RFU of each 5'-isomiR) to [sum RFU of 5' isomiR (-3), 5' isomiR (-2), 5' isomiR (-1), 5' isomiR (0) and 5' isomiR (+1)] is shown. Gray, healthy controls; Blue, patients with NSCLC. Data are presented as mean ± SD and Protocol II was used (Please see the Methods for details). isomiRs, miRisoforms; miR/miRNA, microRNA; RFUs, relative fluorescent units; NSCLC, non-small cell lung cancer.

24.18, respectively (Table III). By contrast, the Cq values for non-specific targets ranged from 27.39 to 31.82. The values in Table III are represented by a graph in Fig. 2A. The reverse primers c1-R, i1-R and i2-R were specific to their genuine targets and their levels of cross-reactivity to other relevant 5'-isomiRs were very low. In addition, a mixture of miR-1246|0|0|, miR-1246|-1|0| and miR-1246|-2|0|, at 2 amol/μl each, was quantified using c1-R, i1-R, or i2-R (Table IV). Similar Cq values were obtained for each RT primer. A detection limit of 0.015625 amol/μl was obtained (R=0.998; Fig. 2B).

Quantification of miR-1246 5'-isomiRs in serum samples by RT-qPCR. The 5'-end-specific primers were first evaluated by qPCR of the pooled serum samples. In case of the 5'-isomiR-specific primer, c1-R, a single peak was observed in the melting curve of qPCR when chemically synthesized RNA was used, whereas two peaks were observed when serum samples were used. Therefore, c2-R was used as a specific primer for 5' isomiR (0) in qPCR of serum miRNAs (Table II). The specificity of c2-R in qPCR is shown in Table III. The sera of 20 patients with NSCLC and that of 20 control subjects were analyzed using RT-qPCR (Fig. 3). Köhler *et al* (37) reported higher level of total miR-1246 in the sera of patients with NSCLC. Therefore, the present study first quantified the levels of miR-1246 in these sera using the MiR-X miRNA First-Strand Synthesis and TB Green qRT-PCR system, which is a poly A-tailing-mediated RT-qPCR method. Total miR-1246 levels were significantly higher in patients with NSCLC (P=0.0047).

Next, levels of the 5'-isomiR (0), 5'-isomiR (-1) and 5'-isomiR (-2) of miR-1246 were quantified and compared between the patient and control groups (Fig. 3). The levels of 5'-isomiR (0) and 5'-isomiR (-1) were significantly lower in the patient group with P-values of 0.00080 and 0.0053, respectively. By contrast, 5'-isomiR (-2) showed a tendency to be increased in the patient group; however, the increase was not statistically significant (P=0.11). A correlation analysis was performed between the total level of miR-1246 and the level of 5'-isomiR (-2) as shown in Fig. 4. Significant positive correlations were

Table III. Evaluation of specificities of 5'-end-specific primers using chemically synthesized miR-1246 5'-isomiRs.

	Reverse primers [specificity]			
Synthesized miRNA	c1-R [canonical]	i1-R [5' (-1)]	i2-R [5' (-2)]	c2-R [canonical]
miR-1246|0|0|	23.36±0.18	27.39±0.81	29.75±0.70	23.52±0.21
miR-1246|-1|0|	28.25±0.11	23.65±0.05	29.32±1.12	28.04±0.58
miR-1246|-2|0|	31.82±0.10	28.38±1.73	24.18±0.03	27.31±0.95

Three independent experiments were performed. Data are presented as Cq means ± SEs. isomiRs, miRisoforms; miR/miRNA, microRNA.

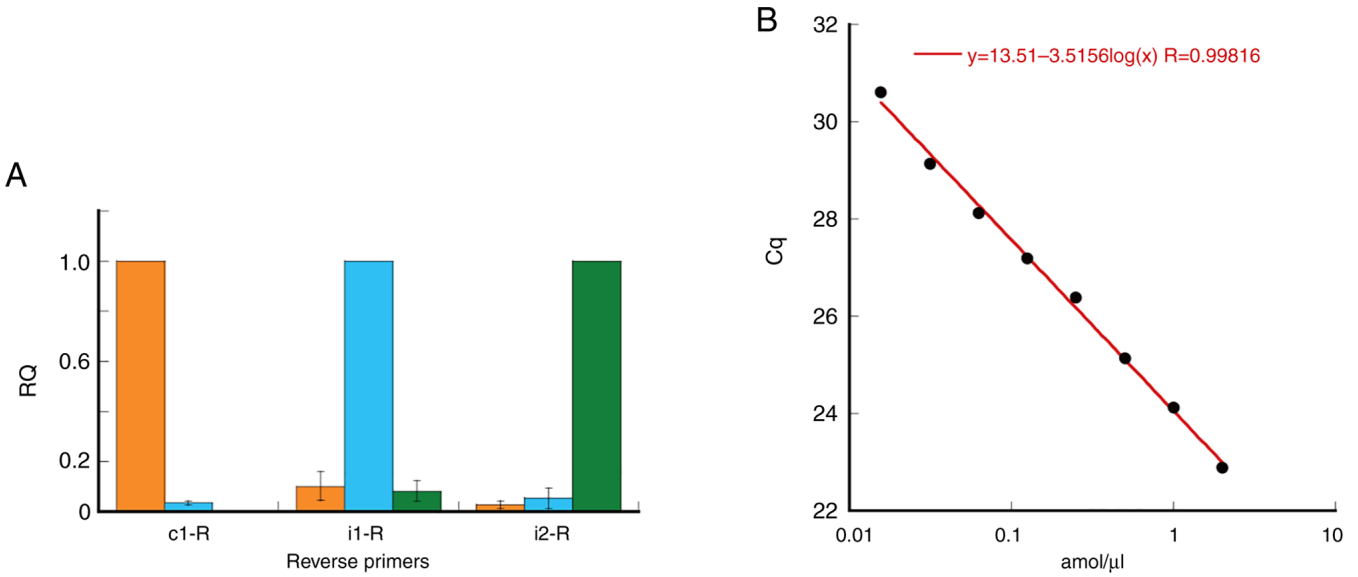

Figure 2. Evaluation of qPCR primers in specific quantification of miR-1246 5'-isomiRs. (A) The relative quantity (RQ) using values in Table III. Orange bar, miR-1246|0|0|; blue bar, miR-1246|-1|0|; green bar, miR-1246|-2|0|. (B) Detection limit of the 5'-isomiR-specific quantification. Two-fold serial dilutions of a 2 amol/µl solution of miR-1246|0|0| were quantified using c1-R. The regression coefficient was 0.998 and Protocol II was used (Please see the Methods for details). qPCR, quantitative PCR; miR/miRNA, microRNA; isomiRs, miRisoforms.

observed between the total level of miR-1246 and the level of 5'-isomiR (-2) in the control (ϱ=0.74, P=0.0012) and patient group (ϱ=0.60, P=0.0087).

Discussion

Circulating miRNAs are attractive candidate biomarkers for the diagnosis and prognosis of cancer and numerous publications during the last decade have led to high expectations for their use. However, this remains challenging due to the low reproducibility of their quantification (24,25). Difficulty in reproducible quantification is caused by the presence of isomiRs. Further, the ability of quantification platforms to discriminate sequences at the ends of miRNAs is variable. Neither of the two commercially available miRNA quantification methods, i.e., stem-loop RT qPCR or poly A-tailing-mediated RT-qPCR, can accurately discriminate sequences at the ends of miRNAs (22,23). Two-tailed RT-qPCR is an excellent method for quantifying the total amounts of relevant isomiRs derived from a unique pre-miRNA (38). This method is efficient when the relevant isomiRs function cooperatively with common target mRNAs (5,17). However, the expression profiles of some isomiRs differ between cell and tissue types (3), races and sexes (39). These profiles can be used to distinguish between different cancer cell types and subtypes (34,40). Certain 5'-isomiRs can act on individual target mRNAs; therefore, 5'-end-specific quantification is required in such cases (5,18,21). Dumbbell PCR is a unique method that discriminates among terminal sequences of miRNAs using two dumbbell-shaped adapters (41); however, in the present study, it was difficult to design specific primers for serum miR-1246.

The present study first used a semi-quantitative method for fragment analysis to demonstrate the ratio of each 5'-isomiR in serum miR-1246 (Fig. 1A). The relevant 5'-isomiRs of miR-1246 were simultaneously reverse-transcribed and amplified using common primers. It is necessary that ligation efficiencies to a 5' adaptor be the same among the relevant 5'-isomiRs in semi-quantitative analysis. The Cq values of chemically synthesized miR-1246|-0|0|, miR-1246|-1|0| and miR-1246|-2|0| were almost similar when specific primers c1-R, i1-R and i2-R were used, respectively (Table III). The 5'-isomiR (-2) was the most abundant in the pooled serum from healthy volunteers and the sera of 20 patients with NSCLC

Table IV. Evaluation of specificities of 5'-end-specific primers using a mixture of chemically synthesized miR-1246 5'-isomiRs.

Synthesized miRNAs	Reverse primers		
	c1-R	i1-R	i2-R
Mixture (l0l0l, l-1l0l, l-2l0l)	23.47±0.21	24.02±0.18	24.14±0.11

Data are presented as Cq means ± SEs. isomiRs, miRisoforms; miR/miRNA, microRNA.

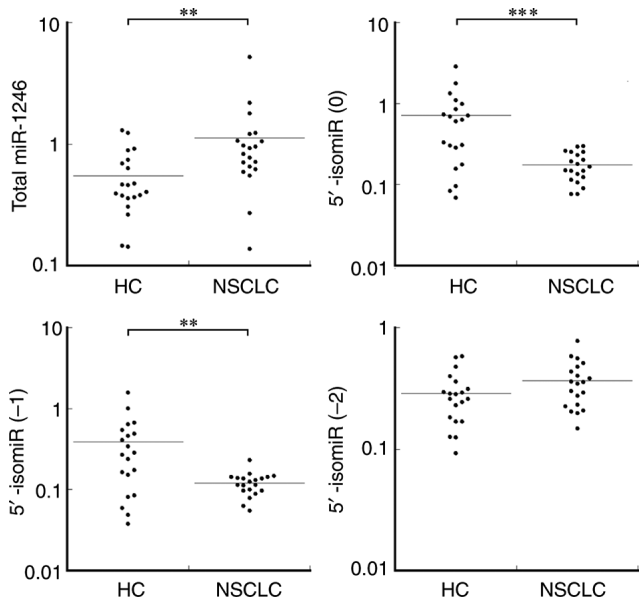

Figure 3. Quantification of miR-1246 in serum samples by RT-qPCR. The total levels of miR-1246 and that of each 5'-isomiR of miR-1246 were quantified. Mann-Whitney U-test was performed. **P<0.01, ***P<0.001. miR/miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HC, healthy control subjects; NSCLC, non-small cell lung cancer.

and healthy controls. The authors previously performed deep sequencing of serum RNA obtained from a patient with stage 1 NSCLC at three time points; prior to surgical resection, post-7 weeks and post-12 months (31). Recurrence was observed five months after the 12-month-sampling in this patient. In the present study, miR-1246l-2l0l was the most abundant and its levels increased markedly 12 months after surgical resection (Table SI). Using RNA sequencing, Umu *et al* (15) showed that miR-1290, another candidate marker of cancer possessing the same seed sequence as that of miR-1246l-2l0l, is expressed at high levels in human serum (42,43). In the present study, according to the RNA sequencing data, expression of miR-1290 in the serum of patients with NSCLC was very low.

miR-1246 is abundant in serum exosomes (15,26-30) and is a fragment of spliceosomal snRNA U2 (RNU2-1f) (44-46). The *RNU2-1* gene is located on chromosome 17 and exhibits copy number polymorphism (47-49). It is unclear how the transcription and processing of snRNA U2 are regulated. Using poly-A-tailing-mediated RT-qPCR, Köhler *et al* (37) showed

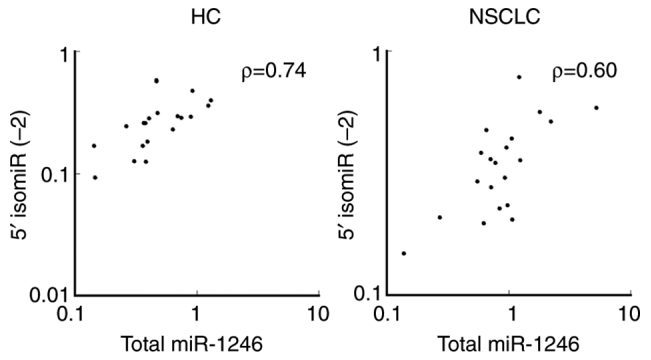

Figure 4. Correlation analysis between levels of total miR-1246 and 5'-isomiR (-2). NSCLC (P<0.01), HC (P<0.01). Spearman's rank correlation coefficient test was used. miR/miRNA, microRNA; NSCLC, non-small cell lung cancer; HC, healthy control subjects.

increased total amount of RNU2-1f in the sera of patients with NSCLC, which is consistent with the results of the present study (Fig. 3). Levels of miR-1246 5'-isomiR (-2) showed a tendency to be increased in patients with NSCLC than in control subjects, although the associated P-value was insignificant (Fig. 3). Significant positive correlations were observed between the total levels of miR-1246 and the levels of 5'-isomiR (-2) in control subjects and patients with NSCLC (Fig. 4). These results implied that an increase in total miR-1246 in patients with NSCLC depended on an increase in 5'-isomiR (-2) rather than an increase in canonical sequence. Additional samples will need to be analyzed to clarify the profiles and functions of each miR-1246 5'-isomiR. Differences in the expression profiles between race or sexes will need to be assessed as only Japanese participants were included in the present study. Future studies should also investigate the correlation between expression of total miR-1246 and each 5'-isomiR with the clinical features and survival. Additionally, the present study performed target prediction for miR-1246 isomiRs (Table SII). The predicted target genes differed significantly between miR-1246l0l0l and miR-1246l-2l0l, suggesting that it is essential to confirm the actual 5'-end of a focused miRNA to identify its real targets (22).

In conclusion, a 5'-isomiR of miR-1246, which is two bases shorter than the canonical sequence registered in the microRNA database, was the most abundant sequence in patients with NSCLC as well as in healthy donors. Significant positive correlations were observed between the total levels of miR-1246 and the level of the 5'-isomiR, but not that of the canonical sequence. These results suggested that the increase in levels of serum miR-1246 in patients with NSCLC depends on that of the 5'-isomiR.

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Availability of data and materials

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

Authors' contributions

TA conducted experiments and wrote the manuscript. TA and MU designed the study and interpreted experimental results. TA and MU confirm the authenticity of all the raw data. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Faculty of Medicine (approval no. H26-010) and the Ethics Committee of the Faculty of Health Sciences (approval no. 2019-45), Kyorin University. Signed informed consent was obtained from all the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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