Original Article

Exosome can prevent RNase from degrading microRNA in feces

Yoshikatsu Koga[1], Masahiro Yasunaga[1], Yoshihiro Moriya[2], Takayuki Akasu[2], Shin Fujita[2], Seiichiro Yamamoto[2], Yasuhiro Matsumura[1]

[1]*Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa;* [2]*Department of Surgery, National Cancer Center Hospital, Tokyo, Japan*

ABSTRACT

Background: Because the stability of miRNA in feces has not been clarified, we examined the stability of miRNA in feces.

Methods: RNase was added into culture media of HT-29 cells and fecal homogenates. The relative quantifications of miRNA were analyzed by real-time RT-PCR.

Results: Cellular miRNA or exosomal miRNA were protected from RNase by the cellular membrane or the exosome; meanwhile, free miRNA was degraded immediately and completely by RNase.

Conclusion: The present study revealed that exosome or cellular membrane could prevent RNase from degrading miRNA inside the exosome or cells even in a dreadful condition, as in feces.

KEY WORDS   exosome, colonocyte, fecal miRNA test, miRNA, cancer screening

Introduction

MicroRNAs (miRNAs), which are small (18-25 nucleotides) noncoding RNA molecules, regulate the activity of specific mRNA targets and play a major role in cancer. The function of miRNA is the downregulation of multiple target gene expressions by degrading the mRNA or blocking its translation into protein through RNA interference (1,2). The let-7, miR-34 family, miR-126, miR-143, miR-145, and the miR-200 family are considered to be tumor suppressor miRNAs in colorectal cancer (CRC) (3-7). Because the expression level of tumor suppressor miRNAs in cancer tissue was lower than in normal tissue, these tumor suppressor miRNAs may become candidates for future miRNA-based cancer therapy (8). On the other hand, since the expression level of the oncogenic miRNAs, such as miR-17-92 cluster, miR-21, and miR-135, in cancer tissue was higher than in normal tissue, these oncogenic miRNAs could be used for a marker of prognosis or poor response to chemotherapy (9-14).

Exosomes are nanoparticles, 50-100 nm in diameter, and are released from cells into extracellular matrixes through fusion of multivesicular bodies with the plasma membrane (15,16). Recent reports indicate that miRNAs are circulating stably in bloodstream wrapping in exosomes, which can prevent RNase from degrading the miRNAs (17-21). Therefore several methods for miRNA-based early cancer detection using serum, plasma, and urine are reported (21-23). Also, several studies are available of the possible use of the miRNA-based method for CRC screening in serum (24,25) and in feces (26).

We have been developing new screening methods for CRC by applying molecular biological tools to exfoliated colonocytes isolated from naturally evacuated feces (27-29). In the past few years especially, we have reported the fecal RNA test, including the CRC-related gene expression analysis (30) and the CRC-related miRNA expression analysis (31). Within this context, we investigate the

stability of miRNA in feces.

Materials and Methods

Cell line and fecal samples

The human colorectal cancer cell line HT-29 (American Tissue Culture Collection, Rockville, MD) was cultured in the Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B at 37°C in a humidified atmosphere of 5% CO2: 95% air.

Naturally evacuated fecal samples were obtained from 3 healthy volunteers with no endoscopical abnormalities. Volunteers were instructed to evacuate at home into a disposable 5 × 10-cm polystyrene tray (AsOne, Osaka, Japan) and to bring the fecal sample to our laboratory at 4°C. The samples were then immediately prepared for the next step.

Isolation of exfoliated colonocytes from feces using EpCAM beads

EpCAM (epithelial cell adhesion molecule) beads (JSR, Tsukuba, Japan), immunomagnetic beads conjugated with EpCAM monoclonal antibody (mAb) (clone B8-4), were used for isolation of colonocyte from feces (32).

Fecal samples were processed as described previously (28). Briefly, one gram of fecal sample was homogenized with a buffer (40 mL) consisting of Hanks' solution, 10% fetal bovine serum (FBS), and 25 mM HEPES buffer (pH 7.35) at 200 rpm for 1 min using a Stomacher system (Seward, Thetford, UK). The homogenate was filtered through a nylon filter (pore size, 512 μm), and following the addition of 40 μL of EpCAM beads, the sample mixture was incubated for 30 min under gentle rolling conditions at room temperature. The mixture on the magnet was incubated on a shaking platform for 15 min at room temperature. The supernatant was then removed, and the colonocytes in the pellet were stored at −80°C until RNA extraction.

Isolation of exosome from culture media or feces using CD63 beads

CD63 beads (JSR), immunomagnetic beads conjugated with CD63 mAb (R&D systems, Minneapolis, MN), were used for isolation of exosome from culture media or feces.

Ten microliters of CD63 beads were applied to 1 mL of culture media of HT-29 cells, and the sample mixture was incubated for 30 min under gentle rolling conditions at room temperature. The mixture on the magnet was incubated on a shaking platform for 15 min at room temperature. The supernatant was then removed, and the exosomes in the pellet were stored at −80°C until RNA extraction.

Isolation of exosome from feces was processed in the same manner as described above. The exosomes isolated from feces using CD63 beads were stored at −80°C until RNA extraction.

Extraction of total RNA

Fecal samples were homogenized as described previously (33,34), and total RNA was extracted from all homogenates using a miRNeasy Mini Kit (Qiagen, Valencia, CA), in accordance with the manufacturer's instructions. Briefly, one gram of feces was homogenized with 5 mL of Isogen (Nippon Gene, Toyama, Japan), using an IKA Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) at 6,000 rpm for 2 min. The homogenates were centrifuged at 15,000 rpm for 5 min at 4°C. The supernatants were transferred into a new tube, up to 5 mL more Isogen was added, and 1.5 mL of chloroform was then added.

HT-29 cells, exosomes isolated by CD63 beads, and colonocytes isolated by EpCAM beads were also homogenized with 1 mL of Isogen, and to the homogenates 0.2 mL of chloroform were added.

One milliliter of culture media was also homogenized with 3 mL of Isogen-LS (Nippon gene), and to the homogenates 0.2 mL of chloroform were added.

All of the tubes were shaken vigorously for 30 sec, and centrifuged at 15,000 rpm for 15 min at 4°C. The aqueous phase was transferred into a new tube. One-and-a-half volume of 100% ethanol was added, and the tube was vortexed for 15 sec. The mixture was poured onto a miRNeasy spin column (Qiagen), and the columns were centrifuged at 10,000 rpm for 15 sec at room temperature. The remaining steps were done according to the manufacturer's instructions. Each sample was eluted in 100 μL of RNase-free water. The total RNA was electrophoresed using a Cosmo-I microcapillary electrophoresis (Hitachi High-Technologies, Tokyo, Japan), and the concentrations of total RNA was determined using a NanoDrop UV spectrometer (LMS, Tokyo, Japan). The RNA samples were stored at −80°C until analysis.

cDNA synthesis and real-time RT-PCR

For miRNA expression analysis, cDNAs for U6, miR-16, and miR-21 were synthesized. For this purpose, we used the commercially available TaqMan MicroRNA Assay (Applied Biosystems, Foster, CA).

cDNA for miRNA was synthesized using a TaqMan MicroRNA RT Kit (Applied Biosystems) in accordance

with the manufacturer's instructions. The reaction mixture consisted of 2 μL (or 5 ng) of total RNA, 0.5 μL of 10 × RT buffer, 1 μL of 5 × RT primer, 0.05 μL of dNTPs (100 mM), 0.06 μL of RNase Inhibitor (20 U/μL), and 0.33 μL of MultiScribe Reverse Transcriptase (50 U/μL) in a final reaction volume of 5 μL.

The reaction mixture for real-time PCR consisted of 4 μL of a template cDNA, 10 μL of TaqMan Fast Universal PCR Master Mix (Applied Biosystems), and 1 μL of 20 × primer/probe mixture in a total reaction volume of 20 μL. Real-time RT-PCR was performed with precycling heat activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec and annealing/extension at 60°C for 30 sec in an Applied Biosystems 7500 Fast Real-Time PCR System.

Susceptible to RNase degradation

To evaluate the susceptibility to RNase, RNA extracted from HT-29 cells was treated using RNase (Qiagen, final concentration: 5 μg/mL) at 4°C or 37°C for 0, 5, 10, 20, and 30 min. After the treatment, all samples were electrophoresed using a Cosmo-I microcapillary electrophoresis, the concentrations of total RNA were evaluated using a NanoDrop UV spectrometer, and the expressions of miRNA from HT-29 cells were analyzed using real-time RT-PCR.

Analysis of RNA protection from RNase

HT-29 cells (5×10^5 cells) were plated into a 10-cm cell culture plate (Corning, Corning, NY). After an exchange for 10 mL of fresh medium the next day, HT-29 cells were cultured for 48 hr. The HT-29 cells were then incubated at 37°C for 0, 30, 60, and 90 min after addition of RNase (final concentration, 5 μg/mL). The culture media and cells were processed as described above, and free miRNA, exosomal miRNA, and cellular miRNA could be obtained. Three replicates were performed in each sample.

One gram of fecal sample from 3 volunteers was put into Stomacher Lab Blender Bags (Seward) and incubated at 37°C for 0, 30, 60, and 90 min after the addition of RNase (final concentration, 5 μg/mL). The fecal samples were processed, and fecal miRNA, exosomal miRNA, and colonocyte miRNA could be obtained as described above.

Statistical analysis

The miRNA expression analyses were conducted using the comparative Ct (threshold cycle) method. The relative quantification for each miRNA was analyzed using a two-sided t-test. Statistical analyses were performed using StatView Ver. 5 for Windows (Abacus Concepts, Berkeley, CA). $P<0.05$ was considered statistically significant.

Results

Degradation of naked RNA from HT-29 cells using RNase

Total RNA extracted from HT-29 cells was treated, using 5 μg/mL of RNase, and electrophoresed. Two peaks, 18S and 28S ribosomal RNA (rRNA), were observed in the total RNA without treatment of RNase (Fig 1A). On the other hand, no rRNA peak was observed in the total RNA treated with RNase. Small RNAs, including miRNA or degrading RNA, were observed in all samples. miRNA expressions treated with RNase at 4°C were significantly lower than those without treatment (U6: $P=0.002$; miR-16: $P=0.0006$; miR-21: $P=0.003$) (Fig 1B). Same as above, miRNA expressions treated with RNase at 37°C were significantly lower than those without treatment (U6: $P=0.003$; miR-16: $P=0.006$; miR-21: $P=0.01$) (Fig 1C). As a consequence, naked RNA was degraded by 5 μg/mL of RNase at both 4°C and 37°C for only 5 min.

miRNA protected by exosome or cellular membrane from RNase in HT-29 cells

To examine how miRNA was protected from RNase *in vitro*, we cultured HT-29 cells in the medium containing RNase; cellular miRNA extracted from the cells, exosomal miRNA from the exosomes, and free miRNA from the culture media were then analyzed. Cellular miRNA was sufficiently conserved under the treatment of RNase for 90 min (Fig 2A). Exosomal miRNA was conserved under the treatment of RNase for 30 min; however, the miRNA was degraded thereafter (Fig 2B). Free miRNA was degraded by the treatment of RNase within 30 min (Fig 2C). Cellular miRNA was sufficiently protected from RNase by cellular membrane. Exosomal miRNA was partially protected by exosome. On the other hand, free miRNA in the culture media was degraded immediately by RNase.

Effects of RNase on miRNA in exosome or colonocyte in feces

We also examined the susceptibility of miRNA to RNase degradation in feces. Colonocyte miRNA extracted from the fecal colonocyte, exosomal miRNA extracted from the fecal exosomes, and fecal miRNA extracted from the fecal homogenates were analyzed. Ct values of U6 in colonocyte miRNA, exosomal miRNA, and fecal miRNA without treatment of RNase were 31.14 (26.57-36.13) (mean (range)), 33.23 (30.40-35.15), and 32.60 (31.08-34.29), respectively (Table 1). Ct values of miR-16 were 28.60 (25.71-30.83), 29.69 (28.79-31.01), and 30.36 (29.47-31.05), respectively. Also, Ct values of miR-21 were 27.23 (23.83-29.00), 27.92 (26.27-30.46), and 29.32 (28.16-30.68), respectively. Colonocyte miRNA and exosomal miRNA

A
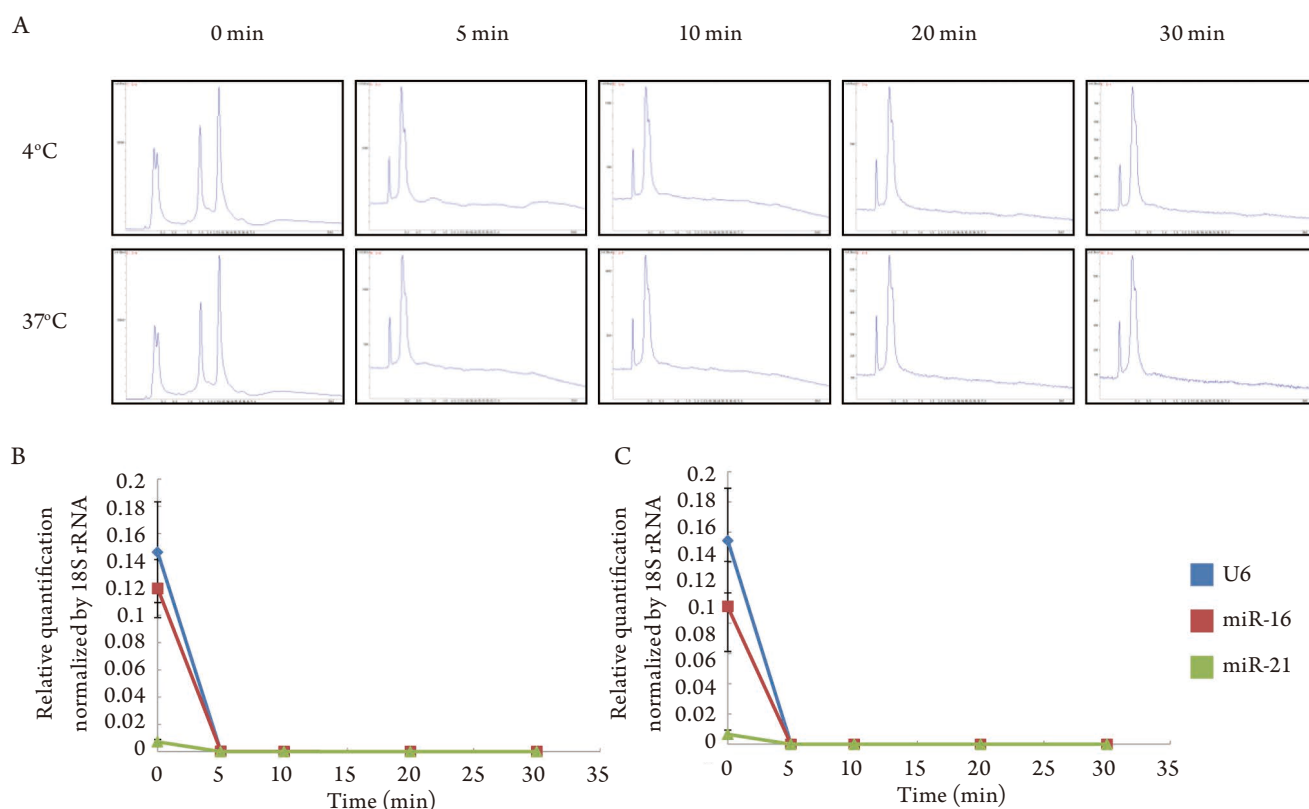
Figure 1 Degradation of naked RNA using RNase. (A) Electropherogram of total RNA treated with RNase. The total RNA is treated with 5 μg/mL of RNase for 0, 5, 10, 20, and 30 min at 4°C and 37°C. Two peaks, 18S and 28S ribosomal RNA (rRNA), are observed in total RNA without RNase treatment. (B) Relative quantification (RQ) of each miRNA treated with RNase at 4°C. The total RNA is treated with 5 μg/mL of RNase for 0, 5, 10, 20, and 30 min at 4°C. RQ of each miRNA is normalized by 18S rRNA (C) RQ of each miRNA treated with RNase at 37°C. The total RNA is treated with 5 μg/mL of RNase for 0, 5, 10, 20, and 30 min at 37°C. RQ of each miRNA is normalized by 18S rRNA.

were not susceptible to RNase degradation (Fig 3A and 3B). On the other hand, fecal miRNA was degraded efficiently by the treatment of RNase (Fig 3C). In the feces, miRNA was sufficiently protected from RNase by cellular membrane and exosome.

Discussion

In the clinical samples, RNA is degraded rapidly by RNase existing in any body fluids such as sweat, sputum, or blood. The effects of RNase should be, therefore, considered in the RNA-based analysis on clinical samples. Although several storage buffers inhibiting the effect of DNase and RNase were available, we have been investigating the CRC screening method based on the analysis using the colonocytes isolated from feces. In our preliminary study, the colonocytes could not be isolated from feces stored in the storage buffers. Therefore we have investigated the

suitable storage condition of fecal samples for our screening test.

Recently it was reported that miRNA was secreted from tumor cells via exosome and was transported to endothelial cells by paracrine induction (35). This indicates that exosome is not only a secretory tool, but that it also supports miRNA. We have been investigating the CRC screening method (30,31). And then we thought that fecal miRNA (free miRNA) from fecal homogenates, exosomal miRNA from fecal exosomes, and colonocyte miRNA from fecal colonocytes might be candidates for the fecal miRNA test. Exfoliated colonocytes were isolated from feces by EpCAM beads, using a previously published method (28,32). Exosomes could be isolated using both the centrifugation method (19,35) and the cell isolation method by anti-CD63 mAb conjugated immunomagnetic beads (36) In the present study, HT-29 cells cultured in the media containing RNase were analyzed, and fecal homogenates were treated
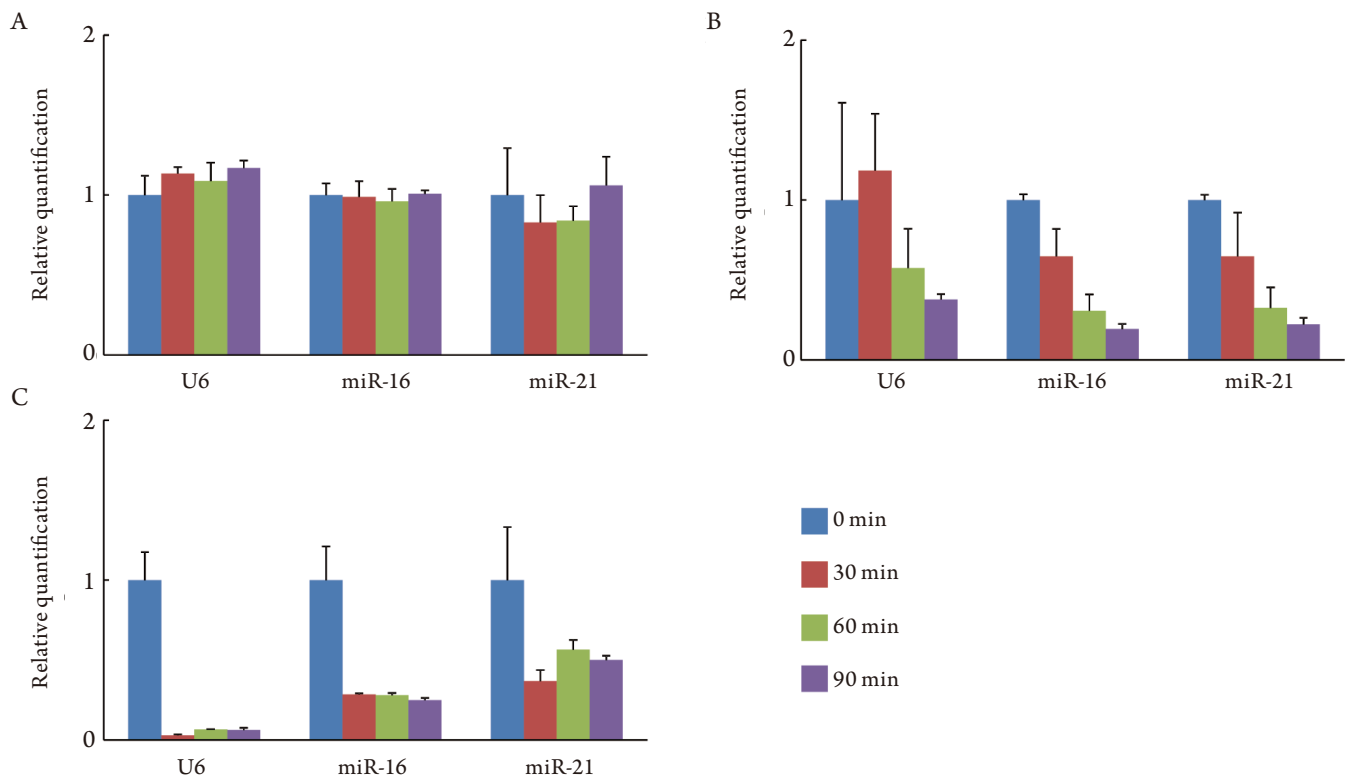
Figure 2 RQ of each miRNA in HT-29 cells treated with RNase. (A) RQ of each miRNA in cellular RNA treated with RNase. HT-29 cells are treated with 5 μg/mL of RNase for 0, 30, 60, and 90 min at 37°C. RQ of each group is compared with that of a no-treatment group. (B) RQ of each miRNA in exosomal RNA treated with RNase. Exosomes are treated with 5 μg/mL of RNase for 0, 30, 60, and 90 min at 37°C. RQ of each group is compared with that of a no-treatment group. (C) RQ of each miRNA in free RNA treated with RNase. Culture media are treated with 5 μg/mL of RNase for 0, 30, 60, and 90 min at 37°C. RQ of each group is compared with that of a no-treatment group. Mean ± SD.

Table 1 Ct value of each miRNA in colonocyte miRNA, exosomal miRNA, and fecal miRNA

	Ct value (Mean (range))		
miRNA	Colonocyte miRNA	Exosomal miRNA	Fecal miRNA
U6	31.14 (26.57-36.13)	33.23 (30.40-35.15)	32.60 (31.08-34.29)
miR-16	28.60 (25.71-30.83)	29.69 (28.79-31.01)	30.36 (29.47-31.05)
miR-21	27.23 (23.83-29.00)	27.92 (26.27-30.46)	29.32 (28.16-30.68)

by RNase. Although free miRNA (fecal miRNA) was degraded rapidly, cellular miRNA (colonocyte miRNA) was highly conserved. In the culture media, exosomal miRNA was conserved for a 30-min treatment of RNase, but degraded for a 90-min treatment. On the other hand, the fecal exosome could be conserved for a 90-min treatment of RNase. These indicated that cellular membrane prevented RNase from degrading miRNA in cells, but that the exosome partially prevented RNase from degrading miRNA in exosome.

In this study, U6, miR-16, and miR-21 were analyzed because U6 and miR-16 were used for internal control

as an expression of miRNA in several reports (31,37) and miR-21 was one of the miRNAs important for CRC carcinogenesis (38,39). The expression of miR-21 in the CRC tissue was higher than that in the normal colorectal mucosa; however, no significant difference was seen between the early stage of CRC and the advanced stage of CRC regarding the expression of miR-21 (31). Recently fecal-based RNA tests have been noticed because of their simplicity and cost-effectiveness (33,34,40), however, fecal miRNA was unstable under the existence of RNase. For the clinical use of fecal miRNA, it was therefore necessary to store the fecal sample under strict conditions. On the
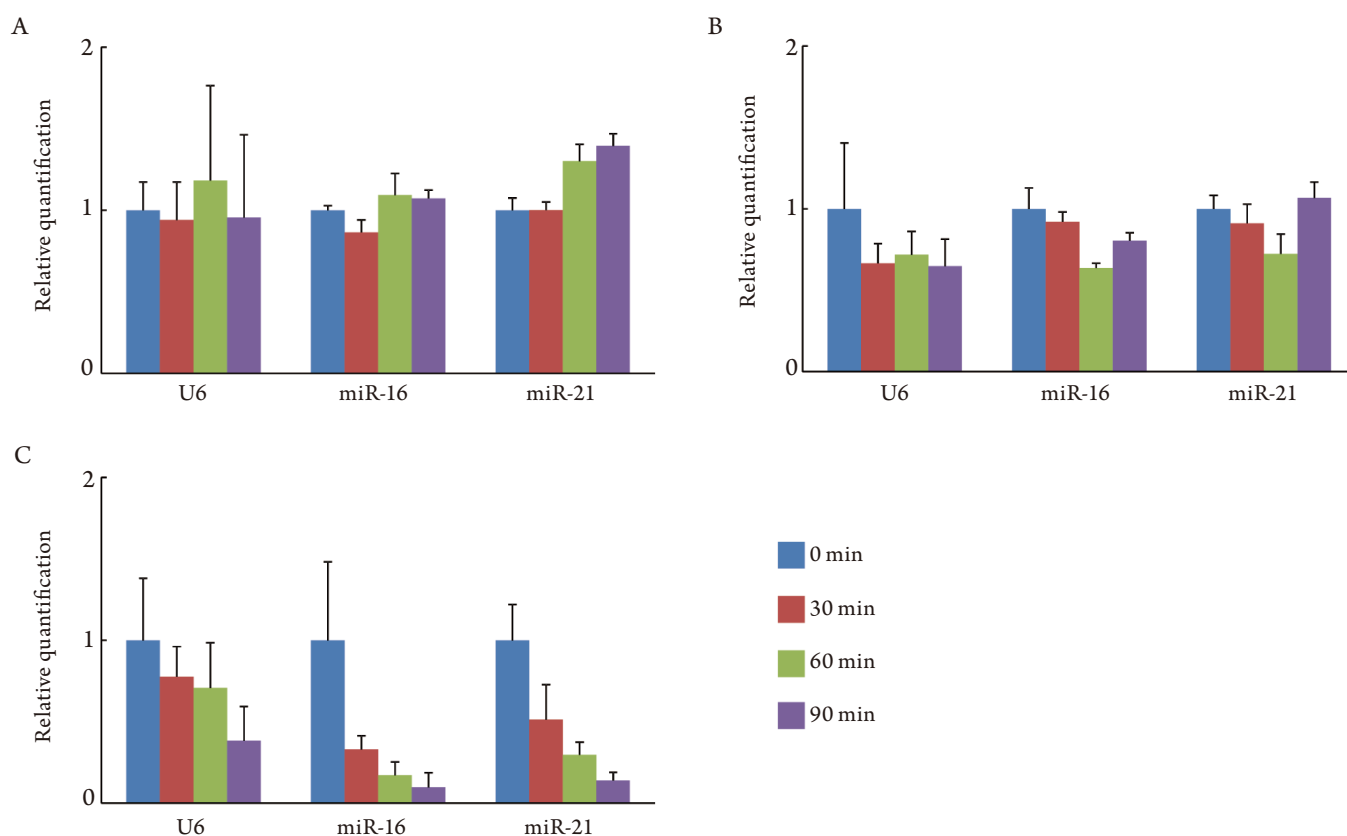
Figure 3 RQ of each miRNA in fecal samples treated with RNase. (A) RQ of each miRNA in cellular miRNA treated with RNase. Exfoliated colonocytes are treated with 5 μg/mL of RNase for 0, 30, 60, and 90 min at 37°C. RQ of each miRNA is compared with that of a no-treatment group. (B) RQ of each miRNA in exosomal miRNA treated with RNase. Exosomes are treated with 5 μg/mL of RNase for 0, 30, 60, and 90 min at 37°C. RQ of each group is compared with that of a no-treatment group. (C) RQ of each miRNA in fecal RNA treated with RNase. Fecal samples are treated with 5 μg/mL of RNase for 0, 30, 60, and 90 min at 37°C. RQ of each group is compared with that of a no-treatment group. Mean ± SD.

other hand, exosomal miRNA or colonocyte miRNA were protected from RNase by exosome or cellular membrane. This information may be important for the clinical use of fecal miRNA in future CRC mass screening. In the present study, we examined miRNA protection from RNase in fecal samples precisely and could show that exosomal miRNA is more stable than free miRNA in a deadful condition like in feces.

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