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Circulating muscle-specific microRNA, miR-206, as a potential diagnostic marker for rhabdomyosarcoma

Mitsuru Miyachi, Kunihiko Tsuchiya, Hideki Yoshida, Shigeki Yagyu, Ken Kikuchi, Akiko Misawa, Tomoko Iehara, Hajime Hosoi *

Department of Pediatrics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-Cho, Kawaramachi-Hirokoji, Kamigyo-Ku, Kyoto 602-8566, Japan

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ABSTRACT

Presently there is no serum biomarker of rhabdomyosarcoma (RMS). Several studies have shown that profiles of microRNA (miRNA) expression differ among tumor types. Here we evaluated the feasibility of using muscle-specific miRNAs (miR-1, -133a, -133b and -206) as biomarkers of RMS. Expression of muscle-specific miRNAs, especially miR-206, was significantly higher in RMS cell lines than in other tumor cell lines, as well as in RMS tumor specimens. Further, serum levels of muscle-specific miRNAs were significantly higher in patients with RMS tumors than in patients with non-RMS tumors. Normalized serum miR-206 expression level could be used to differentiate between RMS and non-RMS tumors, with sensitivity of 1.0 and specificity of 0.913. These results raise the possibility of using circulating muscle-specific miRNAs, especially miR-206, as landmark biomarkers for RMS.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma, accounting for about 5% of pediatric malignancies. The treatment strategy of rhabdomyosarcoma differs from that of other tumors. For example, gross total resection of the primary tumor before chemotherapy leads to an improvement of prognosis [1,2]. Thus, even tentative diagnosis of RMS before the pathological diagnosis can help surgeons to prepare for gross total resection. However, it is difficult to make a tentative diagnosis of RMS before surgery because no specific serum tumor marker has been identified in RMS so far and radiological findings provide little information for differentiating RMS from other small round tumors such as Ewing sarcoma family tumor, undifferentiated sarcoma and malignant lymphoma, each of whose treatment strategies vary. Thus, it is important to identify serum tumor markers and to make a tentative diagnosis of RMS before biopsy.

The recent discovery of small (16–29 nucleotides) non-protein-coding RNAs, called microRNAs (miRNAs), has provided new insights into cancer diagnosis. Several studies have shown that profiles of miRNA expression differ between normal tissue and tumor tissue and vary among tumor types [3–5].

Tumor-associated messenger RNAs (mRNAs) were found in serum and plasma of cancer patients [6,7]. However, these mRNAs were unstable and difficult to detect. Recent findings suggest that cell-free circulating miRNAs are stably and abundantly present in serum and plasma [8]. Tumor-specific miRNAs were found in the serum or plasma of colon, breast and prostate cancer patients [8–11].

A small number of muscle-specific miRNAs (miR-1, -133a, -133b and -206) have been identified and shown to have an important role in myogenesis, embryonic muscle growth and cardiac growth and function [12–14]. Other studies have shown that muscle-specific miRNAs are more abundantly expressed in myogenic tumors, such as RMS and leiomyosarcoma than in other tumor types [5,15]. In this study, we evaluated the feasibility of using serum muscle-specific miRNAs extracted from the patient's serum as a non-invasive diagnostic test for rhabdomyosarcoma.

2. Materials and methods

2.1. Cell lines

Sixteen cell lines were used for this study, including human rhabdomyosarcoma cell lines (Rh30 [16], SCMC-RM2 [17], RD

Abbreviations: RMS, rhabdomyosarcoma; miRNA, microRNA; mRNA, messenger RNA; ROC curve, receiver-operating characteristics curve; PRE, pretreatment reexcision; NB, neuroblastoma; EWS, Ewing sarcoma; MRT, malignant rhabdoid tumor; C_t, cycle threshold of PCR amplification.

* Corresponding author. Fax: +81 75 252 1399.

E-mail addresses: mmiyachi@koto.kpu-m.ac.jp (M. Miyachi), tsuchiya@koto.kpu-m.ac.jp (K. Tsuchiya), hide0519@koto.kpu-m.ac.jp (H. Yoshida), shigeky@koto.kpu-m.ac.jp (S. Yagyu), ken-k@koto.kpu-m.ac.jp (K. Kikuchi), amisawa@koto.kpu-m.ac.jp (A. Misawa), iehara@koto.kpu-m.ac.jp (T. Iehara), hhosoi@koto.kpu-m.ac.jp (H. Hosoi).

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[18], RMS-YM [19], CT-TC [20], Rh18 [21] and Rh41 [22]), neuroblastoma cell lines (IMR32 [23], GOTO [24], SK-N-SH [25] and KP-N-RT [26]), Ewing sarcoma cell lines (KP-EWS-YI [27], KP-EWS-AK and KP-EWS-SH) and malignant rhabdoid tumor cell lines (G401 [28] and MRT-YM [29]). KP-EWS-AK and KP-EWS-SH were our previously established cell lines. KP-EWS-AK cell line is derived from primary Ewing sarcoma of right scapula. KP-EWS-SH cell line is derived from bone marrow metastasis of Ewing sarcoma. The cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

2.2. Patient characteristics

A total of 55 participants including 10 patients with RMS, 28 patients with other pediatric tumors and 17 healthy subjects were recruited. Patient characteristics are summarized in Supplementary Tables 1–3.

Tumor analyses were done for all of the subjects whose tumors were available: 7 RMS tumors and 14 other pediatric tumors. The RMS patients (8.1 ± 5.2 years) and patients with other pediatric tumors (6.6 ± 6.1 years) did not significantly differ in age ($p = 0.499$). The sex distribution in the RMS group was 3:4 and in the other pediatric tumor group was 6:8 ($p = 1.0$).

Serum analyses were done for all of the subjects whose sera were available: 8 RMS patients, 23 non-RMS patients and 17 healthy subjects. The ages of RMS patients (10.0 ± 5.1 years) and patients with other pediatric tumors (6.6 ± 5.3 years) were not significantly different ($p = 0.195$). The healthy subjects were adults with an average age of 33.5 ± 4.9 years. The sex distribution was 4:4 in the RMS group, 11:12 in the other pediatric group and 9:8 in the control group ($p = 0.95$).

2.3. Sample collection

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. Informed consent was received from the healthy volunteers and the parents of patients. Patients were identified on the basis of histological examination of tumor specimens. Tumor specimens were surgically resected and immediately stored at −80 °C. Peripheral blood was obtained from each patient before any therapy and surgery. Blood samples were stored at −20 °C until miRNA extraction. To avoid contamination of serum miRNA by the miRNA from white blood cells, serum was centrifuged at 18,000*g* for 10 min [30,31] before miRNA extraction. The same centrifugation step was done before miRNA was extracted from culture supernatants of the cell lines to avoid contamination of supernatant miRNA by miRNAs from the cell lines.

2.4. RNA extraction

Total RNA was extracted from the cell lines, culture supernatants of cell lines, tumor specimens and sera (200 μl) using a mirVana PARIS kit (Ambion) following the manufacturer's protocol.

2.5. miRNA quantification by quantitative real-time RT-PCR

miRNA in tumor specimens and serum samples was quantified with Taqman quantitative RT-PCR (Applied Biosystems). Each reaction was primed using a gene-specific stem-loop primer. Assays containing the RT stem-loop primer and the PCR primers and probes were used. RNA was transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems). The reaction was performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). The PCRs were carried out in a final volume of 20 μl using a 7300 Real-Time PCR System (Applied Biosystems). Reactions consisted of 1.33 μl cDNA, TaqMan Universal PCR Master mix, No AmpErase UNG (Applied Biosystems), 0.2 μM TaqMan Probe, 1.5 μM forward primer and 0.7 μM reverse primer. The PCRs were initiated with a 10 min incubation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The expression levels of miRNAs were normalized to miR-16 [32,33] and were calculated utilizing the $\Delta\Delta C_t$ method [34].

2.6. Statistical analysis

Expression levels of miRNAs were compared using the Mann–Whitney *U* test. Receiver-operating characteristics (ROC) curves were established to evaluate the diagnostic value of miRNAs for differentiating RMS tumors from non-RMS tumors. The Spearman rank order correlation test was used to examine correlation relationships. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Expression of muscle-specific miRNAs in cell lines

Expression of muscle-specific miRNAs was significantly higher in RMS cell lines than in neuroblastoma (NB), Ewing sarcoma (EWS) and malignant rhabdoid tumor (MRT) cell lines. miR-206 was most abundantly expressed and miR-1 was least abundantly expressed among muscle-specific miRNAs (Supplementary Fig. 1).

3.2. Expression of muscle-specific miRNAs in tumor specimens

Expression of muscle-specific miRNAs was significantly elevated in RMS tumors (Supplementary Fig. 2). Each muscle-specific miRNA was equally abundantly expressed.

3.3. Expression of muscle-specific miRNAs in culture supernatants of cell lines

Total RNA was extracted from culture supernatants of cell lines. Both miR-16 and muscle-specific miRNAs were detected in the total RNA. Expression of muscle-specific miRNAs was significantly elevated in culture supernatants of the RMS cell lines (Supplementary Fig. 3). miR-206 was most abundantly expressed and miR-1 was least abundantly expressed among muscle-specific miRNAs.

3.4. Expression of muscle-specific miRNAs in serum samples

Serum levels of the muscle-specific miRNAs were significantly higher in patients with RMS tumors than in patients with non-RMS tumors or patients in the control group (Fig. 1, Mann–Whitney *U* test with Bonferroni correction). ROC curve analysis revealed that the serum levels of muscle-specific miRNAs (miR-1, -133a, -133b and -206) were useful biomarkers for differentiating patients with RMS from patients with non-RMS tumors (Fig. 2). The sensitivity and specificity of each miRNA are summarized in Table 1. Normalized serum miR-206 expression level showed the highest sensitivity and specificity among muscle-specific miRNAs. Normalized miR-206 expression level in non-RMS patients was statistically higher than that in the control group (Fig. 1). Using paired tumor and serum samples pooled from six RMS and eight non-RMS patients, tumor miRNA levels were found to be significantly correlated with serum miRNA levels in miR-133a, -133b and -206 (Spearman rank order correlation test; Supplementary Table 4). Importantly, the expression level of each of the muscle-specific miRNAs decreased after treatment of RMS (Supplementary Fig. 4).

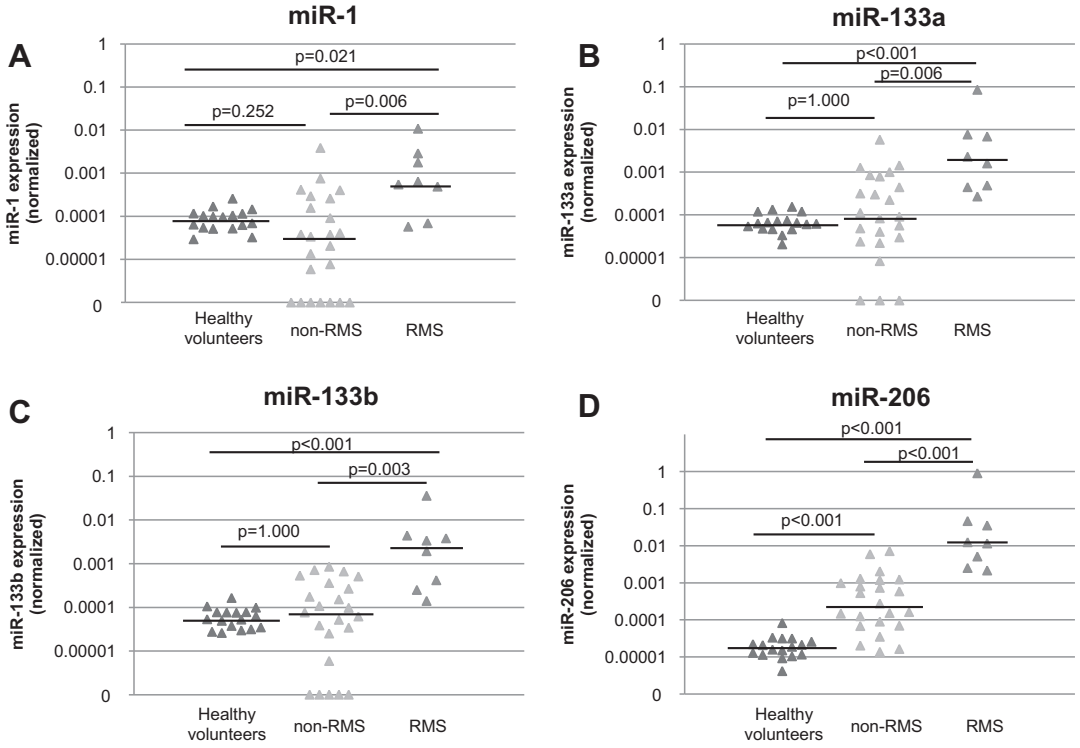

Fig. 1. Circulating levels of muscle-specific microRNAs in sera from pediatric tumor patients and healthy volunteers. Scatter plots of serum levels of (A) miR-1, (B) miR-133a, (C) miR-133b and (D) miR-206 in healthy volunteers, patients with non-RMS tumors and patients with RMS tumors. Expression levels of the miRNAs are normalized to miR-16.

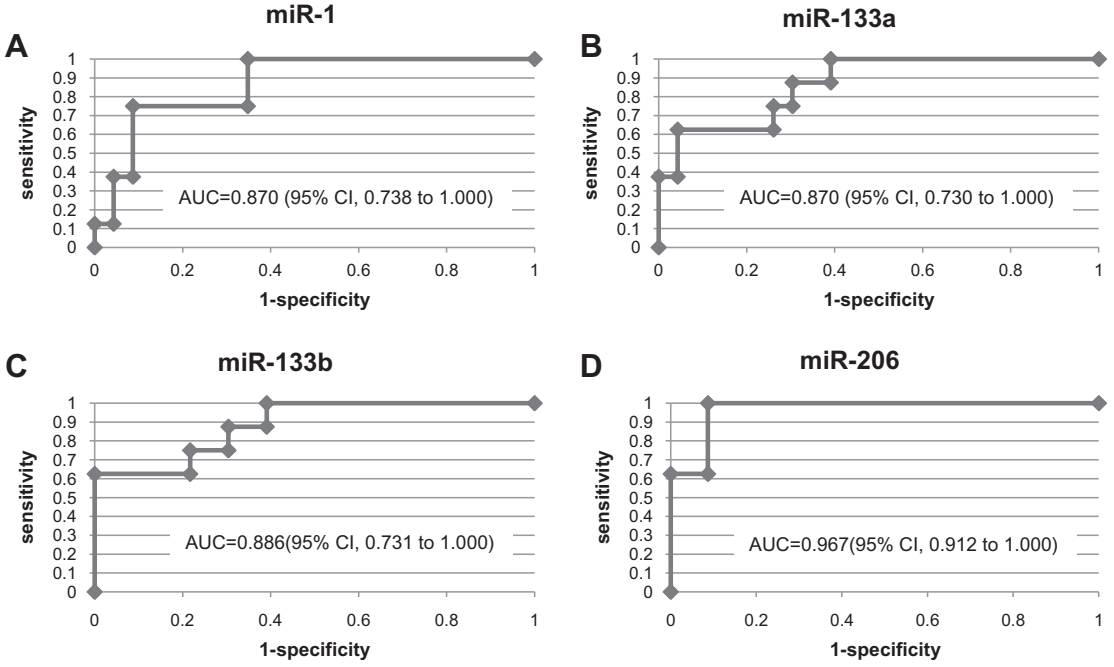

Fig. 2. Receiver-operator characteristics (ROC) curve analyses using serum (A) miR-1, (B) miR-133a, (C) miR-133b and (D) miR-206 for discriminating RMS.

The C_t (cycle threshold of PCR amplification) value of miR-206 is also a useful biomarker for differentiating patients with RMS from patients with non-RMS tumors with ROC curve areas of 0.989 (95% confidence interval (CI), 0.961–1.000), sensitivity of 1.0 and specificity of 0.957 (Fig. 3 and Table 1).

The C_t values of miR-16 in patients with RMS tumors and in patients with non-RMS tumors were not significantly different (Fig. 3). The C_t value of miR-16 was lower in healthy volunteers than in pediatric cancer patients (Fig. 3). The C_t value of miR-16 in pediatric tumor patients was positively correlated with the time

Table 1
Sensitivity and specificity of muscle-specific microRNAs.

miR	Cut-off value	Sensitivity	Specificity
1	4.5×10^{-4}	0.75	0.913
133a	2.8×10^{-4}	0.875	0.695
133b	2.5×10^{-4}	0.875	0.695
206	2.1×10^{-3}	1.0	0.913
206 C_t value	32.9	1.0	0.957

C_t, cycle threshold of PCR amplification.

of storage (Spearman rank order correlation test; Spearman's rank correlation coefficient, 0.598, $p < 0.001$).

4. Discussion

We showed that the expression levels of muscle-specific microRNAs were elevated in each of the RMS cell lines and their culture supernatants. The expression levels were also significantly elevated in RMS tumor and serum samples, as expected. Because no specific serum tumor marker has been identified in RMS so far, these muscle-specific microRNAs could be quite valuable in the diagnosis of RMS. Among the muscle-specific microRNAs, miR-206 was the best marker for RMS prediction. miR-206 is unique among the muscle-specific microRNAs in that it is specifically expressed in skeletal muscle, being absent or expressed at relatively low levels in other tissues. This may explain why miR-206 has the highest specificity and sensitivity among muscle-specific microRNAs.

miR-16 and RNU48 are commonly used as reference miRNAs. miR-16 and RNU48 are stably expressed in breast and colon tissue and their levels are not appreciably changed in malignant breast and colon tissue [32,33]. In addition, the serum level of miR-16 was not significantly different between prostate cancer patients and healthy individuals [8]. Because we did not detect RNU48 in serum, we used miR-16 as a reference miRNA in this study. A potential problem is that the C_t value of miR-16 was higher in the pediatric cancer patients in our study than in the healthy volunteers. However, this may be an artifact due to longer storage of the pediatric cancer samples. In the case of healthy volunteers, miRNA was extracted from serum within a week of serum collection, while in the case of the pediatric cancer patients, it was extracted at times from 1 week to 20 years after collection. Furthermore, the C_t value of miR-16 in pediatric tumor patients was positively correlated with the time of storage. Therefore, we suspect that the high C_t value of miR-16 in pediatric cancer patients is due to degradation of miRNAs. However, we cannot exclude the possibility that the expression of miR-16 decreases in pediatric cancer patients. Further studies are needed to determine which miRNA is best suited as a reference miRNA in pediatric cancer.

The normalized miR-206 expression level in non-RMS patients was statistically higher than that in the control group (Fig. 1). We speculate that this is due to high C_t values of miR-16 in non-RMS patients because the C_t values of miR-206 in non-RMS patients were not significantly different from those of the control group (Fig. 3). The high miR-16 C_t values in non-RMS patients are probably due to degradation of miRNAs.

Recent reports have shown that forced expression of miR-206 in RMS cells promoted myogenic differentiation and blocked tumor growth [35,36]. Therefore, miR-206 is considered to be an oncosuppressor microRNA. Missiaglia et al. also showed that low miR-206 expression in RMS tumors correlates with poor prognosis [36]. Our finding that miR-206 can be detected in RMS patients' sera may make it possible to use the sera to predict the prognosis. Further studies are needed to determine the correlation between serum miR-206 expression level and prognosis.

Gross total resection of the primary tumor before chemotherapy leads to improvement of prognosis in RMS [1,2]. Pretreatment reexcision (PRE) is recommended if gross total resection of the primary tumor is possible within a certain number of days following biopsy. However, PRE can be avoided if tentative diagnosis of RMS

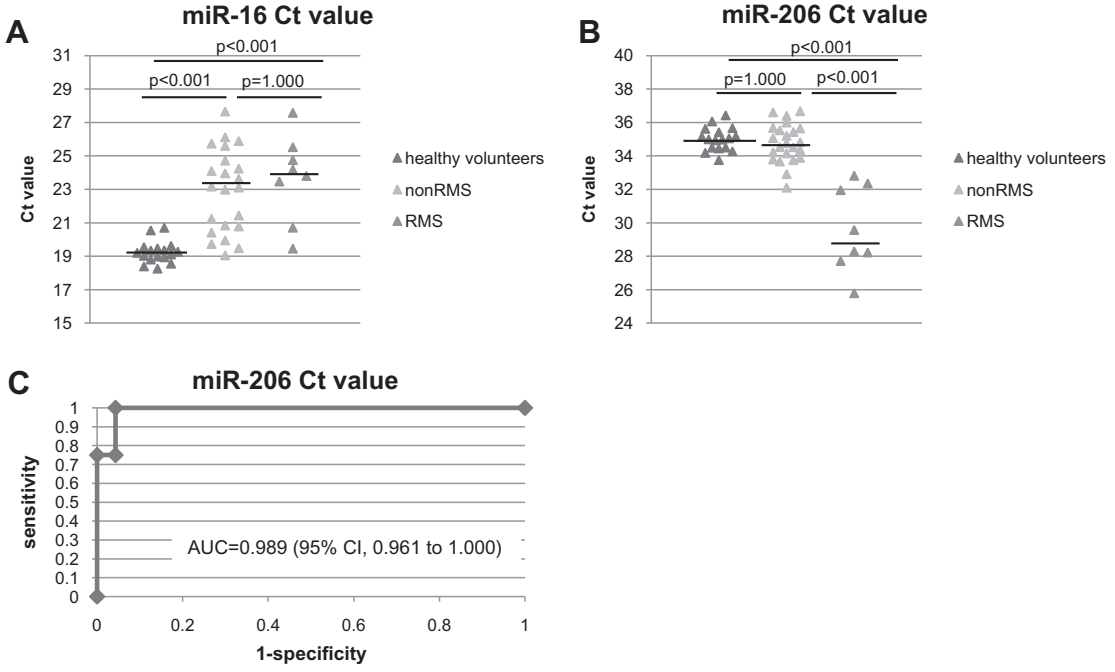

Fig. 3. C_t values of miR-206 and miR-16 in sera from pediatric tumor patients and healthy volunteers. Scatter plots of serum C_t values of (A) miR-206 and (B) miR-16 in healthy volunteers, patients with non-RMS tumors and patients with RMS tumors. (C) Receiver-operator characteristics (ROC) curve analyses using serum miR-206 C_t values for discriminating RMS.

is reliably made and gross total resection is conducted at the first surgery. Detection of serum muscle-specific microRNAs makes it possible to tentatively diagnose RMS and might decrease the rate of PRE. It might also decrease the number of patients with gross residual disease who have a poor prognosis.

Detection of the microRNAs takes only 4 h and costs only about $25. Only 200 μl of serum is required. This test can be a quick, non-invasive and cost-effective way to differentiate RMS from other tumors.

5. Limitations

Several myogenic tumor specimens (leoimyosarcoma, leiomyoma and rhabdomyoma) also express muscle-specific microRNAs [15]. The serum samples of patients with these tumors might contain high levels of muscle-specific miRNAs. However, the incidences of these tumors in children are rare.

Our results are based on a small sample size and a retrospective study. Larger prospective clinical trials are needed to validate these results.

6. Conclusion

Muscle-specific miRNAs were significantly elevated in sera of patients with RMS and can be potential non-invasive biomarkers for RMS diagnosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.015.

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