MicroRNA-6826 and -6875 in plasma are valuable non-invasive biomarkers that predict the efficacy of vaccine treatment against metastatic colorectal cancer

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Abstract. Various vaccine treatments against metastatic colorectal cancer have been developed and applied. However, to improve the efficacy of immunotherapy, biomarkers that can predict the effects are needed. It has been reported that various microRNAs (miRNAs) in peripheral blood may be useful as non-invasive biomarkers. In this study, miRNAs influencing the efficacy of vaccine treatment were screened for in a microarray analysis of 13 plasma samples that were obtained from patients prior to vaccine treatment. To validate the screening results, real-time RT-PCR was performed using 93 plasma samples obtained from patients prior to vaccine treatment. Four candidate miRNAs were selected according to the results of the comprehensive analysis of miRNA expression, which were ranked using the Fisher criterion and the absolute value of the log2 ratio in the screening analysis. The validation analysis showed that in the HLA-A*2402-matched patient group (vaccine-treated group), patients with a high expression of plasma miR-6826 had a poorer prognosis than those with a low expression (P=0.029 and P=0.754, respectively). Moreover, multivariate analysis of the Cox regression model indicated that the expression of miR-6826 was the most significant predictor for overall survival (P=0.003, hazard ratio, 3.670). In conclusion, plasma miR-6826 and miR-6875 may be predictive biomarkers for a poor response to vaccine treatment. Although further clarification is needed regarding the functions of miR-6826 and miR-6875 and their relationship to immune-related molecules, plasma miR-6826 and miR-6875 may be useful negative biomarkers for predicting the efficacy of vaccine treatment.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer in men and the second most common type in women, accounting for ~608,000 deaths annually worldwide (1). The most common cause of death from CRC is metastasis to distant organs. Although the prognosis of metastatic colorectal cancer (mCRC) has been improving owing to chemotherapy and molecular-targeted therapy (2,3), it is not yet satisfactory. Various immunotherapies for CRC have been developed and used, such as personalized peptide vaccination (4) and dendritic cell-based active immunotherapies (5). Recently, programmed cell death 1 (PD-1) antibody has also been receiving increased attention around the world (6). However, useful biomarkers that can predict good clinical outcomes from immunotherapy have not yet been identified (7), and there are few immunological biomarkers, such as the B-cell signature, as exemplified by the expression of the immunglobulin G κ chain in tumor-infiltrating lymphocytes. The development of biomarkers for immunotherapy is desired for the appropriate selection and evaluation of a patient population for clinical trials of cancer at an earlier stage, and for the effective development of cancer vaccine treatments (7,8).
MicroRNAs (miRNAs) are endogenous single-stranded RNA molecules consisting of 18-24 nucleotides that regulate the transcription levels of target genes and are involved in multiple intracellular processes (9,10). Recently, several studies have reported a relationship between the immune response and miRNAs. As such, it is presumed that miRNAs are involved in the immune response. In addition, the role of miRNAs as crucial regulators of innate and adaptive immune responses has been coming to light (11). In the process of tumor progression enhanced by an antitumor immunity microenvironment, miRNAs are considered to be one of the key players in tumor cell escape from immunological surveillance (12,13) in the induction of antitumor T cells (14) and in the immune-mediated recognition of tumor cells (15). As such, in patients in whom the efficacy of vaccine treatment is insufficient, there may be impairment of the immune response due to upregulated or downregulated miRNAs.

It has been reported that various miRNAs in plasma may be useful as non-invasive biomarkers for detecting early CRC or for predicting prognosis and recurrence (16,17). Recently, in our institution, a phase I study in which five epitope peptides [three derived from tumor-associated oncoantigens and two derived from vascular endothelial growth factor receptors (VEGFRs)] were applied to advanced-stage colon cancer patients (18). We subsequently performed a phase II study with the same vaccine regimen in combination with oxaliplatin-containing chemotherapy and further assessed its safety and promising potential to induce cytotoxic T lymphocytes (CTLs) and improve overall survival (OS) (19). In these studies, we found that a high CTL response after vaccination and a skin reaction at the injection site were possible biomarkers for the outcome of vaccine treatment (18). Moreover, a low neutrophil/lymphocyte ratio and a low plasma interleukin 6 level (20) were also possible predictive biomarkers of longer survival in vaccinated patients (19). We also reported the usefulness of tumor miRNA expression for predicting the efficacy of immune-chemotherapy (21).

The purpose of the present study was to explore novel predictive biomarkers that can predict the efficacy of vaccine treatment; we investigated the plasma miRNAs of mCRC patients treated with the phase II study protocol in order to detect liquid biomarkers.

Materials and methods

**Summary of the phase II study.** To evaluate the clinical benefits of cancer vaccination treatment, we conducted a phase II trial that was a non-randomized HLA-A status double-blinded study using five HLA-A*2402-restricted peptides: RNF43-721 (NSQPVWVLCL) (22), TOMM34-299 (KLRQEVKQNL) (23), KOC1 (IMP-3)-508 (KTVNELQNL) (24), VEGFR1-1084 (SYGVLLWEI) (25) and VEGFR2-169 (RFVPDGNRI) (26). The detailed protocol of this phase II study was previously described (19). Briefly, the therapy consisted of a cocktail of five therapeutic epitope peptides in addition to oxaliplatin-containing chemotherapy. Although the peptides used in this study were HLA-A*2402-restricted peptides, all enrolled patients, whose HLA-A*2402 status was double-blinded, were administered the same regime of peptide cocktail and oxaliplatin-containing chemotherapy. The cocktail containing 3 mg of each of the five peptides was mixed with 1.5 ml of incomplete Freund's adjuvant (IFA) and administered subcutaneously into the thigh or axilla regions weekly for 13 weeks; thereafter, the vaccination schedule was reduced to once every 2 weeks.

Patients were eligible for enrollment if they were ≥20 years of age with a histologically confirmed advanced CRC, chemotherapy-naïve, had adequate functions of critical organs and had a life expectancy of ≥3 months. Between February 2009 and November 2012, 96 chemotherapy-naïve CRC patients were enrolled under the concealment of their HLA-A*2402 status.

Among the 96 patients who were enrolled in this study, 93 cases were available for miRNA analysis. Written informed consent for inclusion was obtained from all patients, and the study protocol was approved by the local ethics committee (H20-102, UMIN000001791).

**Patients and plasma.** A total of ninety-three patients (HLA-A*2402-matched, n=48 and HLA-unmatched, n=45) with mCRC who were treated in the phase II study had pretreated plasma available for miRNA analysis. Peripheral blood from each patient was collected in ethylenediaminetetraacetic acid (EDTA) tubes. The blood samples were centrifuged at 400 x g for 15 min at 4°C. The plasma was then aliquoted and stored at -80°C until use.

miRNA microarray. In order to screen for miRNAs involved in the response to vaccine treatment, microarray analysis of miRNA expression was performed using 13 plasma samples collected from mCRC patients prior to vaccine treatment. All of the plasma sample were from HLA-matched patients: five were from patients who survived >3 years and eight were from patients who survived <2 years.

Total RNAs from plasma samples (n=13) were analyzed by miRNA microarray. Total RNA was extracted from the samples using 3D-Gene RNA extraction reagent from a liquid sample kit (Toray Industries, Inc., Tokyo, Japan) according to the manufacturer's protocol. A comprehensive miRNA expression analysis was performed using a 3D-Gene miRNA Labeling kit and a 3D-Gene Human miRNA Oligo Chip (Toray Industries, Inc.), which was designed to detect 2,555 miRNAs registered in the miRBase database (release 20). Individual miRNAs were considered to be present if the corresponding microarray signals were more than the mean ± 2 standard deviation (SD) of the negative control signals, of which the top and bottom 5% ranked by signal intensity were removed. Once a miRNA was regarded to be present, the mean signal of the negative control, of which the top and bottom 5% ranked by signal intensity were removed, was subtracted from the signal of the miRNA. If the signal became a negative value (or was undetected) after subtraction of the background, the value was replaced by the value of the lowest signal intensity on the microarray minus 0.1 on a log2 scale. In order to normalize the signals across the different microarrays tested, quantile normalization was performed (27).

**Selection of miRNAs for validation.** Using the fold-change value and the Fisher criterion, differentially expressed miRNAs

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between the responders (OS ≥3 years) and non-responders (OS <2 years) were classified. OS between 2 years and ≤3 years were excluded to compare a long-term survivor and a short-term survivor.

Validation using qRT-PCR. From 400-µl samples of plasma, total RNA was purified using a miRNeasy Serum/Plasma kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. miR-191 was used as an endogenous internal control (28,29).

We used TaqMan miRNA probes (Applied Biosystems Japan Ltd., Tokyo, Japan) to perform the qRT-PCR assay according to the manufacturer's instructions. In each step, from plasma purification to the qRT-PCR, an equal volume (400 µl) of plasma sample was processed. The total RNA was reverse-transcribed to complementary DNA using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems) and stem-loop RT primers (hsa-miR-135, hsa-miR-6826, hsa-miR-6835 and hsa-miR-6875, in addition to hsa-miR-191 for the internal control) (Applied Biosystems). RT-PCR was performed using the LightCycler® 480 System II (Roche Diagnostics k.k., Tokyo, Japan). The reactions were initiated at 95˚C for 5 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All reactions, including the no template controls, were run in duplicate. The relative expression levels of the target miRNAs were normalized to those of miR-191 according to the ΔΔCt method. For every target miRNA, the relative Ct values were divided into an OS ≥2-year group (responders) and an OS <2-year group (non-responders) which were plotted separately.

Statistical analysis. The obtained values are shown as the mean ± SD. The values beyond the mean ± 3SD were excluded as outliers for each miRNA. The expression levels of plasma miRNAs were compared between the responders and non-responders using Scheffe's or Dunnett's test.

For each miRNA, the cut-off value was set as the median and a survival curve was obtained by the Kaplan-Meier method to evaluate the efficacy of vaccine treatment. P-values were calculated with the log-rank test. The P-value for the relative Ct value between the responders and non-responders was calculated with the t-test. For every miRNA, a survival curve for each HLA-A*2402 status was obtained using the Kaplan-Meier method and analyzed using the log-rank test.

A Cox's proportional hazards model and a logistic regression model were used to estimate the hazard ratios (HRs) for the treatment effect in relation to OS and biomarkers or prognostic clinical information. All statistical analyses were performed with SPSS Statistics 20.0 (SPSS, Inc., Chicago, IL, USA). A value of P<0.05 was considered statistically significant.

Results

miRNA microarray. Ten candidate miRNAs as biomarkers were selected by a comprehensive analysis of the miRNAs, according to the miRNA expression levels ranked using the Fisher criterion between the patients who survived >3 years and those who survived <2 years (Table I). Finally, we selected four miRNAs (miR-135a-3p, miR-6875-5p, miR-6835-5p and miR-6826-5p) for which the expression difference according to the absolute value of the log2 ratio was >1.30 between the long-term survivor and the short-term survivor.

Validation analysis. In the validation phase we defined a responder as OS ≥2 years and a non-responder as OS <2 years. The expression of miR-6826 was significantly higher in the non-responders than that observed in the responders (P=0.002, Fig. 1A). To investigate the efficacy of vaccine treatment, samples were divided according to two HLA statuses. The expression of miR-6826 was also significantly higher in the non-responders than in the responders in the HLA-A*2402-matched group (P=0.003, Fig. 1B). In contrast, there was no significant difference in the expression of miR-6826 between the responders and non-responders in the HLA-A*2402-unmatched group (Fig. 1C). As such, a high

Table I. Selection of the microRNA from the result of the comprehensive analysis of the microarray.

| miRNA name   | OS ≥3 years (n=5) | OS <2 years (n=8) |
|--------------|------------------|------------------|
|              | Mean   | SD    | Mean   | SD    |
| miR-135a-3p  | 148.0  | 131.5 | 49.7   | 39.5  | 1.6 | 1.02
| miR-6875-5p  | 451.4  | 431.7 | 184.3  | 213.9 | 1.3 | 0.61
| miR-6798-5p  | 505.0  | 309.5 | 295.6  | 260.7 | 0.8 | 0.59
| miR-1233-5p  | 1,477.7 | 1,062.1 | 3,007  | 2,600.6 | 1.0 | 0.57
| miR-6124     | 137.9  | 102.4 | 257.7  | 238.6 | 0.9 | 0.57
| miR-1275     | 97.2   | 67.0   | 199.4  | 192.8 | 1.0 | 0.54
| miR-1229-5p  | 126.2  | 85.3   | 244.3  | 234.8 | 1.0 | 0.50
| miR-197-5p   | 41.1   | 26.2   | 90.6   | 101.9 | 1.1 | 0.45
| miR-6826-5p  | 145.3  | 130.3  | 425.9  | 510.7 | 1.6 | 0.44
| miR-6835-5p  | 70.9   | 77.4   | 253.5  | 332.6 | 1.8 | 0.43

Bold indicates the four mRNAs whose expression difference according to the absolute value of the log2 ratio was >1.30 between the long-term survivor and the short-term survivor. OS, overall survival; SD, standard deviation.
expression level of miR-6826 may indicate that the vaccine treatment will have poor efficacy.

The median of each miRNA Ct value was used as the cut-off value to discriminate high and low values. In the subgroup analysis, patients bearing HLA-A*2402, with a lower miR-6826 expression showed a longer OS than patients with a higher miR-6826 expression in the HLA-A*2402-matched group (P=0.048, Fig. 2A). In contrast, in the HLA-A*2402-unmatched group, there was no difference in OS between those with a high or low miR-6826 expression (Fig. 2B). This suggested that miR-6826 could be a useful biomarker for predicting the efficacy of vaccine treatment.

Regarding the expression of miR-6875 and miR-135, there was no significant difference between the patients who survived ≥2 years and those who survived <2 years (Figs. 3 and 4). However, in the subgroup analysis of patients bearing HLA-A*2402, patients with a lower miR-6875 expression had a longer OS than patients with a higher miR-6875 expression (P=0.029, Fig. 5A). In addition, there was no difference in OS between the patients with a lower or higher miR-6875 expression in the HLA-A*2402-unmatched group (Fig. 5B). This suggested that miR-6875 may also be useful as a biomarker for predicting the efficacy of vaccine treatment.

There was no significant difference in OS according to miR-135 expression between the HLA-A*2402-matched group and the HLA-A*2402-unmatched group (Fig. 6). A high expression of miR-6826 and miR-6875 in plasma may indicate a poor response to not only vaccine treatment in combination with chemotherapy, but also to the vaccine treatment by itself.

The Ct value of miR-6835 could not be measured in all samples even after >55 cycles, indicating that the quantity of miR-6835 in plasma was too small (data not shown).

To explore biomarkers for this vaccine therapy, we analyzed immunological parameters, tumor factors, as well as miRNA expression levels by a Cox's proportional hazards model and a logistic regression model. Multivariate analysis

Figure 1. miR-6826 expression in the plasma of responders and non-responders according to overall survival. (A) The expression of miR-6826 was significantly (P=0.002) higher in the non-responders than in the responders (n=90). (B) The HLA-A*2402-matched group (n=46). The expression of miR-6826 was significantly higher in the non-responders than that in the responders (P=0.003). (C) The HLA-A*2402-unmatched group (n=44). There was no significant difference in the expression of miR-6826 between the non-responders and the responders. Bars indicate the median values of each group. OS, overall survival; Y, years.

Figure 2. OS according to the expression of miR-6826. (A) The HLA-A*2402-matched group. Patients with a lower miR-6826 expression had a longer OS than those with a higher miR-6826 expression (P=0.048). (B) The HLA-A*2402-unmatched group. There was no significant difference in the OS of patients with a high or low level of miR-6826 expression. OS, overall survival.
Figure 3. miR-6875 expression in the plasma of responders and non-responders according to overall survival. (A) There was no significant difference between the responders and non-responders (n=91). (B) The HLA-A*2402-matched group (n=46). There was no significant difference between the responders and non-responders. (C) The HLA-A*2402-unmatched group (n=44). There was no significant difference between the responders and non-responders. Bars indicate the median values of each group. OS, overall survival; Y, years.

Figure 4. miR-135 expression in the plasma of responders and non-responders according to overall survival. (A) There was no significant difference between the responders and non-responders (n=89). (B) The HLA-A*2402-matched group (n=45). There was no significant difference between the responders and non-responders. (C) The HLA-A*2402-unmatched group (n=44). There was no significant difference between the responders and non-responders. Bars indicate the median values of each group. OS, overall survival; Y, years.

Figure 5. OS curves according to the expression of miR-6875. (A) The HLA-A*2402-matched group. Patients with a lower miR-6875 expression had a longer OS than those with a higher miR-6826 expression (P=0.029). (B) The HLA-A*2402-unmatched group. There was no significant difference in OS between patients with a high or low level of miR-6875 expression. OS, overall survival.
of the Cox regression model indicated that the expression of miR-6826 was the most significant predictor for OS (P=0.003, HR, 3.670) (Table II). Moreover, the sensitivity of miR-6826 to predict prolonged OS was 100%, and negative predictive value was also 100% in the HLA-A*2402 matched patients (Table III).

Discussion

Many novel vaccine approaches, such as whole tumor cell vaccines, peptide vaccines (30), viral vector vaccines and dendritic cell vaccines, for the treatment of cancer have been developed. However, useful biomarkers that can predict a good clinical outcome from immunotherapy have not yet been identified (7) and few immunological or other biomarkers are available for use in clinical trials of immunotherapy.

To the best of our knowledge, this is the first study performed on the measurement of plasma (or serum) miRNAs for predicting the efficacy of immunotherapy using liquid biopsy. Firstly, we selected four miRNAs as biomarkers for
predicting the efficacy of the vaccine treatment. Next, we validated the results of the comprehensive analysis using qPCR of the plasma miRNAs of 93 patients; in the vaccine-treated group, patients with a high expression of plasma miR-6826 and miR-6875 had a poorer prognosis than those with a low expression. Hence, we concluded that plasma miR-6826 and miR-6875 levels are negative predictive biomarkers for the efficacy of the vaccine treatment. Moreover, multivariate analysis indicated that the expression of miR-6826 was the most powerful predictor for OS, among immunological parameters, tumor factors, and miRNA expression levels. In consideration that the negative predictive value of miR-6826 was 100%, the high value of miR-6826 may be an exclusion criteria in upcoming clinical studies of immunotherapy.

These results also suggested that a high expression level of miR-6826 or miR-6875 may be related to the suppression of immune competence, and may be novel molecular targets for regulating the effects of immunosuppressive factors. miR-6826 was previously found to be upregulated in the serum of patients with pancreatic-biliary cancer, and was a diagnostic marker for pancreatic-biliary cancer (31). In addition, miR-6875 was reported to be a tumor marker for detecting early-stage breast cancer in combination with four other miRNAs (32), although the roles of miR-6826 and miR-6875 on the immune system have not yet been reported and no target mRNA has been reported for miR-6826 or miR-6875 in the miRBase database (release 18). miRNAs have been implicated in adaptive immunity by controlling the development and activation of T and B cells. Dynamic changes in the expression of miRNAs may be important for the regulation of gene expression during antigen-induced T cell differentiation.

Regarding such immune-suppressive miRNAs, miR-155 is implicated in the upregulation of regulatory T cells (Tregs) and myeloid-derived suppressor cells (33,34), which have been implicated in adaptive immunity by controlling the development and activation of T and B cells. Dynamic changes in the expression of miRNAs may be important for the regulation of gene expression during antigen-induced T cell differentiation.

In conclusion, the expression levels of miR-6826 and miR-6875 may be amenable to immune suppression and may be novel molecular targets for regulating the effects of immunosuppressive factors. miR-6826 was previously found to be upregulated in the serum of patients with pancreatic-biliary cancer, and was a diagnostic marker for pancreatic-biliary cancer (31). In addition, miR-6875 was reported to be a tumor marker for detecting early-stage breast cancer in combination with four other miRNAs (32), although the roles of miR-6826 and miR-6875 on the immune system have not yet been reported and no target mRNA has been reported for miR-6826 or miR-6875 in the miRBase database (release 18). miRNAs have been implicated in adaptive immunity by controlling the development and activation of T and B cells. Dynamic changes in the expression of miRNAs may be important for the regulation of gene expression during antigen-induced T cell differentiation.

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