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miR-125b-1 and miR-378a are predictive biomarkers for the efficacy of vaccine treatment against colorectal cancer

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Key words

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Many clinical trials of peptide vaccines have been conducted. However, these vaccines have provided clinical benefits in only a small fraction of patients. The purpose of the present study was to explore microRNAs (miRNAs) as novel predictive biomarkers for the efficacy of vaccine treatment against colorectal cancer. First, we carried out microarray analysis of pretreatment cancer tissues in a phase I study, in which peptide vaccines alone were given. Candidate miRNAs were selected by comparison of the better prognosis group with the poorer prognosis group. Next, we conducted microarray analysis of cancer tissues in a phase II study, in which peptide vaccines combined with chemotherapy were given. Candidate miRNAs were further selected by a similar comparison of prognosis. Subsequently, we carried out reverse-transcription PCR analysis of phase II cases, separating cancer tissues into cancer cells and stromal tissue using laser capture microdissection. Treatment effect in relation to overall survival (OS) and miRNA expression was analyzed. Three miRNA predictors were negatively associated with OS: miR-125b-1 in cancer cells ($P = 0.040$), and miR-378a in both cancer cells ($P = 0.009$) and stromal cells ($P < 0.001$). Multivariate analysis showed that expression of miR-378a in stromal cells was the best among the three predictors (HR, 2.730; 95% CI, 1.027–7.585; $P = 0.044$). In conclusion, miR-125b-1 and miR-378a expression might be considered as novel biomarkers to predict the efficacy of vaccine treatment against colorectal cancer.

Colorectal cancer is the third most commonly diagnosed cancer in males and the second most commonly diagnosed cancer in females, with an estimated 1.4 million cases and 693 900 deaths occurring in 2012.[1] In the past decade, chemotherapy and monoclonal antibody treatment have improved the prognosis of patients with mCRC. However, most of these patients die because the cancer develops tolerance to these agents. There is, therefore, a great need for development of additional treatment.

Immunotherapy has been used to treat mCRC and has the potential to eradicate the disease by activating immune responses.[2] Peptide vaccines are one form of cancer vaccine that induces tumor-specific T-cell responses using tumor-associated antigen-derived peptides. Many phase I and II clinical trials using peptide vaccines have been conducted and these vaccines have provided clinical benefits in a small fraction of patients.[3,4] Currently, immune checkpoint blockade therapies are a very promising cancer immunotherapy. For example, antibodies blocking PD-1 and PD-L1 have shown durable responses in a number of different advanced malignancies.[5,6] Additionally, several recent studies suggest alternative targeting strategies for indoleamine 2,3-dioxygenase blockade at the level of gene expression.[7,8] Furthermore, combination immunotherapy with peptide vaccines and immune-checkpoint blockade therapies that are designed to concurrently activate tumor-specific immune responses and inactivate immunosuppression in the tumor microenvironment may lead to the induction of stronger antitumor responses.[9,10]

We previously reported a phase I and II study in which five epitope peptides were applied to advanced-stage colon cancer patients.[11,12] However, active immunotherapy using epitope peptides requires preservation of the host immune system. Hence, it is desirable to identify predictive biomarkers for the selection of patients who are likely to respond well, and to effectively induce specific CTL to epitope peptides.

miRNAs are endogenously expressed non-coding RNAs, 18–25 nucleotides in length, which regulate gene expression at the post-transcriptional level.[13] miRNAs play roles as crucial modulators of various biological processes such as proliferation, tumor initiation and development in CRC.[14] However, miRNAs are also critical regulators of immune responses,[15] and their aberrant expression or dysfunction in the immune system has been associated with cancer. For example, p53 modulates the tumor immune response by regulating PD-L1 through miR-34 in non-small-cell lung cancer.[16] In another example, miR-20b, -21 and -130b are upregulated in advanced CRC and inhibit PTEN expression, resulting in PD-L1 overexpression.[17] In addition, some miRNAs have been mentioned as possible biomarkers for immunotherapy.[18–20]

Cancer tissues consist of cancer cells and surrounding stromal cells, including inflammatory cells, immunocompetent cells, endothelial cells, and fibroblasts. Stromal cells in the tumor microenvironment play an important role in cancer metastasis.[21] Cancer stroma interacts with cancer tissues through miRNAs, creating a niche for the cancer cells.[22] A study showed that high levels of miR-21 in the stroma predict short disease-free survival in stage II CRC.[23] In a separate study, miR-21 suppression with antisense inhibitors was shown to prevent fibroblast-to-myofibroblast transdifferentiation in response to TGF-β.[24]

The purpose of the present study was to explore miRNAs as novel predictive biomarkers for the efficacy of immunotherapies before their treatment. We previously reported that three miRNAs might be useful biomarkers.[25] As the cellular origin is crucial for determination of miRNAs, in the present study, we used LCM to separate cancer cells and stromal cells for miRNA analysis. Here we demonstrate the results of miRNA microarray analysis and qRT-PCR in CRC cancer and stromal cells. We suggest that miR-125b-1 and miR-378a may serve as novel predictive biomarkers for active immunotherapies.

Materials and Methods

Summary of the phase I study. Detailed protocol of the phase I study was described previously.[11] Briefly, patients were eligible for enrolment when: (i) they had histologically confirmed CRC without indication for surgical resection; (ii) they had failed to respond to prior standard chemotherapy or could not tolerate the standard therapy; and (iii) when they had the *human leukocyte antigen (HLA)-A*2402*-allele by DNA typing. Good Manufacturing Practice grade peptides of RNF43-721,[26] TOMM34-299,[27] KOC1 (IMP-3)-508,[28] VEGFR1-1084,[29] and VEGFR2-169,[30] which are peptides that are recognized by HLA-A*2402-restricted CTL, were used. Dose escalation was carried out in three patient cohorts using doses of 0.5 mg, 1 mg, and 3 mg for each peptide. Each peptide was given to patients s.c. into the thigh or axilla regions on days 1, 8, 15, and 22 in a 28-day treatment course. The determined recommended dose was 3.0 mg, which was followed by carrying out a single injection of a cocktail of the five peptides. The cocktail of the five peptides at a dose of 3 mg was given to six patients. Among 18 *HLA-A*2402*-matched CRC patients who were enrolled in this study, eight primary CRC tissues were available for miRNA microarray analysis.

Summary of the phase II study. To evaluate clinical benefits of cancer vaccination treatment, we conducted a phase II trial, non-randomized, *HLA-A* status double-blind study. Detailed protocol of this phase II study was described previously.[12] Briefly, the therapy consisted of a cocktail of five peptides in addition to oxaliplatin-containing chemotherapy. The peptides used in this study were recognized by HLA-A*2402-restricted CTL; however, the *HLA-A* status of all enrolled patients was masked. The cocktail of 3 mg each of five peptides was mixed with 1.5 mL incomplete Freund's adjuvant and given s.c. into the thigh or axilla regions weekly for 13 weeks, then biweekly after 14 weeks. Patients were eligible for enrolment when they were ≥20 years old with a histologically confirmed advanced CRC, were naïve for chemotherapy, had adequate functions of critical organs, and had a life expectancy of ≥3 months. Between February 2009 and November 2012, 96 CRC patients were enrolled under the masking of their *HLA-A* status. Among these 96 patients, 26 cases were available for miRNA microarray analysis, and 68 primary CRC tissues were available for qRT-PCR analysis.

Sample collection. In the phase I trial, eight primary CRC tissues from 18 patients were obtained by surgery before treatment. Tissues were snap-frozen in liquid nitrogen and stored at −80°C. In the phase II trial, 26 primary CRC tissues were obtained from 96 patients and were stored as frozen tissues; 16 samples were from the *HLA-A*2402*-matched group and 10 samples were from the *HLA-A*2402*-unmatched group. Sixty-eight primary CRC tissues were obtained and stored as FFPE tissues; 34 samples from each of the *HLA-A*2402*-matched and *HLA-A*2402*-unmatched groups. Primary CRC lesions were resected in advance of the vaccine treatment studies at Yamaguchi University Hospital and affiliated hospitals. Written informed consent was obtained from all patients. This study was approved by the Institutional Ethics Review Boards of Yamaguchi University and was conducted in accordance with the Declaration of Helsinki (H20-102, Clinical Trial Registration: UMIN000001791).

Microarray analysis. Total RNA was extracted from frozen tissue using a mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Concentrations and purities of total RNAs were assessed using a spectrophotometer and RNA integrity was verified using an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). OD260/OD280 ratios of each sample were between 2.00 and 2.10, which were accepted as adequate for microarray analysis. The miRNA array analysis was carried out with 1-μg samples of total RNA using the miRCURY LNA™ microRNA Array 6th generation (Exiqon, Vedbaek, Denmark) and the GenePix®4000B (Molecular Devices, Sunnyvale, CA, USA). Relative intensity of each hybridization signal was evaluated using the Microarray Data Analysis Tool Ver3.2 (Filgen, Nagoya, Japan).

Laser capture microdissection. FFPE tumor specimens were sectioned into 15-μm-thick slices, and stained with H&E to enable visualization of histology.[31] LCM was carried out using the LMD system LMD6000 (Leica Microsystems, Tokyo, Japan) to microdissect cancer cells and stromal cells from CRC tissues. At least 30 mm^2 of cancer cells and 45 mm^2 of stromal cells were collected from each FFPE specimen. Depending on the percentage of cancer cells and stromal cells in the specimen, this generally required from two to four sections.

qRT-PCR. Total RNA was extracted from LCM-captured material using a QIAGEN miRNeasy FFPE Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Concentrations and purities of the total RNAs were assessed as described for microarray analysis using a spectrophotometer. Expression of miRNAs in both cancer cells and stromal cells was verified by qRT-PCR analysis using TaqMan® MicroRNA Assays (Life Technologies). cDNA was synthesized in a 7.5-μL reaction volume from total RNA (10 ng) using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). Reactions were incubated at 16°C for 30 min and then at 42°C for 30 min, and were inactivated at 85°C for 5 min. Each cDNA was analyzed in duplicate by qRT-PCR using sequence-specific primers for hsa-miR-125b-1 (ID002378), hsa-miR-224 (ID002099), hsa-miR-486 (ID001278), hsa-miR-147b (ID002262), hsa-miR-196b (ID002215), rno-miR-422b (ID001314), or RNU6B (ID001093) in an Applied Biosystems 7300 real-time PCR instrument (Life Technologies). miR-378a was previously known as miR-422b, and rno-miR-422b has the same sequence as miR-378a (ACUGGACUUGGAGUCAGAAGGC). Each individual assay was carried out in a 20-μL reaction volume with 1.33 μL cDNA, 1.0 μL TaqMan MicroRNA Assay, 10 μL TaqMan™ Universal PCR Master Mix II (Applied Biosystems) with No AmpErase UNG and 7.67 μL nuclease-free water (Applied Biosystems). Reactions were incubated for 10 min at 95°C, and then for 55 cycles with denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. Data were analyzed using SDS software v1.3.1 (Life Technologies). Threshold and baseline settings were set according to protocol recommendations.

***In situ* hybridization.** ISH was carried out on 5-μm-thick sections of FFPE samples using the RiboMap Kit (Ventana Medical Systems, Tucson, AZ, USA) on the Discovery ULTRA automated ISH instrument (Ventana Medical Systems). Probes were 5′ DIG-labelled ISH LNAs (miRCURY-LNA detection probes; Exiqon). Sequences of probe for miR-125b-1 and miR-378a were/5DigN/AGCTCCCAAGAGCCTAACCCGT and/5DigN/CCTTCTGACTCCAAGTCCAGT, respectively.

Sequence of the scramble probe for a negative control was/5DigN/GTGTAACACGTCTATACGCCCA. The ISH steps after the deparaffinization step were carried out based on the standard protocol provided in the manufacturer's RiboMap application note. Briefly, after treatment with proteinase K, the slides were hybridized with the detection probe for 8 h at 52°C. The digoxigenins were detected with a polyclonal anti-DIG antibody and an alkaline phosphatase-conjugated second antibody (Ventana Medical Systems). Signal was detected using the BlueMap NBT/BCIP substrate kit (Ventana Medical Systems). The sections were counterstained with Kernechtrot as a marker stain and covered with a glass coverslip.

Statistical analysis. In microarray analysis, expression signals were calculated by log2 transformation of the normalized data, and differentially expressed miRNAs were detected by using the fold-change value and the Fisher index using Microsoft Excel 2010 according to a previously reported formula.[32] In qRT-PCR, relative quantities of miRNA levels were determined using the 2-ΔΔCT method after normalization with RNU6B as a standard reference.[33] Differences between groups were estimated using the Mann–Whitney *U*-test. Categorical variables were compared by using the chi-squared and Fisher's exact tests. The cut-off value was determined by Youden's index for each ROC analysis. OS was measured in months from the first vaccination to the day of patient death from any cause. Survival curves were analyzed by the Kaplan–Meier method and the log–rank test, and a Cox's proportional hazards model was used to estimate the HR and the 95% CI for the treatment effect in relation to OS and miRNA expression, other biomarkers, and prognostic clinical information. Statistical analyses were done with JMP V11 (SAS Institute Inc.). $P < 0.05$ was considered statistically significant.

Results

Selection of candidate miRNAs to predict the efficacy of vaccination. We first carried out microarray analysis of the expression profiles of 1425 miRNAs using eight frozen tissues from patients enrolled in a phase I study. We classified the patients into two groups of four patients each, a responder group and a non-responder group. Responders were defined according to the following criteria: (i) having a PFS period of more than 150 days; or (ii) having an OS period of longer than 300 days without any additional chemotherapy. Non-responders were defined according to the following criteria: (i) having a PFS period shorter than 90 days; and (ii) having an OS period shorter than 200 days. Subsequently, on the basis of the relative comparison of hybridization signals of individual miRNAs, we selected the top 30 miRNAs showing significant differences in expression levels between the two groups (Table S1). Next, 26 frozen tissues from the phase II study were analyzed using a miRNA microarray. In this analysis, we examined expression levels of the 30 candidate miRNAs and their association with clinical outcome. The expression levels of six miRNAs were significantly associated with prognosis in the *HLA-A*2402*-matched groups. There was no difference in prognosis in *HLA-A*2402*-unmatched groups according to the expression levels of these six miRNAs. Therefore, these miRNAs had the potential to be predictive biomarkers for the efficacy of the vaccine treatment. Expression levels of these six miRNAs and survival analysis are shown in Table 1.

Validation of candidate miRNA expression and association of the miRNA expression levels with clinical outcome. We carried out qRT-PCR analysis of the expression of these six miRNAs in 68 FFPE tissues from the phase II study. The FFPE specimens were separated into cancer cells and stromal cells by LCM. miRNA expression levels were examined in these two types of cells, and association of miRNA expression levels with clinical outcome was evaluated. Characteristics of these 68 patients are listed in Table 2. No difference in any characteristic was found between *HLA-A*2402*-matched and *HLA-A*2402*-unmatched groups. Expression levels of the six miRNAs and survival analysis of the patients according to low or high expression of these miRNAs are shown in Table 3. Three predictors were negatively associated with OS in the *HLA-A*2402*-matched group only: miR-125b-1 in cancer cells ($P = 0.040$), and miR-378a in both cancer cells ($P = 0.009$) and stromal cells ($P < 0.001$). In addition, expression of miR-125b-1 ($P = 0.089$) and miR-486 ($P = 0.092$) in stromal cells tended to be negatively associated with OS. Overall survival of the *HLA-A*2402*-matched and -unmatched groups of patients according to high or low expression of miR-125b-1 or miR-378a is shown in the form of Kaplan Meier curves in Figure 1. These data suggest that miR-125b-1 and miR-378a have the potential to be negative predictive biomarkers for the efficacy of the vaccine treatment.

Univariate and multivariate analysis of the association of 125b-1 and miR-378a expression with OS. Results of univariate and multivariate analyses are shown in Table 4. Expression of miR-378a in both cancer cells (HR, 2.853; 95% CI 1.281–

Table 1. Microarray analysis of candidate miRNA expression and phase II study patient survival analysis (log–rank test)

miR name	Expression in cancer tissue			Cut-off value	*HLA-A*2402*	Group	*n*	*P*-value
	Mean	SD	Median					
196b-5p	689.1	666.5	526.5	400	Matched	Low	7	0.001
						High	9	
					Unmatched	Low	5	0.366
						High	5	
147b	483.6	185.2	460.0	500	Matched	Low	9	0.040
						High	7	
					Unmatched	Low	5	0.366
						High	5	
378a	6225.6	2216.9	6336.7	6500	Matched	Low	9	0.066
						High	7	
					Unmatched	Low	5	0.515
						High	5	
486-5p	345.3	71.0	339.0	365	Matched	Low	11	0.006
						High	5	
					Unmatched	Low	6	0.998
						High	4	
224-5p	343.2	290.8	272.2	270	Matched	Low	8	0.011
						High	8	
					Unmatched	Low	5	0.657
						High	5	
125b-1	604.0	312.0	518.0	550	Matched	Low	11	0.021
						High	5	
					Unmatched	Low	5	0.092
						High	5	

HLA, human leukocyte antigen; miRNA and miR, microRNA; SD, standard deviation.

Table 2. Clinical characteristics of patients in the phase II study whose tissues were analyzed for miRNA using qRT-PCR

Characteristics	*HLA-A*2402* matched (*n* = 34)	*HLA-A*2402* unmatched (*n* = 34)	*P*-value
Sex			
Male	14	19	0.225
Female	20	15	
Age (years)			
Mean	68	64	0.080
SD	8	8	
Range	47–82	38–77	
Unresectable site			
Liver	19	26	0.073
Lung	11	10	0.793
Dissemination	4	3	0.690
Bone	0	2	0.151
Lymph node	5	8	0.355
Other	3	1	0.303
No. involved organs			
One	27	22	0.302
Two	6	8	
Three	1	4	
Primary tumor site			
Colon	22	25	0.431
Rectum	12	9	

HLA, human leukocyte antigen; SD, standard deviation.

6.788: $P = 0.010$) and stromal cells (HR, 3.984; 95% CI 1.661–9.243: $P = 0.003$) was significantly associated with OS. The expression of miR-378a in stromal cells was confirmed to be an independent predictor of OS by multivariate regression analysis (HR, 2.730; 95% CI, 1.027–7.585; $P = 0.044$).

miR-125b-1 and miR-378a localization in tumor tissue. For specific identification of miR-125b-1 and miR-378a expression in tissue sections, we carried out ISH in tissue sections from patients with a poor prognosis. miR-125b-1 was highly expressed in both cancer and stromal cells (Fig. 2a). Within stromal cells, fibroblast-like cells (Fig. 2b) and mononuclear cells (Fig. 2c) showed strong miR-125b-1 signals. A weak miR-378a signal was observed in both cancer and stromal cells (Fig. 2f). Within stromal cells, fibroblast-like cells showed weak miR-378a signals (Fig. 2g). Such weak signals might be a result of the affinity of LNA probes for miR-378a.

Discussion

Findings of the present study showed that miR-125b-1 and miR-378a are negative predictive biomarkers for the efficacy of vaccine treatment against colorectal cancer. To our knowledge, this is the first study to systematically investigate miRNA biomarkers for cancer immunotherapy and to focus on separate analysis of cancer cells and stromal cells within cancer tissue. Previous miRNA microarray analysis by our group showed that miR-196b, miR-378a, and miR-486 are possible predictive biomarkers. However, it should be noted that the sample size was small and that the precise origins of these mRNAs are unknown. In the present study, we used qRT-PCR in an increasing number of samples and used LCM to separate the cancer cells and stromal cells. In addition, we carried out ISH for specific identification of the miRNAs.

First, our results indicated that high miR-125b-1 expression in cancer cells was significantly associated with poor prognosis

Table 3. qRT-PCR analysis of candidate miRNA expression and phase II study patient survival analysis (log–rank test)

miR name	Expression in cancer cells							
	Mean	SD	Median	Cut-off value	*HLA-A*2402*	Group	*n*	*P*-value
196b-5p	135.079×10^{-2}	169.617×10^{-2}	63.862×10^{-2}	108.588×10^{-2}	Matched	Low	24	0.652
						High	10	
					Unmatched	Low	22	0.120
						High	12	
147b	1.237×10^{-2}	1.254×10^{-2}	0.834×10^{-2}	1.527×10^{-2}	Matched	Low	28	0.210
						High	6	
					Unmatched	Low	27	0.046
						High	7	
378a	61.995×10^{-2}	65.306×10^{-2}	40.041×10^{-2}	27.856×10^{-2}	Matched	Low	15	0.009
						High	19	
					Unmatched	Low	13	0.777
						High	21	
486-5p	5.450×10^{-2}	20.571×10^{-2}	2.299×10^{-2}	2.290×10^{-2}	Matched	Low	18	0.165
						High	16	
					Unmatched	Low	16	0.798
						High	18	
224-5p	101.859×10^{-2}	113.0851×10^{-2}	61.063×10^{-2}	68.359×10^{-2}	Matched	Low	20	0.172
						High	14	
					Unmatched	Low	18	0.512
						High	16	
125b-1	0.786×10^{-2}	1.690×10^{-2}	0.289×10^{-2}	0.498×10^{-2}	Matched	Low	27	0.040
						High	7	
					Unmatched	Low	23	0.429
						High	11	
miR name	Expression in stromal cells							
	Mean	SD	Median	Cut-off value	*HLA-A*2402*	Group	*n*	*P*-value
196b-5p	33.801×10^{-2}	40.004×10^{-2}	18.673×10^{-2}	22.450×10^{-2}	Matched	Low	22	0.311
						High	12	
					Unmatched	Low	18	0.218
						High	16	
147b	0.599×10^{-2}	0.535×10^{-2}	0.424×10^{-2}	0.413×10^{-2}	Matched	Low	17	0.290
						High	17	
					Unmatched	Low	17	0.515
						High	17	
378a	43.009×10^{-2}	44.441×10^{-2}	28.802×10^{-2}	43.080×10^{-2}	Matched	Low	23	0.001
						High	11	
					Unmatched	Low	23	0.215
						High	11	
486-5p	7.569×10^{-2}	8.741×10^{-2}	4.725×10^{-2}	4.766×10^{-2}	Matched	Low	19	0.092
						High	15	
					Unmatched	Low	17	0.124
						High	17	
224-5p	19.249×10^{-2}	19.626×10^{-2}	13.315×10^{-2}	10.984×10^{-2}	Matched	Low	16	0.525
						High	18	
					Unmatched	Low	13	0.990
						High	21	
125b-1	2.311×10^{-2}	2.371×10^{-2}	1.554×10^{-2}	1.690×10^{-2}	Matched	Low	20	0.089
						High	14	
					Unmatched	Low	18	0.136
						High	16	

HLA, human leukocyte antigen; miRNA and miR, microRNA; SD, standard deviation.

in HLA-matched patients. Previous studies have shown that miR-125b plays crucial roles in immune system development and immunological host defense.[34] Upregulated expression of miR-125b may enhance type I interferon expression in airway epithelial cells in eosinophilic chronic rhinosinusitis with nasal polyps,[35] and promote classical activation of macrophages.[36,37] miR-125b-1 was also found to be specifically upregulated in patients with ulcerative colitis,[38] and may

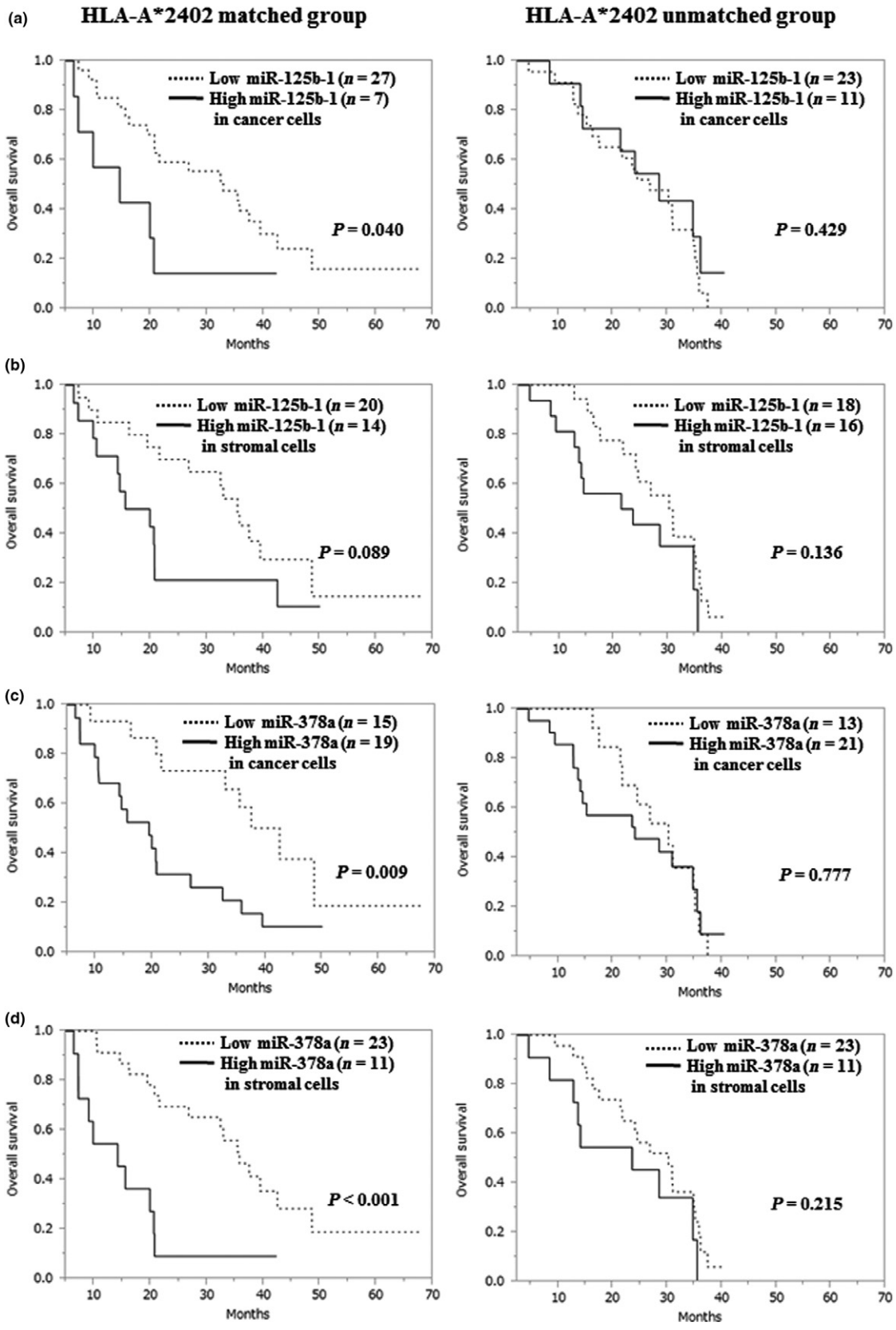

Fig. 1. Overall survival (OS) according to miRNA expression. OS of the *HLA-A*2402*-matched (left side) and -unmatched groups (right side) of phase II study patients according to high or low expression of miR-125b-1 or miR-378a. (a) According to miR-125b-1 in cancer cells. (b) According to miR-125b-1 in stromal cells. (c) According to miR-378a in cancer cells. (d) According to miR-378a in stromal cells. Solid line shows high miRNA expression and dashed line shows low miRNA expression. Statistical analysis was carried out using the log–rank test. HLA, human leukocyte antigen; miRNA and miR, microRNA.

Table 4. Univariate and multivariate analyses of associations between clinical data and overall survival

Factor	Univariate analysis					Multivariate analysis			
	Cut-off	HR	95% CI		*P*-value	HR	95% CI		*P*-value
			Lower	Upper			Lower	Upper	
CRP	>1	1.537	0.619	3.525	0.338				
NLR	>3	1.298	0.542	2.939	0.544				
CEA	>16	1.972	0.871	4.784	0.105				
CA19-9	>21	2.010	0.907	4.514	0.085				
No. involved organs	One or more	2.487	0.960	5.759	0.060				
Relative expression of miR-378a in cancer cells	$>27.856 \times 10^{-2}$	2.853	1.281	6.788	0.010	1.888	0.695	5.031	0.207
Relative expression of miR-378a in stromal cells	$>43.080 \times 10^{-2}$	3.984	1.661	9.243	0.003	2.730	1.027	7.585	0.044
Relative expression of 125b-1 in cancer cells	$>0.498 \times 10^{-2}$	2.586	0.925	6.286	0.068				
Relative expression of 125b-1 in stromal cells	$>1.690 \times 10^{-2}$	1.950	0.876	4.279	0.100				

CA19-9; carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CI, confidence interval; CRP, C-reactive protein; HR, hazard ratio; NLR, neutrophil:lymphocyte ratio.

increase risk of CRC through nuclear factor kappa B (NF-κB) activation.[39,40] However, miR-125b negatively regulates TNF-α expression in neonatal monocytes,[41] and inhibits proper TNF-α production in response to LPS.[42] Hence, miR-125b-1 might play the role of an immune suppressor by increasing immune exhaustion,[43] and resulting in the inhibition of the antitumor immune system. Expression of miR-125b-1 in cancer cells seems to be more important than that in stromal cells (Fig. 1a,b), although strong signals were also observed in stromal cells (Fig. 2b,c). miR-125b-1 might be produced by cancer cells and/or stromal cells, and cross-talk by miRNAs between cancer cells and stromal cells might ultimately work as an immune suppressor.

Second, our results indicated that high expression levels of miR-378a in both cancer cells and stromal cells were significantly associated with poor prognosis in HLA-matched patients. Previous reports showed that miR-378 was downregulated in CRC[44,45] and that miR-378a expression might play an important role as a tumor suppressor gene.[46] In contrast, miR-378a was reported to inhibit cytotoxicity of human NK cells[47] and enhance tumor growth and angiogenesis through repression of the expression of two types of tumor suppressors, Sufu and Fus-1.[48] Hence, after the establishment of tumor microenvironments, the function of miR-378a was thought to be as an immune suppressor. Regarding expression of miR-378a, there were some discrepancies in miRNA expression using ISH, miRNA microarray, and qRT-PCR. Expression level of miR-378a was higher than that of miR-125b-1 in miRNA microarray and qRT-PCR analyses. However, the opposite phenomenon was observed in ISH analysis. The miR-378a signal was weak in the cancer cells and stromal cells, and miR-125b-1 was highly expressed in both cell types. This discrepancy might mean that there were some problems with the affinity of the ISH probe for miR-378a.[49]

In multivariate analysis, miR-378a in stromal cells showed the best performance among the three predictors for selection of patients who are likely to respond well to peptide vaccines (Table 4). We additionally considered whether the combination of miR-125b-1 and miR-378a gave a better predictive power. Because the high expression of miR-125b-1 and miR-378a might be negative predictive factors, we classified 68 patients into three groups according to the number of predictive factors (i.e. high expression of miR-125b-1 or miR-378a in cancer or stromal cells). The OS of each group was shown in the form of Kaplan–Meier curves in Figure S1. There was a significant difference between the "0 factor" group and the "2 factors" group. Hence, the combination of miR-125b-1 and miR-378a in cancer cells and stromal cells might have effective prognostic power.

The link between miR-125b-1 and miR-378a with immunotherapy is unclear, and it is very important to identify the target genes of these two miRNAs. Hence, we carried out RNA sequencing using 22 cancer tissues from a phase II study (data not shown). We classified the 22 samples into three groups according to the expression of each miRNA by qRT-PCR analysis: the high expression group in both cancer and stromal cells, the low expression group in both cell types, and the group of others. Difference in target gene expression between the "high expression group" and the "low expression group" was assessed by log2 ratio and Ingenuity Pathways Analysis (IPA) (Qiagen).[50] Regarding miR-125b-1, receptor activator of NF-κB (RANK) was downregulated in the "high expression group". RANK, a member of the TNF receptor family, and its ligand RANKL play important roles in the immune system. T cells activated as a result of RANKL expression stimulate dendritic cells, expressing RANK, to enhance their survival and enhance induction of T-cell response.[51,52] RANK is a target gene of miR-125b-1 by IPA (Qiagen), and miR-125b-1 might work as an immune suppressor regulating RANK. Regarding miR-378a, RNF125 was downregulated in the "high expression group". RNF125, E3 ubiquitin ligase, is positive regulator of T-cell activation.[53] miR-378a was considered to regulate RNF125 according to IPA and could also be an immune suppressor. Thus, miR-125b-1 and miR-378a might be therapeutic targets.

It must be mentioned that the mean survival time of the "HLA matched-miRNA high" group appeared to be shorter than that of the "HLA unmatched-miRNA high" group (Fig. 1). However, there was no significant difference between their survival curves (Fig. S2). Furthermore, we investigated side-effects in the "HLA matched-miRNA high" group. The incidence of severe AE in each group of miR-378a is shown in Table S2. There was no significant difference in the incidence of AE between the "HLA matched-miRNA high" group and other groups. It is quite unlikely that vaccine treatment led to deleterious effects in "HLA matched-miRNA low" patients.

It is known that the behavior of cancer cells and the development of tumor tissues are modulated by cross-talk through miRNAs between cancer cells and stromal cells such as

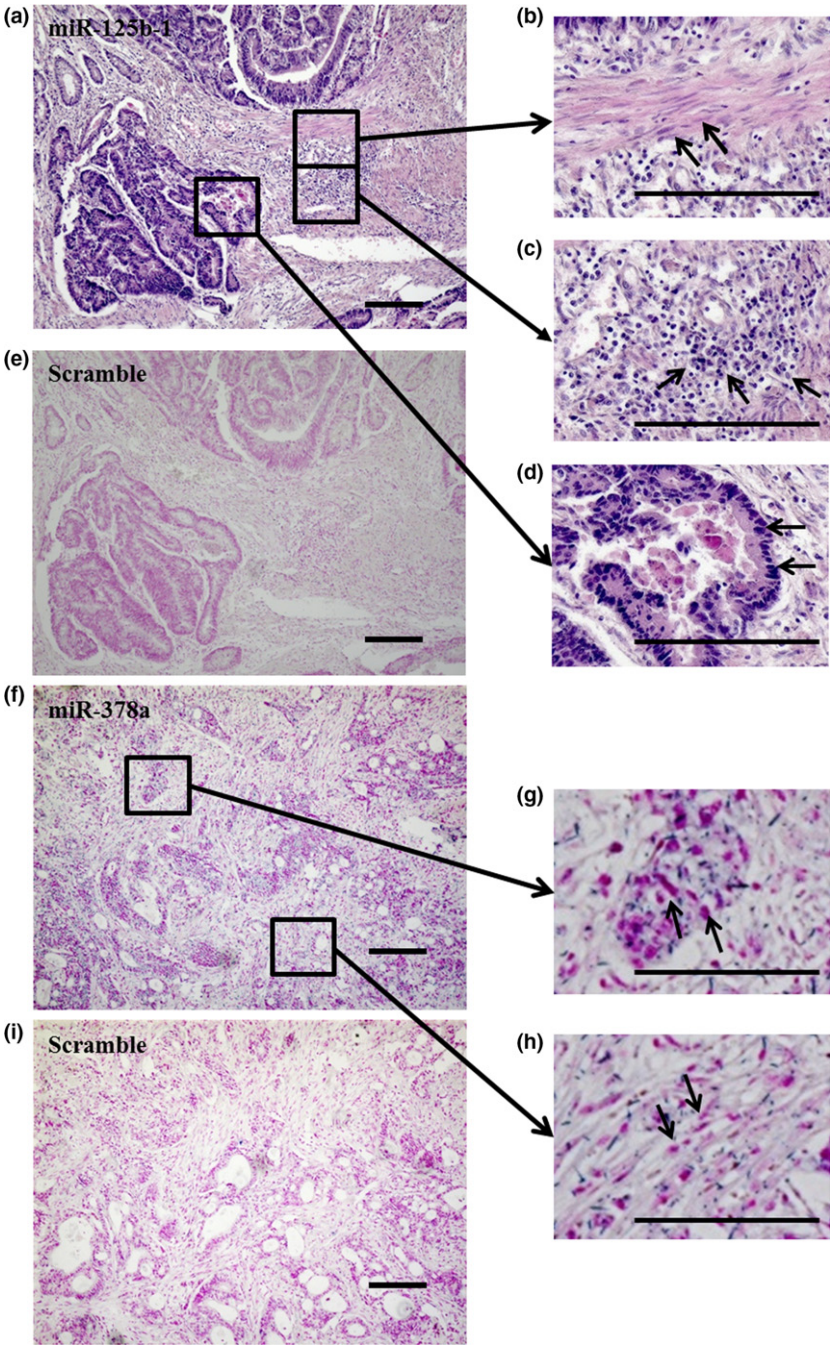

Fig. 2. *In situ* hybridization detecting miR-125b-1 and miR-378a. (a) miR-125b-1 is highly expressed in cancer cells and stromal cells. (b) Arrows indicate miR-125b-1-positive fibroblast-like cells. (c) Arrows indicate miR-125b-1-positive mononuclear cells. (d) Arrows indicate miR-125b-1-positive cancer cells. (e) Scramble probe. (f) A miR-378a signal was observed in both cancer cells and stromal cells. (g) Arrows indicate miR-378a-positive cancer cells. (h) Arrows indicate miR-378a-positive fibroblast-like cells. (i) Scramble probe. Bar, 200 μm. miR, microRNA.

fibroblasts, endothelial cells, pericytes, immune cells, and local and bone marrow-derived stromal stem cells.[54] Although several studies have reported the importance of miRNAs such as miR-21,[23] miR-17-92a and the miR-106b-25 cluster[22] in cancerous stromal cells, the present study is the first to regard the role of miR-125b-1 and miR-378a in colorectal cancer cells and stromal cells for predictive markers of immunotherapy.

In conclusion, miR-125b-1 and miR-378a expression might be considered novel biomarkers to predict the efficacy of immunotherapy in CRC. Although our results are preliminary and the functions of each of the miRNAs in immune responses are still unclear, the applicability of these miRNAs should be confirmed in a future large-scale study as biomarkers to select patients who can expect a better outcome with the vaccine treatment.

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Disclosure Statement

Yusuke Nakamura is a stockholder and a scientific advisor of OncoTherapy Science, Inc. The other authors declare no potential conflicts of interest for this article.

Abbreviations

AE	adverse event
CA19-9	carbohydrate antigen 19-9
CEA	carcinoembryonic antigen
CI	confidence interval
CRC	colorectal cancer
CRP	C-reactive protein
DIG	digoxigenin
FFPE	formalin-fixed paraffin-embedded
HLA	human leukocyte antigen
HR	hazard ratio
ISH	*in situ* hybridization
LCM	laser capture microdissection
LNA	locked nucleic acid
mCRC	metastatic CRC
miRNA	microRNA
NLR	neutrophil lymphocyte ratio
OS	overall survival
PD-1	programmed death-1
PD-L1	PD-ligand 1
PFS	progression-free survival
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative RT-PCR
RNF125	ring finger protein 125
ROC	receiver operating characteristic
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Top 30 miRNAs upregulated or downregulated in a responder group compared with a non-responder group, as assessed using microarray analysis.

Table S2. Frequency of severe adverse events (CTCAE version 3.0) in four groups classified according to miR-378a expression and *HLA-A* status.

Fig. S1. Overall survival (OS) according to number of predictive factors. OS of the *HLA-A*2402*-matched (left side) and -unmatched groups (right side) of phase II study patients according to the number of predictive factors (high expression of miR-125b-1 and/or miR-378a). (a) Predictive factors in cancer cells. (b) Predictive factors in stromal cells. Dotted line shows the survival curve of patients with 0 factors. Solid line shows the survival curve of patients with one factor and the dashed line shows the survival curve of patients with two factors. Statistical analysis was done using the log–rank test. HLA, human leukocyte antigen.

Fig. S2. Overall survival (OS) according to miRNA expression and *HLA-A* status. (a) According to miR-125b-1 in cancer cells. (b) According to miR-125b-1 in stromal cells. (c) According to miR-378a in cancer cells. (d) According to miR-378a in stromal cells. Red line shows *HLA-A*2402*-matched group and black line shows -unmatched group of phase II study patients. Solid line shows high miRNA expression and the dashed line shows low miRNA expression. Statistical analysis was done using the log–rank test. HLA, human leukocyte antigen; miR, microRNA.