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Evaluation of Organoid-Derived Exosomal microRNA as Liquid Biopsy for Colorectal Cancer: A Multicenter Cross-Sectional Study

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ABSTRACT

Exosomal microRNAs (miRNAs) are candidates for liquid biopsies. Organoid culture systems enable long-term expansion of the colon epithelium. This study evaluated exosomal miRNAs from colorectal cancer organoids for liquid biopsy. Organoids were established from normal colon and colorectal cancer tissues. Exosomes were isolated from conditioned media. miRNAs were extracted from exosomes and compared using microarray analysis. Exosomal miRNAs expression levels in the sera of healthy patients and patients with colorectal cancer were compared at a single institution. The multicenter study was validated using miRNAs upregulated in the serum of colorectal cancer patients, along with exosomal miRNAs reported to be upregulated in colorectal adenoma organoids and sera. A total of 44 exosomal miRNAs were commonly expressed in both normal colorectal epithelial cells and colorectal cancer organoids, whereas 59 were exclusively expressed in colorectal cancer organoids. In a single-center cohort study, two exosomal miRNAs (miR-4284 and miR-5100) were upregulated in the serum of colorectal cancer patients. In a multicenter study, four exosomal miRNAs (miR-4284, miR-5100, miR-1246, and miR-1290) were upregulated in the serum of patients with colorectal cancer. The combination of these four exosomal miRNAs had comparable diagnostic performance to carcinoembryonic antigen, with an area under the curve of 0.75 (95% confidence interval: 0.65–0.83) versus 0.79 (95% confidence interval: 0.70–0.87). Combining the four miRNAs with carcinoembryonic antigen improved diagnostic accuracy, with an area under the curve of 0.82 (95% confidence interval: 0.74–0.89). Exosomal miRNAs derived from colorectal cancer organoids can serve as diagnostic biomarkers for colorectal cancer.

Summary

- What is the current knowledge on the topic?
  - Exosomal miRNAs are promising circulating biomarkers for the non-invasive diagnosis of colorectal cancer. Recent advances in organoid culture have enabled the growth of previously unculturable cell types, allowing the extraction of exosomes and exosomal miRNAs. However, few studies have evaluated organoid-derived exosomal miRNAs, and no large-scale investigations have been conducted to assess their clinical utility in liquid biopsy.
- What question did this study address?
  - This study investigated whether miRNAs derived from colorectal cancer organoids could serve as a liquid biopsy biomarker for colorectal cancer diagnosis in a large-scale, multicenter clinical setting.
- What does this study add to our knowledge?
  - We identified four exosomal miRNAs—miR-4284, miR-5100, miR-1246, and miR-1290—as effective diagnostic biomarkers for colorectal cancer. This is the first large-scale study to demonstrate the clinical potential of organoid-derived miRNAs in liquid biopsy.
- How might this change clinical pharmacology or translational science?
  - The findings of this study suggest that organoid culture systems can be utilized to identify candidate miRNAs for liquid biopsy across various tumor types. This approach may contribute to the advancement of non-invasive cancer diagnostics.

1 | Introduction

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related deaths worldwide [1]. Fecal occult blood test using immunoassays [2] is widely used for CRC screening, but it has limitations in terms of sensitivity and participation rates [3–5]. Although colonoscopy is more accurate, its invasiveness, cost, and low adherence make it unsuitable for large-scale screening [6]. Carcinoembryonic antigen (CEA) is used as a non-invasive marker; however, its low sensitivity and elevated levels due to smoking and aging limit its utility as a stand-alone diagnostic marker [7, 8].

MicroRNAs (miRNAs) have recently gained attention as cancer biomarkers. MiRNAs are small non-coding RNAs, approximately 20 nucleotides in length, that are present in body fluids, such as blood, urine, breast milk, and saliva, either in free or encapsulated form within small extracellular vesicles (exosomes) measuring 50–100 nm in size [9, 10]. Exosomal miRNAs are more stable than serum miRNAs, making them promising non-invasive biomarkers [11]. Elevated levels of specific exosomal miRNAs have also been reported [9]. However, the clinical application of exosomal miRNAs remains challenging owing to issues such as low cancer specificity, complex extraction methods, and lack of large-scale patient sample studies.

In 2009, a three-dimensional (3D) organoid culture system was established, enabling long-term culture of normal colonic epithelium and colorectal tumors [12]. Because organoid cultures closely mimic the morphological and functional characteristics

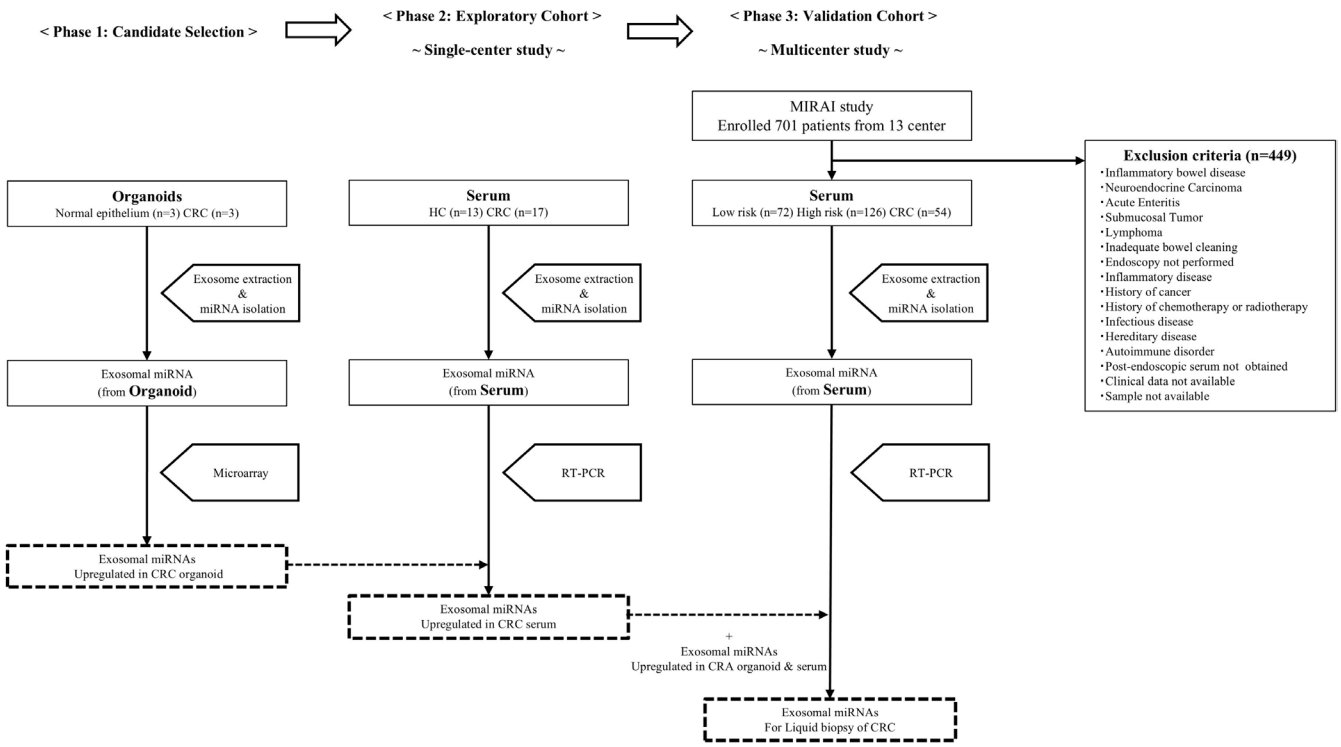

FIGURE 1 | Study design and workflow. This study followed a three-phase workflow to identify and validate exosomal miRNAs as biomarkers for CRC. CRC, colorectal cancer; miRNA, microRNA; RT-PRC, real-time polymerase chain reaction, MIRAI study; liquid biopsy using miRNAs associated with intestinal disease. The study was approved by the ethics committee in October 2021.

of in vivo tissues, [13–15] exosomal miRNAs derived from organoids are expected to serve as highly accurate biomarkers of cancer. In our previous study, we identified exosomal miRNAs (miR-1246, miR-1290, miR-4284, and miR-4323) specific to colorectal adenomas using an organoid culture system [16]. Based on these findings, the aim of this study was to identify exosomal miRNAs secreted by CRC organoids and evaluate their potential as liquid biopsy candidates using serum samples from patients with CRC.

2 | Methods

2.1 | Study Design

This study was conducted in three phases (Figure 1). In the first phase, 3D organoids were established from normal colon epithelium and CRC tissues, and the exosomes secreted from each organoid were isolated. The miRNAs were extracted from the isolated exosomes, and microarray analysis was performed to compare the normal colon epithelium and CRC to identify CRC-derived exosomal miRNAs. In the second phase, the expression levels of candidate serum exosomal miRNAs identified in the first phase were assessed using real-time polymerase chain reaction (RT-PCR) using serum from patients with CRC and healthy individuals at a single center (exploratory cohort). Third, the usefulness of miRNAs as a biomarker was verified in a cross-sectional large-scale multicenter study. Candidate miRNAs were those found in phase 2 (exploratory cohort) and those from colorectal adenoma organoids and sera, as previously reported [16].

2.2 | Patients

In the first phase, three CRC organoids were established from patients with advanced CRC who underwent surgical resection at the Tohoku University Hospital, and three normal colorectal epithelial organoids were established from the normal epithelium of patients who underwent endoscopic resection of colorectal adenomas at the same institution. The clinical characteristics of patients with established organoids are presented in Table S1. In the second phase, serum samples from healthy individuals and patients with CRC archived at Tohoku University Hospital were used. Healthy serum samples were obtained from 13 patients with no endoscopic evidence of tumorous lesions and no history of cancer or chronic inflammation. Serum samples from 17 patients were included, excluding those from patients with appendiceal cancer, neuroendocrine tumors, or multiorgan cancer. The clinical characteristics of the patients are summarized in Table S2. In the third phase, serum samples were collected from patients aged 15 years or older who underwent colonoscopy between November 2021 and September 2023 in the Liquid Biopsy Using miRNAs Associated with Intestinal Disease (MIRAI) study, which was a multicenter study conducted across 13 centers and included 701 patients. After excluding patients that met the exclusion criteria (Figure 1), 252 patients were enrolled in this study. Based on the European Union guidelines, [17] eligible patients were classified into three groups: 72 in the low risk group, 126 in the high-risk group, and 54 in the CRC group. The low risk group was designated as healthy controls. The clinical characteristics of the patients are summarized in Table 1. Ethical approval for phases 1 and 2 of this study was obtained from the Ethics Committee of the Tohoku Medical Megabank Organization (Reception No. 2015–4-011) and the Ethics Committee of Tohoku University Hospital (Reception Nos. 2017–1-346 and 2023–1-558). Phase 3 was approved by the Ethics Committee of Tohoku University Hospital (Reception No. 2022–1-249) in October 2021, and informed consent was obtained from all participants.

2.3 | Establishment of Organoid Cultures

Organoid cultures were established with slight modifications from previously reported protocols [18, 19]. Both CRC and normal colonic epithelia were cultured using tissues washed with phosphate-buffered saline (PBS), resuspended in Matrigel, and seeded in 24-well plates. The culture medium contained epidermal growth factor, Noggin, A83-01, SB202190, and a base medium (Advanced DMEM/F12, GlutaMAX, B27 supplement, and penicillin/streptomycin). The medium was changed every 2–3 days, and the organoids were passaged every 7 days. For CRC, the protocol described above was used without additional modifications. For the normal colonic epithelium, R-spondin-1, Afamin/Wnt3a, and Y-27632 were added.

TABLE 1 | Clinical characteristics of patients in the multicenter study (Phase 3).

	Low risk ($n=72$)[a]	High risk ($n=126$)[b]	CRC ($n=54$)[c]
Age (≥65 years), *n* (%)	37 (51.4)	82 (65.1)	32 (59.3)
Sex (Male), *n* (%)	36 (50.0)	82 (65.1)	26 (48.1)
Diabetes, *n* (%)	61 (84.7)	95 (75.4)	40 (74.1)
Smoking, *n* (%)	5 (6.9)	25 (19.8)	10 (18.5)
Stage, *n* (%)			
I	N/A	N/A	11 (20.4)
II	N/A	N/A	15 (27.8)
III	N/A	N/A	11 (20.4)
IV	N/A	N/A	12 (22.2)
Unknown	N/A	N/A	5 (9.2)

Abbreviations: CRC, colorectal cancer; N/A, not applicable.
[a]Low risk was defined as the absence of any criteria that would categorize patients in the high-risk group.
[b]High risk was defined as the presence of any of the following criteria: adenomas ≥10 mm, high-grade dysplasia, villous component, ≥3 adenomas, serrated lesions ≥10 mm, or serrated lesions with dysplasia.
[c]CRC was classified as stage I or higher cancer.

2.4 | Exosome Extraction From Organoid-Conditioned Medium and RNA Isolation

Exosomes were extracted from organoid-conditioned medium using ultracentrifugation [20]. After several passages, the conditioned medium was collected and centrifuged at 300 *g* for 10 min, followed by centrifugation at 2000 *g* for 30 min to remove the cells. The supernatant was filtered through 0.2 μm filters and ultracentrifuged at 100,000 *g* for 70 min to pellet the exosomes, which were then washed with PBS. The vesicles were then subjected to a second ultracentrifugation step at 100,000 *g* for 70 min. Finally, the exosome pellet was resuspended in 100 μL of PBS and stored at −80°C. RNA was isolated from exosomes using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol of the manufacturer.

2.5 | Microarray Analysis

Microarray analysis (Agilent SurePrint G3 Human miRNA Microarray Kit 8 × 60 K, ver. Human_miRNA_V21.0; Agilent Technologies) and 2 ng of total RNA from the exosome fractions were labeled using a miRNA labeling reagent (Low Input Quick Amp Labeling Kit, RNA Spike-In Kit; Agilent Technologies). Labeled RNA was hybridized according to the instructions of the manufacturer. After hybridization, the array was washed and scanned using a DNA microarray scanner (Agilent Technologies). The raw data were numerically converted using a feature extraction software (ver. 10.7; Agilent Technologies) and the transformed data were analyzed using the GeneSpring software (ver. 12.5; Digital Biology).

2.6 | Exosome Isolation From the Serum of Patients and RNA Extraction

Whole blood was centrifuged at 1900 *g* for 10 min and at 16,000 *g* for 10 min at 4°C to obtain serum from which exosomal miRNAs were isolated. Exosomes were isolated from the serum using the polymer precipitation method with ExoQuick (System Biosciences, Palo Alto, CA, USA). Specifically, 400 μL of serum was thoroughly mixed with 100 μL of ExoQuick solution and incubated at 4°C for 30 min. The mixture was centrifuged at 1500 *g* for 30 min, the supernatant was discarded, and the remaining pellet was centrifuged again at 1500 *g* for 5 min. The exosome pellet was resuspended in 100 μL of D-PBS. RNA was extracted from the exosomes using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the protocol of the manufacturer.

2.7 | Quantitative RT-PCR Analysis of miRNAs

Serum concentrations of candidate miRNAs were quantified by RT-PCR using the TaqMan miRNA Reverse Transcription kit and TaqMan miRNA Assay kit (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed in duplicate using a LightCycler 96 system (Roche, Basel, Switzerland). The miR-16 was used as an internal control, and miRNA expression levels were normalized to miR-16 using the 2^−ΔCt method.

2.8 | Statistical Analysis

Statistical analyses were performed using JMP Pro software (version 17.0; SAS Institute, Cary, NC, USA). In the second phase, the expression levels of exosomal miRNAs in the serum of healthy controls and patients with CRC were compared using unpaired *t*-tests after log transformation and normalization of the data. Statistical significance was set at $p < 0.05$. Similarly, in the third phase, the miRNA expression levels between the control and CRC groups were compared using unpaired *t*-tests following log transformation and normalization. Comparisons between groups for each miRNA were performed using the Benjamini–Hochberg method to adjust for the false discovery rate (FDR). An FDR-adjusted value of less than 0.05 was considered statistically significant. Univariate analyses of the clinical factors associated with elevated miRNA and CEA levels were conducted using the chi-square test or Fisher's exact test. Multivariate analysis was then performed using multiple logistic regression, limited to factors with $p < 0.2$ in the univariate analysis. The diagnostic performance of each miRNA was assessed using receiver operating characteristic (ROC) curves and area under the curve (AUC).

3 | Results

3.1 | Analysis of Exosomal miRNA Derived From Normal Epithelium and CRC Organoids

Microarray analysis was used to compare the differential expression of the three exosomal miRNAs derived from normal colorectal epithelial organoids and three exosomal miRNAs derived from CRC organoids. A total of 87 miRNAs were identified in normal colorectal epithelium-derived exosomes and 103 were identified in CRC-derived exosomes. Of the total, 44 miRNAs were expressed in both exosomes derived from normal colorectal epithelium and CRC, and 59 were expressed only in exosomes derived from CRC organoids (Figure S1A). Among the 44 commonly expressed miRNAs, 40 were upregulated and 4 were downregulated in the CRC group relative to the normal colorectal epithelium group (Figure S1B). Six miRNAs (miR-3162-5p, miR-6127, miR-5100, miR-642a-3p, miR-6879-5p, and miR-1202) showed a fold change > 1.5 and were considered candidate miRNAs for liquid biopsy. Among the 59 miRNAs expressed only in CRC-derived exosomes, 10 with high expression levels (miR-4484, miR-6760-5p, miR-8069, miR-671-5p, miR-5196-5p, miR-197-5p, miR-8485, miR-4284, miR-6851-3p, and miR-6858-3p) were selected as candidate miRNAs for liquid biopsy. A list of the candidate miRNAs is shown in Table 2. Of the 16 candidate miRNAs, 8 (miR-6127, miR-5100, miR-642a-3p, miR-1202, miR-4484, miR-5196-5p, miR-671-5p, and miR-4284) were selected for the second phase based on primer sequence availability.

3.2 | Evaluation of Candidate miRNAs in the Exploratory Cohort

This exploratory cohort study was conducted at a single center. The expression levels of candidate exosomal miRNAs in the serum identified in phase 1 were confirmed by RT-PCR. Two exosomal miRNAs (miR-4284 and miR-5100) were significantly

upregulated (miR-4284: $p < 0.01$; miR-5100: $p < 0.01$), whereas miR-5196-5p was significantly downregulated ($p < 0.01$) in the serum of patients with CRC compared with healthy controls (Figure 2).

3.3 | Evaluation of Candidate miRNAs in the Validation Cohort

In the validation cohort, we selected two miRNAs (miR-4284 and miR-5100) identified in the exploratory cohort, along with four exosomal miRNAs (miR-1246, miR-1290, miR-4284, and miR-4323) that were previously reported to be upregulated in organoids and sera derived from patients with colorectal adenomas [16]. Compared with the control group, the CRC group showed significant upregulation of CEA and four miRNAs (miR-4284, miR-5100, miR-1246, and miR-1290; Figure 3).

To compare the expression levels of exosomal miRNAs and CEA in the serum of patients with CRC, we analyzed the relationship between the expression levels of exosomal miRNAs and CEA and clinical factors using univariate and multivariate

TABLE 2 | Exosomal miRNA expression comparison between CRC organoids and normal colorectal epithelial organoids using microarray analysis.

Exosomal miRNA					
Expressed in CRC and normal epithelium			**Expressed only in CRC**		
Systematic name	**$\log_2$ FC**	***p***	**Systematic name**	**$\log_2$ FC**	***p***
hsa-miR-3162-5p	2.86	0.051	hsa-miR-4484	8.77	0.01
hsa-miR-6127	2.74	0.069	hsa-miR-6760-5p	7.91	0.019
hsa-miR-5100	2.48	0.16	hsa-miR-8069	7.47	0.013
hsa-miR-642a-3p	2.19	0.13	hsa-miR-671-5p	7.41	0.035
hsa-miR-6879-5p	1.79	0.2	hsa-miR-5196-5p	7.38	0.018
hsa-miR-1202	1.54	0.3	hsa-miR-197-5p	6.77	0.02
			hsa-miR-8485	5.34	0.02
			hsa-miR-4284	4.88	< 0.01
			hsa-miR-6851-3p	4.86	< 0.01
			hsa-miR-6858-3p	4.81	< 0.01

Abbreviations: CRC, colorectal cancer; FC, fold change; miRNA, microRNA.

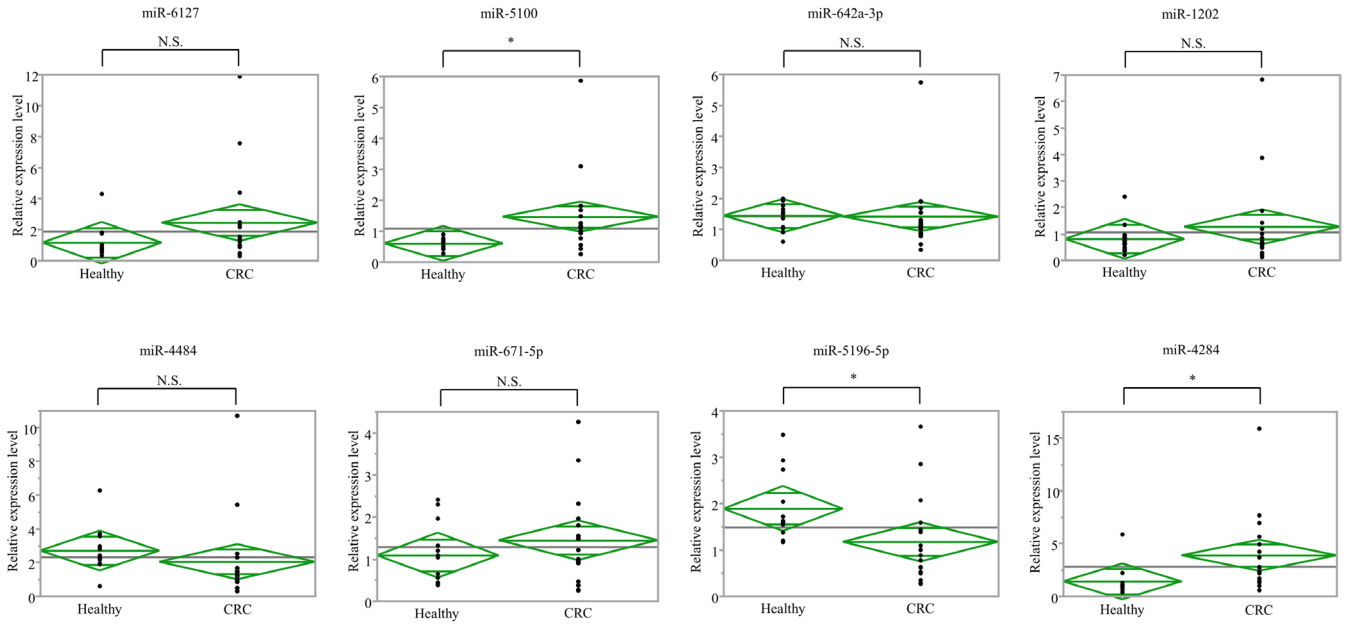

FIGURE 2 | Expression levels of exosomal miRNAs and CEA in patients with CRC compared with healthy individuals in a single-center cohort (phase 2). Statistical analysis was performed using unpaired *t*-tests. *$p < 0.05$. miRNA, microRNA; CEA, carcinoembryonic antigen; CRC, colorectal cancer; NS, not significant.

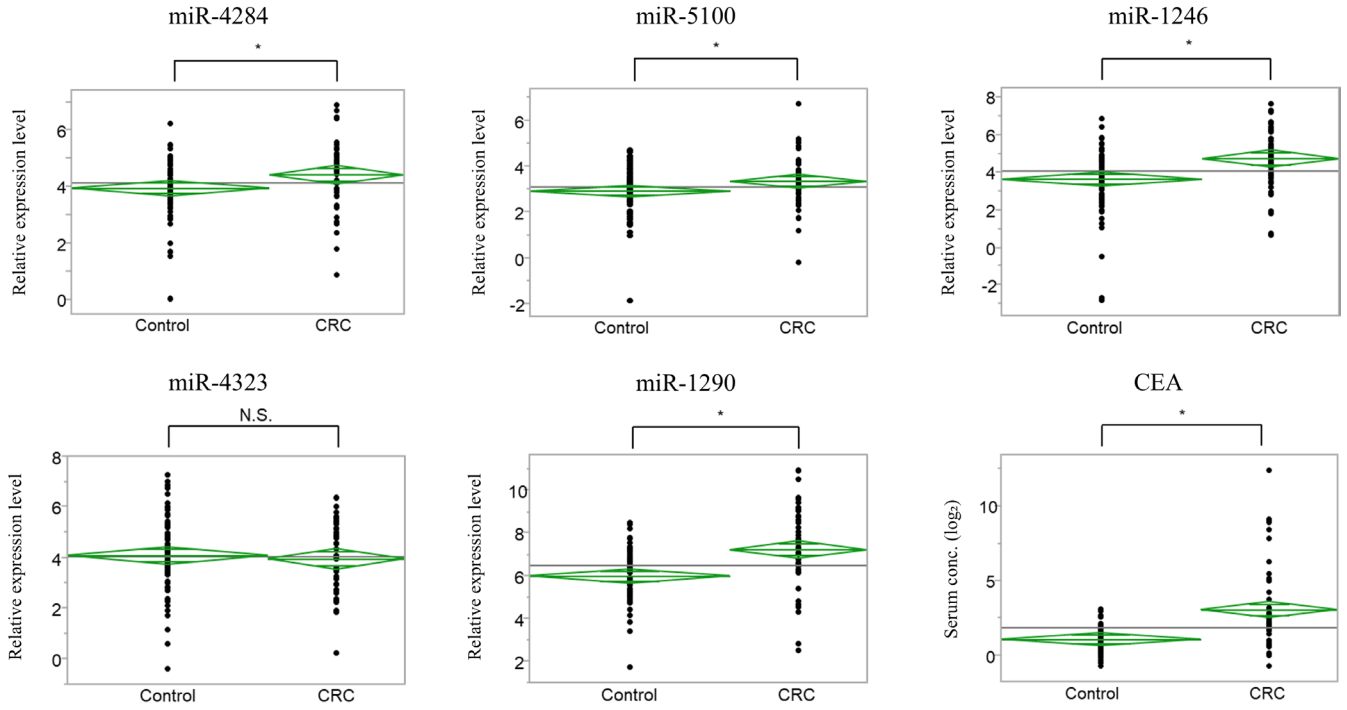

FIGURE 3 | Expression levels of exosomal miRNAs and CEA in patients with CRC compared with controls in a multicenter study (phase 3). Statistical analysis was performed using the *t*-test, with false discovery rate adjustment applied using the Benjamini–Hochberg method. *$p < 0.05$. miRNA, microRNA; CEA, carcinoembryonic antigen; CRC, colorectal cancer; NS, not significant.

analyses (Table 3). The miRNA risk group was defined as having at least three of the four exosomal miRNAs (miR-4284, miR-5100, miR-1246, and miR-1290) exceed the cutoff values determined by the Youden index in each ROC analysis. The cutoff values for miR-4284, miR-5100, miR-1246, and miR-1290 were 4.54, 3.53, 3.85, and 6.45, respectively. Elevated CEA levels were defined as values exceeding the standard threshold (5.0 μg/mL). We analyzed 49 CRC cases, excluding five cases with unknown classifications, and compared them with 72 cases in the control group. The multivariate analysis showed that the only clinical factor associated with the miRNA risk group was tumor stage. Regarding the relationship between the miRNA risk group and tumor stage, the odds ratios compared with the control group were 1.83 (95% confidence interval [CI]: 0.93–3.62, $p = 0.08$) for the high-risk group, 3.80 (95% CI: 1.46–9.89, $p < 0.05$) for stage I/II, and 13.7 (95% CI: 4.36–42.9, $p < 0.05$) for stage III/IV. The clinical factors associated with elevated CEA levels were age (≥ 65 years), smoking, and tumor stage.

3.4 | Diagnostic Ability of Candidate miRNAs as Biomarkers

We analyzed the diagnostic ability of candidate miRNAs that were upregulated in the CRC group using ROC curves. In the comparison between the control and CRC groups, the AUC values for serum exosomal miR-4284, miR-5100, miR-1246, and miR-1290 were 0.64 (95% CI: 0.53–0.74), 0.62 (95% CI: 0.52–0.71), 0.71 (95% CI: 0.61–0.79), and 0.75 (95% CI: 0.65–0.83), respectively. The combination of four exosomal miRNAs showed an AUC of 0.75 (95% CI: 0.65–0.83), and CEA had an AUC of 0.79 (95% CI: 0.70–0.87). Combining the miRNAs with CEA improved diagnostic accuracy to an AUC of 0.82 (95% CI: 0.74–0.89; Figure 4A).

Validation revealed that miRNA expression was not associated with clinical factors other than the tumor stage (stage I/II: $p < 0.01$; stage III/IV: $p < 0.01$) (Table 3). In contrast, CEA expression was significantly affected by clinical factors such as age ($p < 0.01$), smoking ($p < 0.01$), and tumor stage (stage I/II: $p = 0.03$; stage III/IV: $p < 0.01$) (Table 3). Therefore, we analyzed the ROC curves to assess how age and smoking affect the diagnostic performance of these two biomarkers. In patients aged ≥ 65 years, the combined AUC for the four serum exosomal miRNAs was 0.77 (95% CI: 0.63–0.86), whereas the AUC for CEA was 0.85 (95% CI: 0.73–0.92; $p = 0.27$). In patients < 65 years, the combined AUC for the four exosomal miRNAs was 0.76 (95% CI: 0.60–0.87), whereas the AUC for CEA was 0.74 (95% CI: 0.58–0.85; $p = 0.81$). There was a tendency for CEA levels to be lower in patients < 65 years. In smokers, the combined AUC for the four exosomal miRNAs was 0.86 (95% CI: 0.52–0.97), whereas the AUC for CEA was significantly lower at 0.48 (95% CI: 0.20–0.77; $p < 0.05$). In non-smokers, the AUCs for the miRNAs and CEA were 0.73 (95% CI: 0.61–0.82) and 0.82 (95% CI: 0.72–0.89), respectively ($p = 0.13$). The diagnostic performance of serum exosomal miRNAs for CRC was superior to CEA in the smoking group (Figure 4B).

4 | Discussion

Studies on miRNAs as potential liquid biopsy markers have mainly focused on miRNAs extracted from fixed samples, biopsy tissues, or serum, with few reports evaluating exosomal miRNAs derived from organoids [16, 21]. Organoid cultures closely mimic

TABLE 3 | Clinical factors associated with the elevated exosomal miRNA risk group and levels of CEA.

	Exosomal miRNA risk group ($n=87$)				High expression of CEA ($n=44$)			
		Univariate[a]	Multivariate[b]			Univariate[a]	Multivariate[b]	
	n (%)	*p*	OR (95% CI)	*p*	*n* (%)	*p*	OR (95% CI)	*p*
Age								
<65 years	34/101 (33.7)	0.69			10/101 (9.90)	<0.01	Reference	<0.01
≥65 years	53/146 (36.3)				34/146 (23.3)		5.8 (2.11–15.9)	
Sex								
Male	45/141 (31.9)	0.23			21/141 (14.9)	0.18	Reference	0.19
Female	42/106 (39.6)				23/106 (21.7)		1.68 (0.77–3.69)	
Diabetes								
No	68/198 (34.3)	0.62			33/198 (16.7)	0.4		
Yes	19/49 (38.8)				11/49 (22.5)			
Smoking								
No	70/207 (33.8)	0.37			31/207 (15.0)	0.01	Reference	<0.01
Yes	17/40 (42.5)				13/40 (32.5)		5.88 (2.07–16.7)	
Classification								
Low risk	15/72 (20.8)	<0.01	Reference		5/72 (6.94)	<0.01	Reference	
High risk	41/126 (32.5)		1.83 (0.93–3.62)	0.08	18/126 (14.3)		1.62 (0.55–4.81)	0.38
Stage I/II	13/26 (50.0)		3.80 (1.46–9.89)	<0.01	6/26 (23.1)		4.45 (1.16–17.1)	0.03
Stage III/IV	18/23 (78.3)		13.7 (4.36–42.9)	<0.01	15/23 (65.2)		20.1 (5.35–75.7)	<0.01

Abbreviations: CEA, carcinoembryonic antigen; CI, confidence interval; miRNA, microRNA; OR, odds ratio.
[a]Chi-square test or Fisher's exact probability test.
[b]Multiple logistic regression analysis. Factors with $p<0.2$ in the univariate analysis were included in the multivariate analyses.

the in vivo tissue characteristics, [13–15] allowing the evaluation of exosomal miRNAs secreted by CRC cells in a more physiologically relevant environment. In this study, we showed that miR-4284 and miR-5100 were upregulated in the serum of patients with CRC. miR-4284 is upregulated in non-small cell lung and pancreatic cancers, [22, 23] whereas miR-5100 is upregulated in pancreatic cancer and squamous cell carcinoma [22, 24]. miRNAs have not been previously reported in CRC, making them potential novel candidates for liquid biopsy in CRC.

Circulating miRNAs that are used in liquid biopsies include serum and exosomal miRNAs. Although serum miRNAs can be easily extracted directly from the serum, they are unstable in the presence of blood RNases and are affected by sample conditions [25]. In contrast, serum exosomal miRNAs are stable in blood [11] and are less affected by sample processing, storage, or transportation. Multicenter studies require the handling of large numbers of samples, making it essential to use stable biomarkers such as exosomal miRNAs. Therefore, we focused on exosomal miRNAs, which are stable in blood, and extracted exosomes using a polymer precipitation method, which is easier to perform than ultracentrifugation. Few large-scale studies have used this method; hence, our results pave the way for the clinical application of exosomal miRNA biomarkers.

In this study, the combination of four exosomal miRNAs showed a diagnostic performance comparable to that of CEA, and combining these miRNAs with CEA further improved the accuracy. Combining miRNAs with other markers enhances the diagnostic performance [26–28] which is consistent with our findings. Further exploration of these combinations may improve the accuracy of CRC diagnosis.

The exosomal miRNAs identified in this study significantly correlated with tumor progression. Compared with that in the

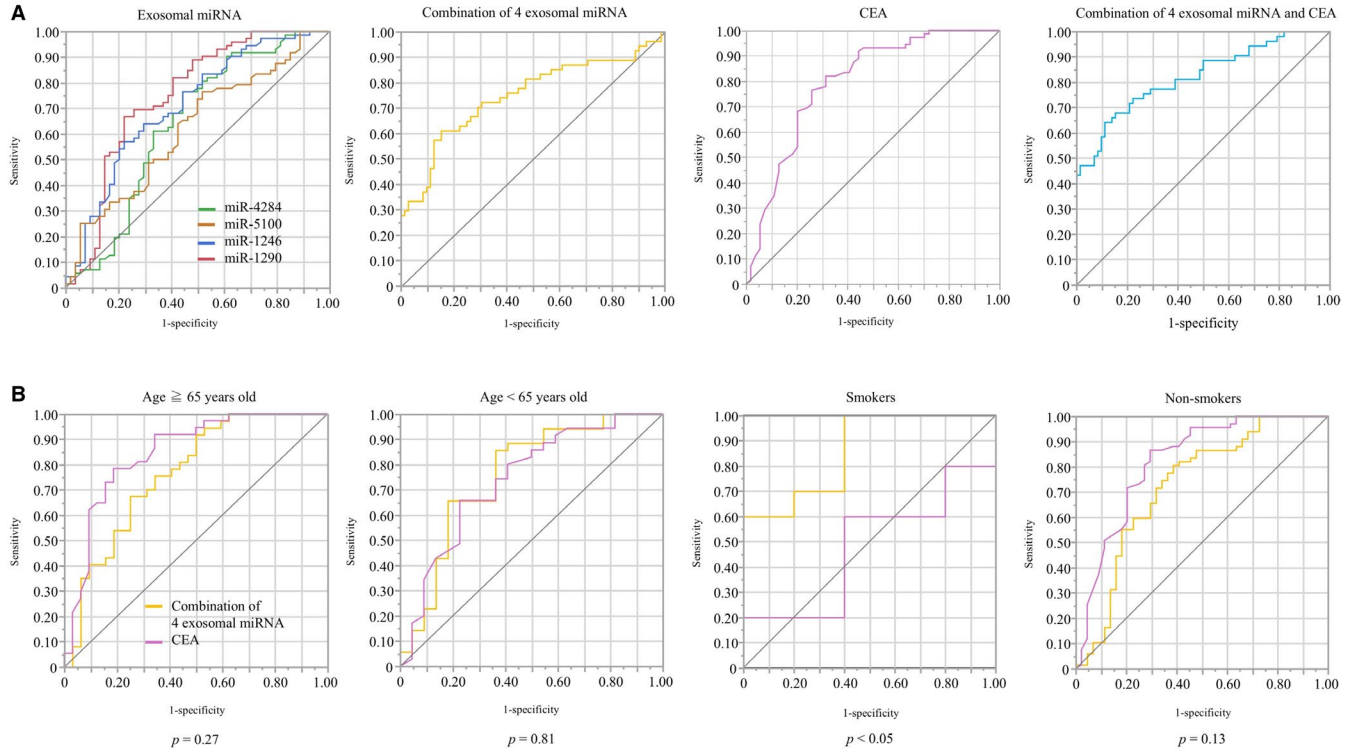

FIGURE 4 | ROC curve analyses comparing the diagnostic performance of exosomal miRNAs and CEA in patients with CRC. (A) ROC curves comparing individual exosomal miRNAs, their combination, CEA, and the combination of four exosomal miRNAs with CEA. (B) ROC curves stratified by age (≥65 vs. <65 years) and smoking status (smokers vs. non-smokers) compared with the combination of four exosomal miRNAs and CEA. A significant difference was observed in smokers ($p < 0.05$). ROC, receiver operating characteristic; miRNA, microRNA; CEA, carcinoembryonic antigen; CRC, colorectal.

control group, miRNA expression was significantly elevated in both stage I/II and III/IV groups. In the high-risk group, no significant differences were observed, although the miRNA expression tended to be higher. The high-risk group included a broad range of cases, from small polyps to large mucosal cancers, which may explain the lack of distinction. A more detailed classification could facilitate improved stratification. Although smoking and aging have been reported to affect the miRNA expression profiles, [29, 30] the exosomal miRNAs identified in this study were unaffected by factors other than tumor progression. This could be because the candidate miRNAs were directly secreted by CRC organoids, reducing the impact of environmental factors. In contrast, age and smoking have long been known to influence CEA levels, and similar results were observed in the present study. Notably, the diagnostic performance of serum exosomal miRNAs for cancer was superior to that of CEA in smokers. Our findings suggest that exosomal miRNAs, when differentiated from conventional biomarkers, can enhance screening accuracy.

This study had several limitations. First, the small number of organoid cultures may have limited the comprehensive identification of exosomal miRNAs secreted by the organoids. Second, when selecting candidate miRNAs from exosomes derived from organoids, we prioritized those with a fold change (FC) of 1.5 or higher for miRNAs common to both CRC and normal organoids, as well as the top 10 miRNAs exclusively secreted by CRC. Since this approach did not encompass all miRNAs, other potential candidate miRNAs may have been overlooked. Third, in this study, the diagnostic performance of miRNAs was superior to that of CEA in the smoking group. However, given the limited number of smokers, larger-scale studies are necessary to confirm this finding. Finally, the cutoff values used in the validation cohort ideally should have been derived from the exploration cohort. However, because miRNA expression levels were assessed using the ΔΔCT method, it was not possible to apply the same cutoff values across cohorts. While using overlapping samples between cohorts could have been an issue, our study design did not incorporate such an approach.

In conclusion, this study demonstrated that a small set of miRNAs with defined cutoff values could effectively differentiate CRC. Future advancements in technology may further enhance screening accuracy. Organoid-based exosomal miRNA research, as demonstrated in this study, holds promise for advancing tumor biomarker development.

Author Contributions

A.S., M.K., Y.K., and A.M. wrote the manuscript. A.S. and M.K. designed the research. M.T., S.T., S.O., E.N., T.K., M.O., Y.S., T.M., H.C., N.Y., J.K., D.O., T.H., M.S., M.I., K.H., H.S., H.I., H.N., Y. Shimoyama, T.N., R.M., H. Shiga, and Y. Kakuta performed the research. Y.K. and A.M. analyzed the data.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.