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Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer

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Background: Functional microRNAs (miRNAs) in exosomes have been recognised as potential stable biomarkers in cancers. The aim of this study is to identify specific miRNAs in exosome as serum biomarkers for the early detection of recurrence in human colorectal cancer (CRC).

Methods: Serum samples were sequentially obtained from six patients with and without recurrent CRC. The miRNAs were purified from exosomes, and miRNA microarray analysis was performed. The miRNA expression profiles and copy number aberrations were explored using microarray and array CGH analyses in 124 CRC tissues. Then, we validated exosomal miRNAs in 2 serum sample sets (90 and 209 CRC patients) by quantitative real-time RT–PCR.

Results: Exosomal *miR-17-92a* cluster expression level in serum was correlated with the recurrence of CRC. Exosomal *miR-19a* expression levels in serum were significantly increased in patients with CRC as compared with healthy individuals with gene amplification. The CRC patients with high exosomal *miR-19a* expression showed poorer prognoses than the low expression group ($P<0.001$).

Conclusions: Abundant expression of exosomal *miR-19a* in serum was identified as a prognostic biomarker for recurrence in CRC patients.

Colorectal cancer (CRC) is the second most common cause of cancer mortality worldwide (Soerjomataram *et al*, 2012). Despite surgical resection and pre- and postoperative chemotherapy and molecular targeting therapy, relapse is common in patients with CRC (Akiyoshi *et al*, 2012; Amano *et al*, 2014). Recurrence, particularly liver metastasis, clearly contributes to the survival of patients with CRC. Many studies have examined potential biomarkers of recurrence in CRC in an attempt to improve patients' prognosis, and microRNAs (miRNAs), the small noncoding RNAs that are associated with the development of cancer, have been shown to be potential biomarkers in various types of cancers (Calin and Croce, 2006; Cortez *et al*, 2012; Komatsu *et al*, 2014).

Exosomes secreted from sheep reticulocytes were first reported in1983 (Pan and Johnstone, 1983), and the mechanism of exosome-mediated miRNA transfer was verified in 2007 (Valadi *et al*, 2007). Exosomes have the capacity to envelop specific miRNAs, maintain the integrity of contents (such as nucleic acids) in circulation and function as a tool for invasive access while reflecting the condition of donor cells (Palma *et al*, 2012; Ge *et al*, 2014; Kowal *et al*, 2014). Through these mechanisms, exosomal contents can be transmitted from donor cells to recipient cells.

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Thus, exosomal miRNAs can contribute to cancer development. Recent studies have shown that detection of specific exosomes may represent a novel diagnostic tool; indeed, ongoing studies are investigating the application of exosome contents as biomarkers and capsules for delivery of therapeutic drugs (van der Meel *et al*, 2014; Yoshioka *et al*, 2014b). Thus, exosomal miRNAs may represent both biomarkers and potential therapeutic targets (Hoshino and Matsubara, 2013). In terms of exosomal function, exosome-mediated cell-to-cell communication may play an important role in the development of cancer. Several reports have shown that specific exosomal miRNAs are related to the phenotypes of certain types of cancer and therefore may function as meaningful biomarkers (Tanaka *et al*, 2013; Ogata-Kawata *et al*, 2014; Xue *et al*, 2014). We previously revealed that a specific exosomal miRNA is a biomarker to predict the recurrence of liver cancer (Sugimachi *et al*, 2015). Moreover, many studies have investigated the application of miRNAs as molecular therapies in the treatment of CRC (Hoshino and Matsubara, 2013; Li and Rana, 2014).

Distant metastasis mediated through the hematogenous route directly contributes to the poor prognosis in patients with CRC (Field and Lipton, 2007; (Amano *et al*, 2014). In addition, CRC cells secrete exosomes into the circulation. Therefore, the aim of this study was to identify serum exosomal miRNAs as biomarkers of recurrence in human CRC. Among the miRNAs that have been shown to be associated with CRC, we focussed on miRNAs with copy number aberrations as evaluated by array comparative genomic CGH (aCGH) in this study because these miRNAs should be robust, stable molecular targets. Finally, we examined the expression of exosomal miRNAs using microarray and quantitative real-time reverse transcription–PCR (qRT–PCR).

MATERIALS AND METHODS

Patients and sample collection. All serum and tissue samples were obtained from patients who underwent primary tumour resection at Kyushu University Beppu Hospital and affiliated hospitals between 1992 and 2007. Written informed consent was obtained from all patients. The study was approved by the institutional review board, the Ethics and Indications Committee of Kyushu University. We used a total of 227 serum samples from CRC patients, of which 6 samples were used for microarray and 221 samples were used for qRT-PCR. Serum samples were also obtained from 28 healthy volunteers for qRT–PCR. A total of 8 tumour tissues and 4 nearby normal tissues from CRC patients were used for aCGH, copy number analysis and miR microarray. All serum samples were redundant blood obtained for the purpose of examination during preoperative and follow-up phases. All patients had a histological diagnosis of CRC and were closely followed up every 3 months. The average overall survival time after resection was 4.64 years in the 209 patients from which samples were used for qRT–PCR. Tumour recurrence was observed in 132 of 165 patients with average interval of 4.82 years. We treated patients in accordance with the Japanese Society of Cancer of the Colon and Rectum Guidelines for the Treatment of Colorectal Cancer. Tumour tissue and normal tissue were immediately cut from the resected colorectal tissue and placed in RNAlater (Ambion, Palo Alto, CA, USA) or embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until RNA extraction. Clinicopathological factors and clinical stage were classified using the TNM system of classification. All data for the samples, including patient age, gender, tumour size and depth, lymphatic invasion, lymph node metastasis, vascular invasion, liver metastasis, peritoneal dissemination, distant metastasis, clinical stage and histological grade, were obtained from the clinical and pathological records.

Isolation of exosomes from serum. For the ultracentrifuge methods, serum of 1.5 ml for microarray and 1.0 ml for qRT–PCR were ultracentrifuged at 100 000 **g** for 70 min at $4\,^{\circ}C$. The pellets were washed with 11 ml of phosphate-buffered saline as described elsewhere (Sugimachi *et al*, 2015). Exosomes were extracted from a very small volume of serum using a Total Exosome Isolation Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The serum volume was fixed at 100 μl for validation by qRT–PCR of samples from 90 patients with CRC and 12 healthy volunteers.

Transmission electron microscopy. The isolated exosomes fraction was dissolved in HEPES buffer, and a drop of the suspension was placed on a sheet. A carbon-coated copper grid was floated on the drop for 10 s. The grid was then removed, and excess liquid was drained from the edge of the grid using a piece of clean filter paper. The grid was touched onto a drop of 2% uranyl acetate or phosphotungstic acid, pH 7.0, for $\sim$5 s, and excess liquid was removed. The grid was allowed to dry for several minutes and was then examined using a JEM1400 microscope (JEOL, Akishima, Japan) (Tanaka *et al*, 2013).

Extraction of total RNA and miRNA. Frozen tissue specimens were homogenised, and total RNA was extracted using a modified acid-guanidine-phenol-chloroform method. The RNA was extracted from exosomes in serum, using miRNeasy mini kit (Qiagen, Venlo, Netherlands) according to the manufacture's protocol. Exosomes purified from a certain volume of serum were diluted with 1 ml of Qiazol Lysis Reagent (Qiagen). After 5 min of incubation at room temperature, 10 μl of 0.1 nM *cel-miR-39* mimic was added to each sample followed by vortexing for 30 s. Subsequent extraction and cartridge work were carried out according to the manufacturer's protocol (Kosaka *et al*, 2010) (Yamamoto *et al*, 2011).

miRNA microarray analysis. The quality of total RNAs from the exosome fraction was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). No DNA contamination was observed. Exosomal miRNA expression profiles were examined using a miRNA microarray analysis system (3D-Gene, Toray, Tokyo, Japan).

Twelve samples (8 from cancer tissues and 4 from normal epithelia) were selected and used for the microarray. The concentration and purity of the total RNAs were assessed using a spectrophotometer, and RNA integrity was verified using an Agilent 2100 Bioanalyzer. Total RNA (100 ng) was directly labelled with cyanine3-CTP (Cy3), without fractionation or amplification, using an Agilent protocol that produces precise and accurate measurements spanning a linear dynamic range from 0.2 amol to 2 fmol of input miRNA. Total RNAs (50 ng) from 12 samples were competitively hybridised to a miRNA array (Agilent Microarray) according to the manufacturer's protocol. A list of miRNAs contained in the array is available from version 14.0 of the Sanger miRNA database (http://microrna.sanger.ac.uk). For data analysis, the intensity of each hybridisation signal was evaluated using Feature Extraction Software (Agilent Technologies) that examines multiple probes and multiple features per probe and studies the measurements and errors for each miRNA. The observed values were imported into GeneSpring GX version 11.0 (Agilent Technologies). Generated miRNA profiles were normalised to the amount of input total RNA.

aCGH and copy number analysis. A total of 124 colorectal tissues were prepared for aCGH analysis. For genome profiling, an Agilent Human Genome Microarray Kit 244K (Agilent Technologies) was used according to the manufacturer's instructions. The raw copy number data for each sample provided by aCGH were analysed using GISTIC algorithm (Mermel *et al*, 2011).

qRT–PCR analysis of miRNAs. Exosomal miRNA expression was assayed using qRT–PCR in serum sample of CRC patients. The cDNA was synthesised from total RNA using Taqman MicroRNA primers specific for *hsa-miR-16a*, *hsa-miR-17a*, *hsa-miR-18a*, *hsa-miR-19a*, *hsa-miR-19b*, *has-miR-20a and hsa-miR-92a* (Applied Biosystems, Tokyo, Japan) and a Taqman Micro-RNA Reverse Transcription Kit (Applied Biosystems). Relative quantification of miRNA expression was calculated using the 2-ΔΔCt method. The *miR-16a* was used as an internal control because it has been reported to be a reliable endogenous control for analysis of miRNA by RT–PCR in humans (Davoren *et al*, 2008). The raw data were presented as the relative quantity of target miRNA, normalised with respect to *miR-16a* and compared with a reference sample. Real-time monitoring of PCR products of samples from 90 patients with CRC and 12 healthy volunteers was performed using an ANI PRISM 7300 (Applied Biosystems) and Taqman Universal PCR Master Mix (Applied Biosystems) following the manufacturer's protocol. Real-time monitoring of PCR products of samples from 209 patients with CRC and 16 healthy volunteers was performed using LightCycler480 (Roche Applied Science, Basel, Switzerland) and Taqman Universal PCR Master Mix (Applied Biosystems) following the manufacturer's protocol. The amplification protocol included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s (Yoshioka *et al*, 2014a).

Table 1. Exosomal microRNAs in serum synchronised with liver metastasis

Rank	MicroRNAs	Fold change
Increased microRNAs		
1	hsa-miR-1288	3.9
2	hsa-miR-1204	2.91
3	**hsa-miR-4437**	2.86
4	**hsa-miR-23a**	2.7
5	hsa-miR-548v	2.59
6	hsa-miR-302c	2.51
7	hsa-miR-642b	2.51
8	hsa-miR-3618	2.41
9	hsa-miR-16–2*	2.34
10	**hsa-miR-19a**	2.3
11	**hsa-miR-19b**	2.25
12	hsa-miR-1246	2.23
13	hsa-miR-3681*	2.16
14	**hsa-miR-92a**	2.15
15	**hsa-miR-320a**	2.05
16	hsa-miR-3150a-5p	2.03
17	hsa-miR-761	2.03
18	hsa-miR-4478	2.02
Decreased microRNAs		
1	**hsa-miR-19b**	8.2
2	hsa-miR-320c	6.18
3	hsa-miR-199a-3p, –199b-3p	5.01
4	hsa-miR-191	4.6
5	hsa-miR-320d	4.56
6	hsa-miR-151b	4.18
7	hsa-miR-185	3.98
8	hsa-miR-146a	3.79
9	hsa-miR-17	3.64
10	**hsa-miR-23a**	3.59
11	hsa-miR-223	3.53
12	hsa-miR-151–5p	3.48
13	hsa-miR-103a	3.31
14	hsa-miR-92b	3.22
15	hsa-miR-107	3.19
16	hsa-miR-15b	3.08
17	hsa-miR-23b	3.02
18	hsa-miR-130a	2.9
19	hsa-let-7i	2.67
20	hsa-miR-30b	2.66
21	**hsa-miR-320a**	2.63
22	hsa-miR-15a	2.62
23	**hsa-miR-92a**	2.57
24	hsa-miR-4485	2.53
25	hsa-miR-320e	2.5
26	hsa-miR-34b*	2.48
27	**hsa-miR-19a**	2.39
28	hsa-miR-27a	2.36
29	hsa-miR-106a	2.34
30	hsa-miR-142–5p	2.29
31	hsa-miR-221	2.23
32	hsa-miR-181a	2.23
33	hsa-miR-3622b-5p	2.22
34	hsa-miR-320b	2.2
35	hsa-miR-21	2.2
36	hsa-miR-484	2.17
37	hsa-miR-1193	2.16
38	hsa-miR-26a	2.15
39	hsa-miR-425	2.1
40	hsa-miR-126*	2.09
41	hsa-miR-1825	2.08
42	hsa-miR-4306	2.07
43	hsa-miR-20a	2.07
44	hsa-miR-18b*	2.04
45	**hsa-miR-4437**	2.03
46	hsa-miR-30a	2.01

Candidate microRNAs associated with liver metastasis are underlined.

Statistical analysis. For continuous variables, data were expressed as mean ± s.d. Survival curves were plotted according to the Kaplan–Meier method and the generalised log-rank test was applied to compare the survival curves. Differences between groups were estimated using the χ^2 test, Student's *t*-test, repeated-measures analysis of variance (ANOVA), log-rank test and Cox regression model. All tests were analysed using JMP 5 software

Table 2. MiRNA clusters that reflect genomic amplification in CRC tissue

MiRNAs	Chromosome	Correlation	Amplification or deletion
miR-200b	1	0.616	Amplification
miR-583	5	0.607	Deletion
miR106b	7	0.914	Amplification
miR-25	7	0.948	Amplification
miR-93	7	0.916	Amplification
miR-96	7	0.786	Amplification
miR-598	8	0.806	Deletion
miR-939	8	0.929	Amplification
miR-1234	8	0.92	Amplification
miR-607	10	0.701	Deletion
miR-148b	12	0.741	Amplification
miR-15a	13	0.781	Amplification
miR-16	13	0.754	Amplification
miR-17	13	0.701	Amplification
miR-18a	13	0.767	Amplification
miR-19a	13	0.753	Amplification
miR-19b	13	0.754	Amplification
miR-20a	13	0.704	Amplification
miR-92a	13	0.727	Amplification
miR-624	14	0.618	Deletion
miR-744	17	0.945	Deletion
miR-633	17	0.786	Amplification
miR-122	18	0.906	Deletion
miR-150	19	0.64	Amplification

Abbreviations: CRC = colorectal cancer; miRNA = microRNA.
Candidate microRNAs associated with liver metastasis are underlined

(JMP, Cary, NC, USA), and the findings were considered significant when the *P*-value was <0.05.

RESULTS

Identification of exosomal RNA in serum. To verify our ultracentrifugation method for isolation of exosomes, we examined exosome using transmission electron microscopy. We captured images of round microvesicles having diameters of $\sim$50 nm in the exosome-rich fraction (Supplementary Figure 1A and B). Exosomal RNA was purified and miRNAs were observed as products of $\sim$25 nucleotides in length (Supplementary Figure 1C).

Relationship between serum exosomal miRNAs and the progression of CRC. The schematic explanation of procedure to select exosomal miRNAs associated with CRC recurrence is shown in Supplementary Figure 2. We examined the relationship between serum exosomal miRNAs and CRC progression using four serum samples that were sequentially obtained from a CRC patient with recurrence of liver metastasis during pre- and postoperative phases of primary resection and pre- and postoperative phases of hepatectomy as well as two serum samples that were sequentially obtained from a CRC patient without recurrence during pre- and postoperative phases of primary resection. Exosomal RNA extracted from each serum sample was used for microarray profiling. Eighteen exosomal microRNAs were increased and 46 exosomal miRNAs were decreased by more than two-fold in serum from the patient with liver metastasis as compared with the patient without recurrence (Table 1). Interestingly, the expressions of 6 exosomal miRNAs (i.e., miR-19a, miR-19b, miR-23a, miR-92a, miR-320a and miR-4437) were synchronised with the development of liver metastasis. Therefore, these six miRNAs were candidate miRNAs associated with liver metastasis in CRC. Among the six miRNAs, miR-19a and miR-92a were significantly upregulated in CTC patients compared with healthy control, and were therefore further evaluated.

miRNA cluster exhibited genomic amplification in CRC tissue. Of the 417 miRNAs analysed, changes in the expression of 58 miRNAs were shown to be related to alternations in a genomic locus. Twenty-four miRNAs had correlation coefficients of over 0.5, and *hsa-miR-25-106b* and *hsa-miR-17-92a* clusters were identified (Table 2).

Expression of the exosomal *miR-17-92a* cluster in serum. Three of six serum exosomal miRNAs exhibiting expression correlating with CRC in microarray analysis were located within the *miR-17-92a* locus. As mentioned above, *mir-17-92a* clusters were

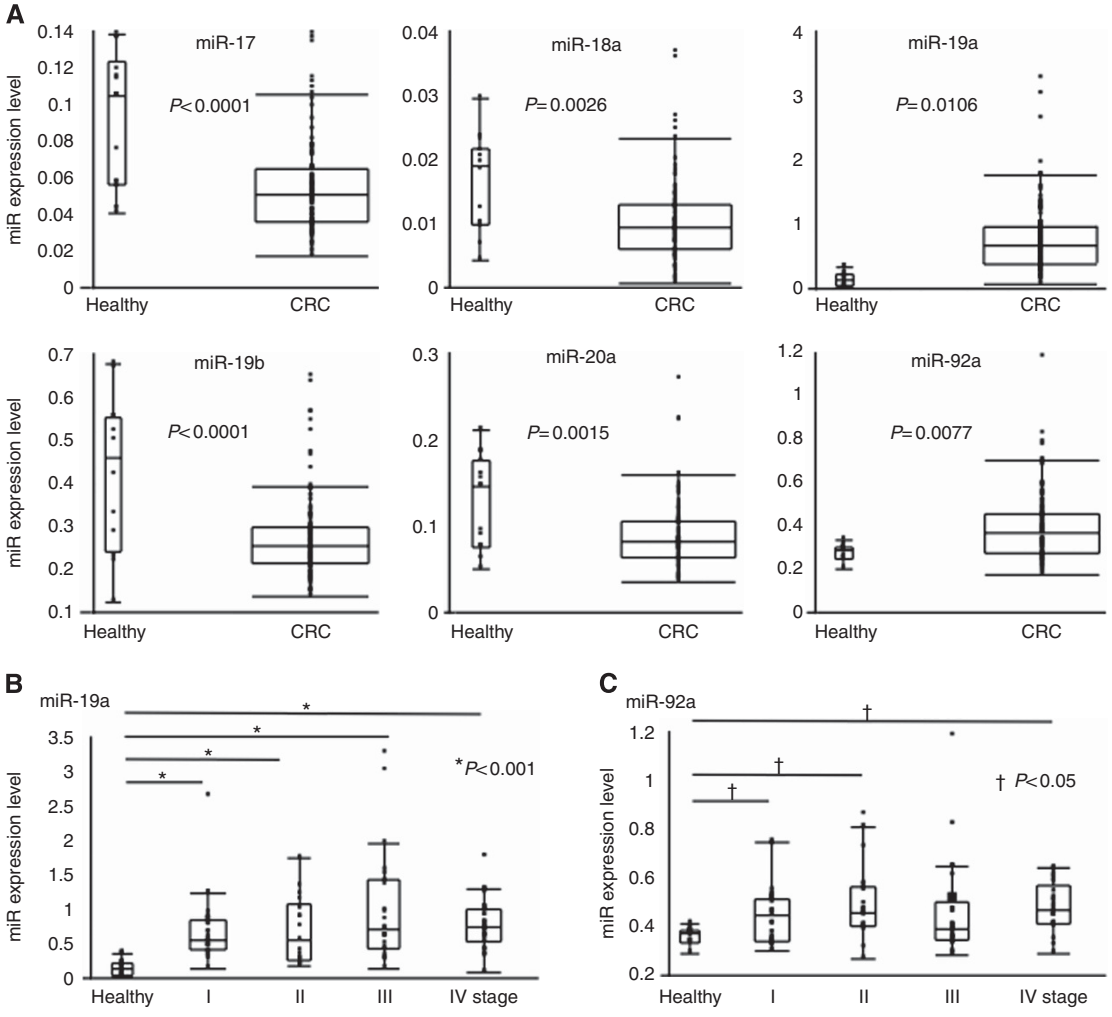

Figure 1. Expression of six microRNAs in the *miR-17-92* cluster. Quantitative RT–PCR using Taqman miRNA assays was used to investigate the expression of the six miRNAs in exosomes purified from serum. The obtained values were normalised to *hsa-miR-16a* as an internal control. (**A**) Expression of 6 serum exosomal miRNAs in 6 healthy volunteers and 90 patients with CRC. (**B**) Expression of serum exosomal *miR-19a* in healthy individuals and patients with different stages of CRC. (**C**) Expression of serum exosomal miR-92a in healthy individuals and patients with different stages of CRC.

upregulated by genetic amplification in CRC tissue. Therefore, we selected miRNAs within the *miR-17-92a* cluster as candidate miRNAs associated with CRC. Expression of these six exosomal miRNAs (i.e., *miR-17*, *miR-18a*, *miR-19a*, *miR-19b*, *miR20a and miR-92a*) in serum was assessed by qRT–PCR in 102 serum samples from 90 patients with CRC and 12 healthy volunteers. Of the six serum exosomal miRs analysed, *miR-19a* and *miR-92a* were significantly increased in CRC patients compared with healthy volunteers (Figure 1A), whereas the other four miRs did not show significant difference. Then, the expression of exosomal *miR-19a* and *miR-92a* in each stage of CRC patient was explored (Figures 1B and C). Interestingly, those exosomal miRNAs were upregulated in both early and advanced stages of CRC compared with healthy controls.

Expression of exosomal *miR-19a* and clinicopathological characteristics. For clinicopathological analysis, we classified the 209 CRC serum samples into 2 groups using the average of *miR-19a* expression level as determined from 16 healthy volunteers. Patients in the high exosomal *miR-19a* expression group ($n=133$) had more frequent occurrence of malignancy-related factors, including serosal invasion ($P=0.0224$), lymphatic permeation ($P=0.0028$), lymph node metastasis ($P=0.0206$), liver metastasis ($P=0.0486$) and higher TNM stage ($P=0.0005$), as compared with the low exosomal *miR-19a* expression group ($n=76$; Table 3).

Prognostic significance of exosomal *miR-19a* expression. Using the two exosomal *miR-19a* expression groups described in the previous section, we analysed the association between *miR-19a* expression and survival rates. We found that high exosomal *miR-19a* expression was significantly associated with poorer survival as compared with low exosomal *miR-19a* expression ($P=0.0004$; Figure 2A). In 164 samples from patients with CRC (excluding stage IV), disease-free survival rate were significantly lower in patients with high exosomal *miR-19a* expression than in patients with low exosomal *miR-19a* expression ($P=0.0002$; Figure 2B). In addition, univariate and multivariate analysis showed that serum exosomal *miR-19a* expression was an independent risk factor for overall survival (Table 4a) and disease-free survival (Table 4b) in CRC patients.

Table 3. Relationship between clinicopathological factors and exosomal miR-19a expression level in serum

	Low expression group		High expression group		
Clinicopathological factors	**Number of cases**	**%**	**Number of cases**	**%**	***P*-value**
Age (years)					
<65	31	40.8	56	42.1	0.8527
⩽65	45	59.2	77	57.9	
Gender					
Male	32	42.1	48	36.1	0.3895
Female	44	57.9	85	63.9	
Tumour size (cm)					
⩽3.0	28	36.8	38	28.6	0.2159
>3.0	48	63.2	95	71.4	
Histological type					
Well	43	56.6	80	60.2	0.6138
Moderate and others	33	43.4	53	39.8	
Serosal invasion					
Present	29	38.2	31	23.3	0.0224*
Absent	47	61.8	102	76.7	
Lymphatic permeation					
Present	19	25.0	61	45.9	0.0028*
Absent	57	75.0	72	54.1	
Venous permeation					
Present	39	51.3	86	64.7	0.0584
Absent	37	48.7	47	35.3	
Liver metastasis					
Present	7	9.2	26	19.6	0.0486*
Absent	69	90.8	107	80.4	
Lymph node metastasis					
Present	23	30.3	62	46.6	0.0206*
Absent	53	69.7	71	53.4	
Carcinoembryonic antigen (CEA; ng ml^{-1})					
⩽5.0	49	64.5	66	53.7	0.1334
<5.0	27	35.5	57	46.3	
CA19-9 (U ml^{-1})					
⩽37	57	76.0	85	70.8	0.4301
<37	18	24.0	35	29.2	
Stage					
I–II	51	67.1	56	42.1	0.0005*
III–IV	25	32.9	77	57.9	

*$P<0.05$.

DISCUSSION

The aim of this study was to identify specific serum exosomal miRNAs as biomarkers reflecting the progression of CRC. We hypothesised that CRC cells secreted exosomes containing miRNAs into the blood in order to transport signals to recipient cells. Our findings indicate that serum exosomal *miR-19a* could be a potential biomarker to predict recurrence of CRC.

In this study, analysis of serum exosomal miRNA expression profiles revealed that six6 serum exosomal miRNAs were regulated concordant with CRC progression. We confirmed that the expression of serum exosomal *miR-19a* was higher in patients with CRC than in healthy controls using two different sets of serum samples. Interestingly, regulation of miRNA expression has been shown to be dependent on the location of miRNAs within cancer-associated loci that exhibit genomic alternations, epigenetic regulation and abnormalities of essential proteins involved in miRNA maturation (Calin and Croce, 2006). Genomic alternations have been shown to cause abnormal miRNA expression. In addition, *hsa-miR-25-106b* and *hsa-miR-17-92a* clusters have cancer-promoting function (Tan *et al*, 2014). In particular, the *hsa-miR-17-92a*, a well-known oncogenic miRNAs cluster, has been shown to be upregulated in various types of cancers, such as of colon cancer, lung cancer and lymphoma (Hayashita *et al*, 2005; Diosdado *et al*, 2009; Olive *et al*, 2010). These findings supported our hypothesis that CRC cells secrete exosomal miRNAs to survive. Cells overexpressing *miR-19a* because of genomic alternations may secrete *miR-19a*-rich exosomes containing *miR-19a*. It has been reported that miR-19a promotes proliferation and invasion of cancer cells by gain- or loss-of-function experiments (Zhang *et al*, 2012; Feng *et al*, 2014). The downstream signalling pathway of miR-19a has not been clarified yet, but several reports have shown that *miR-19a* plays an oncogenic role by repressing tumour suppressor gene PTEN in lymphoma cells, gastric cancer cells and bladder cancer cells (Olive *et al*, 2009; Liang *et al*, 2011; Wang *et al*, 2013; Feng *et al*, 2014). We showed here that high expression of exosomal *miR-19a* was associated with poor prognosis in patients with CRC, suggesting that exosomal *miR-19a* may be a potential marker of poor prognosis in CRC. Indeed, in our data set, we found that high levels of exosomal *miR-19a* in serum of CRC patients were associated with tumour recurrence. Interestingly, *miR-19a* increased in patients with early-stage CRC as well as those with advanced stage. Those results were concordant with the previous study that reported serum miRNAs including miR-19a could be a biomarker for early detection of CRC (Zheng *et al*, 2014). Those results suggested that miR-19a might function at

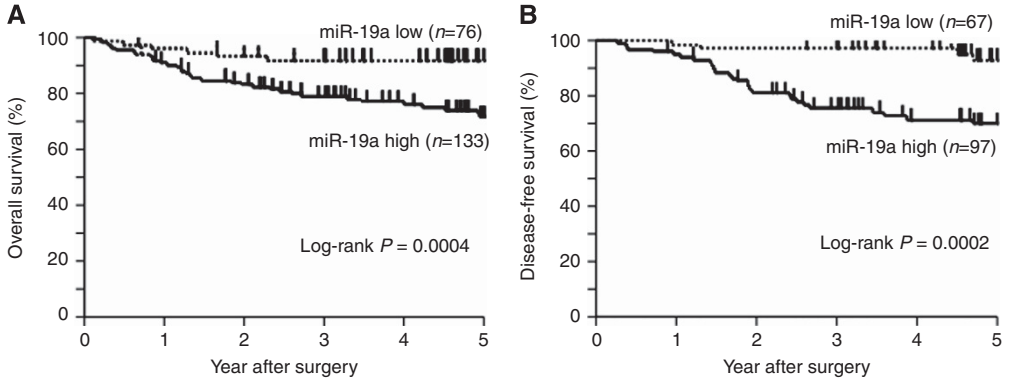

Figure 2. Kaplan–Meier survival curves for CRC patients classified according to the *miR-19a* expression level. (**A**) Overall survival curve of 209 patients with CRC. Two groups were divided according to the average exosomal *miR-19a* expression level in serum of healthy individual. Patients with high expression of exosomal *miR-19a* in serum had significantly poorer prognoses than patients with low expression of exosomal *miR-19a*. (**B**) Disease-free survival curve for 164 patients with CRC.

Table 4. Univariate and multivariate analysis for overall survival and disease-free survival of CRC patients

	Univariate analysis			Multivariate analysis		
Factors	**RR**	**95% CI**	***P*-value**	**RR**	**95% CI**	***P*-value**
(a) Overall survival						
Tumour depth (⩽MP, ⩽SS)	9.87	3.05–60.4	<0.0001*	4.22	1.02–29.3	0.0473*
Tumour size (<3.0 cm, ⩽3.0 cm)	3.79	1.53–12.6	0.0022*	1.24	0.45–4.52	0.7033
Lymph node metastasis (absent, present)	3.07	1.71–5.69	0.0001*	1.51	0.82–2.88	0.1859
Venous permeation (absent, present)	5.39	2.47–14.1	<0.0001*	2.44	1.03–6.74	0.0414*
Liver metastasis (absent, present)	8.16	4.42–14.8	<0.0001*	5.28	2.72–10.3	0.0001*
Exosomal miR-19a expression (low, high)	4.15	1.90–10.9	0.0001*	2.49	1.12–6.61	0.0236*
(b) Disease-free survival						
Tumour depth (⩽MP, ⩽SS)	9.87	3.05–60.4	<0.0001*	4.22	1.02–29.3	0.0473*
Tumour size (<3.0 cm, ⩽3.0 cm)	3.79	1.53–12.6	0.0022*	1.24	0.45–4.52	0.7033
Lymph node metastasis (absent, present)	3.07	1.71–5.69	0.0001*	1.51	0.82–2.88	0.1859
Venous permeation (absent, present)	5.39	2.47–14.1	<0.0001*	2.44	1.03–6.74	0.0414*
Liver metastasis (absent, present)	8.16	4.42–14.8	<0.0001*	5.28	2.72–10.3	0.0001*
Exosomal miR-19a expression (low, high)	4.15	1.90–10.9	0.0001*	2.49	1.12–6.61	0.0236*

Abbreviations: CI = confidence interval; CRC = colorectal cancer; MP = muscularis propria; RR = relative risk; SS = subserosa. *P<0.05.

early stage of colorectal oncogenesis. Recently, Zheng *et al* (2014) reported that a four-miRNA panel (miR-19a-3p, miR-223-3p, miR-92a-3p and miR-422a) for serum samples could successfully differentiate CRC from colon adenoma and healthy control. Those data were consist with our results that miR-19a and miR-92a were significantly upregulated in serum of CRC patients, and our present data even proved that those serum miRs were functional secreted in exosome.

We observed a relationship between miR-19a expression and CRC progression, but the mechanisms through which cancer cells secreted exosome-containing specific miRNAs have been unknown in detail. Further studies are required to clarify the relationship between serum exosomal *miR-19a* and intercellular signalling in CRC.

In conclusion, serum exosomal *miR-19a* was upregulated in CRC independent of clinical staging. Serum exosomal *miR-19a* may be a potential marker of prognosis and a therapeutic target in CRC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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