Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis & Regeneration

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Circulating Exosomal microRNAs as Biomarkers of Colon Cancers

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Introduction

- Colorectal cancer (CRC) is one of the major causes of cancerrelated deaths worldwide

- Systematic methods for diagnosing pathological conditions - reduction in mortality rates?

- Implementation of fecal occult blood test and flexible sigmoidoscopy – screening methods

- Carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA)

MicroRNAs

- endogenous, small, non-coding RNAs
- their tissue-specific expression contributes to the precise control of various biological processes
- Dysfunction of miRNAs is found in various cancers
- the endogenous expression profiles of miRNAs can be used to classify cancer types
- high levels of expression in cancer tissues
- suitable diagnostic or prognostic markers
- are secreted from various cells, including cancer cells, into body fluids via exosomes

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Exosomes

- small membrane vesicles that embed protein, lipids, mRNAs, and miRNAs

- exosomal miRNAs in body fluids - useful diagnostic biomarkers?

- microarray-based profiling of exosomal miRNAs in sera from HCs and primary CRC patients were performed

- miRNA profiles in exosomes from colon cancer cell lines were also examined

-The exosomal miRNA signatures differed between CRC patients and controls

- eight miRNAs (let-7a,miR-1224-5p, miR-1229, miR-1246, miR-150, miR-21, miR-223 and miR-23a) that were significantly elevated in serum exosomes from primary CRC patients and were down-regulated after surgery were identified

- CRC-associated elevation of seven of these exosomal miRNAs was validated using quantitative real-time RT-PCR (qRT-PCR)

- exosomal miRNA signatures reflect pathological changes in CRC patients and are applicable for the development of diagnostic strategies for detection of primary CRCs

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Materials and Methods

Clinical samples

- Serum samples from 88 CRC patients (aged 35 to 65 years) with a primary tumor

- Serum samples from 29 of the patients after surgical resection of the primary tumor from 2003 to 2004

- Serum samples from 13 different CRC patients (aged 45 to 70 years) with a primary tumor

- Surgical specimens of primary colon cancer and surrounding noncancerous Regions

- Sera from 19 individuals (aged 35 to 65 years) that underwent a complete physical screening (HC Sample)

- Serum samples were stored at -20°C until use

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Preparation of exosome- enriched fractions

- Exosome fractions were prepared by a step-wise centrifugation-ultracentrifugation method:

1) Culture was centrifuged at 500xg for 5 min to remove the cell debris

2) Then centrifuged at 16,500xg for 20 min

3) The cleared supernatant was passed through a 0.20 mm filter

4) Ultracentrifuged at 120,000xg for 70 min

5) The pellets were washed with phosphate-buffered saline (PBS)

6) Then resuspended in 250 ml of PBS as exosome-enriched fractions

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Preparation of total exosomal RNA

- The exosome fraction was mixed with 750 µl of Trizol-LS reagent (Invitrogen)

- the aqueous phase was collected by adding chloroform

- After the addition of ethanol to the aqueous phase, total RNA was purified

- The RNA sample was dried using a speed-vac centrifugal concentrator and then dissolved in 10 ml of nuclease-free water

-The concentration of RNA was determined using a NanoDrop spectrophotometer

- The quality of RNA was analyzed using an Agilent 2100 Bioanalyzer and small RNA chips

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MiRNA microarray analysis

- Total RNAs were labeled with pCp-Cy3 and hybridized to a human miRNA oligonucleotide microarray

- After hybridization, the array was washed and scanned

- After numerical conversion of the raw data the transformed data were analyzed

- The signal intensities of the spots on the microarray were normalized to the total signal intensity of the array and are shown as percentages

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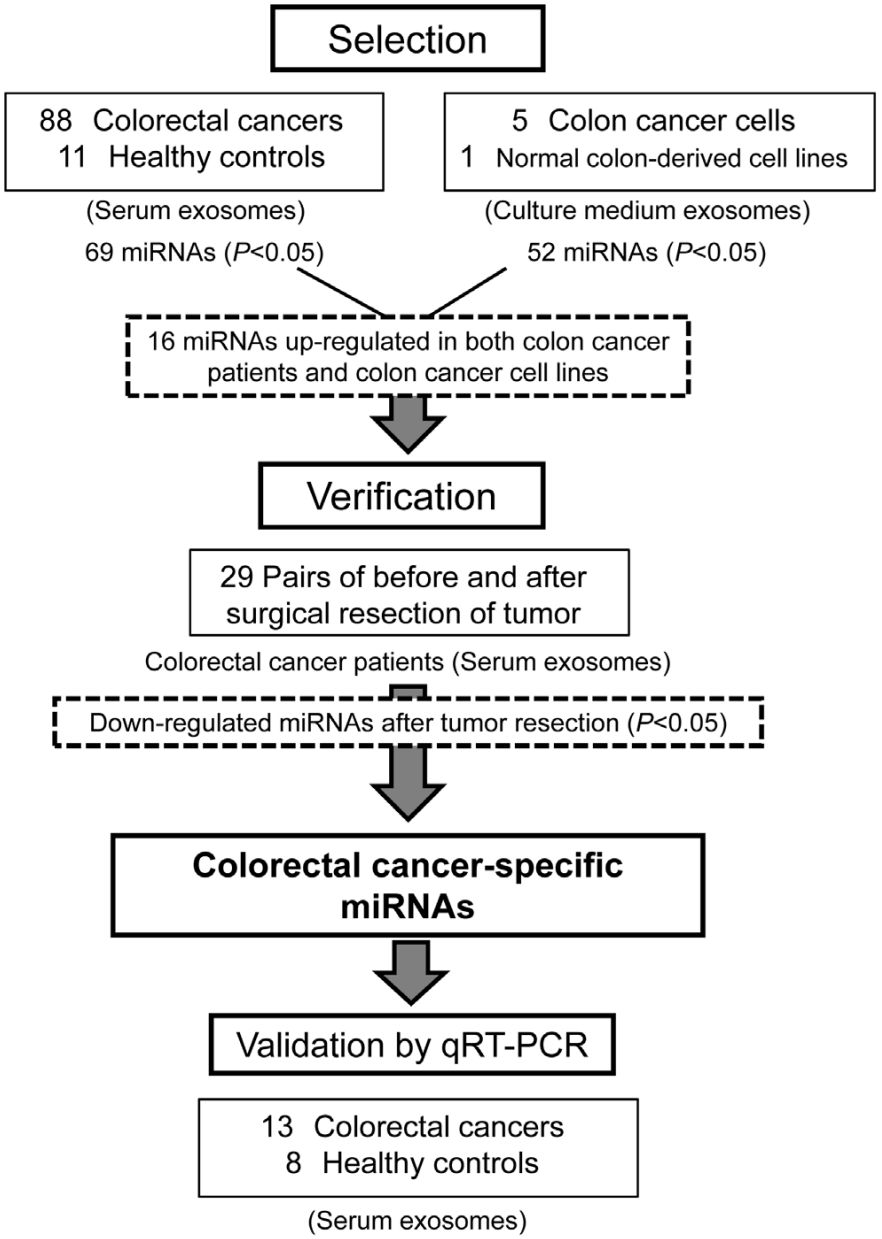
qRT- PCR

- Exosomal miRNAs were reverse transcribed using MultiScribe reverse transcriptase and miRNA-specific primers

- The miRNAs were quantified by real-time PCR

- miR-451 was used as an internal standard (detectable in all samples and its normalized intensities were not significantly different between HC and CRC serum exosomes)


Selection
88 Colorectal cancers
11 Healthy controls
(Serum exosomes)
69 miRNAs (P<0.05)
5 Colon cancer cells
1 Normal colon-derived cell lines
(Culture medium exosomes)
52 miRNAs (P<0.05)
16 miRNAs up-regulated in both colon cancer patients and colon cancer cell lines
Verification
29 Pairs of before and after surgical resection of tumor
Colorectal cancer patients (Serum exosomes)
Down-regulated miRNAs after tumor resection (P<0.05)
Colorectal cancer-specific miRNAs
Validation by qRT-PCR
13 Colorectal cancers
8 Healthy controls
(Serum exosomes)

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Statistical analysis:

- A two-sided paired Student's t-test (Welch's t-test) was used to determine the statistical significance of differences in microarray signal intensities

- Hierarchical clustering analysis (Ward's method)

- The receiver operating characteristic (ROC) curve and area under the curve were analyzed to assess the possibility of using a selected miRNA ratio as a diagnostic marker for CRC

- The Jonckheere-Terpstra test was used to determine the correlation between CRC tumor/node/metastasis stages and miRNA levels

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Results:

Identification of exosomal miRNAs that are elevated in CRC by microarray analysis

- Microarray-based screening was used to detect miRNA levels in exosomal fractions of serum samples from CRC and HC patients, culture media from a normal colon-derived cell line and five different human colon cancer cell lines

- The exosome-enriched fractions were prepared using a step-wise centrifugation-ultracentrifugation Method

- The exosomal miRNA profiles of the five colon cancer cell lines and the FHC cell line were distinct from the endogenous cellular miRNA profiles

- The miRNA profiles of the exosome fractions of serum samples from 88 primary CRC patients (including TNM clinical stages I, II, IIIa, IIIb, and IV) and 11 HCs were determined using microarrays

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Table 1. Characteristics of the HC and CRC patients included in the study.

	HC	CRC				
Clinical stage		**TNM I**	**II**	**IIIa**	**IIIb**	**IV**
	(n = 11)	**(n = 20)**	**(n = 20)**	**(n = 20)**	**(n = 16)**	**(n = 12)**
Age in years, mean (SD)	51.0 (9.2)	52.9 (7.8)	57.7 (7.9)	54.6 (7.2)	54.8 (8.6)	56.9 (4.7)
Range	35–64	38–65	35–65	36–63	36–65	48–64
Sex, n (%)						
Female	3 (27.2)	9 (45.0)	7 (35.0)	9 (45.0)	4 (25.0)	4 (33.3)
Male	8 (72.7)	11 (55.0)	13 (65.0)	11 (55.0)	12 (75.0)	8 (66.7)
Number of exosomal miRNAs detected, mean (SD)	62.5 (20.6)	80.9 (18.0)	76.6 (23.7)	74.7 (19.5)	78.3 (18.6)	71.2 (12.1)
Range	61–124	66–111	61–124	66–111	61–124	66–111
Total signal intensities of exosomal miRNAs, mean (SD)	1954 (877)	2037 (784)	1935 (918)	1924 (796)	1878 (854)	1817 (925)

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A total of 164 miRNAs were detected and 69 miRNAs were expressed at significantly higher levels in CRC patients than HCs

52 miRNAs were secreted at significantly higher levels from all five colon cancer cell lines than from the FHC cells

A comparison of the 69 up-regulated miRNAs from CRC patients and the 52 up-regulated miRNAs from the colon cancer cell lines revealed that 16 miRNAs were present in both sets

The endogenous expression levels of these miRNAs were then measured in the FHC and colon cancer cell lines, as well as colon
cancer and matched non-cancerous tissues from four additional patients

None of these miRNAs were expressed in the cancer tissues or cancer cell lines at significantly higher levels than the matched non-cancerous tissues or the FHC cell line (exception: miR-181d: up-regulated in both)

→ Secretion of miRNAs is not dependent on their cellular expression levels

→ upregulated endogenous miRNAs in cancer cells are not necessarily substrates for exosome-mediated secretion

Colon cancer cells appear to secrete a subset of miRNAs into extracellular spaces via exosomes

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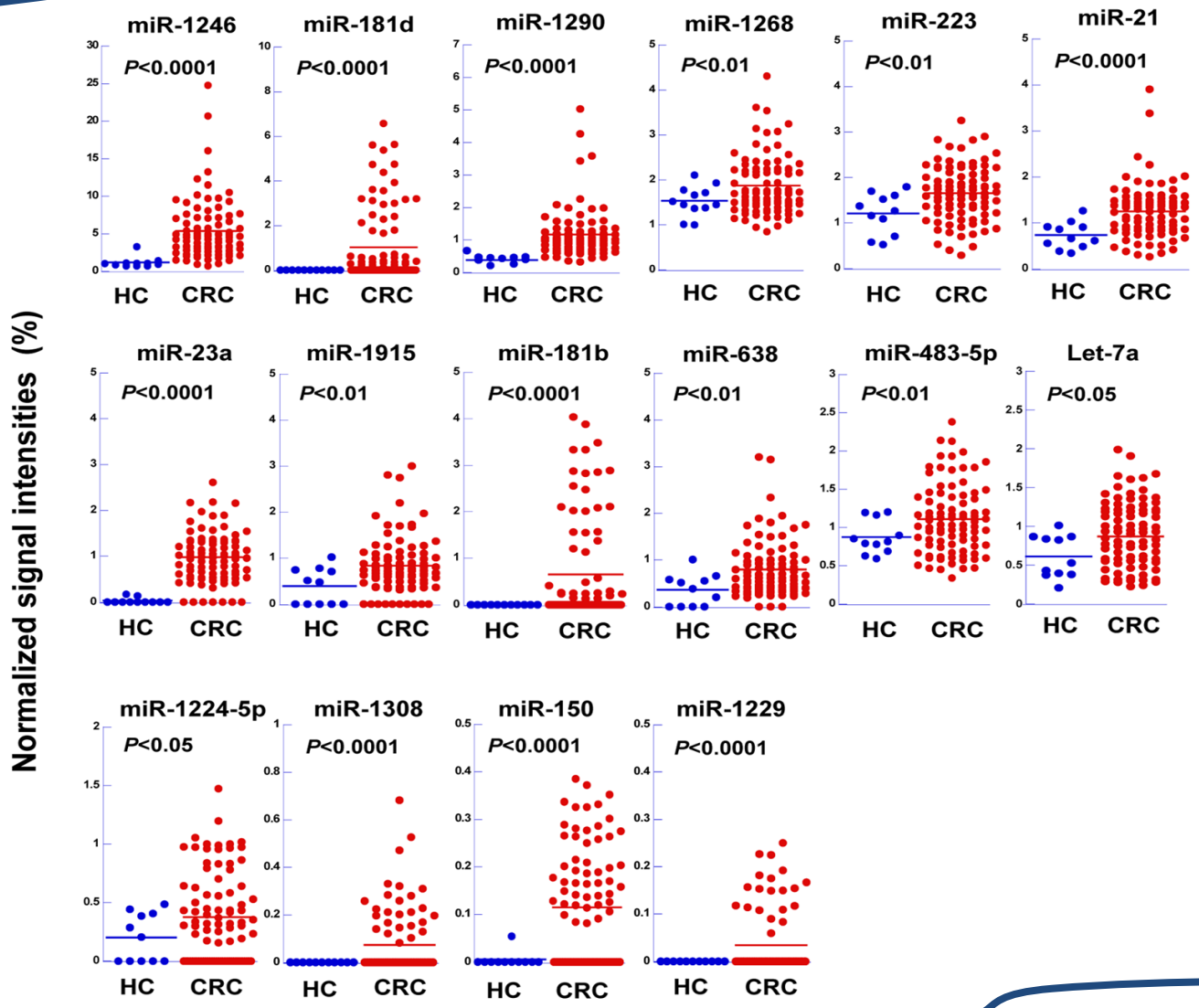
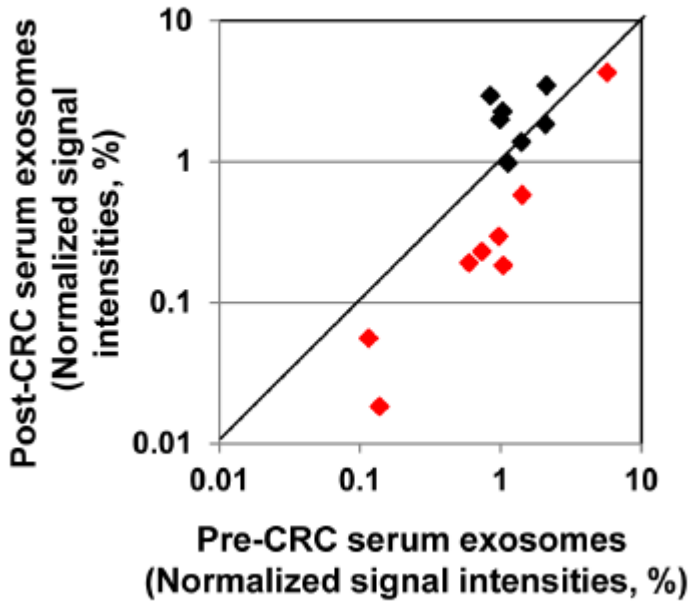
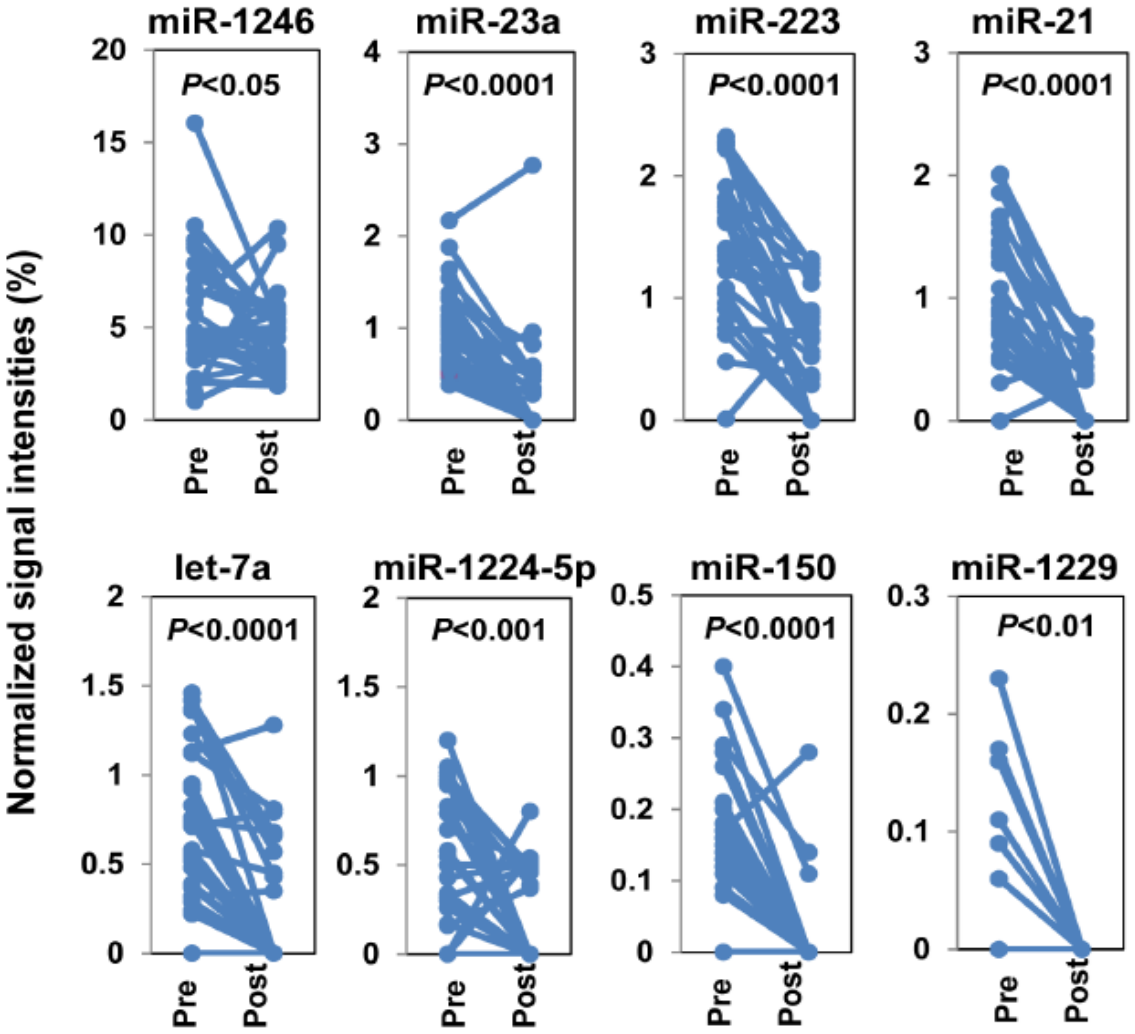


A


Post-CRC serum exosomes
(Normalized signal intensities, %)
10
1
0.1
0.01
0.01
0.1
1
10
Pre-CRC serum exosomes
(Normalized signal intensities, %)


Normalized signal intensities (%)
miR-1246
P<0.05
miR-23a
P<0.0001
miR-223
P<0.0001
miR-21
P<0.0001
let-7a
P<0.0001
miR-1224-5p
P<0.001
miR-150
P<0.0001
miR-1229
P<0.01
Pre
Post

Relationship between the serum exosomal levels of 16 miRNAs and the clinical stages of colon cancer

- the relationship between the 16 commonly up-regulated miRNAs and clinical stages of CRC were analyzed by separating the CRC patients on the basis of their TNM stage

Down-regulation of eight miRNAs after removal of primary tumors

- microarray analyses were used to determine the levels of these miRNAs in exosome-enriched fractions of matched serum samples collected after surgical resection of primary tumors (n= 29 patients)

- Expression levels of eight of the miRNAs were significantly reduced after removal of the primary tumors

→ These eight miRNAs may be derived from exosomes secreted by cancer cells

→ Their levels may reflect the colon cancer status of patients

Potential serum exosome miRNAs for application as diagnostic biomarkers

- The cutoff values of the eight miRNAs that were up-regulated in colon cancer and down-regulated after tumor resection were analyzed using a ROC curve

- The true positive rates of miR-1246 and miR-23a for identification of the 88 CRC patients were 95.5% and 92.0%

- The true positive rates of miR-21, miR-150, let-7a, miR-223, miR-1224-5p, and miR-1229 for identification of CRC were 61.4%, 55.7%, 50.0%, 46.6%, 31.8%, and 22.7%

- It is noted that a combined usage of 8 miRNAs did not show more diagnostic power than those in miRs-1246 and - 23a

- By comparison, the sensitivities of CEA and CA19-9, which are known biomarkers of CRC, were 30.7% and 16.0%

Biomarkers	HCs	CRC patients					Cut-off value	Over cut-off value		AUC
		Stage					%	(n)		
		I	II	IIIa	IIIb	IV		CRC	HC	
	Number of samples									
	11	20	20	20	16	12				
CEA							5 (ng/ml)	27		
CA19-9							37 (U/ml)	14		
hsa-let-7a							0.90	44	1	0.670
hsa-miR-1224-5p							0.50	28	0	0.610
hsa-miR-1229							0.06	20	0	0.614
hsa-miR-1246							1.45	84	1	0.948
hsa-miR-150							0.08	49	0	0.758
hsa-miR-21							1.08	54	1	0.798
hsa-miR-223							1.72	41	1	0.716
hsa-miR-23a							0.31	81	0	0.953

Figure 4. ROC curve analysis of eight miRNAs in serum exosomes of HCs and CRC patients. The signal intensities of the miRNAs are shown as percentages of the total signal intensity. The cut-off values of the eight miRNAs that were up-regulated in colon cancer and down-regulated after tumor resection were analyzed using a ROC curve. Black boxes indicate patients over the cut-off value of the biomarkers or miRNA levels. The normalized intensities of undetectable miRNAs in serum exosomes were calculated as 0.

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- Therefore, they re-evaluated the expression levels of the eight miRNAs in serum exosomes by qRT-PCR analyses of an additional sample set that included

+ eight Hcs
+ seven stage I CRC patients
+ and six stage II CRC patients

- The levels of seven of the miRNAs, namely let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a, were significantly higher in serum exosomes from CRC patients than those from HCs

- Statistically significant increases in expression levels of these miRNAs were even observed for the early stage (TNM stage I) CRC samples

→ These results suggest that serum exosomal miRNAs may be useful for the early detection of primary CRCs

In summary, attempts have recently been made to use miRNAs in serum or plasma as diagnostic biomarkers of various cancers.

-The unique properties of exosomes,

- including their ability to embed specific miRNAs

- their stability in circulation

- their reproducible detection and

- the fact that they reflect the properties of cancer cells may render them useful for the development **of highly sensitive diagnostic strategies for rapid and non-invasive monitoring** of the pathological condition of cancer patients.

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Thank you for your attention!