Circulating miR-103 and miR-720 as novel serum biomarkers for patients with colorectal cancer

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Abstract. Circulating microRNAs (miRNAs) have been reported as a biomarker for human malignancies, including colorectal cancer (CRC). The purpose of this study was to identify a novel biomarker for CRC through examination of serum miRNAs from the patients with CRC. Microarray analysis of miRNA expression was performed using paired pre- and post-operative serum from 10 CRC patients. miR-103 and miR-720 decreased significantly in the post-operative serum when compared to pre-operative serum. With an extended scale validation by qRT-PCR (quantitative real-time polymerase chain reaction) in 30 CRC patients, we confirmed that serum miR-103 and miR-720 decreased significantly after surgery (P=0.0004, and P=0.0274, respectively). Next, we examined serum miR-103 and miR-720 levels in 32 non-cancer patients and 84 CRC patients, and we found that expression of these two miRNAs was significantly higher in CRC patients than non-cancer patients. Furthermore, clinical and pathological survey indicated that high expression of miR-103 was significantly associated with histological differentiation grade, and lymphatic invasion and high expression of miR-720 was significantly associated with male gender and lymph node metastasis. Our data suggest that circulating miR-103 and miR-720 show potential as novel serum biomarkers for CRC.

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Abbreviations: CRC, colorectal cancer; miR, miRNA, microRNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction

Key words: miR-103, miR-720, colorectal cancer

Introduction

With the recent advances in chemotherapy, the prognosis for patients with metastatic colorectal cancer (CRC) has been considerably improved (1). However, CRC is still one of the most common malignancies worldwide and is a major cause of cancer-related deaths (2). Prognosis of this disease depends on tumor stage. The 5-year overall survival rates range from 93% for stage I patients to 8% for stage IV patients (3). Thus, early detection of CRC is important to reduce the mortality of this disease.

Representative tumor markers for CRC, carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 are not reliable in early detection of CRC. A previous study showed that serum p53 antibody test was a more sensitive tumor marker to detect early stage of CRC rather than CEA and CA19-9 (4). However, its feasibility as a screening test remained to be explored. Fecal occult blood test (FOBT) is employed as a screening test to detect CRC, but only 50-60% of early CRC is positive for FOBT. Currently, CRC is primarily diagnosed through colonoscopy. However, this procedure is invasive and expensive, and additionally requires bowel preparation, and may be associated with medical complications. Therefore, an optimal screening test that is easy to perform, noninvasive, acceptable, and can select those who have neoplastic lesions is required to improve the detection of CRC.

MicroRNAs (miRNAs) are newly discovered class of 22 nucleotide noncoding RNA molecules that regulate the translation and stability of specific target mRNAs through base-printing to partially complementary sites on target mRNAs that usually reside within the 3' untranslated regions (5). miRNAs have been considered to play an important role in the multistep processes of carcinogenesis either by oncogenic or tumor suppressive function (6). The association

with tumorigenesis indicates their potential as diagnostic markers (7).

Circulating miRNAs are stably detected in plasma or serum and serve as biomarkers for several diseases, making them potentially useful noninvasive markers for early diagnosis or in monitoring of cancer progression (8,9). Previous studies revealed that miRNAs were potential diagnostic or prognostic tools for CRC. For example, miR-92 or miR-21 is significantly elevated in plasma of CRC patients, and can be a potential noninvasive molecular marker for CRC detection (10,11). We have recently found that serum miR-199a-3p is significantly higher in CRC patients than non-cancer patients, suggesting that circulating miR-199a-3p could be a biomarker for CRC (12). In this study, we explored other novel circulating miRNAs in CRC patients and evaluated its feasibility as a noninvasive diagnostic test for efficient detection of CRC.

Materials and methods

Patients and samples. Informed consent was obtained from CRC patients and non-cancer patients for the use of their blood samples. Venous blood samples were collected from the CRC patients (n=114) and non-cancer patients (n=32) from April 2011 to June 2013. In 30 of the CRC patients, blood samples were taken before and 7th day after surgery. No cancer patients received chemotherapy or radiotherapy before blood sampling. The blood samples were obtained from Osaka University, and its associated hospitals. Whole blood was collected, centrifuged at 1,000 rpm at 4˚C for 15 min. The supernatant fluids were centrifuged at 15,000 rpm at 4˚C for 10 min. The supernatant fluids were stored at -80˚C until RNA extraction. This study was conducted under the supervision of the ethics board of Osaka University Hospital.

RNA extraction. Small RNA was enriched from all serum samples using the mirVana PARIS RNA isolation kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Briefly, 400 μl of serum was thawed on ice and centrifuged at 15,000 rpm for 15 min to remove cell debris. Next, 300 μl of supernatant was lysed with an equal volume of 2X denaturing solution. For normalization of sample-to-sample variation during the RNA isolation procedures, 20 fmol of synthetic *C. elegans* miRNA cel-miR-39 was added to each denatured sample. Small RNAs were then enriched and purified following manufacturer's protocol. The concentration of all RNA samples were quantified by Nano Drop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

miRNA microarray analysis. miRNA microarray experiments were carried out by using Agilent human miRNA microarray cataloged in the Sanger database version 12.0 (design ID 021827). About ten nanogram aliquots of total RNA with cel-miR-39 was used for making miRNA probes according to the Agilent protocol (version 2.3). Microarrays were performed for paired pre- and post-operative serum from 10 CRC patients. Briefly, total RNA was dephosphorylated with calf intestine alkaline phosphatase, denatured with dimethyl sulfoxide, and labeled with pCp-Cy3 using T4 RNA ligase using the miRNA Labeling Reagent and Hybridization kit. Probes were hybridized at 55˚C for 20 h with rotation. Then the slides were washed by Gene Expression Wash Buffer 1 at room temperature for 5 min and by Gene Expression Wash Buffer 2 at 37˚C for 5 min. After hybridization and washing, the slides were scanned using an Agilent scanner (G2505C). Images were extracted using Agilent Feature Extraction software (version 10.7.3.1) and Agilent GeneSpring GX software (version 10.0.2). Differences in miRNA expression between the 10 pairs was determined if the fold change of cel-miR-39 normalized expression values was >2.0 and the P-value was <0.05 using paired t-test for further analysis. The microarray raw data are available in Gene Expression Omnibus (GEO;http://www.ncbi.nlm.nih.gov/geo) under accession number GSE55139.

qRT-PCR. For microRNA based RT-PCR assays, 2.5 μl of enriched small RNAs from serum samples were reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, San Diego, CA, USA) according to manufacturer's instructions in a total reaction volume of 7.5 μl. A 1:20 dilution of RT products was used as template for the PCR stage. PCR reaction was performed in triplicates using TaqMan 2X Universal PCR Master Mix according to the manufacturer's instructions. Each reaction was performed in a final column of 20 μl containing 1.33 μl of the cDNA and 1 μl of Taqman miRNA assay mix. The amplification profile was: denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Each sample was run in triplicates for analysis. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. The 7900 Sequence Detection System 2.3 (Applied Biosystems) software was used to compute the relative change in RNA expression by the 2-ΔΔCt method with 95% confidence intervals.

Primers. The miRNA-specific primer sequences, including miRNA-103, miR-720, cel-miR-39, and RNU6B were designed based on the miRNA sequences obtained from the miRBase. The primer sequences are: hsa-miR-103, 5'-AGCAGCAUUG UACAGGGCUAUGA-3'; hsa-miR-720, 5'-UCUCGCUGGG GCCUCCA-3'; hsa-miR-21, 5'-UAGCUUAUCAGACUGAUG UUGA-3'; cel-miR-39, 5'-UCACCGGGUGUAAAUCAGC UUG-3'; RNU6B, 5'-CGCAAGGATGACACGCAAATTCG TGAAGCGTTCCATATTTTT-3'.

Statistical analysis. The significance of serum miRNA level was determined by Mann-Whitney test, Wilcoxon test and χ^2 test where appropriate using the Graph Pad Prism 6 (San Diego, CA, USA). The sensitivity, specificity, and accuracy were calculated according to the standard formulas. Receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) were established for discriminating patients with CRC. P-value of <0.05 was considered statistically significant.

Results

Results of miRNA microarray analysis. In comparison of CRC patient serum between pre- and post-operation (n=10; 4 stage II CRCs and 6 stage III CRCs) by the miRNA array, we identified miRNAs, most of which showed a decrease in

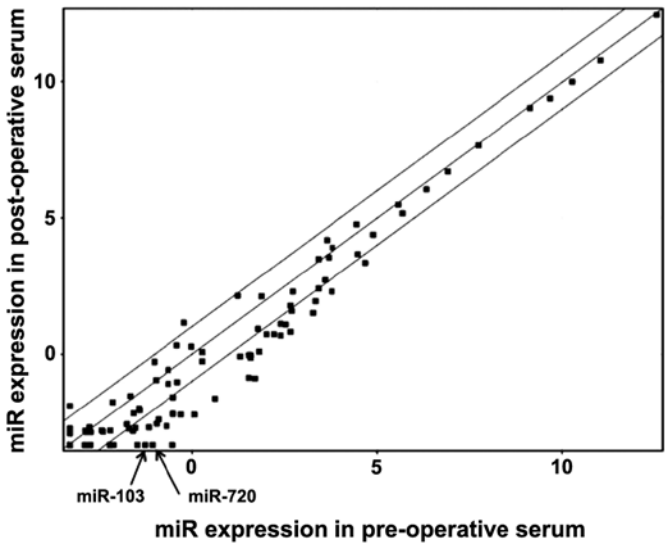

Figure 1. Results of miRNA array analysis. By comparison of patient serum between pre- and post-operation (n=10) by miR array, two miRNAs (miR-103, miR720) decreased in the post-operative serum as compared to the pre-operative serum (P=0.037 and P=0.037, respectively). The arrow and circle indicate miR-103 and miR-720.

the post-operative serum (Fig. 1). Among them, we focused on the two miRNAs, miR-103 and miR-720 whose expression showed a large decrease after surgery with significant P-value (4.09-fold and 4.68-fold decrease; P=0.037 and P=0.037, respectively, Fig. 1).

Confirmation of results obtained by miRNA array by qRT-PCR. We then attempted to confirm the results obtained by miRNA array by qRT-PCR in extended samples of CRC patients (n=30). As shown in Fig. 2, significant decrease in miRNA levels was noted in the post-operative serum for both miRNAs; P=0.0004 for miR-103, and P=0.027 for miR-720.

Expression of miRNAs in serum of normal and CRC patients. We examined serum miR-103 and miR-720 levels in 32 non-cancer patients and 84 CRC patients. As shown in Fig. 3, both miRNAs were significantly higher in CRC patients than non-cancer patients (P=0.0067 for miR-103, and P=0.030 for miR-720, respectively). ROC curve was drawn for serum miR-103 and miR-720, which yielded 0.662 for miR-103 and 0.630 for miR-720 as a value of AUC (Fig. 3). In discriminating CRC from non-tumor control subjects, the sensitivity and specificity of miR-103 were 55.9% and 75.0% at a cut-off point of 0.00081; these values for miR-720 were 58.3% and 56.3% at a cut-off point of 0.0016.

Serum miR-21 expression in non-cancer and CRC patients. As a reference we examined miR-21 as a putative circulating miRNA. Using the same serum sets of normal and CRC patients, we found that serum miR-21 levels increased significantly in CRC patients as compared to non-tumor control patients (P=0.003). AUC of the ROC curves was 0.675. When a cut-off point was set at 0.0107, the sensitivity was 54.7% and the specificity was 84.4% in discriminating CRC from non-tumor control subjects (Fig. 4).

Expression of miR-103 and miR-720 in patients serum and clinicopathological characteristics. CRC patients were divided into two groups by a median expression. Clinical and pathological survey indicated that high expression of miR-103 was significantly associated with poor differentiation and lymphatic invasion as compared to low miR-103 expression group (P=0.044 and P=0.040, respectively; Table I). High expression of miR-720 was significantly associated with male gender and lymph node metastasis as compared to low miR-720 expression group (P=0.001 and P=0.048, respectively; Table I).

Expression of miR-103 and miR-720 in normal mucosa and CRC tissue samples. We examined miR-103 and miR-720

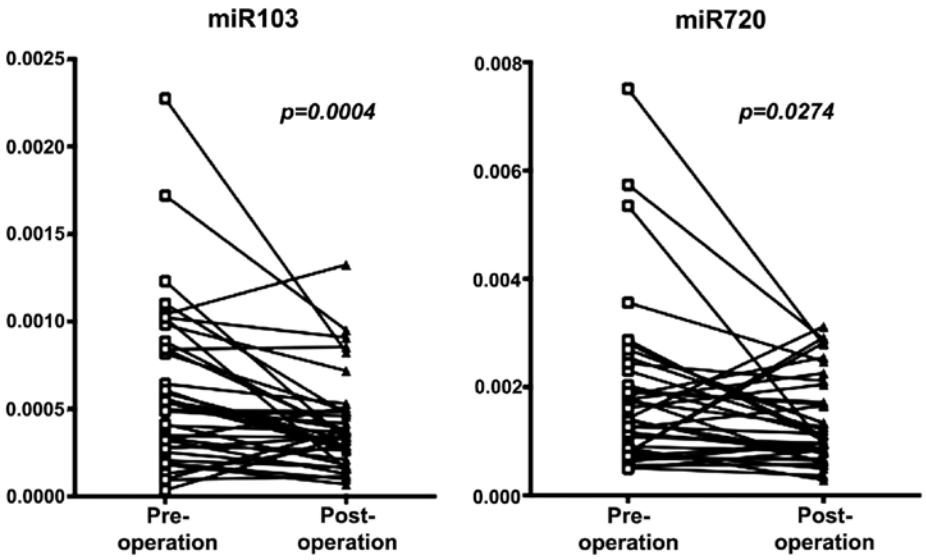

Figure 2. Confirmation of results obtained by miRNA array by qRT-PCR. We confirmed the results obtained by miRNA array by qRT-PCR in serum samples of CRC patients (n=30). A significant decrease in miRNA levels was noted in the post-operative serum when compared with pre-operative serum for both miRNAs; P=0.0004 for miR-103, and P=0.027 for miR-720.

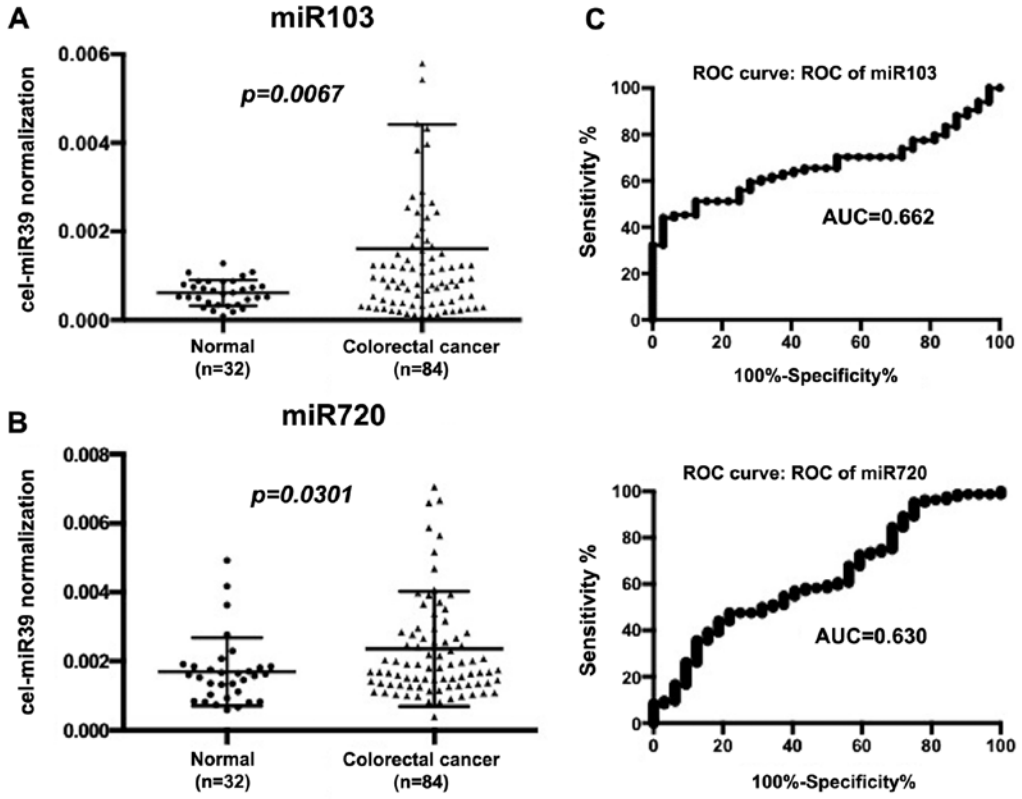

Figure 3. Comparison of serum miRNAs in non-tumor (n=32) and CRC patients (n=84). (A) Serum miR-103 expression in non-tumor and CRC patients. A significant increase in CRC patients was noted (P=0.0067). (B) Serum miR-720 expression in non-tumor and CRC patients. A significant increase in CRC patients was noted (P=0.030). (C) ROC curve was drawn for serum miR-103 and miR-720, which yielded 0.662 for miR-103 and 0.630 for miR-720 as a value of AUC. In discriminating CRC from non-tumor control subjects, the sensitivity and specificity of miR-103 were 55.9% and 75.0% at a cut-off point of 0.00081; these values for miR-720 were 58.3% and 56.3% at a cut-off point of 0.0016.

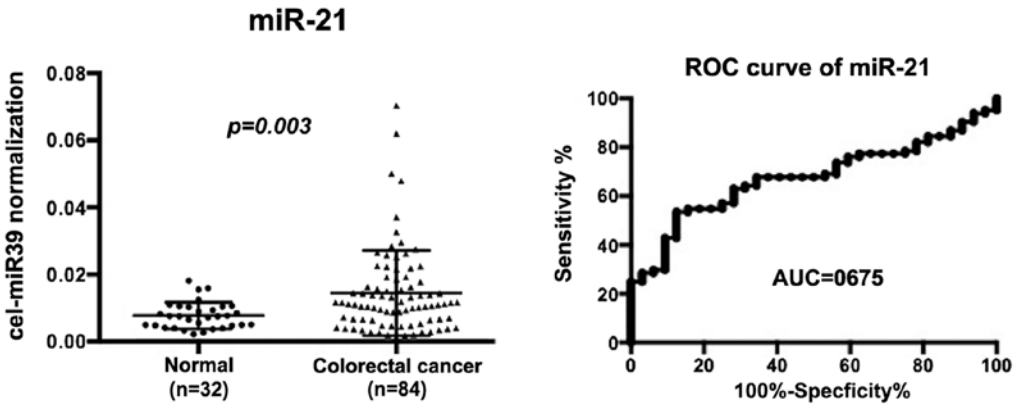

Figure 4. Serum miR-21 expression in non-cancer and CRC patients. Using the same serum sets of normal and CRC patients, we found that serum miR-21 levels increased significantly in CRC patients as compared to non-tumor control patients (P=0.003). AUC of the ROC curves was 0.675. When a cut-off point was set at 0.0107, the sensitivity was 54.7% and the specificity was 84.4% in discriminating CRC from non-tumor control subjects.

expression in normal colonic mucosa (n=10) and CRC tissue samples (n=10). Both miRs were significantly lower in CRC tissue samples than in normal colonic mucosa (P=0.034 for miR-103, and P=0.0052 for miR-720) (Fig. 5).

Discussion

In this study, we explored to identify a novel serum marker for CRC by comparison of patient serum before and after operation, using microarray analysis of miRNAs. miRNAs were mostly reduced after surgery to various degrees (Fig. 1). Among them, miR-103 and miR-720 showed relatively large reduction after surgery. We validated the results by qRT-PCR in extended number of CRC patients (Fig. 2). These findings suggest that the two miRNAs could be derived from main CRC tumors, either directly or indirectly and that they may be useful to monitor the disease progression.

We demonstrated that the serum levels of miR-103 and miR-720 increased in CRC patients compared with non-cancer patients (Fig. 3). The ROC curves of miR-103 and miR-720 to

Table I. Relation between miR-103, miR-720 and clinicopathological features.

	miR-103			miR-720		
Factors	High	Low	P-value	High	Low	P-value
Gender			0.165			0.001[a]
Male	31	25		35	21	
Female	11	17		7	21	
Differentiation			0.044[a]			0.503
tub1	12	21		15	18	
tub2 por muc	30	21		27	24	
Tumor size			1.000			1.000
≥35 mm	22	22		22	22	
<35 mm	20	20		20	20	
Serosal invasion			0.172			1.000
Positive	30	24		15	27	
Negative	12	18		15	27	
Lymph node metastatis			0.820			0.048[a]
Positive	19	18		23	14	
Negative	23	24		19	28	
Lymphatic invasion			0.040[a]			0.827
Positive	25	18		21	22	
Negative	13	24		21	20	
Venous invasion			0.474			0.095
Positive	14	11		16	9	
Negative	28	31		26	33	

Well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous carcinoma. [a]Statistically significant.

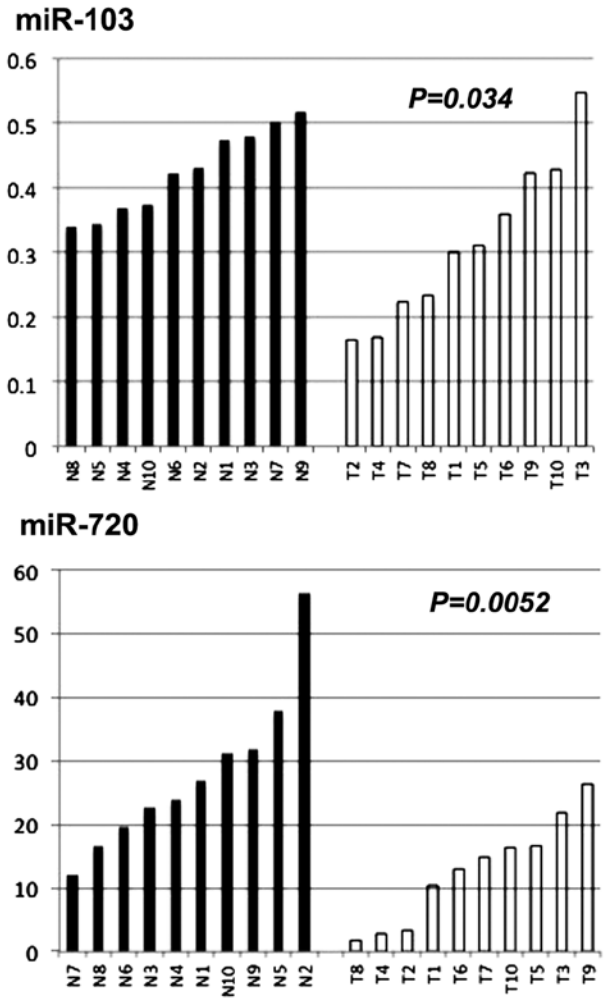

Figure 5. Expression of miR-103 and miR-720 in normal mucosa and CRC tissue samples. We additionally examined miR-103 and miR720 expression in normal colonic mucosa (n=10) and CRC tissue samples (n=10). Both miRs were significantly lower in CRC tissue samples than in normal colonic mucosa (P=0.034 for miR-103, and P=0.0052 for miR-720).

distinguish cancer patients from non-cancer patients appeared to have limitation in sensitivity and specificity. To estimate its value, we examined serum miR-21 in the same series of non-tumor and CRC patients. Serum miR-21 is well known as a serum biomarker for CRC (13). We found that diagnostic power of miR-103 was similar to that of miR-21 in our series but that miR-720 showed limited specificity as compared to miR-21. Thus, miR-103 might be better biomarker for CRC than miR-720.

Clinicopathological survey showed that high expression of serum miR-103 was associated with poor differentiation and lymphatic invasion. Chen *et al* showed that, miR-103 promoted metastasis of CRC by targeting the metastasis suppressors DAPK and KLF4. This study also showed that miR-103 had a role in downregulating E-cadherin, claudin-3, and occludin, which sensitized tumor cells to EMT-inducing signals, and led to local invasion (14). Additionally, we found that high expression of serum miR-720 was associated with lymph node metastasis. The finding is consistent with a report by Wang *et al* that high level of miR-720 in CRC tissues correlated with the tumor size, lymphatic metastasis, distant metastasis, and poor prognosis (15).

In analysis of miR-103 and miR-720 derived from the tissue samples, we found that the expression levels of the two miRNAs, especially miR-720 were significantly lower in CRC tissue samples than in normal colonic mucosa (Fig. 5). The contradictory miRNA expression levels between the tumor tissues and the serum have been demonstrated as to several miRNAs, but this phenomenon remains unexplained and needs to be further explored (16,17). A previous *in vitro* study showed that miRNA profiles in a conditioned medium were different from those in cells, and it is thus suggested that the secreted miRNAs represent a class of signaling molecules in mediating intercellular communication (18). The precise mechanism of miRNA packaging and secretion is largely unknown, and investigation of the mechanism is a future issue of miRNA research (19).

In conclusion, we identified miR-103 and miR-720 from differential expression profile between pre- and post-operation as novel serum biomarkers for CRC. Further investigation of serum miR-103 and miR-720 in patient prog-

nosis and monitoring therapeutic efficacy of chemotherapy is essential.

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