Serum miR-210 as a potential biomarker of early clear cell renal cell carcinoma

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Abstract. Early detection and treatment are critical in the management of renal cell carcinoma (RCC). However, there is no standard serum biomarker to facilitate early diagnosis or prognostic stratification in patients with RCC. Recent reports suggest that circulating microRNAs (miRNAs) have great potential as biomarkers for diagnosis and prognosis in patients with several types of cancers. Further, many studies using miRNA microarray analysis demonstrated that miR-210 expression in clear cell carcinoma (CCC), which is the largest subtype of RCC, was significantly upregulated in tumor tissue. Therefore, we investigated whether serum miR-210 could be a useful biomarker for the diagnosis and progression of CCC. This study included 34 CCC patients and 23 healthy controls (HC). First, we analyzed tissue miR-210 levels in tumor tissues and matched normal tissues from the 34 CCC patients. Second, we investigated the serum miR-210 levels in the 34 CCC patients and the 23 HC patients. Real-time polymerase chain reaction (PCR) was used to measure miRNA levels. Moreover, we examined the correlation between serum miR-210 levels and the clinicopathological parameters. Among patients with CCC, expression of miR-210 was higher in tumor tissues compared to normal tissues (P<0.001). Serum miR-210 levels were higher in CCC patients compared to HCs (P=0.001). Receiver operating characteristic (ROC) curve analysis showed an area under the ROC curve (AUC) of 0.77 (95% confidence interval, 0.65-0.89) and a sensitivity and specificity of 65 and 83%, respectively. In addition, there was no significant association between serum miR-210 levels and age, sex, tumor size or existence of metastasis at diagnosis among the 34 CCC patients. In conclusion, serum miR-210 upregulation may occur in the early stage of CCC and serum miR-210 can be a useful biomarker for early CCC in humans.

Introduction

Renal cell carcinoma (RCC) is a common urological neoplasm of the adult kidney. The diagnostic modalities and therapeutic techniques for RCC continue to improve and the overall incidence and mortality of RCC has increased in the last 20 years (1). The 5-year survival rate is ~98% for stage I disease and ~50% for stage III disease (2), which underscore the importance of early detection and treatment of RCC. However, early detection of often difficult, because early-stage renal tumors are often asymptomatic and non-palpable. Therefore, the identification of non-invasive biomarkers for early-stage RCC would be of benefit. However, no accurate biomarker for RCC currently exists (3).

Recent studies suggest that microRNAs (miRNAs), which are non-protein-coding small RNAs, are involved in cancer progression and metastasis. MicroRNAs are ~22 nucleotides in length and regulate gene expression at the post-transcriptional level by binding to the untranslated region (3'UTR) of target mRNAs, leading to translational inhibition and/or mRNA degradation (4). Specific expression profiles of miRNAs in tissue have been reported in a variety of cancers, including RCC (5). Early studies suggested that miRNAs were strictly intracellular molecules, but more recent studies suggest that miRNAs are highly stable and abundant in the serum, urine and other body fluids. This is because exosomes likely protect miRNAs against degradation by RNase (6,7). Interestingly, serum miRNA levels are similar in men and women and do not vary with patient age (8).

Thus, circulating miRNAs might be good non-invasive biomarkers for diagnostic and prognostic considerations in a variety of cancers. Indeed, several studies have reported that specific circulating miRNAs were useful for distinguishing patients with cancer (e.g., colorectal cancer, breast cancer, prostate cancer) from healthy controls (HCs) (6,9,10). However, the number of studies regarding circulating miRNAs in patients with RCC is small.

Several studies using miRNA microarray analysis demonstrated that miR-210 expression in clear cell carcinoma (CCC), which is the largest subtype of RCC, was significantly

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upregulated in tumor tissues and cell lines (11). In addition, some groups reported that miR-210 upregulation played an important role in tumorigenesis in various types of human cancers (12,13).

The goal of this study was to determine whether circulating miR-210 was a useful diagnostic biomarker for distinguishing CCC patients from HCs.

Materials and methods

Patients and sample collection. The study was approved by the ethics committee of Tottori University Hospital, Japan and all the patients provided written informed consent. We prospectively collected tissue and serum samples from patients undergoing radical nephrectomy or nephron-sparing surgery for renal tumors. Sample collection was performed between 2011 and 2013 at the Department of Urology, Tottori University Hospital. Thirty-four patients with histologically confirmed CCC and 23 HCs with no previous history of any cancer were included in this analysis. Detailed clinicopathological parameters of patients are summarized in Table I.

Tissue samples were obtained from tumor tissues and matched normal tissues from the same kidney specimen in patients with CCC. Immediately after resection, tissue samples were frozen in liquid nitrogen and stored at -80°C.

Blood samples were obtained prior to surgery. Serum was separated after centrifugation (3,000 rpm, 10 min) and stored at -80°C.

Total RNA isolation. Total RNA was extracted from tissue using the mirVana™ miRNA isolation kit (Ambion, USA) and from 200 µl of serum using the microRNA extractor SP kit (Wako, Japan) according to the manufacturer's recommendation (final elution volume, 50 µl). RNA quantity and purity were determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, USA). The RNA samples were stored at -80°C until reverse transcription (RT) reaction.

Quantitative real-time PCR. Complementary DNA (cDNA) was synthetized from total RNA using the TaqMan MicroRNA RT kit (Applied Biosystems) and using miRNA-specific RT primers from the TaqMan MicroRNA assay (Applied Biosystems). RT reaction mixtures were incubated for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. Quantitative real-time PCR was performed using the TaqMan MicroRNA assay on the ABI PRISM 7900HT system (Applied Biosystems). All experiments were performed as specified in the manufacturer's protocols.

Statistical methods. Analysis of the real-time PCR data was done using SDS software, version 2.4 (Applied Biosystems). MicroRNA levels in tissue were normalized against miR-145 and microRNA levels in serum were normalized against miR-16. We confirmed that the expression of miR-145 in tissue and miR-16 in serum were not significantly different when comparing patients and HCs. The relative expression levels of miR-210 were determined by the equation: $2^{-\Delta CT}$ ($\Delta CT=C_{T\ miR-210} - C^{T\ miR-145\ or\ 16}$). Statistical analyses were performed using PASW statistics 18 (SPSS, Chicago, IL,

USA). Sensitivity, specificity and area under curve (AUC) for serum microRNA levels were determined using receiver operator characteristic (ROC) analysis. The relationship between clinicopathologic parameters and microRNA levels was examined using the Mann-Whitney U or Kruskal-Wallis test, as appropriate. P-values of <0.05 were considered to represent statistical significance.

Results

Validation in tissue samples. Using real-time PCR, we first assessed tissue miR-210 levels normalized against miR-145 in 34 pairs of tumor tissues and matched normal tissues obtained from patients with CCC. Tissue miR-210 levels were significantly higher in tumor tissues than in normal tissues (P<0.001; Fig. 1A). There was no difference in the CT values for miR-145 when comparing tumor tissues and normal tissues (P=0.236; Fig. 1B). In 31 cases (92%), the miR-210 level in tumor tissues was increased by >2-fold when compared with that in normal tissues.

Validation in serum samples. We assessed serum miR-210 levels normalized against miR-16 in 34 CCC patients and 23 HCs. The 34 serum samples from CCC patients perfectly matched up with tissue samples described above. Serum miR-210 levels were significantly higher in CCC patients than in HCs (P=0.001; Fig. 2A). There was no significant difference

Table I. Clinicopathological parameters.

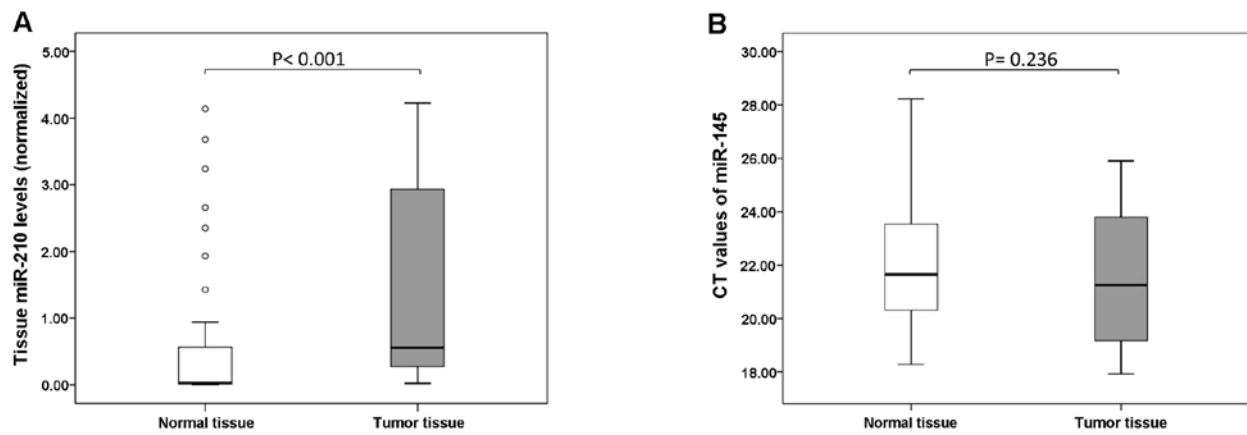|	Serum samples	
	CCC (n=34)	HC (n=23)
Sex		
Male	26	11
Female	8	12
Age (years)		
Mean	66.5	53.5
Range	29-86	36-84
Pathological stage		
pT1a	17	-
pT1b	8	-
pT2a	3	-
pT2b	1	-
pT3a	4	-
pT3b	1	-
pT4	0	-
Lymph nodes metastasis	2	-
Distant metastasis	5	-
Grade		
G1	9	-
G2	24	-
G3	1	-

Figure 1. (A) Tissue miR-210 levels normalized against miR-145 in 34 pairs of tumor tissues and matched normal tissues taken from patients with clear cell carcinoma (CCC). The tissue miR-210 levels were significantly higher in tumor tissues than in normal tissues (P<0.001). (B) There was no significant differences in CT values of miR-145 when comparing tumor tissues and matched normal tissues.
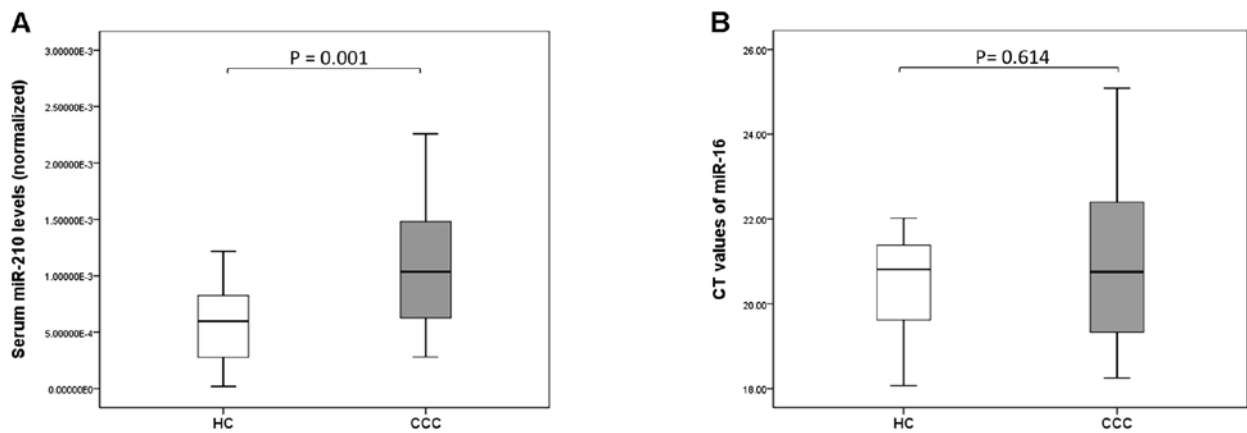

Figure 2. (A) Serum miR-210 levels normalized against miR-16 in 34 CCC patients and in 23 healthy controls (HCs). Serum miR-210 levels were significantly higher in CCC patients than in HCs (P=0.001). (B) There was no significant difference in CT values of miR-16 when comparing CCC patients and HCs.
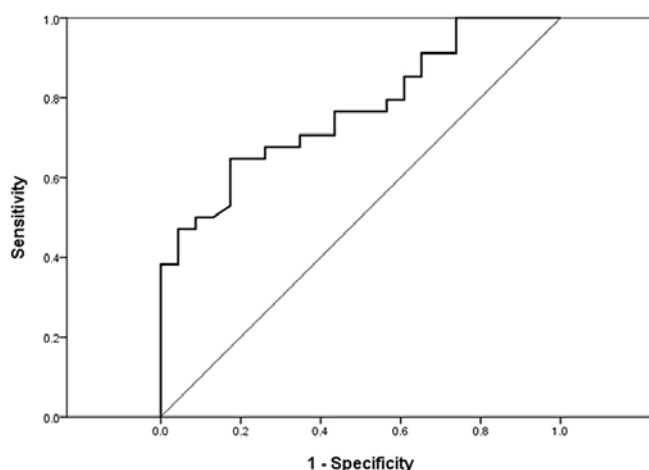

Figure 3. Receiver operating characteristic (ROC) analysis of serum miR-210 showed an area under the ROC curve (AUC) of 0.77 (95% confidence interval, 0.65-0.89) with a sensitivity and specificity of 65 and 83%, respectively.

in the CT values of miR-16 when comparing CCC patients and HCs (P=0.614; Fig. 2B).

ROC curve analysis indicated that the serum miR-210 level might serve as a useful biomarker for differentiating patients with CCC from those with HCs; the AUC was 0.77 (95% confidence interval, 0.65-0.89) and the sensitivity and specificity was 65 and 83%, respectively (Fig. 3).

We analyzed the relationship between serum miR-210 levels and clinicopathological parameters. There was no significant association between serum miR-210 levels and age, sex, tumor size, or existence of metastasis at diagnosis (Fig. 4). Although serum miR-210 level tended to be higher in patients with metastasis at diagnosis when compared with patients without metastasis (P=0.067; Fig. 4D), this difference did not reach the level of statistical significance.

Discussion

Previous studies have described the potential use of circulating microRNA as a non-invasive biomarker for various cancers [e.g., miR-29a and miR-92 in colorectal cancer (9); miR-195 in breast cancer (14); miR-17-5p, miR-21, miR-106a and miR-106b in gastric cancer (15); and miR-141 and miR-26a in prostate cancer (6,16)]. In the case of RCC, several recent
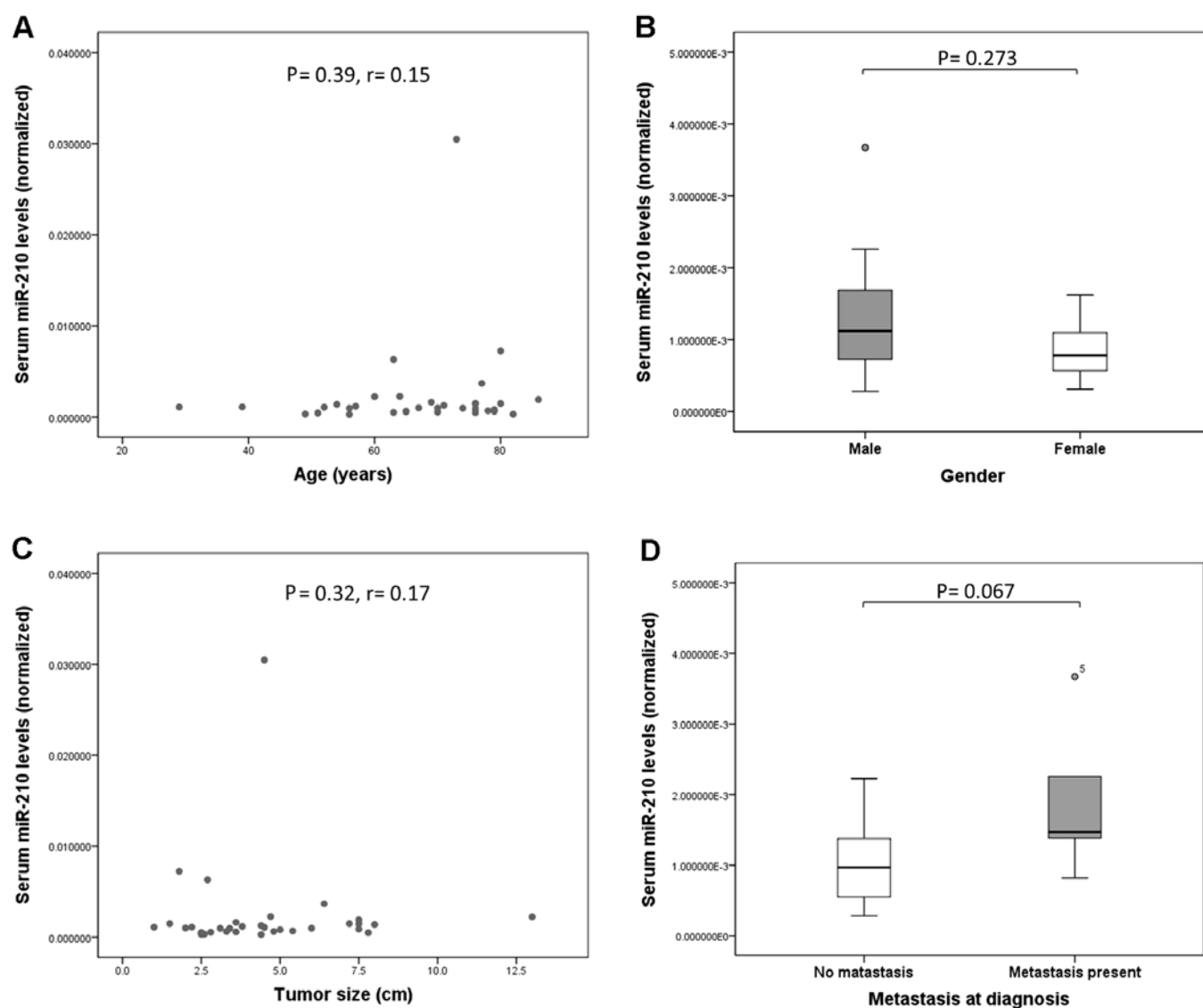
Figure 4. Analysis of the correlation between serum miR-210 levels and clinicopathological parameters. There was no significant association between serum miR-210 levels and age (A), gender (B), tumor size (C), or existence of metastasis at diagnosis (D).
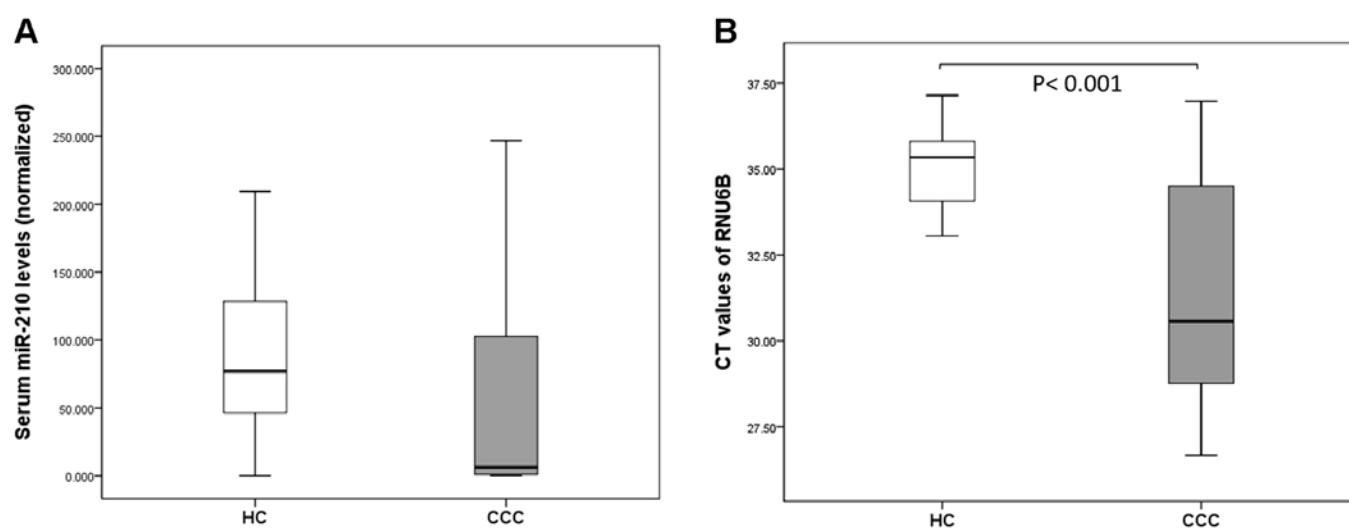

Figure 5. (A) There were no significant differences in serum miR-210 levels normalized against RNU6B when comparing 34 CCC patients and 23 HCs. (B) CT values of miR-16 in 34 CCC patients and in 23 HCs. CT values of RNU6B were significantly lower in CCC patients than in HCs (P<0.001). Moreover, there was marked variability of the Ct values when comparing CCC patients and HCs.

studies using miRNA microarray analysis showed different microRNA expression profiles when comparing tumor tissues and matched normal tissues. However, some of the data regarding the number and type of up-/downregulated microRNAs is conflicting (17-19). Regardless, several recent studies have described the use of circulating microRNA as a new biomarker for RCC.

The present study showed that serum miR-210 levels were significantly higher in CCC patients than in HCs. Furthermore, there was no correlation between serum miR-210 levels and clinicopathological parameters. These results indicate that upregulation of serum miR-210 may occur in the early stage of CCC and can serve as a potential biomarker of early diagnosis in CCC.

Four studies have investigated the utility of circulating microRNAs as a diagnostic biomarker for RCC. Wulfken et al were the first to report that the serum miR-1233 level was increased in 84 patients with RCC from a multicenter cohort (AUC, 0.588; sensitivity, 77.4%; specificity, 37.6%). Moreover, they investigated 13 samples from patients with angiomyolipoma or oncocytoma whose serum miR-1233 levels were similar to those of patients with RCC (20). Redova et al also demonstrated that serum miR-378 and miR-451 were potential biomarkers for RCC. When the utility of miR-378 and miR-451 was evaluated in an independent cohort of 90 patients with RCC and 35 HCs, the combination of serum miR-378 and miR-451 enabled identification of RCC with relatively high accuracy rate (AUC 0.86; sensitivity, 81%; specificity, 83%) (21). Hauser et al confirmed that serum miR-378 was significantly increased in 25 CCC patients (P=0.006), but they did not detect a difference in the level of this biomarker when comparing 117 patients with RCC versus 123 HCs (22). Zhao et al reported that tissue miR-210 levels in 33 CCC patients were significantly higher in tumor tissue than in adjacent non-tumoral renal parenchyma (P=0.004). Serum miR-210 levels were also significantly higher in 68 CCC patients than in 42 HCs (P<0.001) (AUC, 0.87; sensitivity, 81.0%; specificity, 79.4%). Furthermore, serum miR-210 levels in patients with CCC decreased by 1 week after surgical resection of the tumor (23).

The present study normalized the assessed RNA values in tissue and serum against those of miR-145 and miR-16, respectively, which is a different approach from that used by Zhao et al. There is no consensus regarding the optimal normalization gene to use for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of circulating mRNAs (24). The present study did not utilize RNU6B or 5s rRNA for normalization of qRT-PCR data because some studies have reported that RNU6B and 5s rRNA were not stable in human tissues or body fluids. In their study targeting serum miRNA in gastric cancer patients, Song et al demonstrated that RNU6B could not be detected in almost half of the serum samples; in the other half of patients, it was detected with Ct values of >40. Moreover, RNU6B was less stably expressed than let-7a and miR-16 in normal and cancerous human solid tissues (25). Although we actually examined the same study using RNU6B for normalization, there was marked variation in Ct values when comparing CCC patients and HCs (Fig. 5). Therefore, we selected other miRNAs (miR-16, miR-103a, miR-122 and miR-145) for the purposes of normalization, based on previous reports (21,26). Then, we determined which of these miRNAs

was expressed in similar amounts when comparing CCC patients and HCs. As a result of these investigations, miR-145 and miR-16 were selected for use as normalizing genes in the present study.

MiR-210 is upregulated in various types of human cancers, suggesting its important role in tumorigenesis (27). Jung et al reported that plasma miR-210 levels in human breast cancer were associated with trastuzumab sensitivity, tumor presence and lymph node metastasis (28). Although the mechanism of upregulation of miR-210 is still unclear, some groups have reported that miR-210 is induced under-hypoxic conditions via hypoxia-inducible factors (HIFs) in various cancer cell lines (28). It is well known that HIF1α and HIF2α accumulate in CCC as a result of abrogated ubiquitin-mediated degradation due to loss or deficiency of the *von Hippel-Lindau tumor suppressor (VHL)* tumor suppressor gene (29). Nakada et al demonstrated that miR-210 was highly expressed in RCC cell lines and that its expression clearly correlated with the accumulation of hypoxia-inducible factor 1α (HIF1α) under normoxia as well as under hypoxia, suggesting that miR-210 upregulation in CCC was most likely due to accumulation of HIF1α. Further, they confirmed that restoration of VHL expression in the VHL-deficient cell line led to the degradation of HIF1α and suppressed the expression of miR-210, suggesting that miR-210 expression is regulated via the VHL-HIF1α pathway (11). Since VHL inactivation is observed in >70% of CCC patients, its inactivation may occur in the early stage of tumorigenesis. This raises the possibility that the expression of miR-210 is upregulated and plays a role in the early stage of tumorigenesis in CCC tissue and that serum miR-210 is upregulated in the human peripheral blood in the early stages of CCC.

For these reasons, serum miR-210, which can be easily measured on an outpatient basis, could be a useful biomarker for early diagnosis and facilitate earlier treatment and, hence, improves outcomes. Further studies with a larger number of patients are warranted to validate these results.

In conclusion, early detection and treatment of RCC is critical to improve outcomes. Serum miR-210 is a useful biomarker for early CCC.

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