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Original Article

Expression of miR-221 and miR-18a in patients with hepatocellular carcinoma and its clinical significance

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Purpose: Recently, microRNA (miRNA) has been evaluated to provide a new diagnostic and therapeutic modality hepatocellular carcinoma (HCC) and other tumors. They are small non-coding RNA molecules that function as transcriptional and post-transcriptional regulators of gene expression by silencing target genes. The aim of this study was to evaluate the clinical significance of microRNA-18a, 221 (miR-18a, miR-221) expression in HCC formalin-fixed paraffin-embedded (FFPE) tissue.
Methods: miR-18a and miR-221 expressions were assessed by reverse transcription and real-time quantitative reverse transcription polymerase chain reaction in 50 pairs of FFPE HCC and the adjacent noncancerous liver tissues. And we evaluated the expression level in HCC tissues as compared with their adjacent noncancerous counterparts. And the relationship between miR-18a, miR-221 level and clinicopathological data and survival rates were analyzed.
Results: miR-221 and miR-18a were overexpressed in HCC tissue as compared with their adjacent noncancerous liver tissue ($P<0.001$). miR-221 expression was found to be correlated with larger tumor size ($P=0.048$). miR-18a expression was correlated with modified Union for International Cancer Control stage ($P=0.05$). The overall survival ($P=0.02$) of HCC patients with high miR-221 expression was significantly poorer compared to those patients with low expression. Multivariate analyses demonstrated that miR-221 may be a poor prognostic factor of HCC patients.
Conclusion: High expression of miR-221 in FFPE tissues could provide significance for prognosis of HCC patients. Although, miR-18a expression was significantly upregulated in HCC tissues, they are not correlated with prognosis. Further large prospective studies are needed to determine their clinical significance.

Keywords: Hepatocellular carcinoma, MicroRNA, miR-18a, miR-221

INTRODUCTION

Hepatocellular carcinoma (HCC) is hard to predict in an early stage. Despite various and effective treatments, it does not have a good prognosis, unlike other cancers. According to Korean Central Cancer Registry 2017, mortality rates of HCC in men and women rank the 2nd and the 5th, respectively [1]. Treatment for this cancer includes surgical resection, liver transplantation, and locoregional ablation. However, most patients also have liver cirrhosis with an already degraded liver condition when they are diagnosed with HCC. Therefore, those patients who cannot proceed surgical resection and radiofrequency ablation need various kinds of remedy [2]. Recently, understanding the molecular biology based on basic research of HCC has activated translational research which can be used for diagnosis and treatment of HCC.

Among small RNAs, microRNA (miRNA) is one of the popular research subjects recently. It is a small single-stranded non-coding RNA combined with 3′-untranslated lesion (UTR) of target messenger RNA (mRNA) to prevent or destroy translation of target RNA. It is related to post-transcriptional gene silencing. It can control the expression of target mRNA [3]. In the biosynthesis process, the transcribed miRNA precursor is made by Drosha, a premature

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miRNA whose structure is similar to a hairpin and a type of RNA cleavage enzyme III that moves from the nucleus to the cytoplasm. It is processed by Dicer, a type of RNA cleavage enzyme III to form mature miRNA. These mature miRNAs play a role by forming RNA-induced silencing complex and binding them to complementary positions of the 3′-UTR of the target mRNA [4].

More than 2,000 kinds of miRNAs have been found in human cells. They control more than 30% of the entire genome and play an important role in the generation, regeneration, cytodifferentiation and proliferation of normal cells. They are known to play a crucial role in inflammation and cancerization of cells, even in pathosis [5]. Recently, many reports have revealed the correlations and physiological relations between cancers including HCC and prognosis. Especially, overexpression of microRNA (miR)-21, miR-221, and miR-222 has been found in HCC patients than in people with healthy livers. However, HCC tissues rarely express miR-122a, miR-145, miR-199a, and miR-223, unlike normal tissues [4]. Reports on correlations of clinicopathologic prognosis with miR-221 and miR-18a are insufficient. Therefore, the objective of this study was to determine correlations of prognosis with expression levels of miRNA-221 and miRNA-18a in patients with HCC.

METHODS

Patients and tissue samples

Samples of total of 50 patients who underwent surgical resection of HCC in Soonchunhyang University Cheonan Hospital from 2001 to 2009 were analyzed. Distant metastasis was not found at the time of diagnosis. Outpatient tracking after surgery was possible, 50 patients whose paraffin-embedded tissues were preserved relatively well were selected and their medical records were referred to analyze prognostic factors. All clinicopathological standards followed primary HCC, third edition, June 2007, Korea. Tumor differentiation (grade) followed the Edmondson and Steiner nuclear grading system. Tumor stage followed TNM classification of the American Joint Committee on International Union against Cancer, Okuda staging, Barcelona Clinical Liver Cancer staging, and modified Union for International Cancer Control (UICC) staging. The Institutional Review Board of the Soonchunhyang University Cheonan Hospital approved this study (IRB No. SCHCA 2022-06-024-009). The informed consent was waived.

Real-time qRT-PCR for miRNA, data normalization

RNA was purified from formalin-fixed paraffin-embedded (FFPE) tissues using the MirVana miRNA Isolation Kit (Applied BioSystems, Foster City, CA, USA) according to manufacturer instructions. The expression patterns of the miRNA species tested and a housekeeping gene, U6sn, were quantitatively assayed using reverse transcription and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Stem-loop complementary DNAs (cDNAs) were synthesized using looped reverse transcription primers specific for each miRNA. All the materials were purchased by Exiqon (Vedbaek, Denmark). In real-time PCR assays, forward primers specific for each cDNA and a reverse primer universal for all cDNAs were used. We examined the expression of miR-18a and miR-221. All samples were analyzed twice to confirm reproducibility. miR-18a, miR-221 and U6sn were reliable amplified in FFPE tissues. Amplified miRNAs showed specific melting temperature, confirming the accuracy and specificity of the method used. Real-time qRT-PCR was conducted on an ABI Prism 7700 apparatus (Applied Biosystems). Data were analyzed with the ABI Prism 7700 SDS software (Applied Biosystems). The expression of each miRNA was normalized to U6sn RNA internal control. The levels of miRNAs expression were normalized after subtracting the threshold cycle (Ct) value of the U6sn RNA internal control from that of each miRNA Ct value for samples ($\triangle$Ct = l Ct miRNA [samples] – Ct U6snl). In order to compare the levels of miRNA expression between the samples tested, the $\triangle\triangle$Ct value was determined using the formula ($\triangle\triangle$Ct = $\triangle$Ct miRNA [sample A] – $\triangle$Ct miRNA [sample B]). Then, the relative level of miRNA in cancer samples was compared to normal samples by setting the miRNA expression in normal samples value to 1 and determining the fold change in expression against this value using the following formula $2^{\triangle\triangle Ct}$.

Statistical analysis

All of the data were analyzed by PASW Statistics version 26.0 (IBM Corp., Armonk, NY, USA) and with $P < 0.05$ as the threshold of statistical significance. The chi-square and Fisher exact tests were used to compare the levels of miR-221 and miR-18a expression and the various clinicopathological characteristics between the groups. Survival curves for overall survival (OS) and disease-free survival (DFS) were calculated using the Kaplan-Meier method and were compared by the log-rank test. Multivariate analysis of prognostic relevance was evaluated by multivariate Cox regression analysis.

RESULTS

Clinicopathologic characteristics of patients and correlations between miR-221 and miR-18a

Among 50 patients of HCC, there were 39 males and 11 females. Their average age was 53 years. There were 44 (88%) patients suf-

Table 1. Characteristics of patients with hepatocellular carcinoma per miR-18 expression

Variable		No.	miR-18 expression, No. (%)			Mean ± SD	P-value[b]
			Low group	High group	P-value[a]		
Age (yr)	≤ 53	28	17 (60.7)	11 (39.3)	0.347	5.77 ± 5.71	0.369
	> 53	22	17 (77.3)	5 (22.7)		4.28 ± 5.86	
Sex	Male	39	25 (64.1)	14 (35.9)	0.466	5.31 ± 6.23	0.566
	Female	11	9 (81.8)	2 (18.2)		4.42 ± 3.83	
Preoperative TACE	No	19	10 (52.6)	9 (47.4)	0.131	5.82 ± 6.25	0.515
	Yes	31	24 (77.4)	7 (22.6)		4.68 ± 5.50	
Child-Pugh score	0	42	28 (66.7)	14 (33.3)	> 0.99	5.50 ± 6.09	0.118
	≥ 1	8	6 (75.0)	2 (25.0)		3.11 ± 3.16	
Liver cirrhosis	Absence	33	22 (66.7)	11 (33.3)	> 0.99	4.65 ± 4.02	0.524
	Presence	17	12 (70.6)	5 (29.4)		6.02 ± 8.24	
Etiology	HBV	42	29 (69.0)	13 (31.0)	0.839	4.92 ± 4.99	0.369
	HCV	2	1 (50.0)	1 (50.0)		15.46 ± 18.67	
	None	6	4 (66.7)	2 (33.3)		3.06 ± 1.78	
PIVKA-II	Normal	15	10 (66.7)	5 (33.3)	> 0.99	3.65 ± 3.56	0.148
	Abnormal	35	24 (68.6)	11 (31.4)		5.74 ± 6.42	
Alpha-fetoprotein	Normal	21	16 (76.2)	5 (23.8)	0.454	3.85 ± 5.95	0.194
	Abnormal	29	18 (62.1)	11 (37.9)		6.03 ± 5.55	
Pathological grade	I-II	25	17 (68.0)	8 (32.0)	> 0.99	3.81 ± 3.47	0.112
	III-IV	25	17 (68.0)	8 (32.0)		6.42 ± 7.22	
Tumor size (cm)	≤ 3	24	19 (79.2)	5 (20.8)	0.186	4.97 ± 5.91	0.865
	> 3	26	15 (57.7)	11 (42.3)		5.25 ± 5.74	
Tumor size (cm)	≤ 5	37	26 (70.3)	11 (29.7)	0.731	4.66 ± 5.11	0.443
	> 5	13	8 (61.5)	5 (38.5)		6.40 ± 7.39	
Tumor nodes	Single	46	32 (69.6)	14 (30.4)	0.584	5.20 ± 5.97	0.529
	Multi	4	2 (50.0)	2 (50.0)		4.16 ± 2.59	
Okuda staging	I	44	29 (65.9)	15 (34.1)	0.65	5.48 ± 6.02	0.02[a]
	II	6	5 (83.3)	1 (16.7)		2.46 ± 1.93	
BCLC staging	I-II	34	24 (70.6)	10 (29.4)	0.805	4.92 ± 5.57	0.745
	III-IV	16	10 (62.5)	6 (37.5)		5.53 ± 6.32	
AJCC staging	I-II	40	27 (67.5)	13 (32.5)	> 0.99	4.67 ± 5.29	0.39
	III-IV	10	7 (70.0)	3 (30.0)		6.89 ± 7.41	
Modified UICC staging	I-II	34	26 (76.5)	8 (23.5)	0.122	4.74 ± 5.61	0.528
	III-IV	16	8 (50.0)	8 (50.0)		5.90 ± 6.17	
Portal invasion	No	41	28 (68.3)	13 (31.7)	> 0.99	4.57 ± 5.27	0.276
	Yes	9	6 (66.7)	3 (33.3)		7.60 ± 7.49	
Microvascular invasion	No	17	11 (64.7)	6 (35.3)	0.969	5.56 ± 6.12	0.708
	Yes	33	23 (69.7)	10 (30.3)		4.89 ± 5.65	
Distant metastasis	No	18	15 (83.3)	3 (16.7)	0.153	5.77 ± 8.08	0.621
	Yes	32	19 (59.4)	13 (40.6)		4.75 ± 4.03	

SD, standard deviation; TACE, transarterial chemoembolization; HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; BCLC, Barcelona Clinical Liver Cancer; AJCC, American Joint Committee on Cancer; UICC, Union for International Cancer Control.

[a]P-values were calculated by chi-square test or Fisher exact test. [b]P-values were calculated by Student t-test or Mann-Whitney U test for variables with two levels and one-way analysis of variance or Kruskal-Wallis test for variables with three levels.

Table 2. Characteristics of patients with hepatocellular carcinoma per miR-221 expression

Variable		No.	miR-221 expression, No. (%)			Mean±SD	P-value[b]
			Low group	High group	P-value[a]		
Age (yr)	≤ 53	28	18 (64.3)	10 (35.7)	> 0.99	4.03 ± 3.03	0.439
	> 53	22	14 (63.6)	8 (36.4)		3.36 ± 3.02	
Sex	Male	39	23 (59.0)	16 (41.0)	0.287	3.68 ± 2.92	0.837
	Female	11	9 (81.8)	2 (18.2)		3.92 ± 3.47	
Preoperative TACE	No	19	12 (63.2)	7 (36.8)	> 0.99	4.21 ± 3.32	0.406
	Yes	31	20 (64.5)	11 (35.5)		3.44 ± 2.82	
Child-Pugh score	0	42	28 (66.7)	14 (33.3)	0.436	3.89 ± 3.01	0.437
	≥ 1	8	4 (50.0)	4 (50.0)		2.92 ± 3.09	
Liver cirrhosis	Absence	33	22 (66.7)	11 (33.3)	0.813	3.93 ± 2.99	0.529
	Presence	17	10 (58.8)	7 (41.2)		3.35 ± 3.10	
Etiology	HBV	42	29 (69.0)	13 (31.0)	0.113	3.73 ± 2.95	0.149
	HCV	2		2 (100)		8.24 ± 2.18	
	None	6	3 (50.0)	3 (50.0)		2.26 ± 2.42	
PIVKA-II	Normal	15	9 (60.0)	6 (40.0)	0.949	4.49 ± 3.01	0.255
	Abnormal	35	23 (65.7)	12 (34.3)		3.41 ± 2.99	
Alpha-fetoprotein	Normal	21	16 (76.2)	5 (23.8)	0.219	3.99 ± 3.02	0.615
	Abnormal	29	16 (55.2)	13 (44.8)		3.55 ± 3.04	
Pathological grade	I-II	25	18 (72.0)	7 (28.0)	0.377	3.48 ± 3.03	0.553
	III-IV	25	14 (56.0)	11 (44.0)		3.99 ± 3.03	
Tumor size (cm)	≤ 3	24	18 (75.0)	6 (25.0)	0.207	4.23 ± 2.93	0.265
	> 3	26	14 (53.8)	12 (46.2)		3.27 ± 3.06	
Tumor size (cm)	≤ 5	37	27 (73.0)	10 (27)	0.043	3.63 ± 2.93	0.704
	> 5	13	5 (38.5)	8 (61.5)		4.03 ± 3.34	
Tumor nodes	Single	46	29 (63.0)	17 (37.0)	> 0.99	3.52 ± 2.79	0.341
	Multi	4	3 (75.0)	1 (25.0)		6.19 ± 4.71	
Okuda staging	I	44	29 (65.9)	15 (34.1)	0.654	3.87 ± 3.02	0.415
	II	6	3 (50.0)	3 (50.0)		2.73 ± 3.02	
BCLC staging	I-II	34	22 (64.7)	12 (35.3)	> 0.99	4.03 ± 3.28	0.261
	III-IV	16	10 (62.5)	6 (37.5)		3.11 ± 2.30	
AJCC staging	I-II	40	25 (62.5)	15 (37.5)	0.73	3.77 ± 3.00	0.876
	III-IV	10	7 (70.0)	3 (30.0)		3.59 ± 3.22	
Modified UICC staging	I-II	34	23 (67.6)	11 (32.4)	0.64	3.62 ± 2.97	0.712
	III-IV	16	9 (56.2)	7 (43.8)		3.97 ± 3.17	
Portal invasion	No	41	26 (63.4)	15 (36.6)	> 0.99	3.68 ± 3.02	0.795
	Yes	9	6 (66.7)	3 (33.3)		3.98 ± 3.15	
Microvascular invasion	No	17	11 (64.7)	6 (35.3)	> 0.99	3.54 ± 2.84	0.739
	Yes	33	21 (63.6)	12 (36.4)		3.83 ± 3.13	
Distant metastasis	No	18	11 (61.1)	7 (38.9)	> 0.99	4.58 ± 3.34	0.162
	Yes	32	21 (65.6)	11 (34.4)		3.26 ± 2.75	

SD, standard deviation; TACE, transarterial chemoembolization; HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; BCLC, Barcelona Clinical Liver Cancer; AJCC, American Joint Committee on Cancer; UICC, Union for International Cancer Control.
[a]P-values were calculated by chi-square test or Fisher exact test. [b]P-values were calculated by Student t-test or Mann-Whitney U test for variables with two levels and one-way analysis of variance or Kruskal-Wallis test for variables with three levels.

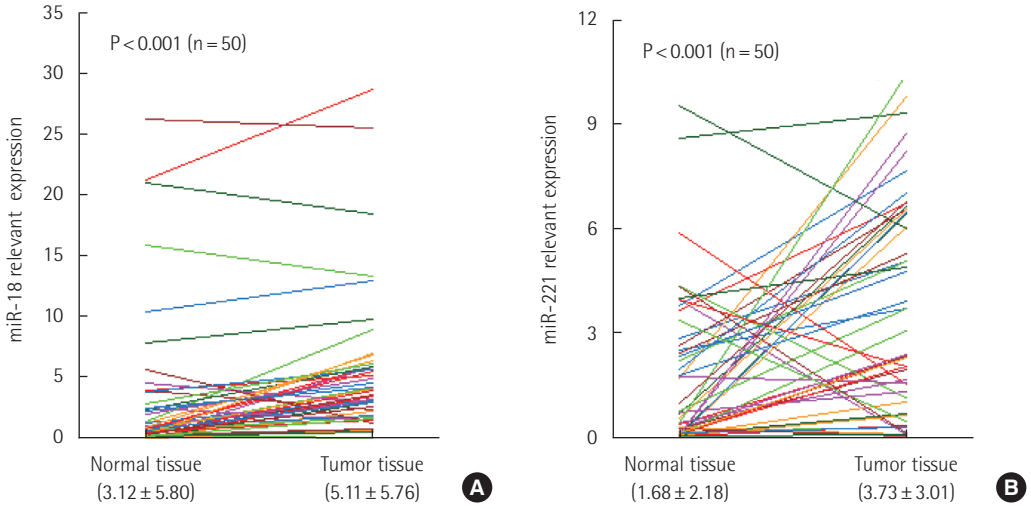


Fig. 1. Individual plot of microRNA (miRNA) expression between normal and tumor tissue. (A) miR-18 and (B) miR-221.

fering from hepatitis B virus (HBV) or hepatitis C virus. Regarding histologic grade, 25 (50%) had grade I or II, 44 (88%) had stage I on Okuda staging, 40 (80%) had stage I or II of American Joint Committee on Cancer staging, and 34 (68%) had stage I or II of modified UICC staging. Nine (18%) and 33 (66%) had portal venin invocation and microvascular invasion, respectively. Expression of miR-18 was statistically and closely related to Okuda stage (P = 0.02). miR-221 was closely related to tumor size (P = 0.043) (Table 1).

Comparison of expression levels of miRNA-221 and miRNA-18a in HCC tissues with those in adjacent tissues of cancer

Real-time qRT-PCR was performed to analyze expression levels of miRNA-221 and miRNA-18a in 50 pairs of HCC tissues and their adjacent tissues. After normalization, relative expression mean ± standard deviation (mean ± SD) levels of miRNA 221 and miRNA-18a in HCC tissues were 5.11 ± 5.76 and 3.73 ± 3.01, respectively, which were significantly (P < 0.001) higher than those in adjacent tissues of cancers (mean ± SD: 3.12 ± 5.80 and 1.68 ± 2.18, respectively) (Table 2, Fig. 1).

Cox proportional hazard regression analysis and survival analysis of target patients' OS and DFS

High expression of miR-221 was related to OS. OS was also related to protein induced by vitamin K absence or antagonist II (PIVKA-II) level, tumor size, pathologic grade, and pathologic stage in univariate analysis. Among them, high miR-221 expression showed a significant association with OS (hazard ratio [HR], 2.85) in multivariate analysis (P = 0.015). PIVKA, tumor size, portal vein invasion, and distant metastasis were closely related to DFS. The Kaplan-Meier analysis and log-rank test demonstrated that the OS of low expression groups of miR-221 were significantly better than that of the high expression groups (P = 0.020) (Tables 3 and 4, Fig. 2).

DISCUSSION

HCC is one of the aggressive malignancies and the second cause of cancer-related mortality in Korea [1]. A lot of molecular events and biomarkers have been reported to uncover etiology of HCC. However, they do not meet the sensitivity or specificity of biomarkers such as alpha-fetoprotein and PIVKA [5]. Recently, many studies have reported that miRNA expression is correlated with tumorigenesis and progression in HCC [4-8]. The dysregulation of miRNA in HCC has been observed in various studies, including upregulated miRNAs and downregulated miRNAs [9]. Although miR-18a and miR-221 are well-known overexpressed miRNAs in HCC, studies regarding their relations with prognosis are insufficient. Therefore, we tried to determine whether miR-18a and miR-2 were actually overexpressed in HCC and investigated their prognostic roles in this study. We evaluated the dysregulation of miR-221 and miR-18a in surgically resected HCCs using FFPE tissues and determined their expression levels as clinically relevant biomarkers in the context of their potential. Expression patterns of these miRNAs were also investigated in normal livers of cancer-free individuals to assess their differential expression levels of patients with HCC.

Although miR-18a is known as multifunctional miRNA and dual functional role in cancer development, it is mostly overexpressed in different types of malignant tumors [10]. The association of miR-18a with cancer progression was first reported by Ota et al. [11]. Since then, many studies have reported that amplifica-

Table 3. Univariate Cox proportional hazard regression analysis for overall and disease-free survival (n=50)

Variable		Overall survival		Disease-free survival	
		HR (95% CI)	P-value	HR (95% CI)	P-value
miR-18 expression	Low	1		1	
	High	1.81 (0.84–3.91)	0.131	1.07 (0.51–2.25)	0.859
miR-221 expression	Low	1		1	
	High	2.39 (1.11–5.13)	0.025[a]	1.43 (0.7–2.89)	0.326
Age (yr)	≤ 53	1		1	
	> 53	1.32 (0.62–2.82)	0.466	1.50 (0.75–2.98)	0.247
Sex	Female	1		1	
	Male	1.63 (0.61–4.33)	0.326	1.95 (0.8–4.74)	0.14
Preoperative TACE	No	1		1	
	Yes	0.77 (0.36–1.65)	0.505	1.15 (0.56–2.34)	0.7
Child-Pugh score	0	1		1	
	≥ 1	1.26 (0.48–3.34)	0.642	1.13 (0.47–2.75)	0.78
Liver cirrhosis	Absence	1		1	
	Presence	1.33 (0.61–2.86)	0.471	0.89 (0.43–1.83)	0.742
Etiology	None	1		1	
	HBV	0.55 (0.19–1.60)	0.27	0.51 (0.19–1.33)	0.166
	HCV	0.43 (0.05–3.88)	0.453	0.83 (0.16–4.29)	0.821
PIVKA-II	Normal	1		1	
	Abnormal	3.70 (1.27–10.73)	0.016[a]	2.88 (1.24–6.70)	0.014[a]
Alpha-fetoprotein	Normal	1		1	
	Abnormal	1.10 (0.51–2.37)	0.81	1.16 (0.58–2.34)	0.672
Pathological grade	I-II	1		1	
	III-IV	2.98 (1.33–6.69)	0.008[a]	1.68 (0.84–3.36)	0.139
Tumor size (cm)	≤ 3	1		1	
	> 3	3.33 (1.45–7.68)	0.005[a]	3.02 (1.46–6.25)	0.003[a]
Tumor size (cm)	≤ 5	1		1	
	> 5	3.17 (1.44–6.97)	0.004[a]	3.07 (1.43–6.59)	0.004[a]
Tumor nodes	Single	1		1	
	Multi	2.27 (0.67–7.61)	0.186	1.00 (0.24–4.19)	0.998
Okuda staging	I	1		1	
	II	2.18 (0.81–5.86)	0.123	1.65 (0.63–4.30)	0.309
BCLC staging	I-II	1		1	
	III-IV	1.82 (0.84–3.95)	0.128	1.65 (0.82–3.32)	0.163
AJCC staging	I-II	1		1	
	III-IV	2.67 (1.15–6.16)	0.022[a]	3.48 (1.61–7.50)	0.001[a]
Modified UICC staging	I-II	1		1	
	III-IV	2.28 (1.06–4.91)	0.036[a]	1.51 (0.74–3.08)	0.256
Portal invasion	No	1		1	
	Yes	2.34 (0.98–5.58)	0.055	3.11 (1.41–6.84)	0.005[a]
Microvascular invasion	No	1		1	
	Yes	0.48 (0.22–1.03)	0.061	0.54 (0.27–1.08)	0.081
Distant metastasis	No	1		1	
	Yes	0.49 (0.23–1.07)	0.072	0.45 (0.22–0.90)	0.024[a]

HR, hazard ratio; CI, confidence interval; TACE, transarterial chemoembolization; HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; BCLC, Barcelona Clinical Liver Cancer; AJCC, American Joint Committee on Cancer; UICC, Union for International Cancer Control.
[a]Statistically significant, $P < 0.05$.

Table 4. Multiple Cox proportional hazard regression analysis

Variable	Overall survival	
	HR (95% CI)	P-value
High miR-221 expression	2.85 (1.23–6.61)	0.015
PIVKA	3.23 (0.92–11.27)	0.066
Tumor size of >3 cm	1.77 (0.66–4.70)	0.254
AJCC staging of III-IV	1.49 (0.61–3.61)	0.379

HR, hazard ratio; CI, confidence interval; miR-221, microRNA 221; PIVKA-II, protein induced by vitamin K absence or antagonist II; AJCC, American Joint Committee on Cancer.

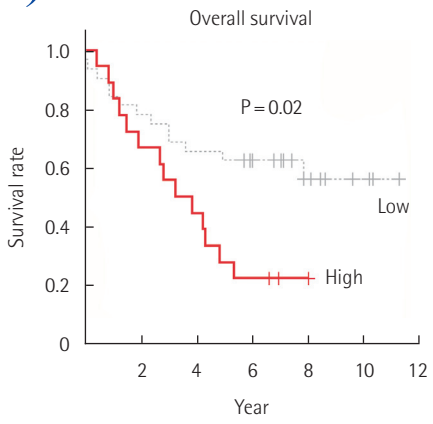
tion of miRNA-18a is related to cancer progression. A miR-221 is one of highly homologous miRNAs. It frequently acts with miR-222 as a gene cluster. Overexpression of miR-221 has been found in several types of cancers [12]. In HCC, overexpression of miR-221 leads to tumor progression, cell growth, and inhibition of apoptosis. miR-221 and miR-18a were noticeably upregulated in HCCs than in adjacent noncancerous tissues in our study. Levels of miR-18a and miR-221 were higher in HCC tissues than in adjacent noncancerous tissues. We found that high expression of miR-221 was correlated with poor OS and poor prognosis in patients with HCC. It was also closely related to Okuda stage. Although the expression level of miR-18a was not correlated with survival or recurrence, we found that the level of miR-18a was significantly associated with tumor stage. Several recent studies have suggested that upregulation of miR-21, miR-155, miR-221, and miR-222 can result in inhibition of target tumor suppressors during early preneoplastic stage for malignant transformation in HCC development [4].

Although the prognostic value and mechanism are not fully understood yet, many recent studies have been reported from a prognostic point of view. Some recent studies have reported meaningful findings regarding a prognostic role of miR-221, showing correlation with a poor survival [13-15]. In our study, overexpression of miR-221 was correlated with shorter OS and poor prognosis in patients with HCC. These results were confirmed not only by univariate analysis, but also by multivariate analysis. There were close relations between Okuda stage and miR-221. In contrast, miR-18a has not been reported to have a significant association with prognosis or survival in HCC. In a recent study using The Cancer Genome Atlas dataset, there was no significant correlation between survival and expression level of miR-18a in several types of cancers including HCC [16]. We investigated the prognostic value of miR-18a in this study. Those with a low expression of miR-18a showed to tendency to have better survival and prognosis. However, such relationship did not reach statistical significance. Although miR-18a did not show statistically significance in survival, it was significantly associated with tumor size.

Recently, one meta-analysis found that overexpression of miR-221 was significantly associated with poor OS and DFS [7]. Subgroup analysis for OS showed that high miR-221 expression was closely related to poor prognosis in both Asian (HR, 2.04; 95% confidence interval [CI], 1.51–2.76) and non-Asian patients (HR, 1.76; 95% CI, 1.28–2.44), suggesting that overexpression of miR-221 could predict poor prognosis in both ethnic groups. Sohn et al. [17] have also reported that exosomal miR-221 levels are higher in HCC patients than in patients with liver cirrhosis. Another study has also shown that expression levels of miRNAs in liver tissues or circulation are correlated with disease severity and survival of HCC patients. Single miRNAs and miRNA panels associated with shorter survival include miR-21, miR-221, and two 20-mer miRNA signature profiles. miR-26a, miR-26b, miR-122, miR-125b, and miR-203 are associated with longer survival. Among these miRNAs, miR-21 was the most replicated one, with a HR ranging from 1.4 to 2.2 in predicting long-term progression of HBV-HCC [5].

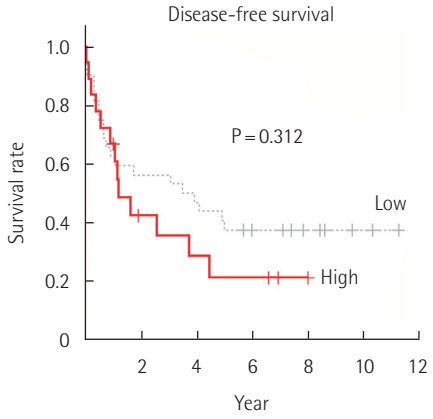
In contrast to our study results, Morita et al. [18]. found that an increase in miR-18a was correlated with higher relevant tumor marker levels, a large tumor size, and a high recurrence rate. They also found that in HCC cells, miR-18a regulated tumor necrosis factor alpha-induced protein 3 (TNFAIP3) expression. miR-18a was related to increases of malignant progression of many cancers, such as cervix, lung, and breast cancers. Many pathways could explain this situation. For example, lung cancer shows downregulation of IRF2, which has apoptotic and antiproliferative activities. A positive correlation between miR-18a and NF-κB has been found. However, it was not subjected to further investigation. Due to difficulties in accessibility and biopsy of liver tissues, studies about circulating miRNAs in plasma or serum from patients with HCC have increased dramatically in recent years [6]. Potential single miRNAs and miRNA panels have been proposed as early diagnostic biomarkers for HCC. There are some reports that circulating miRNAs including miR-18a, miR-21, miR-101, miR-122, miR-139, miR-223, and some miRNA panels might have diagnostic utility in distinguishing HBV-HCC patients from patients with chronic HBV infection or liver cirrhosis [5]. However, early diagnosis of HCC, crucial for treatment outcome, remains challenging.

In HCC which has heterogenetic features, a representative factor highly correlated with prognosis is metastasis. In our study, 32 of 50 patients had distant metastasis. Distant metastasis showed a statistically significant association with DFS (P = 0.024) and a tendency to be correlated with OS (P = 0.072). To date, mechanism of miRNAs related metastasis in HCC has been uncertain and studies about miRNAs' role in metastatic HCC have been rare. A re-



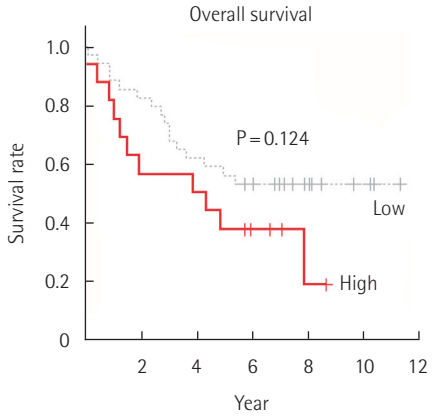
No. at risk (%)	Baseline	1 yr	3 yr	5 yr	10 yr
Low miR-221 expression	32 (96.9)	27 (84.4)	24 (68.7)	20 (62.5)	3 (56.2)
High miR-221 expression	18 (100)	16 (83.3)	10 (55.6)	5 (27.8)	Lost all

A



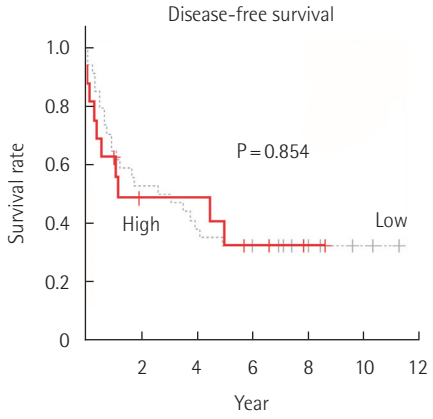
No. at risk (%)	Baseline	1 yr	3 yr	5 yr	10 yr
Low miR-221 expression	32 (96.9)	20 (62.5)	18 (56.2)	13 (37.5)	2 (37.5)
High miR-221 expression	18 (100)	12 (66.7)	5 (35.4)	3 (21.2)	Lost all

B



No. at risk (%)	Baseline	1 yr	3 yr	5 yr	10 yr
Low miR-18 expression	34 (100)	30 (88.2)	25 (67.6)	19 (55.9)	3 (52.9)
High miR-18 expression	16 (93.8)	13 (75.0)	9 (56.2)	6 (37.5)	Lost all

C



No. at risk (%)	Baseline	1 yr	3 yr	5 yr	10 yr
Low miR-18 expression	34 (100)	22 (64.7)	17 (50.0)	11 (32.4)	2 (32.4)
High miR-18 expression	16 (93.8)	10 (62.5)	6 (48.6)	5 (32.4)	Lost all

D

Fig. 2. MicroRNA (miR)-221 survival curve for overall survival rate (A) and disease-free survival rate (B). miR-18 Survival curve for overall survival rate (C) and disease-free survival rate (D). Survival rates were calculated by Kaplan-Meier estimates.

cent study has shown that miR-494-3p plays a role in promoting HCC migration and invasion whereas miR-126-3p has an opposite effect [19]. miR-10b can also promote migration and invasion of HCC through RhoC, uPAR, and MMPs [20]. miR-34a has also been suggested to be able to induce recruitment of Treg cell and promote venous metastases of HBV-positive HCC [21]. In the present study, correlations of metastasis with miR-18a and miR-221 and the mechanism of metastasis related to them these were not investigated. However, it was confirmed that miR-18a and miR-221 were related to prognosis through our study. Additional research is needed to determine their correlations with metastasis.

In conclusion, our results demonstrate that expression levels of miR-221 and miR-18a in HCC tissues are correlated with tumor stage and poor prognosis of HCC patients. miR-221 might be able to be used as a promising prognostic marker for HCC. However, this study has some limitations. First, this study had a small sample size which might weaken the statistical power. Second, only two targets in miRNAs were selected and investigated. Third, the cutoff value could be different with other studies. Thus, further large prospective studies are needed to determine potential targets of miRNAs and their clinical and therapeutic significance in HCC.

CONFLICT OF INTEREST

Hae Il Jung is an editorial board members of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

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