

miR-615-3p expression level in bone marrow is associated with tumor recurrence in hepatocellular carcinoma

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Received October 30, 2014; Accepted January 14, 2015

DOI: 10.3892/mco.2015.514

Abstract. The significance of disseminated tumor cells (DTCs) in bone marrow (BM) on tumor recurrence has not been investigated in patients with hepatocellular carcinoma (HCC). The aim of the present study was to clarify the molecular characteristics of DTCs that affect postoperative recurrence based on microRNA (miRNA) expression profiles in clinical HCC patients undergoing curative resection. DTCs were prospectively collected from the BM of preoperative HCC patients using immunomagnetic beads and subjected to miRNA microarray analysis. Microarray analysis of nine HCC patients (n=5 patients with postoperative HCC recurrence, n=4 patients without HCC recurrence) demonstrated that *miR-615-3p* is significantly upregulated in the DTCs of patients with recurrence compared to the DTCs from patients without recurrence. *In vitro* experiments demonstrated that the *miR-615-3p* expression level is significantly correlated with malignant characteristics in HCC cells. These data suggest that *miR-615-3p* in DTCs may play an important role in postoperative HCC recurrence, which suggests that *miR-615-3p* is a potential target molecule for regulating postoperative HCC recurrence.

Introduction

Hepatocellular carcinoma (HCC) is a common cancer and the third most frequent cause of cancer-related mortalities worldwide (1,2). The prognosis of HCC patients remains poor even following curative treatment, owing to the high incidence of post-treatment recurrence. Additionally, no effective chemotherapy regimens have been established for treating HCC.

Furthermore, mechanisms by which HCC recurs are not fully understood (3). To improve the prognosis of HCC patients, the mechanism underlying HCC recurrence requires clarification and new, effective therapies based on that mechanism require development.

Disseminated tumor cells (DTCs) represent the beginning of systemic disease arising from localized cancer (4,5). Over the past 20 years, evidence has been accumulating that the presence of DTCs in bone marrow (BM) is a prognostic indicator for patients with various types of cancer. However, recent studies indicate that all the patients with DTCs do not necessarily develop recurrence and/or metastasis (6,7). In a recent study we demonstrated that DTCs are observed in BM even in patients with early-stage gastric cancer (8). These findings indicate that DTCs from different individuals may have varying characteristics that influence the capacity to develop cancer recurrence and/or metastasis.

microRNAs (miRNAs) constitute a class of small non-coding RNAs that function as a novel class of global, post-transcriptional gene regulators by binding to partially complementary sequences in the 3' untranslated regions of downstream target mRNAs (9-11). Studies have indicated that miRNAs determine cancer metastasis and recurrence through regulating multiple target genes in numerous types of cancer, including HCC (12-15). Furthermore, our recent study showed that miRNA profiles in DTCs in BM differ significantly between colorectal cancer patients with and without metastasis and that the expression level of specific miRNAs correlates significantly with malignant potential and long-term prognosis (16).

These findings led us to consider that an investigation of the miRNA expression profiles in DTCs from the BM of HCC patients may be useful to identify the mechanisms of HCC recurrence and metastasis based on miRNA-regulated DTC characteristics, which will in turn lead to the development of novel, mechanism-dependent effective therapies. To clarify the mechanism underlying postoperative HCC recurrence, BM samples were prospectively collected from preoperative HCC patients and their miRNA expression profiles were analyzed. Using patient clinical data, an attempt was made to identify the miRNA expression pattern responsible for postoperative recurrence by comparing miRNA expression profiles from the BM of patients with and without postoperative recurrence.

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Key words: hepatocellular carcinoma, disseminated tumor cells, bone marrow, microRNA, *miR-615-3p*

Table I. Characteristics of the HCC patients enrolled in the study.

No.	Gender	Age, years	Risk factor	Child-Pugh classification	Multiplicity	Maximum tumor size, cm	Tumor thrombus	Postoperative recurrence ^a
1	M	37	HBV	A	Solitary	1.1	-	-
2	F	69	HBV	A	Solitary	2.0	-	+
3	M	70	HCV	A	Multiple	1.7	+	-
4	M	72	HCV	B	Solitary	3.0	-	-
5	M	65	-	A	Multiple	5.0	-	+
6	M	60	HCV	A	Solitary	0.9	-	-
7	M	40	HBV	A	Multiple	8.0	+	+
8	M	76	-	A	Solitary	1.8	-	+
9	M	47	HBV, HCV	A	Multiple	3.5	-	-

^aPostoperative HCC recurrence was investigated during 2 years of observation after surgery. HCC, hepatocellular carcinoma; M, male; F, female; HBV, hepatitis B virus; HCV, hepatitis C virus.

Materials and methods

Cell lines. The human HCC cell lines PLC/PRF/5, HuH7, HLE and HLF were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂.

HCC patients. The study enrolled nine HCC patients who had undergone curative hepatic resection for HCC at the Department of Surgery, Osaka University Hospital (Osaka, Japan) between May 2010 and August 2010. The patient characteristics are shown in Table I. Curative resection was defined as the complete removal of all the macroscopically evident tumors. All the patients were postoperatively followed at regular intervals of 3-4 months to check for postoperative recurrences. The Institutional Review Board of Osaka University Hospital approved the study protocol (IRB #09142) and a signed consent form was obtained from each patient.

Drugs. 5-Fluorouracil (5-FU) and cisplatin (CDDP) were kindly supplied by Kyowa Hakko Kogyo Co., Ltd., (Tokyo, Japan) and Pfizer Japan, Inc. (Tokyo, Japan), respectively.

BM samples. BM was aspirated under general anesthesia immediately before surgery, as previously described (17). The BM aspirate was obtained from the ilium of each patient using a BM aspiration needle. The mononuclear cell fraction was isolated and RNA was extracted from the BM sample.

Magnetic-activated cell sorting (MACS). Cell sorting was performed as previously described (16,18). Briefly, the mononuclear cell fraction was isolated using density-gradient centrifugation. Each fraction was separated by MACS. First, cluster of differentiation 14 (CD14)-allophycocyanin and CD45-fluorescein isothiocyanate antibodies (Miltenyi Biotec, Auburn, CA, USA) were applied to detect human monocytes and macrophages, respectively, and the sorted cells were

removed as host-derived cells. Subsequently, using the epithelial cell adhesion molecule (EpCAM)-phycoerythrin antibody (Miltenyi Biotec), EpCAM-positive cells were sorted from the remaining fraction and used as viable epithelial tumor cells.

Transfection. The oligonucleotide hsa-miR-615-3p (pre-miR-615-3p) and the antisense oligonucleotide inhibitor of hsa-miR-615-3p (anti-miR-615-3p) were purchased from Ambion Inc., (Austin, TX, USA). Pre/anti-miR-615-3p were transfected using Lipofectamine[®] RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Each scrambled oligonucleotide was transfected in the same way as a matched negative control.

RNA extraction. Total RNA was isolated from cell lines and BM samples with Trizol reagent (Invitrogen) as previously described (19). RNA quality was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 and 280 nm wavelengths.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for miRNA expression. RT was performed with the TaqMan MicroRNA RT kit (Applied Biosystems, Foster City, CA, USA) and real-time RT-qPCR was performed with TaqMan MicroRNA assays (Applied Biosystems) using the ABI7900HT system (Applied Biosystems). The expression of the target miRNA was normalized relative to that of the endogenous control, RNU48. Data were analyzed according to the comparative Ct method (20).

RT-qPCR for mRNA expression. RT was performed as previously described (21). Real-time RT-qPCR was performed using designed oligonucleotide primers and the LightCycler 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). To detect the amplification products, the LightCycler-DNA master SYBR-green I (Roche Diagnostics) was used as described previously and the amount of target gene expression was calculated (22). The expression of the target gene was normalized relative to the expression of

GAPDH, which was used as an endogenous control. The designed PCR primers were as follows: E-cadherin forward, 5'-TGCCCAGAAAATGAAAAAGG-3'; and reverse primer, 5'-GTGTATGTGGCAATGCGTTC-3'; N-cadherin forward, 5'-TGAAACGCCGGATAAAGAACG-3'; and reverse primer, 5'-TGCTGCAGCTGGCTCAAGTCAT-3'; vimentin forward, 5'-TCTGGATTCACTCCCTCTGG-3'; and reverse primer, 5'-TGCACTGAGTGTGTGCAATTT-3'; fibronectin forward, 5'-CAGTGGGAGACCTCGAGAAG-3'; and reverse primer, 5'-TCCCTCGGAACATCAGAAAC-3'; and *GAPDH* forward, 5'-GTCCGAGTCAACGGATTTGGT-3'; and reverse primer, 5'-GCCATGGGTGGAATCATATTGG-3'.

miRNA microarray analysis. Extracted total RNA was labeled with Hy5 using the miRCURY LNA Array miR labeling kit (Exiqon, Vedbæk, Denmark). The labeled RNAs were hybridized onto 3D-Gene Human microRNA Oligo chips containing 941 antisense probes printed in duplicate spots (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase microRNA database (<http://www.mirbase.org/>). Following stringent washes, fluorescent signals were scanned with the ScanArray Express Scanner (PerkinElmer, Waltham, MA, USA) and analyzed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA, USA). The raw data for each spot were normalized by substitution with a mean intensity of the background signal, which was determined by the signal intensities of all the blank spots with 95% confidence intervals. Measurements of the duplicate spots with signal intensities greater than two standard deviation (SD) of the background signal intensity were considered valid. The relative expression level of miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments following normalization by their equivalently adjusted median values.

Cell proliferation assay. Cells were uniformly seeded (3×10^3 /well) in triplicate into 96-well dishes (day 0). Cells were counted using the Cell Counting kit-8 (Dojindo, Kumamoto, Japan) on days 1-3. Plate absorbance was measured in a microplate reader at 450 nm.

Growth inhibition assay. The growth inhibition assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Co., St. Louis, MO, USA) assay, as described previously (23). Briefly, cells were incubated for 72 h in several concentrations of 5-FU and CDDP. After re-incubation for 4 h with MTT solution, an acid-isopropanol mixture was added to dissolve the resulting formazan crystals. Plate absorbance was measured in a microplate reader at a wavelength of 550 nm with a 650 nm reference and the results were expressed as a percentage of absorbance relative to that of the untreated controls.

Invasion assay. The invasion assay was performed using transwell culture chambers (BD Biosciences, Bedford, MA, USA) according to the manufacturer's instructions. The upper chamber was loaded with cell suspension and the lower chamber was loaded with 10% FBS. After a 24-h incubation, cells that had invaded the undersurface of the membrane were

counted under a microscope. Three microscopic fields were randomly selected for cell counting.

Statistical analysis. Data are presented as mean \pm SD. Between-group differences were assessed using the χ^2 test and continuous variables were compared using Student's t-test. Statistical analysis was performed using JMP software version 9.0.2 (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-615-3p expression is higher in the recurrence group compared to the non-recurrence group. Previously, we reported that postoperative HCC metastasis is represented by recurrence within a 2-year postoperative period (24). During 2 years of observation after surgery, postoperative HCC recurrence was identified in four of nine patients (the recurrence group); the five patients without recurrence were termed the non-recurrence group. To investigate the miRNA expression profiles in DTCs and their regulation of postoperative HCC recurrence, the miRNA from DTCs isolated preoperatively from the BM of the recurrence group was compared against the non-recurrence group. Cluster analysis revealed distinct miRNA expression profiles between the two groups; the expression levels of three out of 941 miRNAs (0.3%) exhibited a mean change of >1.5 -fold in the recurrence group compared to the non-recurrence group (Fig. 1). When including adequate expression quantities and excluding miRNA*s, *miR-615-3p* was identified as a candidate miRNA in DTCs regulating HCC recurrence. Subsequently, further *in vitro* investigations were performed focusing on the effect of *miR-615-3p* on malignant characteristics of HCC cells.

miR-615-3p alters proliferative activity. To evaluate the effect of *miR-615-3p* on cell proliferation, pre-*miR-615-3p* and anti-*miR-615-3p* were transfected into PLC/PRF/5 and HuH7 cells, which exhibited the lowest and highest expression of *miR-615-3p* among the four HCC cell lines, respectively (Fig. 2A). RT-qPCR confirmed that *miR-615-3p* expression was significantly increased and decreased in cells transfected with pre-*miR-615-3p* and anti-*miR-615-3p*, respectively. The cell proliferation assay demonstrated that PLC/PRF/5 and HuH7 cells overexpressing *miR-615-3p* were significantly more proliferative compared to the controls and that *miR-615-3p*-suppressed cells exhibited decreased cell proliferation compared to the controls (Fig. 2B and C).

miR-615-3p induces resistance to chemotherapeutic agents. The growth inhibition assay was also used to examine the resistance to chemotherapeutic agents, such as 5-FU and CDDP. PLC/PRF/5 cells transfected with pre-*miR-615-3p* and anti-*miR-615-3p* were significantly more and less resistant to the two drugs, respectively, compared to the control cells (Fig. 3A and B). HuH7 cells exhibited a pattern similar to that of PLC/PRF/5 cells, although *miR-615-3p*-overexpressing HuH7 cells did not show more resistance compared to the control cells (Fig. 3C and D).

Expression level of miR-615-3p is associated with epithelial-mesenchymal transition (EMT). The EMT, a highly

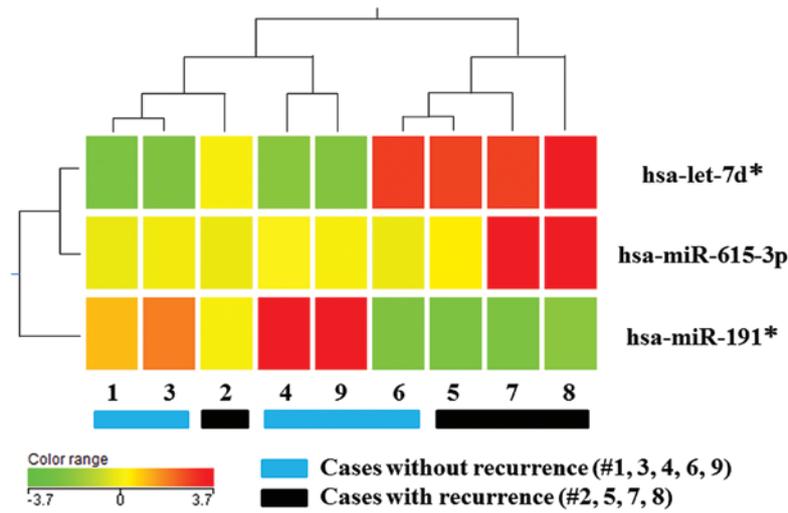


Figure 1. Heatmap of the microRNA (miRNA) profile in disseminated tumor cells from hepatocellular carcinoma patients with and without recurrence. The miRNA levels were determined using miRNA microarray analysis. Cluster analysis showed three miRNAs (*hsa-let-7d**, *hsa-miR-615-3p* and *hsa-miR-191**) that were significantly differentially expressed between the two groups, with a >1.5-fold change. The heatmap summarizes the expression levels of these three miRNAs, which range from low (green) to high (red). Patients 2, 5, 7 and 8 had recurrence and patients 1, 3, 4, 6 and 9 had no recurrence.

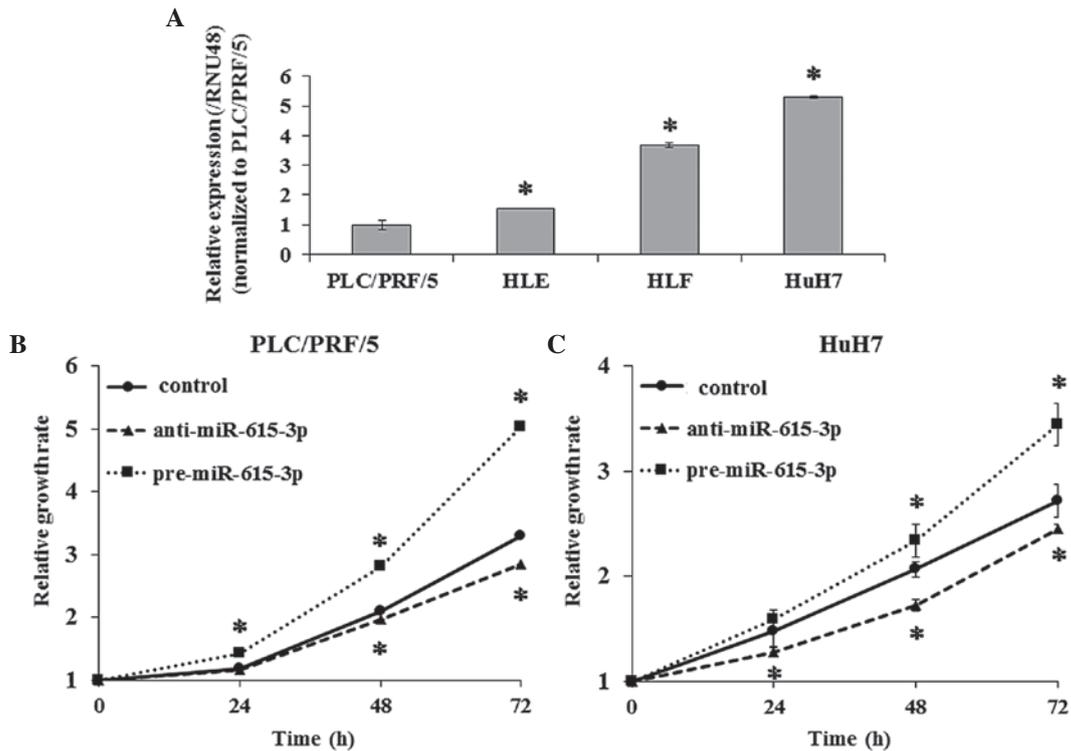


Figure 2. Expression level of *miR-615-3p* in hepatocellular carcinoma (HCC) cell lines and its effect on cell proliferation. The expression level of *miR-615-3p* was examined by reverse transcription-quantitative polymerase chain reaction in four HCC cell lines: PLC/PRF/5, HLE, HLF and HuH7. (A) PLC/PRF/5 and HuH7 exhibited the lowest and highest expression levels of *miR-615-3p* among the four cell lines, respectively. The effect of *miR-615-3p* on cell proliferation was investigated using a cell proliferation assay in (B) PLC/PRF/5 and (C) HuH7 cells. Cell proliferative capacity is enhanced by transfection with pre-*miR-615-3p* and suppressed by transfection with anti-*miR-615-3p*. Experiments were performed in triplicate and data are expressed as the mean \pm standard deviation. * $P < 0.05$ compared to (A) PLC/PRF/5 and (B and C) each control.

conserved developmental program activated during mesoderm formation and neural crest development, is indicated in promoting the dissemination of malignant cells from primary tumors (25). In this context, whether changing the *miR-615-3p* expression affects EMT in HCC cells was investigated. PLC/PRF/5 cells were transfected with pre-*miR-615-3p* and

HuH7 cells with anti-*miR-615-3p*. Control PLC/PRF/5 cells exhibited a well-defined epithelial polygonal shape, while pre-*miR-615-3p*-transfected PLC/PRF/5 cells exhibited a clear morphological change characterized by a spindle-like fibroblastoid appearance (Fig. 4A). Consistent with this finding, a significantly greater expression of N-cadherin, fibronectin and

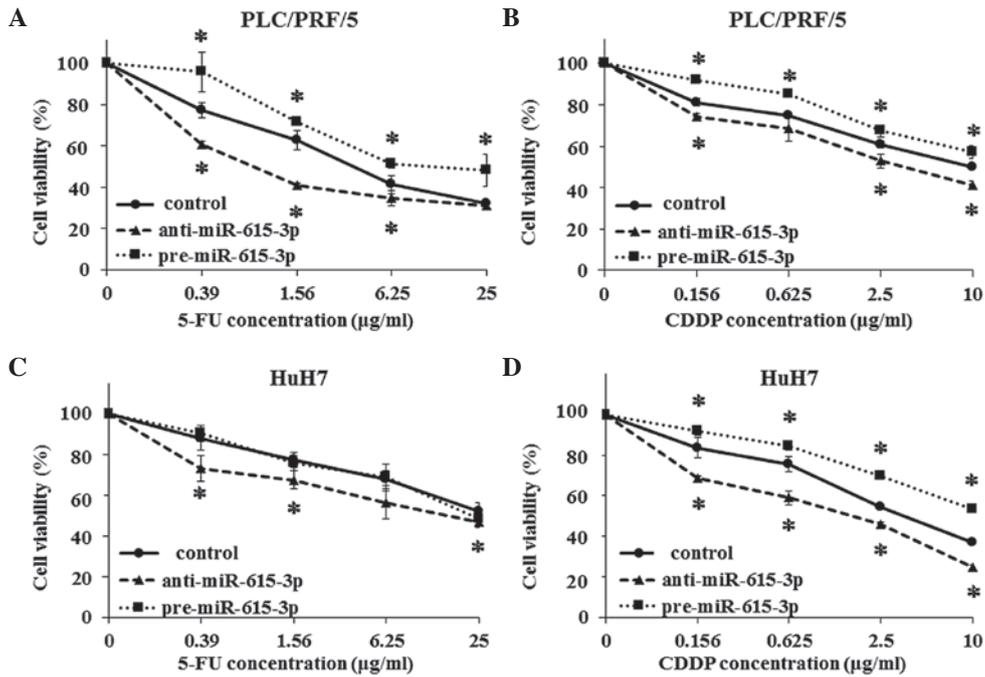


Figure 3. Evaluation of hepatocellular carcinoma cell resistance to chemotherapeutic agents following modulation of *miR-615-3p* expression. Resistance to (A and C) 5-fluorouracil (5-FU) and (B and D) cisplatin (CDDP) was investigated by the growth inhibition assay in (A and B) PLC/PRF/5 and (C and D) HuH7 cells following transfection with pre-*miR-615-3p* and anti-*miR-615-3p*. Resistance of PLC/PRF/5 cells to 5-FU and CDDP was enhanced by transfection with pre-*miR-615-3p* and suppressed by transfection with anti-*miR-615-3p*. In HuH7 cells, pre-*miR-615-3p* transfection induced resistance to CDDP only, while anti-*miR-615-3p* transfection attenuated resistance to the two drugs. Experiments were performed in triplicate and data are expressed as the mean \pm standard deviation. * $P < 0.05$ compared to each control.

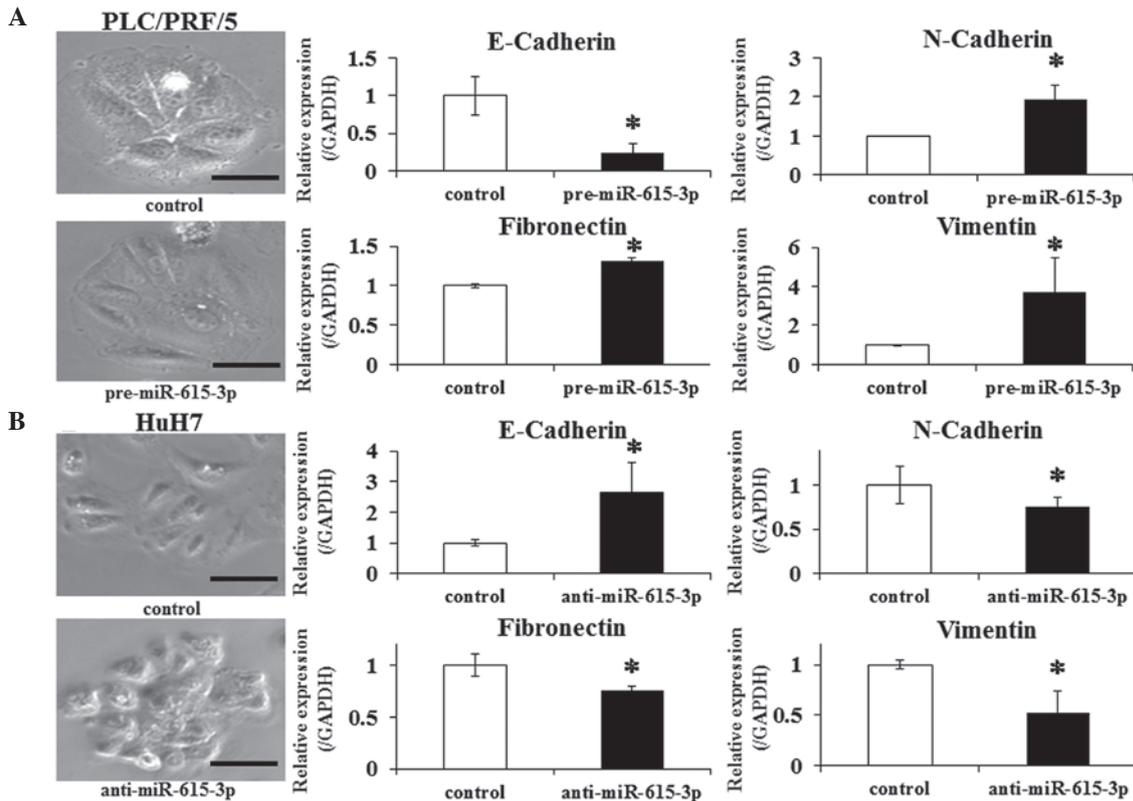


Figure 4. Effect of *miR-615-3p* on epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma cells. Morphological findings were observed in (A) PLC/PRF/5 following pre-*miR-615-3p* transfection and (B) HuH7 following anti-*miR-615-3p* transfection. Expression levels of genes associated with EMT (such as E-cadherin, N-cadherin, fibronectin and vimentin) were also examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in (A) PLC/PRF/5 following pre-*miR-615-3p* transfection and in (B) HuH7 following anti-*miR-615-3p* transfection. The images (left) are representative photographs of the morphological appearance and the bar charts (right) show comparisons of the expression levels of the EMT-associated genes examined by RT-qPCR. Experiments were performed in triplicate and data are expressed as the mean \pm standard deviation. * $P < 0.05$ compared to each control. Scale bar, 50 μm .

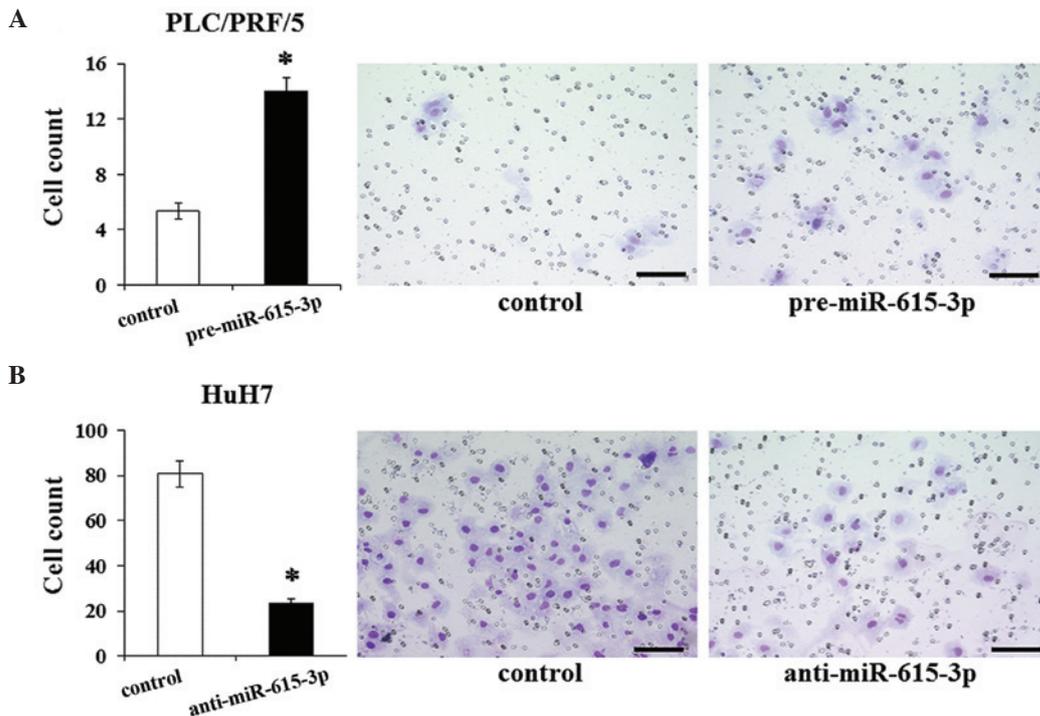


Figure 5. Evaluation of the invasive capacity in hepatocellular carcinoma cells following modulation of the *miR-615-3p* expression. Invasive capacity was investigated by the invasion assay in (A) PLC/PRF/5 cells following pre-*miR-615-3p* transfection and in (B) HuH7 cells following anti-*miR-615-3p* transfection. The images (right) show representative photographs of each sample and the bar charts (left) depict the numbers of invaded cells following modulation of the *miR-615-3p* expression level compared to the controls. Experiments were performed in triplicate and data are expressed as the mean \pm standard deviation. * $P < 0.05$ compared to each control. Scale bar, 100 μm .

vimentin, and lower expression of E-cadherin was observed in the transfected cells compared to the control cells (Fig. 4B). The changes of the morphological appearance and expression of the above genes were also examined in HuH7 cells transfected with anti-*miR-615-3p*. The transfected HuH7 cells exhibited a pattern of the changes that was opposite to the pattern observed in PLC/PRF/5 cells (Fig. 4B). These observations indicated that the *miR-615-3p*-overexpressing cells promote EMT-like phenotypes in HCC cell lines.

miR-615-3p promotes invasive capacity. The association of *miR-615-3p* with EMT led us to consider the effect of *miR-615-3p* on the invasive capacity of cells. The invasive capacity was examined in HCC cells following the modulation of their *miR-615-3p* expression level. The invasion assay demonstrated that the invasive capacity of PLC/PRF/5 cells transfected with pre-*miR-615-3p* is significantly greater than that of the control cells (Fig. 5A). The *miR-615-3p*-suppressed HuH7 cells exhibited a significantly less invasive capacity compared to the control cells (Fig. 5B). These results suggested that *miR-615-3p* influences the invasive capacity, which was consistent with the results of the EMT features induced by *miR-615-3p*.

Discussion

In the present study, microarray data were used to compare miRNA expression profiles of DTCs in BM collected preoperatively from HCC patients with and without postoperative recurrence. DTCs of the HCC patients with recurrence

exhibited distinctly different miRNA expression profiles compared to the patients without recurrence. In particular, *miR-615-3p* expression was significantly increased in the BM of patients with recurrence compared to patients without recurrence. Additional *in vitro* experiments revealed that the expression level of *miR-615-3p* is significantly associated with the malignant characteristics of HCC cells, such as proliferative capacity, chemotherapeutic resistance and acquisition of EMT-like features. These results suggested the possibility that DTCs from different individuals may have varying characteristics that affect their capacity to develop recurrence/metastasis, which supported the concept that characteristics of existing DTCs, rather than the presence or absence of DTCs, may determine cancer recurrence. Notably, *miR-615-3p* induced chemoresistance and acquisition of EMT, which are representative phenotypes of cancer stem cells (CSCs) (26,27). This finding indicates that DTCs from patients with recurrence may have features of CSCs, suggesting consistency with previous studies that CSC features are frequently observed in isolated tumor cells in cancer patients with metastatic lesions (28,29).

Previously, we investigated the prognostic impact of α -fetoprotein (*AFP*) mRNA in BM samples from HCC patients and identified that survival rates did not differ significantly between patients with *AFP* mRNA-positive BM versus *AFP* mRNA-negative BM (17). However, during the same period, another study demonstrated that *AFP* mRNA expression in the BM of HCC patients is of significant prognostic value (30). The controversy appears to be unexpected when considering the possibility that characteristics of DTCs play a role in postoperative tumor recurrence. These previous studies subjected

the entire mononuclear cell fraction to analysis; by contrast, the present study analyzed a sorted fraction of viable epithelial tumor cells. Considering the range of cell characteristics among fractions, differences in the fraction analyzed may be a source of the controversy regarding the prognostic significance of *AFP* mRNA in the whole mononuclear cell fraction of BM.

miR-615-3p, a member of the *miR-615* family, reportedly promotes the phagocytic capacity of splenic macrophages through upregulation of peroxisome proliferator-activated receptor γ (31). Regarding the correlation of *miR-615-3p* with malignant diseases, there has been only one study, which reported that *miR-615-3p* is associated with survival in mantle cell lymphoma (32). However, the mechanism by which *miR-615-3p* influences the prognosis has not been investigated. To validate the results of the present study, it is also necessary to investigate whether modulating the level of *miR-615-3p* expression in an *in vivo* HCC model would cause a change in metastatic capacity. In this context, the present study will help clarify the function of *miR-615-3p* in malignant disease. In addition, considering that no studies have focused on the target genes of *miR-615-3p*, identifying which of its target genes regulate cancer recurrence is required.

The results of the present study potentially lead to several clinical applications in the field of HCC. One is the development of a novel clinical model for predicting the postoperative prognosis in HCC patients by examining the *miR-615-3p* expression level in DTCs in BM. When considering the sample origin for this potential prediction model, peripheral blood may also be used, as circulating tumor cells (CTCs) may be an alternative to DTCs. CTCs are also reportedly associated with hematogenous dissemination and poor prognosis in cancer patients (5,33). When the upregulation of *miR-615-3p* is confirmed in the DTCs and the CTCs of HCC patients with recurrence, prognosis may be more easily predicted by analyzing CTCs instead of DTCs in BM. However, several studies comparing the detection rate of the tumor cells (and their prognostic significance) between DTCs and CTCs have reported that the two cell populations do not always correlate and further investigation is required before CTCs are analyzed as a prognostic alternative to DTCs (34,35). Another possible application is a potential miRNA-targeted therapeutic option against HCC. New therapeutic options targeting specific miRNAs have recently been developed (36,37). The results of the present study may lead to a novel, effective therapeutic option that targets *miR-615-3p* against HCC recurrence; inhibition of *miR-615-3p* may lead to the prevention of recurrence in HCC patients by suppressing DTCs/CTCs with high malignant potential. However, several challenges must be overcome in order to realize such a new therapy, including the drug delivery system, off-target effects and possible toxicities (38).

In conclusion, the results of the present study demonstrated that DTCs in the BM of HCC patients with and without postoperative recurrence exhibit distinct miRNA expression profiles. This finding suggests that the characteristics of DTCs defined by miRNA expression profiles may act in postoperative tumor recurrence. Furthermore, among the miRNAs with altered expression, *miR-615-3p* was significantly upregulated in the DTCs of patients with recurrence and its expression level correlated significantly with malignant characteristics of HCC cells *in vitro*. These results may indicate that the expression

level of *miR-615-3p* in DTCs in the BM is one of the factors that determine postoperative tumor recurrence and suggest that *miR-615-3p* is a potential target molecule for regulating postoperative HCC recurrence.

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