Human Cancer Biology

Clinical Cancer Research

MiR-222 Overexpression Confers Cell Migratory Advantages in Hepatocellular Carcinoma through Enhancing AKT Signaling

Queenie W-L. Wong[1,2], Arthur K-K. Ching[1,2], Anthony W-H. Chan[1], Kwong-Wai Choy[3], Ka-Fai To[1,2], Paul B-S. Lai[4], and Nathalie Wong[1,2]

Abstract

Purpose: This study aims to profile the expressions of 156 microRNAs (miRNA) in hepatocellular carcinoma (HCC) and to characterize the functions of miR-222, the most significantly upregulated candidate identified.

Experimental Design: miRNA expression profile in HCC tumors, matching adjacent cirrhotic livers, and cell lines was conducted using quantitative PCR. Common miR-222 upregulations were further validated in a larger cohort of tumors. The functional effects of miR-222 inhibition on HCC cell lines were examined. The downstream modulated pathways and target of miR-222 were investigated by coupling gene expression profiling and pathway analysis, and by *in silico* prediction, respectively. Luciferase reporter assay was done to confirm target interaction.

Results: We identified a 40-miRNA signature that could discriminate tumors from adjacent cirrhotic liver tissue, and further corroborated common miR-222 overexpression in tumors relative to its premalignant counterpart (55.3%; $P < 0.0001$). Increased miR-222 expression correlated significantly with advanced stage HCC and with the shorter disease-free survival of patients ($P \leq 0.01$). Inhibition of miR-222 in Hep3B and HKCI-9 significantly retarded cell motility ($P < 0.05$). Further investigations suggested that AKT signaling was the major pathway influenced by miR-222. A consistent reduction of AKT phosphorylation in Hep3B and HKCI-9 was shown following miR-222 suppression. The protein phosphatase 2A subunit B (PPP2R2A) was predicted as a putative miR-222 target *in silico*. We found that miR-222 inhibition could augment the tumor protein level and restore luciferase activity in reporter construct containing the PPP2R2A 3′ untranslated region ($P = 0.0066$).

Conclusions: Our study showed that miR-222 overexpression is common in HCC and could confer metastatic potentials in HCC cells, possibly through activating AKT signaling. *Clin Cancer Res; 16(3); 867–75.* ©*2010 AACR.*

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related mortality. Over 560,000 new cases are diagnosed annually, and a roughly similar incidence of deaths has been reported yearly (1). The overall poor survival of HCC patients is primarily attributed to the late disease presentation, which rules out curative surgery for the majority of patients (2). The prognosis for the 10% to 20% of patients eligible for surgical resection remains dismal, as there is a high incidence of postoperative recurrences (3). The generally poor clinical outcome of individuals diagnosed with HCC underscores the importance in obtaining a better understanding of the transcriptional activation of oncogenic signaling pathways, and the control of cancer-associated genes.

MicroRNAs (miRNA) belong to a class of endogenously expressed small noncoding RNAs (19-25 nucleotides in length) that regulate gene expression by either directing mRNA degradation or repressing posttranscriptional protein translation through binding to the 3′ untranslated region (UTR) of targeted gene transcript (4). Increasing evidence suggests that miRNAs can mediate gene expression in a broad spectrum of regulatory pathways and are therefore believed to play essential roles in a range of biological processes. Although the precise function of many of the predicted ~800 human miRNAs is still undefined, several cellular miRNAs have already been

Authors' Affiliations: [1]Department of Anatomical and Cellular Pathology, [2]State Key Laboratory in Oncology in South China, Departments of [3]Obstetrics and Gynaecology, and [4]Surgery, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Nathalie Wong, Department of Anatomical and Cellular Pathology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong, China. Phone: 852-2632-1128; Fax: 852-2637-6274; E-mail: natwong@cuhk.edu.hk.

doi: 10.1158/1078-0432.CCR-09-1840

Translational Relevance

Hepatocellular carcinoma (HCC) is a highly malignant tumor that leads rapidly to death. Recent advances in cancer biology have implicated changes in the endogenous expression of small microRNAs (miRNA) as one of the most important controlling mechanisms in the expression of causative tumor-related genes in human cancers. Nevertheless, our knowledge of miRNA alterations in human HCC is limited. This study defined a distinct 40-miRNA signature in discriminating HCC tumors from adjacent nonmalignant cirrhotic liver tissue. The most significant event observed was the upregulation of miR-222. Increased miR-222 expression correlated with advanced stage HCC tumors and shorter disease-free survival of patients. The functional relevance of miR-222 was found in promoting HCC cell motility through enhancing AKT signaling, and target inhibition on protein phosphatase, PPP2R2A. The elucidation of deregulated miRNAs in HCC is expected to enhance the understanding on the tumor biology and serve as the basis for the further development of biomarkers in disease prognosis and therapies.

established as crucial regulators of cell growth, differentiation, and apoptosis by their control of critical tumor suppressors and oncogenes, for example the *RAS* by Let-7, *BCL2* by miR-15a and miR-16-1, *PTEN* by miR-21, and *E2F1* by miR-17~miR-92 (5–8). Because a growing number of cancer-associated genes are now believed to be regulated by miRNAs, it is not surprising that miRNAs have also been implicated in the pathogenesis of cancer.

In this study, we profiled the HCC genome for deregulated miRNAs expression by quantitative PCR (qPCR) analysis. We identified the altered expressions of a distinct subset of miRNAs that underscored HCC development from the nonmalignant precursor of liver cirrhosis. The presence of cirrhosis was defined as the diffuse nodulation of liver composed of regenerating hepatocellular nodules surrounding the fibrous septa. Histologically, Ishak fibrosis score of 5 was used as a cutoff value for defining cirrhosis. Within the nontumorous liver parenchyma, an increase in fibrous tissue alone was not classified as cirrhosis but was considered as chronic hepatitis. Profound miR-222 overexpression was confirmed in a larger series of primary HCC tumors compared with nontumoral livers, and a strong relationship was established between the high expression of miR-222 with tumor progression and patient survival. In line with the associated clinicopathologic features, we determined a functional role for miR-222 in the cell migratory and the invasive advantages of HCC cells. Downstream investigation by gene expression profiling of miR-222 knockdown cells highlighted AKT signaling as the major pathway influenced by miR-222. Reduced AKT phosphorylation was verified by Western blot, which led us to further explore for protein phosphatases with complementary 3′UTR sequences to miR-222. By computational algorithm, the *PPP2R2A* gene, a component of the protein phosphatase 2A (PP2A), was predicted as a cellular target. We were able to corroborate the target association of miR-222 on the PP2A regulatory subunit B α isoform PPP2R2A by reporter assay and Western blot analysis. Our findings in this study highlight a new pathway for the regulation of AKT signaling in HCC.

Materials and Methods

Profiling of 156 human miRNAs (Applied Biosystems) was done on 20 HCC, 12 matching cirrhotic livers adjacent to the tumor (TN), and three cell lines (Hep3B, HKCI-4, and HKCI-9; ref. 9) by qPCR. Information on patient demographics is shown in Supplementary Table S1. The expression of miR-222 was assessed in a cohort of 99 HCC and 94 TN by Taqman miRNA (Applied Biosystems). Tumorous HCC and adjacent TN tissues were collected from patients who underwent curative surgery at the Prince of Wales Hospital, Hong Kong between 1998 and 2005. Consecutive patient cases with surplus tissues were retrieved from the tissue bank [Chinese University of Hong Kong-New Territories East Cluster (CUHK-NTEC) Clinical Research Ethics Committee ref. CRE-2008.309]. From the cohort of HCC patients studied, three patients received neoadjuvant therapy before curative surgery. One patient was given selective internal radiation therapy using ^{90}yttrium microspheres and two patients were treated with cisplatin/IFN α-2b/Adriamycin/fluorouracil combinatory chemotherapy. All samples were preserved by slow freezing in a block of Tissue-Tek O.C.T (Sakura Finetek) upon collection from the operation theatre. Three normal human liver controls acquired commercially were also included in our profiling study (Ambion, Clontech Laboratory, and Stratagene). Details of the methods used in the functional studies, immunoblottings, oligonucleotide expression array and pathway analysis, target prediction and luciferase reporter, and statistics are described in the supplementary document.

Results

Distinct miRNA pattern discriminates HCC from putative premalignant cirrhotic liver. Profiling of 156 miRNAs suggested the deregulation of 97 miRNAs in HCC tumors and cell lines compared with normal liver controls. Frequent incidences of upregulation of miRNAs, including miR-99a, miR-184, miR-224, and miR-222, were detected, whereas common downregulation was suggested for miR-325, miR-199a*, miR-199a, and miR-223 (Supplementary Fig. S1). To further define the miRNAs involved in HCC development, informatics analysis was deployed to determine and prioritize candidate miRNAs. Using a

Wilcoxon test and limiting the false-positive discovery rate to 0% in significance analysis of microarrays (SAM) analysis, a distinct 40-miRNA expression pattern was defined in HCC tumors compared with nontumoral cirrhotic liver tissue (Fig. 1). Significant deregulation of 23 overexpressed and 17 repressed miRNAs was suggested (Table 1A and B). The 40-miRNA signature also ensured discriminatory clustering of HCC tumors from liver cirrhosis (Fig. 1). Upregulated miR-222 was the most significant event observed among the 40 candidates concerned.

MiR-222 expression in HCC tumors. To further verify the importance of miR-222 in HCC, qPCR analysis was carried out to determine the miR-222 expressions in a cohort of 99 primary HCC tumors and 94 TN (Table 2). Compared with normal livers, increased expressions of miR-222 were detected in as much as 98% of HCC (97 of 99 cases), in which a median 20.16-fold (quartiles, 6.93-125.00) was suggested. Elevated miR-222 expression was also observed in 86% of TN, with a median 4.98-fold (2.69-31.30). Raised expressions of miR-222 were similar in TN arising from a cirrhotic background and chronic hepatitis (P = 0.46). Overall, miR-222 seemed to be upregulated in 55% of HCC (52 of 94 pairs) with increased in expression ranging from 9.21-fold to >100-fold relative to adjacent TN ($P < 0.0001$; Fig. 2Ai).

In correlative analyses with clinicopathologic features of tumors, we found a significant increase of miR-222 expressions in advanced stage HCC (P = 0.014; Fig. 2Aii). In early T_1 tumors (n = 65), miR-222 expressions were

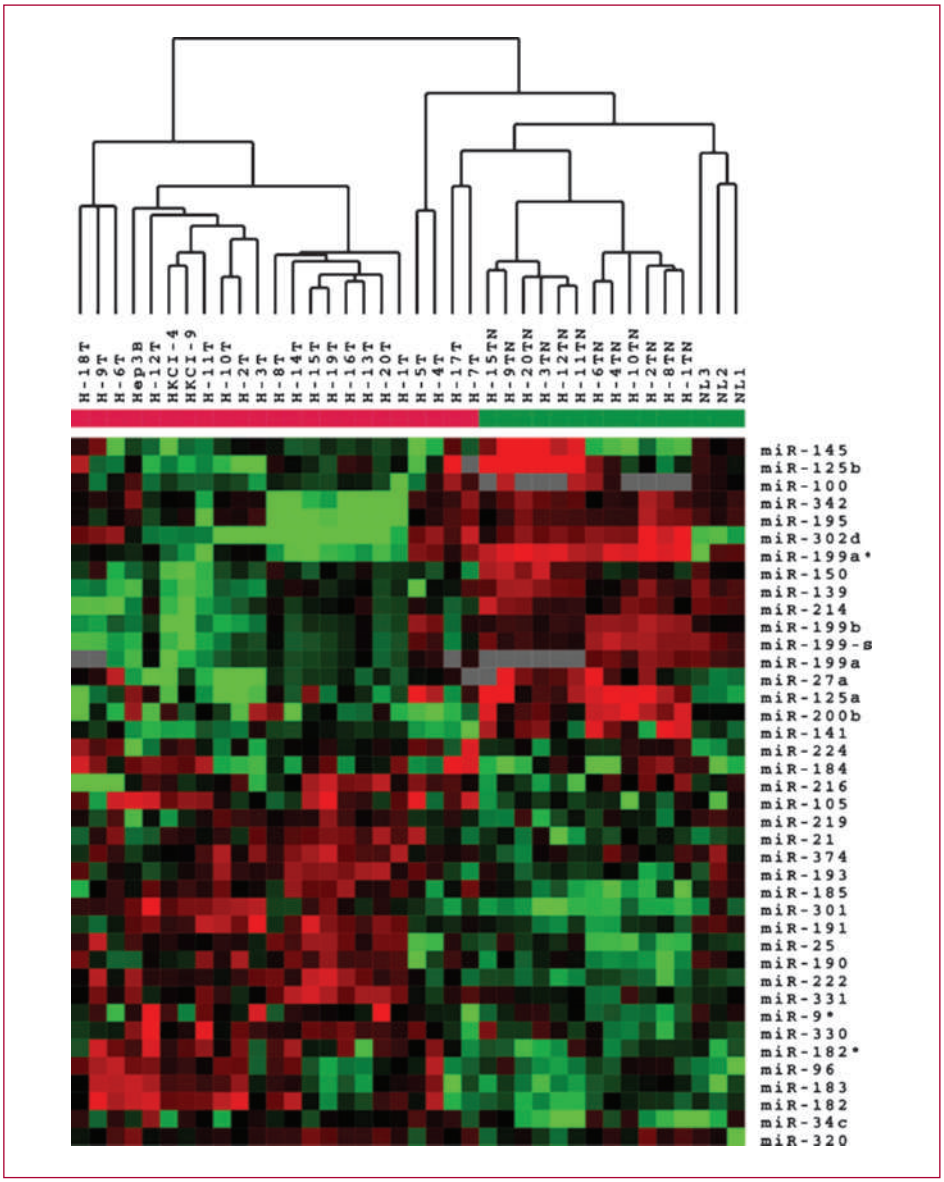

Fig. 1. Hierarchical clustering was done on 40 significant miRNAs derived from the SAM analysis. Two distinct dendrograms that coincided with HCC tumors (red bar) and the surrounding nontumorous liver tissue (green bar) were observed. Red, high expression levels; green, low expression levels. Some tumors were suggested at a closer distance to the cirrhotic liver tissue. These HCC-4, HCC-5, and HCC-7 cases were in fact early-stage T_1 tumors, which may explain in part their closer relation to their premalignant counterparts.

suggested at a median 16-fold (4.14-fold to 61.18-fold), whereas significantly higher miR-222 levels at a median of 35.52-fold (11.60-fold to 371.80-fold) were shown in advanced T_2/T_3 tumors ($n = 34$). In the Kaplan-Meier analysis, high expressions of miR-222 correlated with poorer disease-free survival of patients ($P = 0.011$; hazard ratio, 2.214; 95% confidence interval, 1.188-3.829; Fig. 2Bi) and shorter overall survival ($P = 0.003$; hazard ratio, 3.125; 95% confidence interval, 1.418-5.555; Fig. 2Bii).

Table 1. The 40 significant miRNAs correspond to 23 upregulated and 17 downregulated candidates, and the upregulation of miR-222 was the most significant event observed

Rank	miRs	SAM Score (d)
A. Upregulated genes		
1	miR-222	2.22
2	miR-301	2.20
3	miR-320	1.96
4	miR-25	1.91
5	miR-190	1.81
6	miR-219	1.79
7	miR-331	1.77
8	miR-105	1.76
9	miR-182	1.76
10	miR-9*	1.74
11	miR-330	1.67
12	miR-193	1.65
13	miR-183	1.62
14	miR-185	1.60
15	miR-191	1.60
16	miR-182*	1.60
17	miR-21	1.53
18	miR-374	1.44
19	miR-96	1.42
20	miR-216	1.42
21	miR-34c	1.30
22	miR-224	1.30
23	miR-184	1.23
B. Downregulated genes		
1	miR-199a*	–2.31
2	miR-199a	–2.10
3	miR-141	–1.76
4	miR-139	–1.64
5	miR-125b	–1.63
6	miR-302d	–1.63
7	miR-125a	–1.60
8	miR-27a	–1.58
9	miR-100	–1.53
10	miR-150	–1.51
11	miR-214	–1.51
12	miR-195	–1.49
13	miR-342	–1.48
14	miR-199b	–1.42
15	miR-200b	–1.41
16	miR-145	–1.36
17	miR-199-s	–1.34

Table 2. Demographic information of HCC cases studied for miR-222 expression

	No. of patients ($n = 99$)
Gender	
Male	80
Female	19
Median age (y; range)	57 (48.0-66.5)
Viral HBV infection*	99
Underlying liver disease	
Cirrhosis	81
Chronic hepatitis	18
Staging†	
T_1	65
T_2	17
T_3	17
Macrovascular invasion	
Presence	10
Absence	89
Median overall survival (mo; range)	33.7 (13.7-72.2)
Median disease-free survival (mo; range)	19.5 (5.5-59.2)

*Patients were seropositive for hepatitis B surface antigen (HBsAg).
†The disease stage of tumors was classified according to the American Joint Committee on Cancer tumor-node-metastasis staging criteria (Greene et al., 2002).

MiR-222 inhibition retarded HCC cell motility. Functional investigations of miR-222 by antisense experiments indicated negligible effects on cell viability in Hep3B and HKCI-9 cells ($P \geq 0.38$; Fig. 3A). However, significant inhibitions on cell migratory and invasive capacities were readily detected in anti–miR-222 transfectants. We found a considerable reduction in cell migration in Hep3B (64.5%, $P = 0.008$) and HKCI-9 (52.5%, ic>P = 0.048), and a similar decrease in cellular invasiveness in Hep3B (49.4%, $P = 0.01$) and HKCI-9 (45%, $P = 0.015$) between anti–miR-222 and mock experiments (Fig. 3B and C).

To further investigate the role of miR-222 in HCC cell motility, Phalloidin-stained actin filaments (filamentous actins) were examined in Hep3B cells following miR-222 inhibition. Confocal microscopy revealed a marked reduction of phorbol 12-myristate 13-acetate–induced spiky filopodia formation around the membrane peripherals of anti-miR-222–transfected cells (Fig. 3Dii). The percentage of cells displaying reduced filopodia reached ~40% at

24 hours and remained steady at 45% to 50% by 32 and 48 hours posttransfection ($P = 0.027$; Fig. 3Di).

AKT signaling implicated as downstream modulated pathway of miR-222. To define the potential pathways modulated by miR-222 in the control of HCC cell motility, we performed microarray analysis on anti-miR-222–transfected Hep3B and HKCI-9 cells. Compared with mock transfection, specific deregulations of 691 and 1,350 known genes were identified in Hep3B and HKCI-9, respectively, in response to miR-222 suppression. A concordant upregulation of 44 and downregulation of 99 transcripts was suggested in both cell lines. Hierarchical clustering depicted discrete cluster dendrograms of anti–miR-222 transfectants and mock controls (Fig. 4A). Pathway analysis of common differentially expressed genes by ingenuity pathway analysis further highlighted functional networks, including cell-to-cell signaling, cellular movement, and cell development modulated by miR-222 (Table 3). Cell-to-cell signaling was the most significant event observed in these biological networks, in which AKT signaling was the major pathway implicated (Table 3). This finding prompted us to further investigate the effect of miR-222 inhibition on AKT expression. Although Western blot did not suggest an effect on the total AKT protein, miR-222 inhibition resulted in a consistent reduction of active phosphorylated AKT in Hep3B and HKCI-9 (Fig. 4C).

PPP2R2A, a potential target of miR-222. Based on the effect shown on the level of active AKT, we reasoned that one of the downstream cellular targets of miR-222 was likely involved in the regulation of AKT phosphorylation. To this end, we used the TargetScan algorithm to highlight protein phosphatases with 3′UTR sequences complementary to miR-222. Predicted targets included five phosphatase genes, *PPP2R2A*, *PPP3R1*, *PPP1R15B*, *PTPRZ1*, and *PPP6C*. *PPP2R2A* was the only phosphatase among these potential candidates with a reported role in the control of AKT activity. A putative miR-222 binding site

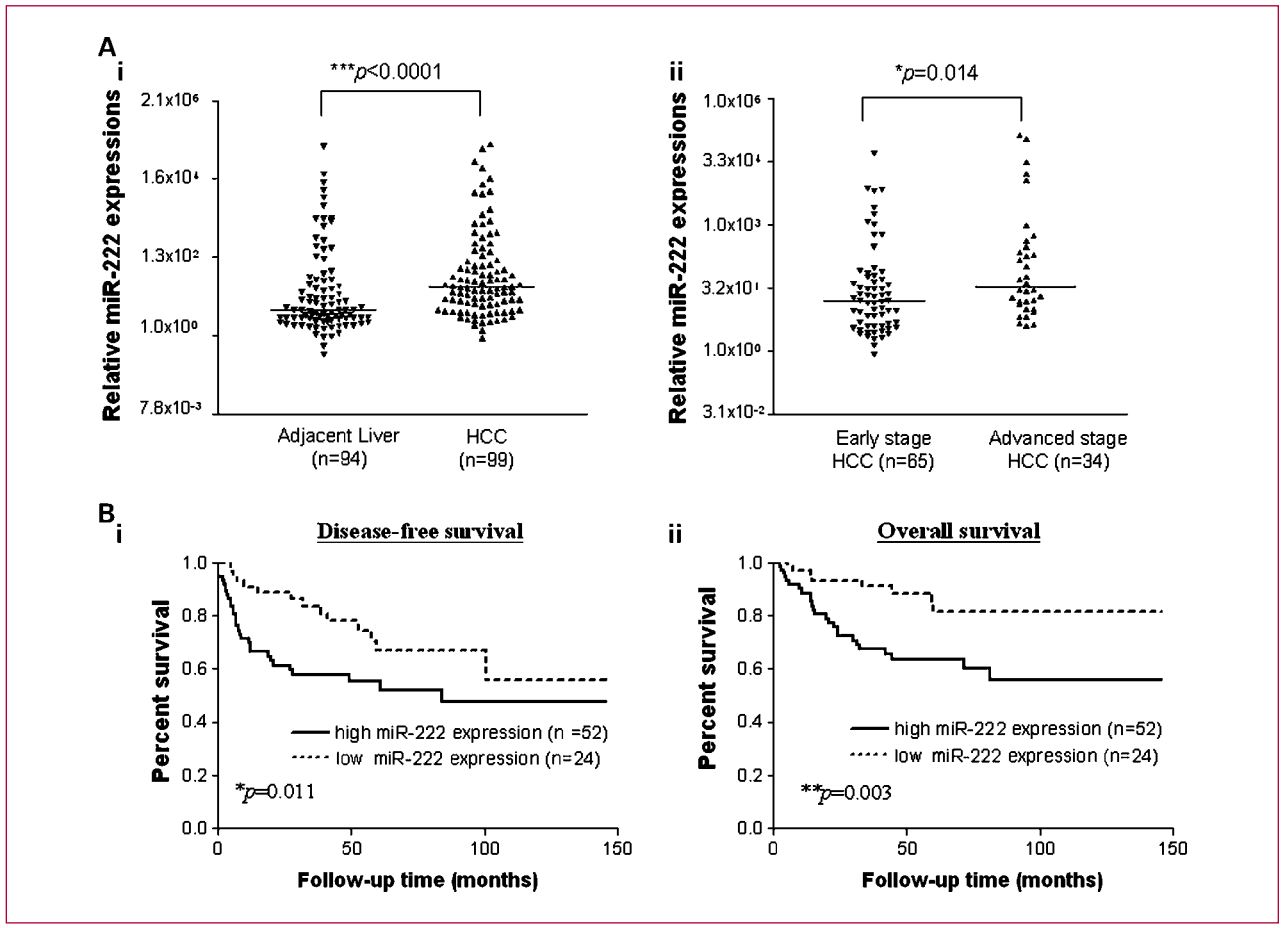

Fig. 2. Ai, a significant upregulation of miR-222 was observed in HCC tumors compared with adjacent nonmalignant liver tissue ($P < 0.0001$). Aii, a significant association of high miR-222 expressions and advanced stage HCC was suggested ($P = 0.014$). Bi and Bii, Kaplan-Meier curves of the disease-free survival and overall survival for patients depending on miR-222 expressions. A high-level expression in the tumor was scored when a gain of 2-fold or more was achieved compared with adjacent nontumoral liver, whereas 2-fold loss or more was considered low expression. The overall survival rate in patients with high miR-222 expression (solid line) was significantly lower than in those with low miR-222 expression (dotted line; $P \leq 0.011$).

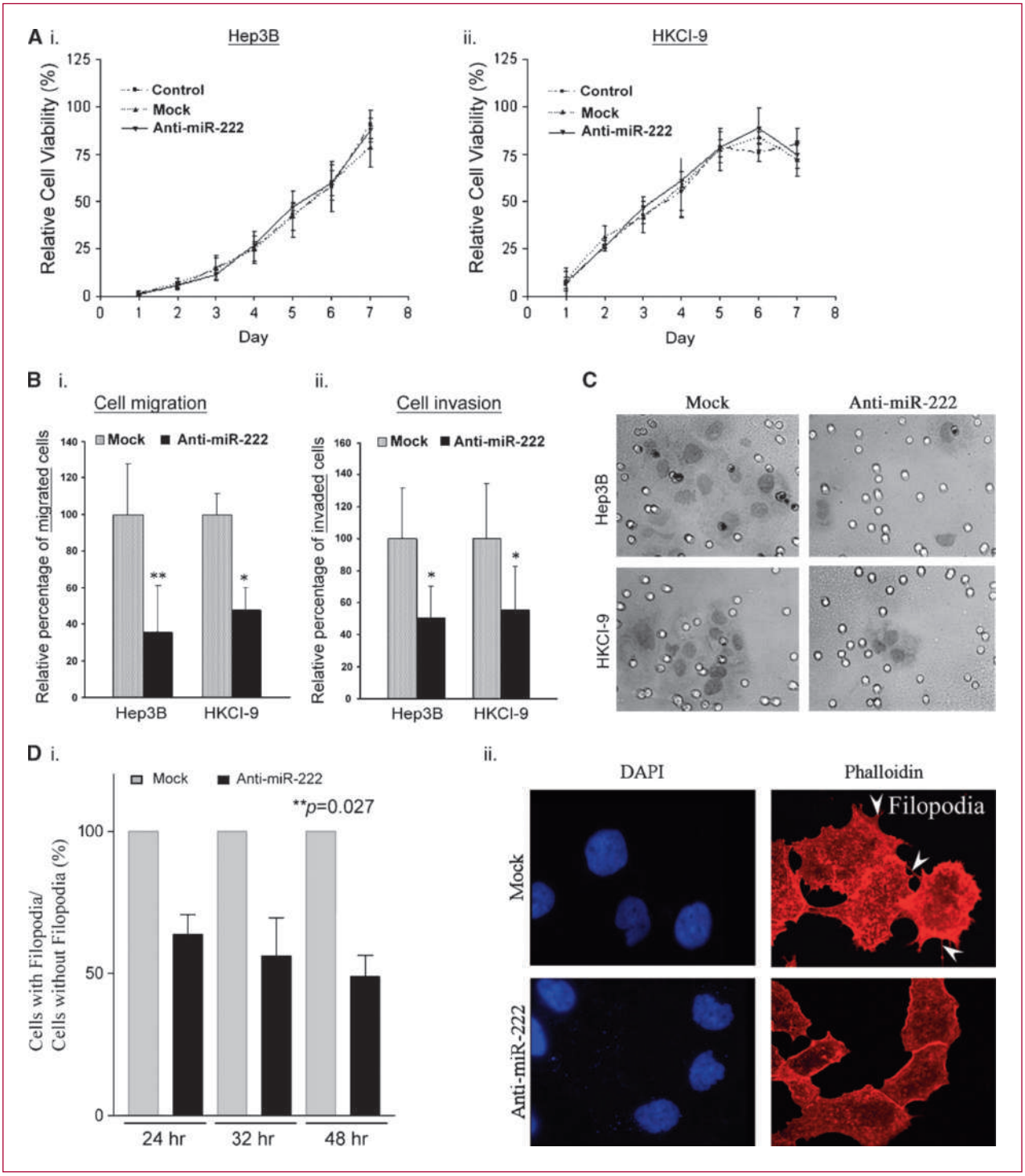

Fig. 3. Ai and Aii, negligible effects on cell viability were suggested between anti–miR-222 and mock control experiments ($P \geq 0.38$). Data from day 1 to day 7 posttransfection were shown. Bi, significant reductions in the chemotactic cell migration toward fetal bovine serum were observed in Hep3B and HKCI-9 cell lines transfected with anti–miR-222 compared with the mock control ($P \leq 0.048$). Bii, a corresponding decrease in the invasive capacity was suggested in Hep3B and HKCI-9 cell lines following miR-222 suppression ($P \leq 0.015$). Columns, mean of three individual experiments; bars, SEM. C, representative image of cells after migration through porous membrane of Transwell chamber is shown. Di, a significant reduction of filopodia was observed in anti–miR-222 transfectants compared with mock control following phorbol 12-myristate 13-acetate stimulation (2 μmol/L) at 24, 32, and 48 h posttransfection ($P = 0.027$). A profound decrease of filopodia was detected at 48 h posttransection. A total of 200 cells were scored for each condition. Columns, mean of three individual experiments; bars, SEM. Dii, representative images of Hep3B transfectants stained by TRITC-conjugated phalloidin to indicate actin filaments and counterstained by 4′,6-diamidino-2-phenylindole (DAPI) to indicate the nucleus is shown.

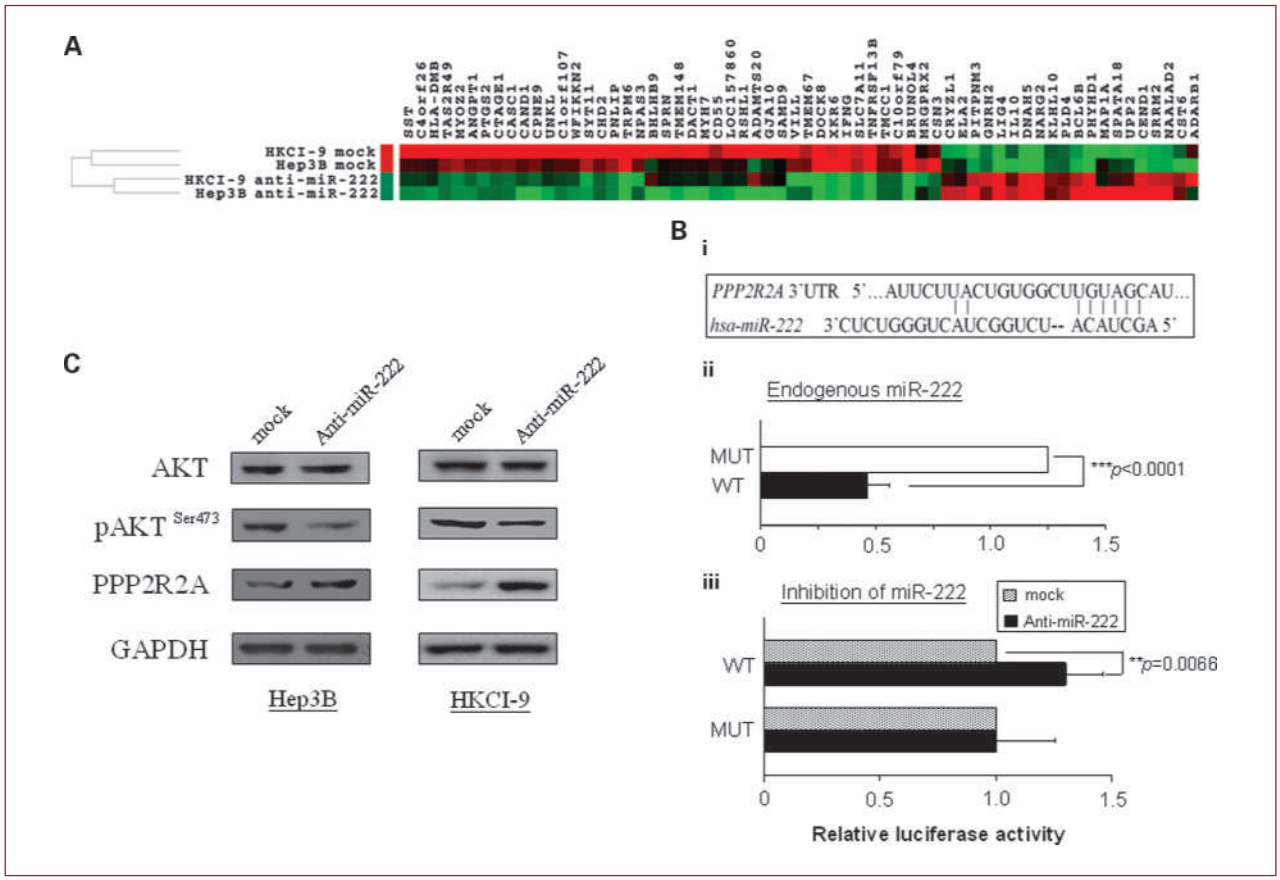

Fig. 4. A, two distinct dendrograms that represent Hep3B and HKCI-9 transfected with anti–miR-222 and mock control are shown. Red, high expression levels; green, low expression levels. Bi, base pairing complement suggested the putative miR-222 binding position at 452 to 457 of the PPP2R2A 3′UTR. The firefly luciferase activity was standardized to *Renilla* control. Bii, a strong inhibition of luciferase activity was observed in Hep3B cells transfected with WT construct ($P < 0.0001$) compared with the DEL construct; MUT, mutant. Biii, a significant increase in luciferase activity was detected in Hep3B cells transfected with anti–miR-222 compared with mock control RNA ($P = 0.0066$). Columns, mean of three individual experiments; bars, SEM. C, Western blot analysis suggested the consistent reductions of phosphorylated AKT in anti-miR-222–transfected Hep3B and HKCI-9 cells compared with mock controls, whereas total protein levels remained unchanged. An increased PPP2R2A protein level was detected in Hep3B and HKCI-9 cell lines after miR-222 inhibition.

that comprised eight matched nucleotides was predicted at the 3′UTR of PPP2R2A (Fig. 4Bi). Introduction of wild-type (WT) construct containing the WT PPP2R2A 3′UTR into Hep3B cells suggested a strong inhibition of luciferase activity compared with the mutant-deleted construct ($P < 0.0001$; Fig. 4Bii). We further validated the specific interaction between miR-222 and the putative binding site of PPP2R2A transcript in the presence of miR-222 inhibition. Transfection of anti–miR-222 into Hep3B was able to restore the luciferase activity of WT construct by 30% ($P = 0.0066$; Fig. 4Biii). By contrast, no effect on the luciferase signal was detected with the deleted construct. We were able to further substantiate the negative regulatory role of miR-222 on PPP2R2A by assessing the protein level of PPP2R2A in Hep3B and HKCI-9. Western blot analysis showed that the rescue of PPP2R2A protein expression could be consistently detected in both cell lines in response to anti–miR-222 transfection compared with the mock control (Fig. 4C).

Table 3. Pathways implicated in the miR-222 inhibition by ingenuity pathway analysis

Rank	Functional Network	Implicated Pathway
1	Cell-to-Cell Signaling	AKT signaling
2	Cellular Movement	Actin cytoskeleton
3	Cell Development	Transcription modulation

Discussion

This study reports on a qPCR-based miRNA profiling in HCC. Using informatic analysis, we were able to identify a 40-miRNA signature that underlined the hepatic transformation from liver cirrhosis. Several studies have recently reported on the miRNA profiling of liver cancer

(10–23), and several deregulated miRNAs identified in this study was common to those previously published. Examples include the upregulation of miR-301, miR-224, miR-21, miR-221, and miR-222, and the downregulation of miR-199a*, miR-199a, miR-195, miR-214, and miR-223. Ura and coworkers (22) reported on a unique 23-miRNA cluster, which could differentiate HCC tumors from noncancerous chronic hepatitis liver tissue, irrespective of HBV or HCV infection. Many of these 23 miRNAs were also found to be deregulated in the 40-miRNA pattern determined in this study, e.g., miR-222, miR-183, miR-199a*, and miR-125b. Identification of the same miRNA deregulations in different studies may further imply their importance in liver tumorigenesis.

The upregulation of miR-222 was the most significant event observed in our study. Using Northern blot analysis, previous studies both by our group and others have shown that expressions of miR-222 were often higher in HCC tumors than in the adjacent nontumoral liver tissue. The prognostic value and functional implications of miR-222 in HCC, however, remain largely undefined. Here, based on qPCR analysis on a large cohort of primary HCC tumors, we were able to establish a significant link between the level of miR-222 expression, and tumor progression and disease-free survival of patients. Consistent with our findings, miR-222 has earlier been implicated in HCC progression from an inverse correlation shown with tumor differentiation (20). Upregulations of miR-222 have been described in prostate cancer, primary glioblastoma, papillary thyroid carcinoma, and breast cancer, and a possible functional role for miR-222 in cell growth and proliferation has been proposed, through its influence on the expression of cell cycle regulatory proteins such as the cyclin-dependent kinase inhibitor p27Kip1 (24–26). In this study, inhibition of miR-222 activity did not seem to exert an effect on cell viability in Hep3B and HKCI-9. However, a substantial reduction in the invasive phenotype and chemotactic migrations was readily observed in both cell lines. Our functional investigations hence suggest an oncogenic role for miR-222 in promoting cellular invasiveness and motility in HCC cells. Phalloidin staining of filamentous actins showed a marked decrease in filopodia formation in anti-miR-222–transfected cells. This finding further affirmed an effect of miR-222 on the motile phenotype of HCC through the structural organization of filopodia at the leading edge of migrating cells.Consistent with this finding, a recent study by Felicetti et al. (27) reported that ectopic expression of miR-222 could elevate the migratory and invasive behaviors of melanoma cells.

Expression profiling of anti-miR-222–transfected Hep3B and HKCI-9 highlighted AKT signaling as a major pathway under the influence of miR-222. In HCC, phosphoinositide 3-kinase/AKT signaling is one of the more important oncogenic pathways and is frequently activated during the liver tumorigenesis (28, 29). Immunohistochemical studies have shown that the common phosphorylation status of AKT correlated with vascular invasion and intrahepatic metastasis in HCC (28, 29). It has also been suggested that AKT phosphorylation is a risk factor for early disease recurrences and poor prognosis of HCC patients (30). *In vitro* functional studies have further shown that the AKT pathway is a critical mediator in the control of HCC cell invasion and motility (31, 32). In this study, we showed that miR-222 inhibition could induce a profound reduction in AKT phosphorylation, which in turn would support the potential role of miR-222 in AKT activation. Because the evident reduction of active AKT was not accompanied by a decrease in total AKT, we reasoned that miR-222 regulated AKT activity in the process of dephosphorylation. We therefore examined several protein phosphatases for the presence of complementary 3′UTR sequences to miR-222, which led us to identify PPP2R2A as a potential downstream target.

PPP2R2A is a regulatory subunit of PP2A (33). PP2A is a well-conserved essential protein serine/threonine phosphatase in eukaryotes and is a well-recognized regulator in the control of AKT activity. Kuo et al. (14) have confirmed a direct interaction between PPP2R2A and AKT by *in vitro* pull-down assay, and have further shown that PPP2R2A is required for targeting PP2A to AKT in prolymphoid FL5.12 cells. More importantly, the ectopic expression of PPP2R2A has been shown to impair phosphorylation at the Ser-473 and Thr-308 residues of AKT (14). In a functional context, PP2A has been implicated in modulating cell motility in lung cancer cells and fibroblasts (34, 35). Here, using reporter assay and Western blot, we showed that miR-222 could target and suppress PPP2R2A protein translation. Given the reported role of PPP2R2A in AKT dephosphorylation, we propose that PPP2R2A is a likely intermediate that interplays between miR-222 and the AKT signaling path.

In conclusion, this study has highlighted a potential prognostic value of miR-222. Although increased levels of miR-222 were more profound in tumors, they could also be detected in the premalignant lesions of adjacent TN. The stepwise elevation of miR-222 expressions from TN to early HCC and late-stage tumors would imply miR-222 activation in the early transcriptional dysregulations of liver carcinogenesis and its contribution to both the developmental and malignant progressions of HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Earmarked Grant from the Hong Kong Research Grants Council (ref. no. CUHK 4410/06M) and in part by a Collaborative Research Fund from the Hong Kong Research Grants Council (ref. no. CUHK 4/CRF/08).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 7/15/09; revised 10/31/09; accepted 11/28/09; published OnlineFirst 1/26/10.

References

1. Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide, version 2.0, IARC CancerBase No. 5. Lyon: IARC Press; 2004.
2. Pang RW, Poon RT. From molecular biology to targeted therapies for hepatocellular carcinoma: the future is now. Oncology 2007;72 Suppl 1:30–44.
3. Poon RT, Fan ST, Lo CM, et al. Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years. Ann Surg 2001;234:63–70.
4. Pillai RS, Bhattacharyya SN, Artus CG, et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 2005;309: 1573–6.
5. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2002; 99:15524–9.
6. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004;64:3753–6.
7. Woods K, Thomson JM, Hammond SM. Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. J Biol Chem 2007;282:2130–4.
8. Meng F, Henson R, Lang M, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 2006;130:2113–29.
9. Chan KY, Lai PB, Squire JA, et al. Positional expression profiling indicates candidate genes in deletion hotspots of hepatocellular carcinoma. Mod Pathol 2006;19:1546–54.
10. Wong QW, Lung RW, Law PT, et al. MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. Gastroenterology 2008;135:257–69.
11. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98:5116–21.
12. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998;95:14863–8.
13. Greene FL, American Joint Committe on Cancer, American Cancer Society. AJCC cancer staging manual. pp. xiv 421–i411. New York: Springer-Verlag; 2002.
14. Kuo YC, Huang KY, Yang CH, Yang YS, Lee WY, Chiang CW. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55α regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. J Biol Chem 2008;283:1882–92.
15. Budhu A, Jia HL, Forgues M, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 2008;47: 897–907.
16. Gramantieri L, Ferracin M, Fornari F, et al. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res 2007;67:6092–9.
17. Jiang J, Gusev Y, Aderca I, et al. Association of MicroRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. Clin Cancer Res 2008;14:419–27.
18. Ladeiro Y, Couchy G, Balabaud C, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008;47:1955–63.
19. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007;133: 647–58.
20. Murakami Y, Yasuda T, Saigo K, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006;25:2537–45.
21. Wang Y, Lee AT, Ma JZ, et al. Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. J Biol Chem 2008;283:13205–15.
22. Ura S, Honda M, Yamashita T, et al. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. Hepatology 2009;49:1098–112.
23. Li S, Fu H, Wang Y, et al. MicroRNA-101 regulates expression of the v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene in human hepatocellular carcinoma. Hepatology 2009;49: 1194–202.
24. Miller TE, Ghoshal K, Ramaswamy B, et al. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. J Biol Chem 2008;283:29897–903.
25. Nikiforova MN, Tseng GC, Steward D, Diorio D, Nikiforov YE. MicroRNA expression profiling of thyroid tumors: biological significance and diagnostic utility. J Clin Endocrinol Metab 2008;93:1600–8.
26. Medina R, Zaidi SK, Liu CG, et al. MicroRNAs 221 and 222 bypass quiescence and compromise cell survival. Cancer Res 2008;68: 2773–80.
27. Felicetti F, Errico MC, Bottero L, et al. The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. Cancer Res 2008;68:2745–54.
28. Li W, Tan D, Zhang Z, Liang JJ, Brown RE. Activation of Akt-mTOR-p70S6K pathway in angiogenesis in hepatocellular carcinoma. Oncol Rep 2008;20:713–9.
29. Chen JS, Wang Q, Fu XH, et al. Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: Association with MMP-9. Hepatol Res 2009;39:177–86.
30. Nakanishi K, Sakamoto M, Yamasaki S, Todo S, Hirohashi S. Akt phosphorylation is a risk factor for early disease recurrence and poor prognosis in hepatocellular carcinoma. Cancer 2005;103:307–12.
31. Krasilnikov M, Ivanov VN, Dong J, Ronai Z. ERK and PI3K negatively regulate STAT-transcriptional activities in human melanoma cells: implications towards sensitization to apoptosis. Oncogene 2003; 22:4092–101.
32. Saxena NK, Sharma D, Ding X, et al. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. Cancer Res 2007;67:2497–507.
33. Cohen PT, Brewis ND, Hughes V, Mann DJ. Protein serine/threonine phosphatases; an expanding family. FEBS Lett 1990;268:355–9.
34. Xu L, Deng X. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. J Biol Chem 2006;281:35567–75.
35. Zheng M, McKeown-Longo PJ. Cell adhesion regulates Ser/Thr phosphorylation and proteasomal degradation of HEF1. J Cell Sci 2006;119:96–103.