

Expression of MSP58 in hepatocellular carcinoma

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Abstract We have investigated the expression and role of the 58-kDa micro-spherule protein (MSP58) in hepatocellular carcinoma (HCC). Immunohistochemistry was performed in 252 samples from patients with HCC to detect the expression level of MSP58. Results indicated that the expression level of MSP58 in the cancer samples was significantly higher than that in adjacent normal tissues. The Wilcoxon–Mann–Whitney test showed significant difference in the expression of MSP58 in patients with serum AFP, tumor size, histological differentiation, and universal integrated circuit card (UICC) stage ($P < 0.001$, $P = 0.004$, $P < 0.001$, $P < 0.001$, respectively). A total of 252 HCC patients were followed up for five consecutive years, and Kaplan–Meier survival analysis demonstrated that the survival time of HCC patients with low expression

of MSP58 was longer than those with high expression during the 5-year follow-up period ($P < 0.001$). Cox regression analysis indicated that high expression of MSP58 (++ or +++), serum AFP ($\geq 25 \mu\text{g/L}$), tumor size ($\geq 3 \text{ cm}$), and UICC stage (III or IV) were the independent poor prognostic factors of HCC ($P = 0.008$, 0.0290 , 0.001 , 0.047 , respectively). Furthermore, down-regulation of MSP58 was introduced to HCC cell lines (HepG2 and Huh7) by plasmid transfection. In vivo and in vitro studies indicated that MSP58si markedly reduced proliferation and promoted the apoptosis of HepG2 and Huh7 cells. In summary, our results demonstrated that MSP58 played an important role in the proliferation and apoptosis of HCC cells and the expression of MSP58 in HCC patients was closely related to the prognosis.

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Abbreviations

MSP58	58-kDa micro-spherule protein
HCC	Hepatocellular carcinoma
AFP	Alpha-fetoprotein
GGT	Gamma-glutamyltransferase
IGF	Insulin-like growth factor
TGF	Transforming growth factor
FHA	Fork-head-associated domain
Bhlh	Basic-helix-loop-helix
UICC	Universal integrated circuit card

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer representing 85 % of liver cancers, represents the fifth most common cancer in the world, and is the third leading cause of cancer-related deaths worldwide [1–3]. In recent years, the HCC incidence is still increasing in developed countries although considerable progress has been made in diagnostic and therapeutic modalities [4, 5]. Even with aggressive treatment, HCC usually has a poor prognosis, with a 5-year survival rate as low as 25–39 % after common treatments, such as surgery, chemotherapy, and radiotherapy [1–3, 5]. The poor prognosis of HCC is also caused by its poorly differentiated phenotype, portal venous invasion, and intrahepatic metastasis [1–3]. The molecular genetics of HCC have recently been extensively characterized [6–9]. Although many molecular markers, including alpha-fetoprotein (AFP), gamma-glutamyltransferase (GGT), insulin-like growth factor (IGF), and transforming growth factor (TGF), have been exploited for detecting HCC, these lack sensitivity and specificity for evaluating the prognosis of HCC patients [1–3, 6–9]. Thus, there is an urgent demand for research into novel molecular markers that can serve as diagnostic and prognostic markers for HCC.

58-kDa micro-spherule protein (MSP58) is a 462-amino acid protein. The molecular structure includes nucleolar localization signal, nuclear localization signal, coiled-coil domain, and fork-head-associated domain (FHA). It had been proved that coiled-coil domain can mediate protein interaction. The FHA domain was found in some proteins of prokaryotic and eukaryotic cell, and these proteins located in the nucleus, involved in a series of cellular activities, such as mitosis, DNA repair, and transcriptional regulation [10–12].

58-kDa micro-spherule protein was also known as MCRS1 or P78 and has been implicated in playing a role in cell proliferation and development of malignancy [12, 13]. Nucleolar MSP58 was initially identified through its interaction with the proliferation-related nucleolar protein p120,

and its overexpression leads to enlargement of nucleoli [10]. MSP58 has been reported to interact with several other proteins. It is reported to play a role in modulation of Daxx-dependent transcriptional repression [11] and is associated with the transcriptional activity of the Basic-helix-loop-helix (bHLH) transcription factor stimulated by retinoic acid (STRA) 13 [14]. Moreover, TOJ3, an avian homologue of MSP58, was a target of the transcription factor v-Jun. Ectopic expression of TOJ3 in avian fibroblasts led to anchorage-independent growth, strongly suggesting that TOJ3 plays an important role in Jun-induced cell transformation and tumorigenesis [12, 15]. MSP58 was also shown to behave as an oncogene, and its transformation activity was inhibited by physical interaction with the PTEN tumor suppressor [16, 17]. Our laboratory had reported that there was different expression of MSP58 in human glioma [18] and colorectal cancer [19], and we had demonstrated that MSP58 regulates colorectal cancer cell proliferation, development, and apoptosis, by the cyclin D1–cyclin-dependent kinase 4-p21 pathway.

Nonetheless, despite these data, the functions of MSP58 in tumors remain largely unknown. In this study, the expression level of MSP58 was detected in 252 samples from patients with HCC. The relationship between expression of MSP58 and survival time during the 5-year follow-up period was evaluated. In addition, we down-regulated the expression of MSP58 in HCC cell lines, *in vivo*, and *in vitro* studies to reveal that MSP58si influences the proliferation and apoptosis of HCC cell lines. Our study provides explicit evidence of the regulation of MSP58 in HCC cells and indicates that MSP58 may be a prognostic marker in HCC patients.

Methods

Patients and specimens

A total of 252 human primary HCC tissues and matched control tissues were obtained from patients who underwent hepatectomy at the Xijing Hospital, between 2001 and 2004. The mean age was 45 years (range 19–78 years), with 101 women and 151 men. None of these patients had received preoperative chemotherapy or radiotherapy. Overall survival, which was defined as the time from the operation to patient death or the last follow-up, was used as a measure of prognosis. The median follow-up period was 32.4 months (range 9–66 months). Patient characteristics are summarized in Table 1. Both the tumor and the corresponding non-tumor tissues not less than 3 cm away from the HCC were sampled, and the diagnosis were confirmed by pathological examination. After surgical resection, all the tissue samples were fixed in 10 % formalin and

Table 1 Relationship between 58-kDa microspherule protein (MSP58) expression and clinicopathological features of 252 patients with hepatocellular carcinoma

	MSP58 (-/+)	MSP58 (++)	MSP58 (+++)	P values
Age				0.547
<65	81 (61.4)	23 (17.4)	28 (21.2)	
≥65	70 (58.3)	20 (16.7)	30 (25.0)	
Gender				0.225
Male	87 (57.6)	24 (15.9)	40 (26.5)	
Female	64 (63.4)	19 (18.8)	18 (17.8)	
Serum AFP				<0.001
<25 µg/L	69 (75.8)	15 (16.5)	7 (7.7)	
≥25 µg/L	82 (50.9)	28 (17.4)	51 (31.7)	
Vascular invasion				0.141
No	40 (66.7)	11 (18.3)	9 (15.0)	
Yes	111 (57.8)	32 (16.7)	49 (25.5)	
Liver cirrhosis				0.820
No	69 (59.5)	19 (16.4)	28 (24.1)	
Yes	82 (60.3)	24 (17.6)	30 (22.1)	
HBV				0.926
Negative	59 (60.2)	15 (15.3)	24 (24.5)	
Positive	92 (59.7)	28 (18.2)	34 (22.1)	
HCV				0.955
Negative	102 (59.6)	30 (17.5)	39 (22.8)	
Positive	49 (60.5)	13 (16.0)	19 (23.5)	
Tumor size (cm)				0.004
<3	41 (73.2)	12 (21.4)	3 (5.4)	
≥3	110 (56.1)	31 (15.8)	55 (28.1)	
Tumor number				0.724
Solitary	116 (60.4)	33 (17.2)	43 (22.4)	
>1	35 (58.3)	10(16.7)	15 (25.0)	
UICC stage				<0.001
I	58 (93.5)	3 (4.8)	1 (1.6)	
II	34 (61.8)	16 (29.1)	5 (9.1)	
III	48 (60.0)	10 (12.5)	22 (27.5)	
IV	11 (20.0)	14 (25.5)	30 (54.5)	
Histological differentiation				<0.001
Well	60 (70.6)	12 (14.1)	13 (15.3)	
Moderate	74 (62.2)	22 (18.5)	23 (19.3)	
Poor	17 (35.4)	9 (18.8)	22 (45.8)	

embedded in paraffin, and consecutive 2-mm sections were cut. The fresh cancer tissues and matched normal tissues of fifteen HCC patients were obtained for western blot analysis. Histological types were assigned according to the WHO classification criteria. The protocols used in the study were approved by the hospital’s Protection of Human Subjects Committee. The use of human tissues in this study was approved by the institutional review board of the

Fourth Military Medical University and was done in accordance with international guidelines, and written informed consent was obtained from each patient.

Immunohistochemical staining [19]

Immunohistochemistry was performed using the Histostain-Plus SP kit. Briefly, the sections were deparaffinized with xylene and rehydrated through gradient ethanol immersion. Endogenous peroxidase activity was quenched by 0.3 % (v/v) hydrogen peroxide in methanol for 20 min. The sections were then blocked with 10 % (v/v) normal goat serum in PBS for 1 h, followed by overnight incubation at 4 °C with the mouse anti-MSP58 antibody diluted (MSP58, Mouse, 1:500, Abnova, Taiwan). After three 5-min washes with PBS containing 0.02 % (v/v) Tween-20 (PBST), the sections were treated with biotinylated antimouse secondary antibody for 20 min at room temperature, followed by three additional 5-min washes with PBST. Then, the specimens were incubated with streptavidin–horseradish peroxidase for 20 min at room temperature followed by repeated wash. Reaction product was visualized with DAB at room temperature for 5 min. Sections were counterstained with hematoxylin for 30 s and rinsed with tap water, immediately dehydrated by sequential immersion in gradient ethanol and xylene, and then mounted with Permount onto coverslips. Images were obtained under a light microscope (Olympus BX51; Olympus, Japan) equipped with a DP70 digital camera.

Immunohistochemical analysis

Sections without the primary antibody were used as negative controls. Clear colorectal cancer samples that previously showed immunoreactivity to the MSP58 antibody were used as positive controls to confirm MSP58 expression. Expression of MSP58 was evaluated as the percentage of positive cells and staining intensity as previously described [20, 21]. The percentage of positive cells was evaluated quantitatively and scored as 0 for staining of ≤1 % of total cells counted, 1 for staining of 2–25 %, 2 for staining of 26–50 %, 3 for staining of 51–75 %, and 4 for staining of >75 % of the cells examined. Intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. A total ‘staining score’ of 0–12 was calculated and graded as negative (–, score 0–1), weak (+, score 2–4), moderate (++, score 5–8), or strong (+++, score 9–12).

Statistical analysis

The relationship between MSP58 expression levels and clinicopathological factors was analyzed using the Wilcoxon–Mann–Whitney test. The overall survival time of 252

HCC patients was defined as the time from the surgery to death due to cancer. The Kaplan–Meier method was used to determine the cumulative probability of survival, and data were analyzed with the log-rank test. Univariate and multivariate statistical analyses were done using the Cox regression model to investigate the effects of patients' characteristics on overall survival. A score was assigned to each variable for the Cox regression analysis [20, 21]. A value of $P < 0.05$ was considered statistically significant.

Cell culture

The human HCC cell lines HepG2, Hep3B, Huh7, Bel7402, sk-Hep1, and normal liver cell line LO2 were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained as recommended [22, 23]. All cells were incubated at 37 °C in a humidified chamber containing 5 % CO₂.

Western blot analysis [19]

Fifteen cases of human HCC tissues or HCC cells were washed twice with Hanks' balanced salt solution and lysed directly in RIPA buffer (50 mM Tris–HCl pH 7.4, 1 % [v/v] Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na₃VO₄). Cell lysate (60 µg) was separated by SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with mouse anti-MSP58 antibody (MSP58, Mouse, 1:500, Abnova, Taiwan). Anti-GAPDH antibody was used for all western blots as a loading control (diluted 1:5,000; Sigma). After three washes for 15 min in TBS–Tween, the membranes were incubated at room temperature for 2 h with horseradish-peroxidase-conjugated antimouse secondary antibody (dilution 1:2,000; Santa Cruz Biotechnology, CA, USA). The membranes were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, CA, USA).

Plasmid construction and cell transfection

pSilencer3.1 (Ambion, Austin, TX, USA) was used according to the manufacturer's protocol for construction of a human MSP58 siRNA vector. The oligonucleotide 5'-GATC CGCA GCTC ATCA TCGA ACTT CTTC AAGA GAGA AGTT CGAT GATG AGCT GTTT TTTG GAAA-3' was used to encode siRNA against MSP58, as described previously [19]. The non-specific siRNA 5'-GATC CGAC TTCA TAAG GCGC ATGC ACTT CAAG AGAG TGCA TGCG CCTT ATGA AGTC TTTT TTGT CGAC A-3' (Shanghai GenePharma Co., Shanghai, China) was used as a negative control. The two resulting plasmids were designated pSilencer3.1-MSP58 and pSilencer3.1-NC. Cell transfection

was carried out with Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) using the manufacturer's protocol. Briefly, cells were plated and grown to 70–90 % confluence without antibiotics and transfected with 1 µg plasmid. For stable transfection, G418 (400 µg/mL) was added to the cells after 24 h of transfection. Mixed clones were screened and expanded for an additional 6 weeks. The HCC cell lines HepG2 and Huh7 transfected with pSilencer3.1-MSP58, pSilencer3.1-NC, and pSilencer3.1 were designated HepG2- or Huh7-MSP58si, HepG2- or Huh7-pSilencer3.1-NC, and HepG2- or Huh7-pSilencer3.1.

Monolayer growth rate

Monolayer culture growth rate was determined as described previously [19] by conversion of MTT (Sigma Chemical Co., St. Louis, MD, USA) to water-insoluble formazan by viable cells. Cultures were assayed every day for 7 days, and absorbance values were determined with an enzyme-linked immunosorbent assay reader (DASIT, Milan, Italy) at 490 nm.

Plate colony formation assay [19]

For colony formation assays, 1×10^3 cells were seeded into 60-mm dishes with 5 mL DMEM supplemented with 10 % FBS (Sigma Chemical Co.) and 400 µg/mL G418 (Merck, Darmstadt, Germany). After 14 days, the resulting colonies were rinsed with PBS, fixed with methanol at –20 °C for 5 min, and stained with Giemsa (Sigma-Aldrich) for 20 min. Only clearly visible colonies (diameter >50 µm) were counted.

Soft agar clonogenic assay

Soft agar clonogenic assays were carried out as described previously [19] to assess anchorage-independent growth, as a characteristic of *in vitro* tumorigenicity. Briefly, cells were detached and plated in 0.3 % agarose with a 0.5 % agarose underlay. The number of foci >100 µm was counted after 17 days.

Tumorigenicity in nude mice [19]

For tumorigenicity assays, four groups of five mice each were injected subcutaneously at a single site with stably transfected cells. Tumor onset was scored visually and by palpitation at the sight of injection by two trained members of the laboratory staff at different times on the same day. Average tumor size was estimated by physical measurement in cm of the excised tumor at the time of death. With the exception of mice with large tumor burdens, animals were killed 4 weeks after injection. Tumors were verified by H&E staining. Blocks were stored for further analysis.

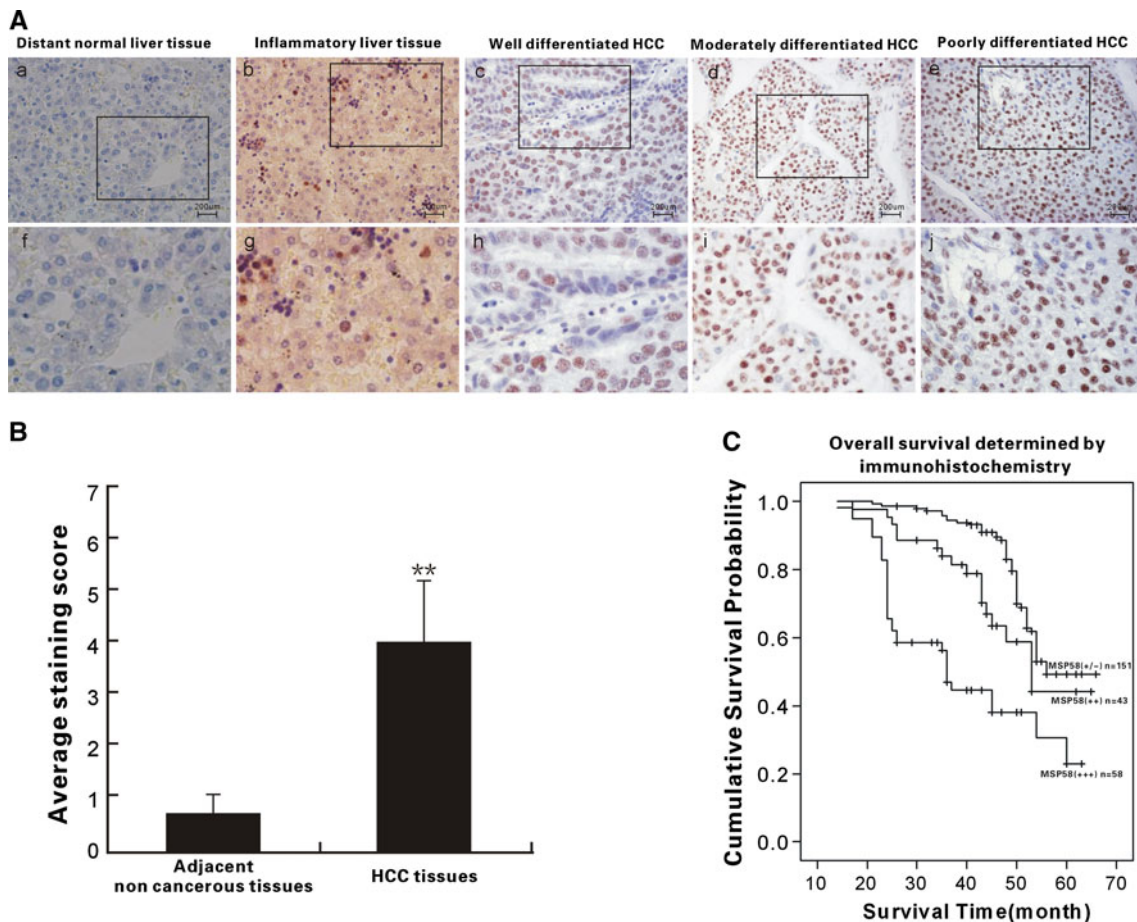


Fig. 1 **A** Immunohistochemical analysis of the MSP58 protein expression in the primary hepatocellular carcinoma surgical specimens. (a) and (f) Normal liver tissue distant from the tumor, scored as MSP58 (-). (b) and (g) In a few of inflammation liver tissues, weak expression of MSP58 was observed, mainly in the nuclear expression, scored as MSP58 (±). (c) and (h) Well-differentiated HCC, scored as MSP58 (+). (d) and (i) Moderately differentiated HCC, scored as MSP58 (++). (e) and (j) Poorly differentiated HCC, scored as MSP58 (+++).

a–e with ×200 magnification; f–j with ×400 magnification. **B** Average staining scores of MSP58 immunostaining in adjacent non-cancerous and HCC tissue specimens. ***P* < 0.01. **C** Cause-specific survival of patients determined by the immunoactivity of MSP58. Cause-specific survival analysis using the Kaplan–Meyer method revealed that 252 HCC patients with relatively low expression of MSP58 had a more favorable prognosis compared with those with high expression (*P* < 0.0001)

Cell cycle analysis [19, 20]

Cells were seeded into 60-mm-diameter plates in complete medium overnight, placed in serum-free medium for 48 h to synchronize the cells, and returned to complete medium for 24 h before harvesting. The suspension was filtered through 50-ml nylon mesh, and the DNA content of stained nuclei was analyzed using a flow cytometer (EPICS XL; Coulter, Miami, FL, USA). The cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Software.

Apoptosis detection with flow cytometer [19, 20]

Untreated and transfected HepG2 and Huh7 cells were trypsinized at 24, 48, and 72 h, washed with cold PBS, and resuspended in PBS. Annexin V-FITC (BD Biosciences, San Jose, CA, USA) at a final concentration of 1 lg/mL,

and 250 ng of propidium iodide was added to the mixture containing 100 μL of cell suspension and binding buffer. The cells were vortexed and incubated for 15 min at room temperature in the dark, followed by incubation with 400 μL of binding buffer for flow cytometric analysis. Each experiment was carried out in triplicate.

Results

Expression of MSP58 in HCC and its correlation with prognosis

The expression of MSP58 by immunoreactivity with the specific MAb was generally localized in both the nucleus and the cytoplasm of HCC cells, and most expression of MSP58 was found in the nucleus (Fig. 1A: a–j). MSP58-positive

expression in HCC was 40.48 % (102/252), significantly higher than 9.52 % (24/252) in the unaffected tissue adjacent to the tumor ($P < 0.01$). MSP58 protein expression in HCC was significantly increased compared with that in normal liver tissues (Fig. 1A: a–j). In addition, its expression level increased from well-differentiated to poorly differentiated tumors (Fig. 1A: a–j). In several cases of inflammation liver tissues, we also found weak expression of MSP58 (Fig. 1A: b, g). By average staining score, non-cancerous cells had significantly lower MSP58 expression than adjacent HCC cells (0.87 ± 0.54 vs 4.13 ± 1.23 , $P < 0.01$) (Fig. 1B). As shown in Fig. 1 and Table 1, 252 HCC patients were subdivided into the following three subgroups based on the expression levels of MSP58, 151 with none to weak expression (59.92 %; –/+), 43 with moderate to locally strong expression (17.06 %; ++), and 58 with strong expression (23.02 %; +++).

The correlation between expression level of MSP58 and patients' characteristics, such as gender, age, tumor size, tumor number, tumor differentiation, liver cirrhosis, HBV, HCV, vascular invasion, serum AFP, and UICC stage, was investigated. MSP58 protein expression was significantly correlated with serum AFP, tumor size, UICC stage, and histological differentiation (Table 1; $P < 0.001$, $P = 0.004$, $P < 0.001$, $P < 0.001$, respectively). And no significant correlation was observed between MSP58 expression and gender, age, liver cirrhosis, HBV, HCV, vascular invasion, and tumor number. Overall survival analysis using the Kaplan–Meier method revealed that the prognosis of 252 HCC patients with high or moderate MSP58 expression was significantly worse than that of those with none or weak MSP58 expression (Fig. 1C; $P < 0.001$).

Univariate and multivariate analyses were conducted using a Cox proportional hazards model to examine the impact of MSP58 expression and other clinical pathological parameters in HCC patients. MSP58 expression, serum AFP, tumor size, histological grade, and UICC stage were significant prognostic factors in the univariate analysis ($P < 0.001$, $P = 0.010$, $P = 0.004$, $P = 0.041$, $P = 0.012$, respectively; Table 2). Multivariate Cox regression analyses showed that high expression of MSP58 (++ or +++) and high serum AFP (≥ 25 $\mu\text{g/L}$), tumor size (≥ 3 cm), and UICC stage (III or IV) were independent poor predictors ($P = 0.008$, $P = 0.029$, $P = 0.001$, $P = 0.047$, respectively). Thus, MSP58 expression may be useful for predicting the overall survival of HCC patients (Table 2).

To further confirm these findings, western blot analysis was performed in 15 freshly obtained HCC tissues and adjacent normal tissues. We found that cancer tissues tended to show higher MSP58 expression compared with the adjacent normal tissues in ten samples (Fig. 2a), which was consistent with the findings in immunohistochemical analysis. However, no significant changes were found in

Table 2 Univariate and multivariate analyses of overall survival in hepatocellular carcinoma

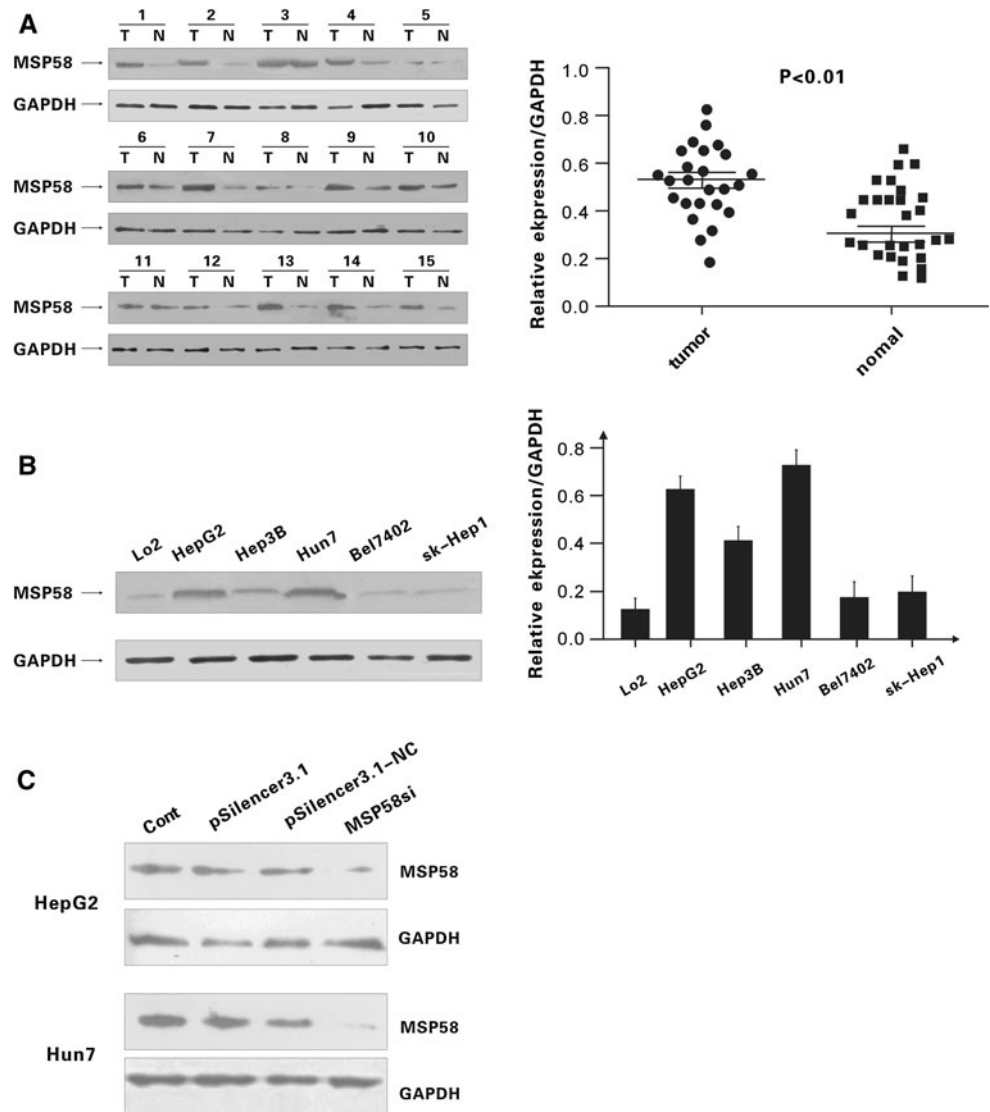
Variables	Univariate analysis		Multivariate analysis	
	Wald chi-square	<i>P</i> values	Risk ratio (95 % confidence interval)	<i>P</i> values
Age (>65 years)	0.203	0.652	0.990 (0.653–1.501)	0.962
Gender (female)	0.977	0.323	1.329 (0.833–2.120)	0.233
Serum AFP (≥ 25 $\mu\text{g/L}$)	6.602	0.010	1.757 (1.058–2.919)	0.029
Vascular invasion (yes)	1.236	0.266	0.691 (0.441–1.085)	0.108
Liver cirrhosis (yes)	0.673	0.412	1.216 (0.494–2.993)	0.670
HBV (positive)	0.630	0.427	1.269 (0.709–2.273)	0.423
HCV (positive)	2.194	0.139	1.030 (0.569–1.863)	0.923
Tumor size (≥ 3 cm)	8.073	0.004	2.258 (1.387–3.676)	0.001
Tumor number (>1)	1.385	0.239	1.039 (0.631–1.711)	0.881
UICC stage (III or IV)	6.311	0.012	1.671 (1.008–2.771)	0.047
Histological grade (moderate or poor)	4.182	0.041	0.667 (0.403–1.105)	0.116
MSP58 (++ or +++)	16.745	<0.001	1.871 (1.180–2.967)	0.008

the remaining five samples. As shown in Fig. 2a, MSP58 protein expression was significantly higher in the HCC tumor tissues than in normal liver tissues ($P < 0.01$). We also found that MSP58 expression was significantly higher in some HCC cell lines than in the normal liver cell lines (Fig. 2b). These results indicated that up-regulated MSP58 expression may play a role in HCC development.

Down-regulation of MSP58 inhibits in vitro proliferation and growth and in vivo tumorigenicity of HCC cells

HepG2 and Huh7 cell lines were used for transfection because relatively high expression of MSP58 was observed in the two HCC cell lines (Fig. 2b). After cell transfection and antibiotic screening for 6 weeks, the expression of MSP58 in stably transfected cells was determined by western blotting. MSP58si appeared to reduce the expression of MSP58 in HepG2 and Huh7 effectively, whereas no effect was observed with the control, pSilencer3.1, and pSilencer3.1-NC vector (Fig. 3c). Expression of MSP58 was not significantly changed in HepG2 and Huh7 cells transfected with empty vector and pSilencer3.1-NC vector.

Fig. 2 a Western blot analysis of MSP58 expression in 15 fresh HCC patients. In 10 patients, the MSP58 protein expression was higher in the tumor tissues than in matched adjacent non-tumorous tissues (patient 1, 2, 4, 7, 9, 10, 12, 13, 14, 15; $P < 0.01$), and no significant difference was observed in the remaining patients. **b** The MSP58 protein levels were significantly higher in the HepG2 and Hun 7 cells than in the normal liver cell line LO2. **c** After stable transfection, the expression of MSP58 in HepG2 and Hun 7 cells was evaluated by western blotting. GAPDH was used as an internal control



Stably transfected HepG2-MSP58si and Huh7-MSP58si were chosen for further cellular assay. When the growth curves of these cell lines were compared in medium containing 10 % FCS, the curves for MSP58si cells were significantly lower than control cells ($P < 0.05$ on days 4–7; Fig. 3a). To determine the effect of MSP58 on the colony-forming ability of HCC cells, we carried out in vitro plate colony formation assays. Compared with control cells, fewer clones were observed in HepG2-MSP58si and Huh7-MSP58si cells ($P < 0.01$; Fig. 3b). We then evaluated the effect of MSP58 suppression on anchorage-independent colony formation in soft agar as an additional assessment of tumorigenicity in vitro. MSP58 down-regulation significantly impaired anchorage-independent colony growth, reducing both colony number and size ($P < 0.01$; Fig. 3c). In contrast, no loss of colony-forming ability was observed

with either pSilencer3.1-NC cells in nude mice. Injected HepG2- or Huh7-MSP58si cells led to significantly smaller tumors than control cells carrying empty vector ($P < 0.05$; Fig. 3d). Thus, both in vitro and in vivo assays suggested that down-regulation of MSP58 might inhibit proliferation, growth, and tumorigenicity of HCC cells.

Down-regulation of MSP58 induces cell cycle arrest and promotes apoptosis of HCC cells

To further investigate the mechanism by which down-regulation of MSP58 inhibits HCC cell growth, we used FACS analysis to study the effects of MSP58 expression on the cell cycle (Table 3). The results of cell cycle analysis showed that 24 h after the release of synchronized cultures, 5.71 % of HepG2-MSP58si cells were in S-phase, compared with

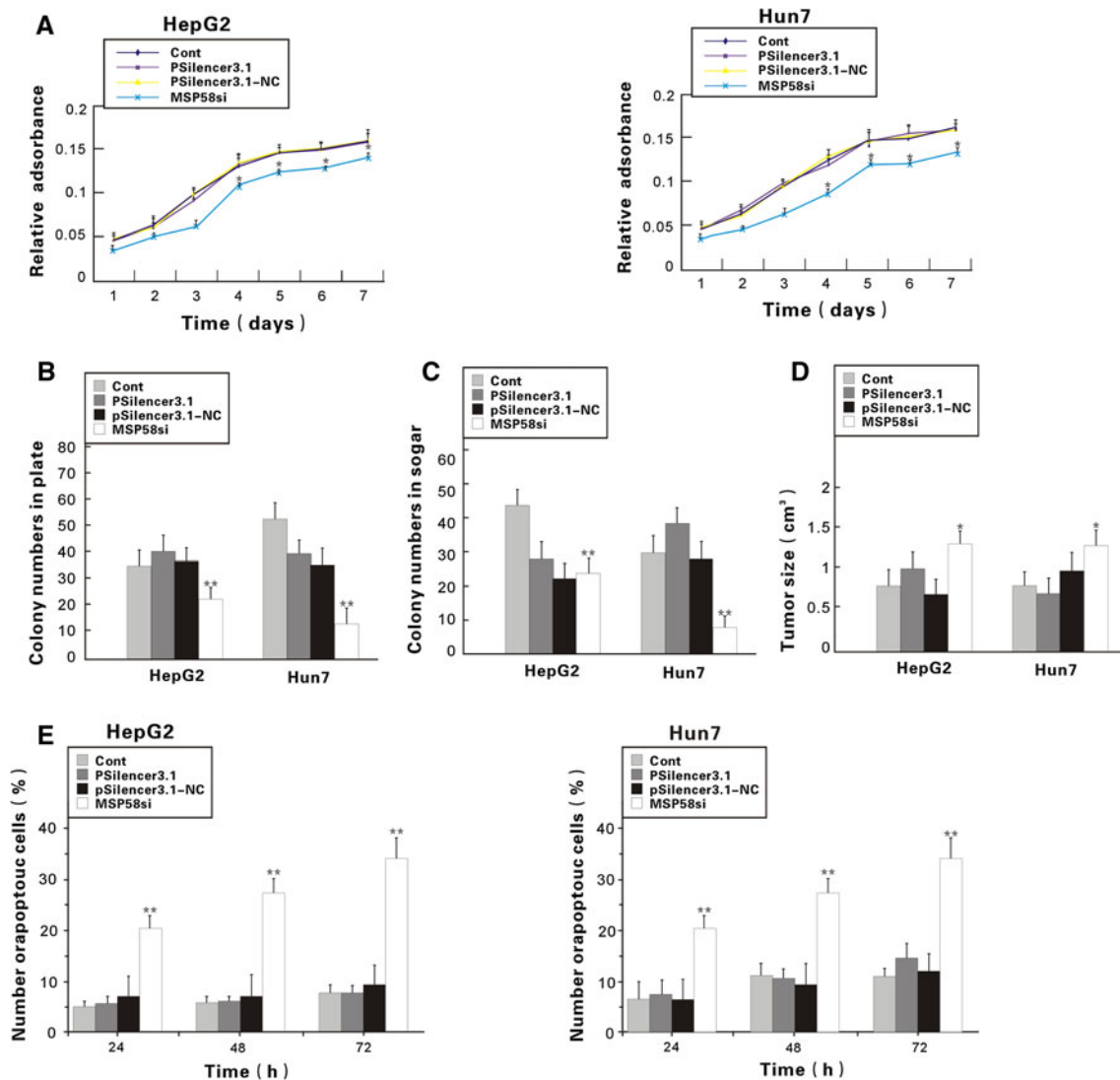


Fig. 3 **a** Monolayer growth rates of HepG2 and Hun 7 cells transfected with the indicated plasmids were determined by MTT assay. Values represent the mean from at least three separate experiments. *Error bars* are standard error of the mean (SEM). * $P < 0.05$. **b** To test plate colony formation of HepG2 and Hun 7 cells transfected with the indicated plasmids, cells were placed in wells with media and incubated for 15 days before counting the number of foci. Values are the mean from at least three separate experiments, each conducted in triplicate, and error bars show SEM. ** $P < 0.01$. **c** Colony formation of HepG2 and Hun 7 cells transfected with the indicated plasmids was carried out by placing cells in media containing soft agar for 17 days. Foci $>100 \mu\text{m}$ were

counted. Values represent the mean of at least three separate experiments, each conducted in triplicate. *Error bars* are SEM. ** $P < 0.01$. **d** Effect of MSP58 siRNA on tumorigenicity of HepG2 and Hun 7 cells in nude mice was calculated by measurement of excised tumors at the time of death. Tumors were verified as HCC by H&E staining. * $P < 0.05$. **e** HepG2 and Hun 7 cells untreated and transfected with pSilencer3.1, pSilencer3.1-NC, and pSilencer3.1-MSP58 were seeded in six-well plates containing 10 % FBS-RPMI-1640 and harvested at 24, 48, and 72 h, respectively, followed by apoptosis assay using the Annexin V-FITC apoptosis detection kit. ** $P < 0.01$

11.81 % of untransfected HepG2 cells: 11.24 % transfected with pSilencer3.1 and 10.77 % transfected with pSilencer3.1-NC ($P < 0.05$); and 4.96 % of Huh7-MSP58si cells were in S-phase compared with 11.52 % of untransfected Huh7 cells: 12.66 % transfected with pSilencer3.1 and 11.42 % transfected with pSilencer3.1-NC ($P < 0.05$).

Additionally, apoptotic rates of HepG2 and Huh7 cells untreated and transfected with pSilencer3.1, pSilencer3.1-NC,

and pSilencer3.1-MSP58 were analyzed by flow cytometer. The results revealed that there was no significant difference in apoptotic rate between untreated and transfected with pSilencer3.1 and pSilencer3.1-NC groups in both HepG2 and Huh7 cells. However, the apoptotic rate of HepG2 and Huh7 cells transfected with pSilencer3.1-MSP58 was significantly increased compared with untreated, pSilencer and pSilencer-NC groups ($P < 0.01$; Fig. 3e).

Table 3 Cell cycle analysis of 58-kDa microspherule protein (MSP58) expression in hepatocellular carcinoma cells

Group	HepG2			Huh7		
	G1	G2	S	G1	G2	S
Cont	54.76 ± 2.62	33.43 ± 2.15	11.81 ± 2.99	58.37 ± 1.79	30.11 ± 2.29	11.52 ± 1.81
pSilencer3.1	55.42 ± 3.06	33.34 ± 2.68	11.24 ± 2.72	57.51 ± 2.17	29.83 ± 2.13	12.66 ± 2.17
pSilencer3.1-NC	54.81 ± 2.84	34.42 ± 2.75	10.77 ± 2.32	58.13 ± 1.98	30.45 ± 1.92	11.42 ± 1.75
MSP58si	61.54 ± 3.20 ^{a,b,c}	32.75 ± 1.63	5.71 ± 1.83 ^{a,b,c}	65.90 ± 2.32 ^{a,b,c}	29.14 ± 1.89	4.96 ± 1.68 ^{a,b,c}

N = 3

^a *P* < 0.05 compared with the control group using one-way ANOVA with a Tukey's post hoc test

^b *P* < 0.05 compared with the pSilencer3.1 group using one-way ANOVA with a Tukey's post hoc test

^c *P* < 0.05 compared with the pSilencer3.1-NC group using oneway ANOVA with a Tukey's post hoc test

Discussion

Our study and other laboratory studies had shown that MSP58 would interact with a number of molecules that have important roles in tumor proliferation and invasiveness. These molecules include PTEN [16, 17], NDRG2 [24], Daxx [11], NRF1 [25], and STRA13 [14]. Recently, MSP58 was shown to behave as a new oncogene and its transformation activity was inhibited by physical interaction with the PTEN [16–18], which had been shown to be a tumor suppressor. Lin [18] found that down-regulated MSP58 expression in U251MG cells could decrease tumor cell growth, migration, and invasion, MSP58Si-U251MG cells also appear G1 phase resistance and obviously decrease the expression of E2F2. So we speculated that PTEN deficiency in MSP58/E2F2 pathway was a key factor, which promoted the malignant proliferation in glioma cells. Ivanova [14] also reported that MSP58 might play an important role in glioma cell proliferation by interacting with STRA13, which synergistically inhibits tumor suppressor genes STAT1 transcription. In previous studies, we identified MSP58 as a novel NDRG2-interacting protein and found that NDRG2 could alter the role of MSP58 in cell proliferation [24]. It had been proved that NDRG2 served as a tumor suppressor gene involved in gastric cancer [26], colorectal cancer [27–29], hepatocellular carcinoma [30, 31], esophageal squamous cell carcinoma [20], and thyroid cancer [32]. We also found that MSP58 regulates colorectal cancer cell proliferation, development, and apoptosis, by the cyclin D1–cyclin-dependent kinase 4-p21 pathway [19]. These findings suggested that MSP58 gene may serve as a new oncogene involved in some human cancer process.

In the present study, we used a relatively large series of clinical tissue samples to explore the role of MSP58 in HCC for the first time. We examined MSP58 protein expression in paired primary HCC samples and HCC cell lines using western blotting. We found that MSP58 expression was up-regulated at both the transcriptional and translational levels in most primary HCC tumor tissues and HCC cell lines. Consistent with these observations,

immunohistochemical analyses also showed that MSP58 expression was increased in most HCC tumor tissues compared with the corresponding non-tumorous liver tissues. These results indicated that up-regulated MSP58 expression may play a role in HCC development.

In the immunohistochemical analysis, increased MSP58 expression in HCC was significantly associated with tumor size, histological differentiation, serum AFP, and UICC stage. The relationship between high MSP58 expression and larger tumor size suggested that the increase in MSP58 expression may help promote the rapid expansion of the HCC tumor. Additionally, most of the poorly differentiated HCC samples were positive for MSP58 expression, but MSP58 expression was profoundly weaker in the moderately and well-differentiated tumor samples. Thus, increased MSP58 expression is correlated with poor differentiation in HCC cells and may further promote HCC progression. The UICC stage of HCC was closely related to tumor size, tumor number serum AFP, and vascular invasion. Therefore, high expression of MSP58 may lead to the expansion of tumor volume of HCC, resulting in poor UICC stage.

Our Kaplan–Meier survival analysis revealed that high MSP58 expression was significantly linked to poor prognosis after surgical resection in the HCC patients (*P* < 0.001). Furthermore, MSP58 expression was an independent prognostic factor for overall survival in the multivariate analysis. These results suggest that MSP58 can serve as a new predictor of prognosis in HCC patients after surgical resection.

Based on the high MSP58 expression in the HepG2 and Huh7 HCC cell lines, we down-regulated MSP58 expression in the two cell lines to further examine the mechanism by which it promotes HCC progression. Down-regulated MSP58 expression significantly suppressed HCC cell proliferation and colony formation. In parallel experiments, we also found that down-regulated MSP58 expression suppressed tumor growth in injectable mouse models. Consistent with our results, a previous study has shown that decreased MSP58 expression in CRC inhibits cellular

transformation and xenograft tumor growth. MSP58si also led to G1 phase cell cycle arrest and induced apoptosis of HCC cells, indicating that MSP58 promotes HCC tumorigenicity by inducing cell cycle arrest and apoptosis.

Taken together, our results revealed that MSP58 is a differentially expressed molecule in HCC, and MSP58 might be as a prognostic biomarker. Our data do provide evidence that MSP58 expression correlates with the proliferation and apoptosis of HCC cells. However, further studies are required to classify the biological functions of MSP58 and elucidate the specific roles of MSP58 in the invasion and metastasis of HCC.

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Conflict of interest The authors declare that they have no conflict of interest.

References

- Luk JM, Liu AM. Proteomics of hepatocellular carcinoma in Chinese patients. *OMICS*. 2011;15(5):261–6.
- Block T, Mehta AS, London WT. Hepatocellular carcinoma of the liver. *Cancer Biomark*. 2010;9(1–6):375–83.
- Washburn K, Half G. Hepatocellular carcinoma and liver transplantation. *Curr Opin Organ Transplant*. 2011;16(3):297–300.
- Kudo M. Adjuvant therapy after curative treatment for hepatocellular carcinoma. *Oncology*. 2011;81(Suppl 1):50–5.
- Lencioni R, Crocetti L. Local-regional treatment of hepatocellular carcinoma. *Radiology*. 2012;262(1):43–58.
- Yu L, Dai Z, Wang Z, Fan J, Zhou J. Prognostic indicators for tumor recurrence after liver transplantation in hepatocellular carcinoma and related molecular targeted therapy. *Oncology*. 2011;81(Suppl 1):116–22.
- Masuda T, Miyoshi E. Cancer biomarkers for hepatocellular carcinomas: from traditional markers to recent topics. *Clin Chem Lab Med*. 2011;49(6):959–66.
- Zhu AX, Duda DG, Sahani DV, Jain RK. HCC and angiogenesis: possible targets and future directions. *Nat Rev Clin Oncol*. 2011;8(5):292–301.
- Tanaka S, Arai S. Molecular targeted therapy for hepatocellular carcinoma in the current and potential next strategies. *J Gastroenterol*. 2011;46(3):289–96.
- Ren Y, Busch RK, Perlaky L, Busch H. The 58-kDa microspherule protein (MSP58), a nucleolar protein, interacts with nucleolar protein p120. *Eur J Biochem*. 1998;253(3):734–42.
- Lin DY, Shih HM. Essential role of the 58-kDa microspherule protein in the modulation of Daxx-dependent transcriptional repression as revealed by nucleolar sequestration. *J Biol Chem*. 2002;277(28):25446–56.
- Bader AG, Schneider ML, Bister K, Hartl M. TOJ3, a target of the v-Jun transcription factor, encodes a protein with transforming activity related to human microspherule protein 1 (MCRS1). *Oncogene*. 2001;20(51):7524–35.
- Du X, Wang Q, Hirohashi Y, Greene MI. DIPA, which can localize to the centrosome, associates with p78/MCRS1/MSP58 and acts as a repressor of gene transcription. *Exp Mol Pathol*. 2006;81(3):184–90.
- Ivanova AV, Ivanov SV, Lerman ML. Association, mutual stabilization, and transcriptional activity of the STRA13 and MSP58 proteins. *Cell Mol Life Sci*. 2005;62(4):471–84.
- Karagiannidis AI, Bader AG, Hartl M, Bister K. TOJ3, a v-jun target with intrinsic oncogenic potential, is directly regulated by Jun via a novel AP-1 binding motif. *Virology*. 2008;378(2):371–6.
- Okumura K, Zhao M, DePinho RA, Furnari FB, Cavenee WK. PTEN: a novel anti-oncogenic function independent of phosphatase activity. *Cell Cycle*. 2005;4(4):540–2.
- Okumura K, Zhao M, Depinho RA, Furnari FB, Cavenee WK. Cellular transformation by the MSP58 oncogene is inhibited by its physical interaction with the PTEN tumor suppressor. *Proc Natl Acad Sci USA*. 2005;102(8):2703–6.
- Lin W, Zhang J, Zhang J, et al. RNAi-mediated inhibition of MSP58 decreases tumour growth, migration and invasion in a human glioma cell line. *J Cell Mol Med*. 2009;13(11–12):4608–22.
- Shi H, Chen S, Jin H, et al. Downregulation of MSP58 inhibits growth of human colorectal cancer cells via regulation of the cyclin D1-cyclin-dependent kinase 4–p21 pathway. *Cancer Sci*. 2009;100(9):1585–90.
- Shi H, Li N, Li S, et al. Expression of NDRG2 in esophageal squamous cell carcinoma. *Cancer Sci*. 2010;101(5):1292–9.
- Shi H, Zhou Y, Liu H, et al. Expression of CIAPIN1 in human colorectal cancer and its correlation with prognosis. *BMC Cancer*. 2010;10:477.
- Xu XM, Yuan GJ, Deng JJ, et al. Inhibition of 12-lipoxygenase reduces proliferation and induces apoptosis of hepatocellular carcinoma cells in vitro and in vivo. *Hepatobiliary Pancreat Dis Int*. 2012;11(2):193–202.
- Zhao JJ, Pan K, Li JJ, et al. Identification of LZAP as a new candidate tumor suppressor in hepatocellular carcinoma. *PLoS ONE*. 2011;6(10):e26608.
- Zhang J, Liu J, Li X, et al. The physical and functional interaction of NDRG2 with MSP58 in cells. *Biochem Biophys Res Commun*. 2007;352(1):6–11.
- Wu JL, Lin YS, Yang CC, et al. MCRS2 represses the transactivation activities of Nrf1. *BMC Cell Biol*. 2009;10:9.
- Choi SC, Yoon SR, Park YP, et al. Expression of NDRG2 is related to tumor progression and survival of gastric cancer patients through Fas-mediated cell death. *Exp Mol Med*. 2007;39(6):705–14.
- Piepoli A, Cotugno R, Merla G, et al. Promoter methylation correlates with reduced NDRG2 expression in advanced colon tumour. *BMC Med Genomics*. 2009;2:11.
- Kim YJ, Yoon SY, Kim JT, et al. NDRG2 expression decreases with tumor stages and regulates TCF/beta-catenin signaling in human colon carcinoma. *Carcinogenesis*. 2009;30(4):598–605.
- Lorentzen A, Vogel LK, Lewinsky RH, et al. Expression of NDRG2 is down-regulated in high-risk adenomas and colorectal carcinoma. *BMC Cancer*. 2007;7:192.
- Ventura-Holman T, Mamoon A, Subauste MC, Subauste JS. The effect of oncoprotein v-erbA on thyroid hormone-regulated genes in hepatocytes and their potential role in hepatocellular carcinoma. *Mol Biol Rep*. 2011;38(2):1137–44.
- Lee DC, Kang YK, Kim WH, et al. Functional and clinical evidence for NDRG2 as a candidate suppressor of liver cancer metastasis. *Cancer Res*. 2008;68(11):4210–20.
- Zhao H, Zhang J, Lu J, et al. Reduced expression of N-Myc downstream-regulated gene 2 in human thyroid cancer. *BMC Cancer*. 2008;8:303.