Biomedicine & Pharmacotherapy 88 (2017) 421–429

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

HCRP1 downregulation promotes hepatocellular carcinoma cell migration and invasion through the induction of EGFR activation and epithelial-mesenchymal transition

Jiawen Xu[a], Xi Zhang[b], Hongliang Wang[c], Shujian Ge[d], Taihong Gao[e], Lin Song[a], Xinxing Wang[f], Hui Li[g], Yejun Qin[a,*,1], Zhenhai Zhang[f,*,1]

[a] Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, 250021, PR China
[b] Department of Breast and Thyroid Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, 250021, PR China
[c] Shandong Academy of Occupational Health and Occupational Medicine, Shandong Academy of Medical Sciences, Jinan, Shandong, 250062, PR China
[d] Department of Science and Education, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, 250021, PR China
[e] Department of Neurosurgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, 250021, PR China
[f] Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, 250021, PR China
[g] Department of Nursing, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, 266071, PR China

ARTICLE INFO

Article history:
Received 11 November 2016
Accepted 2 January 2017

Keywords:
Hepatocellular carcinoma related protein 1
Hepatocellular carcinoma
Epidermal growth factor receptor
Epithelial-to-mesenchymal transition

ABSTRACT

Hepatocellular carcinoma related protein 1 (HCRP1), which is essential for internalization and degradation of ubiquitinated membrane receptors, is downregulated in several tumors and strongly affects the outcomes of cancer patients. It is reported the expression of HCRP1 is inversely related to epidermal growth factor receptor (EGFR) in breast cancer and lead to resistance to cetuximab in ovarian cancer. However, its exact mechanism in the progression of Hepatocellular carcinoma (HCC) remains unknown. Herein, HCRP1 expression and its clinical significance were examined in 101 HCC patients using immunohistochemistry. Cell proliferation, migration and invasion assays were conducted in HCC cell lines. EGFR activation and degradation were then observed after EGF inducing in HCRP1 knockdown HepG2 cells. In addition, we also detected whether epithelial-to-mesenchymal transition (EMT) was involved in the malignancy promoted by HCRP1. The results showed that 59 of the 101 HCC cases exhibited downregulation of HCRP1 expression ($P < 0.01$) as compared to 30 benign liver lesions and 20 normal liver tissues, all of which showed a high level of HCRP1. HCRP1 expression was significantly related to age ($P = 0.017$), pathological grade ($P = 0.003$), tumor encapsulation ($P = 0.037$), recurrence ($P = 0.039$) and death ($P = 0.015$), but unrelated to cirrhosis ($P = 0.216$), tumor size ($P = 0.273$), and distant metastasis ($P = 0.554$). Lower HCRP1 expression was correlated with shorter RFS and OS ($P < 0.001$), and decreased HCRP1 level is an independent prognostic marker in HCC patients ($P < 0.05$). Overexpression of HCRP1 decreased and knockdown increased HCC cell proliferation, migration and invasion. HCRP1 depletion increased EGFR activation and inhibited EGFR degradation. EMT phenotype was promoted after HCRP1 downregulation *via* increase of Snail and Twist1 and activation of Akt phosphorylation in HepG2 cells. Conversely, upregulation of HCRP1 in SMMC-7721 cells led to the opposite effect. In conclusion, our study indicated that downregulation of HCRP1 is a valuable prognostic factor involved in EGFR regulation and acquisition of the mesenchymal phenotype of HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of primary liver cancer and the third leading cause of cancer-related deaths worldwide [1]. Although various therapeutic options are available, most HCC patients are diagnosed at the late stage, largely due to high frequency of recurrence/distant metastasis and lack of advanced diagnostic techniques [2].

* Corresponding authors at: Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University, No. 324 Weiqi Avenue, Jingwu Road, Jinan, Shandong, 250021, PR China.
E-mail addresses: yejunqqin@163.com (Y. Qin), slyyzhangzhenhai@sina.com (Z. Zhang).
[1] The two authors contributed equally to this work and are co-corresponding authors.

http://dx.doi.org/10.1016/j.biopha.2017.01.013
0753-3322/

Therefore, identifying new predictive markers and understanding the molecular genetic mechanisms underlying the metastasis of HCC are critical.

HCC related protein 1 (HCRP1), also called VPS37A, is located at 8p22. It was isolated through positional candidate cloning *via* loss of heterozygosity (LOH) analysis and acted as a subunit of mammalian ESCRT-I, which plays an important role in mediating receptor downregulation [3]. Several studies have shown that low HCRP1 expression is an adverse prognostic predictor in patients with oral and oropharyngeal cancer [4], and loss of its expression can lead to cetuximab, a monoclonal antibody targeting epidermal growth factor receptor (EGFR) resistance in ovarian cancer [5]. Our previous study showed that decreased HCRP1 expression was an independent prognostic predictor and an inverse relationship between HCRP1 and EGFR expression was found in breast cancer patients [6]. HCRP1 depletion could efficiently promote breast cancer metastasis through upregulating EGFR signaling pathway [7]. Few studies have mentioned that the HCRP1 gene is down-regulated in HCC [3,8], however, very little is known about the underlying function of HCRP1 protein in regulating cell growth and invasion.

Epithelial-to-mesenchymal transition (EMT) is a multiple program in which epithelial cells are transmitted to mesenchymal cells, which are considerably more prone to metastasis [9,10]. EMT has been confirmed as a key step in initiating cancer cell migration [11], but whether its progress is involved in HCRP1-induced cell migration and invasion remains unknown. In this study, we detected HCRP1 protein expression in HCC tissues after surgery and HCC cell lines with varying invasiveness. Then, the relationship between HCRP1 expression and common clinical pathological parameters and survival was evaluated. In addition, we also investigated the effects of HCRP1 silencing or upregulation on migration/invasion as well as EGFR regulation and EMT-related factors in HCC cells.

2. Materials and methods

2.1. Patients and tissue specimens

A total of 131 paraffin-embedded sections of liver samples and their related clinical data were collected from the Department of Pathology, Shandong Provincial Hospital, Shandong University from January 2010 to December 2011. The data consisted of 101 cases of HCC, 10 cases of hepatocellular adenoma (HCA), 8 cases of focal nodular hyperplasia (FNH), 12 cases of large regenerative nodules arising in cirrhotic livers, and 20 specimens of normal liver tissues from paracancer tissues (distance of 2 cm from cancer tissues). The age of the patients ranged from 35 to 77 years. The diagnoses were reviewed by two gastrointestinal pathologists based on histology, clinical information, and other related data. The cases were staged and graded according to the Cancer Staging Manual (Seventh Edition) of the American Joint Committee on Cancer [12]. None of the included cases were treated with chemoembolization or systemic chemotherapy before surgery. The patients were postoperatively followed up until January 2016. Informed consent was obtained from all the participants of this study, and the use of tissue specimens was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China).

2.2. Immunohistochemistry

For the immunohistochemical analyses of HCRP1 (Cat.# sc-376978, Santa Cruz Biotechnology, USA), 4 μm-thick sections from the formalin-fixed, paraffin-embedded tissues were used. The samples were deparaffinized in xylene and rehydrated through a graded series of ethanol washes. After the endogenous peroxidase was inhibited and the antigen was retrieved (microwave irradiation in 0.01 M citrate buffer at pH 6.0), the sections were incubated with HCRP1 primary antibody at 4 °C overnight and then with horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako Cytomation, Denmark). After washing, tissues were stained for 5 min with 3,3′-diaminobenzidine (DAB) chromogen and counterstained with hematoxylin (Zhongshan Golden Bridge, Inc), dehydrated and coverslipped. Negative controls were treated without the primary antibody. For HCRP1 immunohistochemical evaluation, a staining score value was calculated as the intensity of cytoplasmic staining (negative: 0, weak:1, moderate:2, strong:3) multiplied by the percentage of positve tumor cells (<10%:1, 11%–50%: 2, >51%:3). The final staining score ≤3 was regarded as low expression, and the score between 4 and 9 was regarded as high expression.

2.3. Cell culture

Human HCC cell lines (SMMC-7721, Bel7404, and HepG2) and normal liver cell line (L02) were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose; Gibco-BRL, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Immunoblotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking in 5% nonfat milk for 1 h at room temperature, the membranes were incubated overnight with primary antibodies against HCRP1 (Cat.# ab85843, Abcam, USA), MMP2 (Cat.# 4022, Cell Signaling, Beverly, MA, USA), MMP9 (Cat.#2270, Cell Signaling, Beverly, MA, USA), p-EGFR (Cat. #3777, Cell Signaling, Beverly, MA, USA), EGFR (Cat. # 4267, Cell Signaling, Beverly, MA, USA), E-cadherin (Cat.# 3195, Cell Signaling, Beverly, MA, USA), vimentin (Cat.# 5741, Cell Signaling, Beverly, MA, USA), Snail (Cat.# 3879, Cell Signaling, Beverly, MA, USA), Twist-1 (Cat.# 49254, Cell Signaling, Beverly, MA, USA), p-ERK (Cat.# 9106, Cell Signaling, Beverly, MA, USA), ERK (Cat.# 9107, Cell Signaling, Beverly, MA, USA), p-Akt (Cat.# 121789, Cell Signaling, Beverly, MA, USA), Akt (Cat.# 2966, Cell Signaling, Beverly, MA, USA) and β-actin (Cat.# A1978, Sigma-Aldrich, USA) at 4 °C. Then, the membranes were incubated for 1 h at 4 °C with the appropriate HRP-conjugated secondary antibodies (Cat.# 111-035-003, Jackson ImmunoResearch Inc, PA, USA for HCRP1; Cat.# 7074, Cell Signaling, Beverly, MA, USA for the other primary antibodies). Protein expression levels were detected *via* enzyme-linked chemiluminescence (Pierce, Rockford, USA).

2.5. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using an RNA Isolation Plus Kit (Cat. #9108, Takara, Dalian, China) according to the manufacturer's instructions. The extracted RNA was then reverse-transcribed to cDNA using PrimeScript® RT Master Mix (Cat.# DRR036A, Takara, Dalian, China) at 37 °C for 15 min, 85 °C for 5 s, and then 4 °C. qPCR was performed for HCRP1 (5′CGTGGACCCGAGGAGGATGA3′, 5′TGAGACAC TCGCTCAGCTTCTTG3′, E-cadherin (5′AAAGACCAAG TGACCACCTTAGAG3′, 5′GAAACAGCAAGAGCAGCAGAAT3′), vimentin(5′GGAGCCCGCTGAGACTTGAA3′, 5′TGCTGTCCCGCCGATT GAGG3′), Snail(5′AGATGAGGACAGTGGGAAAGG3′, 5′TGAAGTA GAGGAGAAGGACGAAGGAG3′), Twist1(5′TCGACTTCCTCTACCA

GGTCCTCC3′, 5′GAGCCGCTCGTGAGCCACATA3′) and GAPDH (5′ ACAGTCAGCCGCATCTTC3′, 5′CTCCGACCTTCACCTTCC3′) in a 10 μL reaction volume using the SYBR® PremixEx Taq™ (Cat.# RR420A, Takara, Dalian, China) and ABI7900HT Real-Time PCR System (Life, Singapore). The thermal cycle conditions were: one cycle at 95 °C for 30 s, 40 cycles of amplification at 95 °C for 5 s, followed by 60 °C for 30 s. The mRNA level was normalized to the geometric mean of GAPDH (conserved gene) mRNA to control the variability in expression levels, and the results were analyzed using the ΔΔCt method.

2.6. Lentiviral vector infection

A lentiviral vector that expressed a constitutively active form of HCRP1 (HANBIO, Shanghai, China) was transfected into SMMC-7721 cells *via* standard infection at a multiplicity of infection of 100 for 24 h. An empty lentiviral vector (HANBIO, Shanghai, China) was used as the negative control. The stably expressed clones were selected in media with puromycin dihydrochloride (Cat. #P9620 Sigma-Aldrich, MO, USA) for two weeks.

2.7. Small interfering RNA (siRNA) interference

HepG2 cells (1×10^5) were seeded onto 6-well plates in triplicate. After overnight incubation, the cells were transfected with various concentrations of HCRP1 siRNA (HANBIO, Shanghai, China). Lipofectamine 2000 (Cat. # 11668-019, Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Cat. # 31985062, Gibco) were used for transfection according to the manufacturers' protocols. The intracellular green fluorescence could be easily observed under a fluorescence microscope (Zeiss, Oberkochen, Germany) when siRNA was successfully delivered into the cells.

2.8. Cell proliferation assay

Cell proliferation was measured with a Cell Counting Kit-8 (CCK-8; Dojindo, Japan), and the cells were seeded onto 96-well microplates at a density of 5×10^3 cells per well. After 1–7 days of culture at 37 °C with 5% CO_2, absorbance at 450 nm was measured with a plate reader. Each group contained six wells, and the measurement was repeated thrice.

2.9. Cell migration and invasion assays

The migration capability of cells was evaluated using Boyden dual chamber assay with 8 μm pore-size membranes (BD Biosciences, New Jersey, USA) in 24-well plates. A total of 3×10^4 cells were suspended in medium without serum and then added to the upper chamber, while serum-positive media was added in the bottom chamber as chemo-attractant. After incubation with 5% CO_2 for 48–72 h at 37 °C, non-migrating cells on the upper side of the insert membrane were removed with cotton swabs, and the insert was fixed in methanol and stained with hematoxylin and eosin. The number of cells was counted in five random mid-power fields and the mean was calculated.

For the invasion assay, Boyden dual chamber assay was performed as described above with several modifications. Briefly, 40 μL growth factor-reduced Matrigel (BD Biosciences, New Jersey, USA) was added on the transwell chamber membranes for 4 h at 37 °C, and assays similar to cell migration assays were subsequently performed.

2.10. EGFR activation

To investigate the relationship between HCRP1 depletion and EGFR signaling pathway, EGF (100 ng/mL, Cat.# cyt-217, East Brunswick, NJ USA), a growth factor could specifically bind to EGFR was treated at different setting time to detect EGFR phosphorylation and expression in HepG2 cells.

2.11. Statistical analysis

All statistical analyses were performed using SPSS 15.0 (IBM, Armonk, NY, USA). The relationship between the expression of HCRP1 and other markers as well as the clinicopathological characteristics of the patients were evaluated using chi-squared test. Analysis of Recurrence-free survival (RFS) and overall survival (OS) were conducted with the Kaplan-Meier method and the log-rank test. Univariate and multivariate Cox regression analyses were performed to investigate the differences in all possible death risk factors. The Student's *t*-test was applied to evaluate the differences in groups as appropriate and the significance level and the statistical significance was determined at $p < 0.05$ (two-tailed).

3. Results

3.1. HCRP1 expression was extremely reduced in HCC

We first detected the expression of HCRP1 in 101 HCC tissues, 10 HCA tissues, 8 FNH tissues, 12 large regenerative nodules arising in cirrhotic livers, and 20 specimens of normal liver tissues from peritumoral tissues *via* immunostaining. All the benign liver lesions showed a high level of HCRP1, whereas 59 of the 101 HCC cases exhibited downregulation of HCRP1 expression ($P < 0.01$, Table 1, Fig. 1A). To further investigate the endogenous expression of HCRP1 in HCC cells, we detected the mRNA and protein levels in three HCC cell lines (SMMC-7721, Bel7404, and HepG2) and human normal liver cell line (L02) *via* qPCR and western blot analysis, respectively. The results demonstrated that HCRP1 expression was significantly lower in HCC cell lines than in normal liver cells (Fig. 1B, C), which was similar to the results of the tissues. Among the HCC cell lines, HepG2 and SMMC-7721, which exhibited high and low expressions of HCRP1, respectively, were chosen for further research.

3.2. HCRP1 expression correlated with clinical parameters and survival

To further explore the role of HCRP1 in HCC, a set of well-known clinicopathological factors was studied. As shown in Table 2, HCRP1 expression was significantly related to age ($P = 0.017$), pathological grade ($P = 0.003$), tumor encapsulation ($P = 0.037$), recurrence ($P = 0.039$) and death ($P = 0.015$), but unrelated to cirrhosis ($P = 0.216$), tumor size ($P = 0.273$), and distant metastasis ($P = 0.554$).

A Kaplan–Meier curve of RFS and OS, stratified using the HCRP1 expression level, was plotted (Fig. 2), and the log-rank test

Table 1
HCRP1 expression in different liver diseases by histopathology.

		HCRP1 expression, n (%)		
Disease Group	Patients, n	High	Low	*P*-value
HCC	101	59 (58.4)	42 (41.6)	<0.001**
HCA	10	10 (100)	0 (0)	
FNH	8	8 (100)	0 (0)	
Cirrhosis	12	12 (100)	0 (0)	
Normal liver	20	20 (100)	0 (0)	

$\chi 2$ test was used to calculate the statistical significance of the variables. **Overall P-value of HCC. $P < 0.01$ between HCC and HCA, HCC and FNH, HCC and Cirrhosis, HCC and normal liver, individually. HCRP1, Hepatocellular carcinoma related protein 1; HCA, hepatocellular adenoma; FNH, focal nodular hyperplasia; Cirrhosis, large regenerative nodules arising in cirrhotic livers.

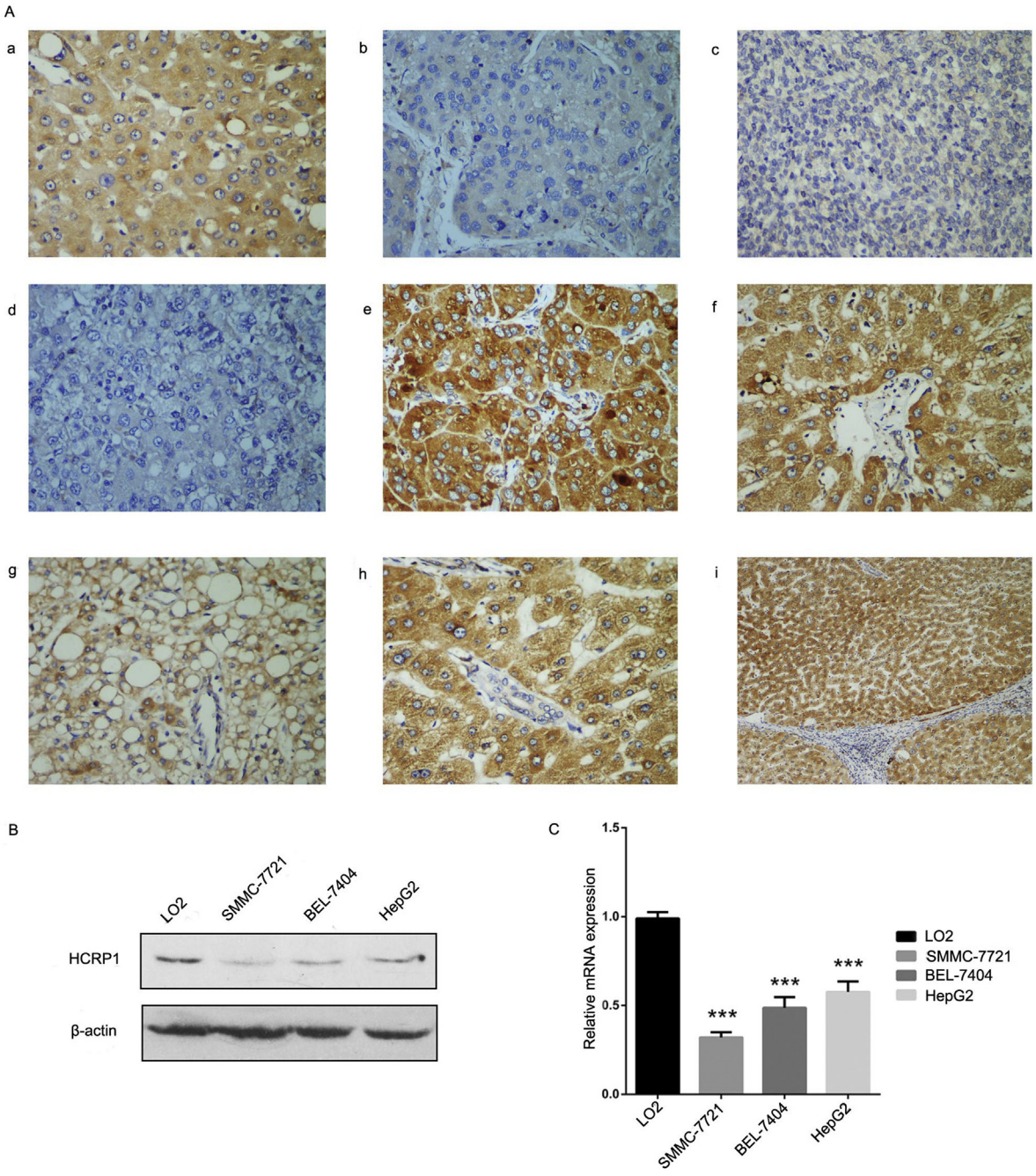

Fig. 1. Expression of HCRP1 in different liver diseases and normal liver tissues by immunohistochemical staining. (A, a) high HCRP1 expression in the well differentiated HCC; (b) low expression in the moderate differentiated HCC; (c and d) low HCRP1 expression in the poor differentiated HCC; (e) high HCRP1 expression in the poor differentiated HCC; (f) high HCRP1 expression in the normal liver tissues; (g) high HCRP1 expression in the HCA; (h) high HCRP1 expression in the FNH; (i) high HCRP1 expression in the cirrhosis liver lesions. (original magnifiation in a, b, d, e, f, g and h, 400×; original magnifiation in c and i, 200×). (B) Western blot analysis of HCRP1 expression in L02, SMMC-7721, Bel-7404 and HepG2 cells. (C). qPCR analysis of HCRP1 expression in L02, SMMC-7721, Bel-7404 and HepG2 cells. *** $P < 0.001$.

presented significant differences between the low and high expression groups ($\chi 2 = 9.959$; $P = 0.005$ for RFS; $\chi 2 = 8.583$, $P = 0.003$ for OS). The five-year survival rates were $43.1\% \pm 6.5\%$ and $67.4\% \pm 7.1\%$ in the low and high expression groups, respectively. In the univariate survival analyses, HCRP1, histological grade, tumor size, tumor encapsulation, and distant metastasis were significantly associated with OS, whereas patient age and cirrhosis did not exhibit any significant correlation. Multivariate survival analysis using the Cox proportional hazards model was then performed for all the significant factors found in the univariate analysis (Table 3). Low HCRP1 expression, as well as high histological grade and capsule involvement, were associated with poor OS, thereby suggesting that decreased expression of HCRP1 could be an independent prognostic marker in HCC.

3.3. *HCRP1 inhibits* in vitro *cell growth and migration/invasion in HCC*

To verify whether HCRP1 modulates cell growth, CCK8 assay was performed. The knockdown and overexpression of HCRP1 were achieved with HCRP1-specific siRNA and HCRP1 lentiviral vector in HepG2 and SMMC-7721 cells, respectively. Empty vectors were used as negative control. As shown in Fig. 3A, the knockdown and expression of HCRP1 in HepG2 cells could effectively boost cell growth, whereas elevated HCRP1 expression in SMMC-7721 cells

Table 2
Correlation of HCRP1 with clinicopathological factors (n = 101).

Factor	Total	HCRP1 Expression		r	P-value
		Low	High		
All case	101	59	42		
Age at surgery					
≥50	72	36	36	0.237	0.017*
<50	29	22	7		
Hepatocirrhosis					
Yes	61	32	29	0.124	0.216
No	40	26	14		
Pathological Grades					
I	17	6	11	−0.295	0.003**
II	62	34	28		
III	22	18	4		
Tumor Size					
<3 cm	26	13	13	−0.110	0.273
3–5 cm	29	16	13		
>5 cm	46	29	17		
Capsule involved					
Yes	45	31	14	−0.208	0.037*
NO	56	27	29		
Distant metastasis					
Yes	29	18	11	−0.060	0.554
No	72	40	32		
Recurrence					
Yes	72	46	26	−0.206	0.039*
No	29	12	17		
Death					
Yes	47	33	14	−0.241	0.015*
No	54	25	29		

* $P < 0.05$.
** $P < 0.01$.

Table. 3
Univariate and multivariate survival analysis of influencing factors (n = 101).

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age at surgery	1.191	0.618–2.295	0.601	–	–	–
Cirrhosis	1.462	0.824–2.597	0.194	–	–	–
Histological grade	2.115	1.316–3.397	0.002**	1.686	1.031–2.757	0.037*
Tumor Size	1.460	1.005–2.122	0.047*	1.252	0.853–1.837	0.252
Capsule involved	2.688	1.488–4.854	0.001**	2.008	1.096–3.676	0.024*
Distant metastasis	1.876	1.040–3.390	0.037*	1.524	0.831–2.801	0.173
HCRP1	0.407	0.217–0.762	0.005**	0.521	0.274–0.991	0.047*

* $P < 0.05$.
** $P < 0.01$.

demonstrated the opposite effect. A transwell assay was performed to determine whether HCRP1 regulated the migration and invasion of HCC. The results confirmed that HepG2 cells transfected with HCRP1-siRNA exhibited significantly increased migration and invasion as compared to the control groups(Fig. 3B). MMP2 and MMP9 proteins, which play important roles in promoting cancer cell migration and invasion, were evaluated by western blot analysis, which showed that their levels were significantly higher in HCRP1-silenced HepG2 cells than in the control groups. In contrast, HCRP1-infected SMMC-7721 cells exhibited opposite results (Fig. 3C). These data confirmed that the depletion of HCRP1 could promote HCC cell proliferation, migration and invasion *in vitro*.

3.4. HCRP1 knockdown facilitates EGF-induced EGFR activation and inhibits EGFR degradation

HCRP1 is known as a subunit of mammalian ESCRT-I which is vital in the sorting and down-regulation of EGFR, and we found HCRP1 depletion could affect EGFR activation and degradation in breast cancer cells. To further make clear the role of HCRP1 on regulating EGFR activity in HCC, we evaluated phosphorylation status of EGFR in HCRP1 knockdown HepG2 cells upon EGF stimulation and the results showed the level of p-EGFR was significantly increased in HCRP1-silenced HepG2 cells at 3 and 5 min time point than in the control groups (Fig. 4A), suggesting HCRP1 reduction could efficiently trigger the EGF-EGFR signaling activation.

We also detected the effects of HCRP1 on EGFR degradation in HepG2 cells transfected with HCRP1 siRNA which were treated with EGF for 0–3 h. Western blot results showed that the EGFR level were remarkably higher in HCRP1-silenced HepG2 at 2 and 3 h (Fig. 4B), indicating knockdown of HCRP1 could play an important role in suppressing EGFR degradation in HCC cells.

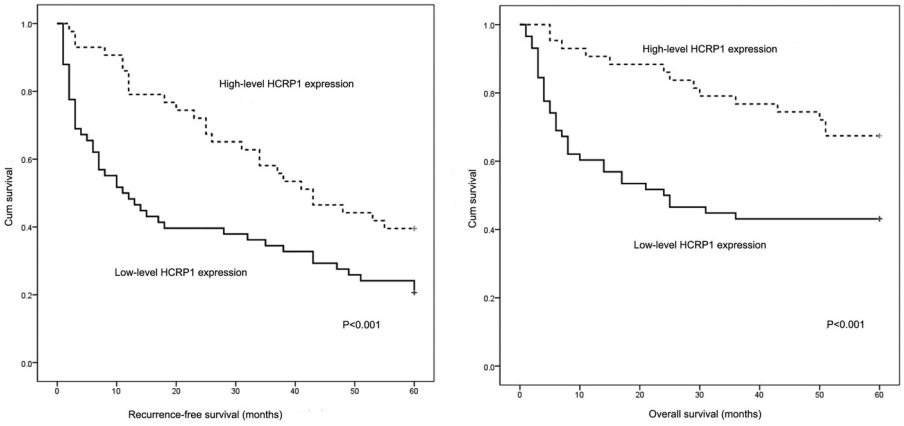

Fig. 2. Kaplan–Merier survival curve of recurrence free survival and overall survival according to HCRP1 expression in HCC patients. $P < 0.001$.

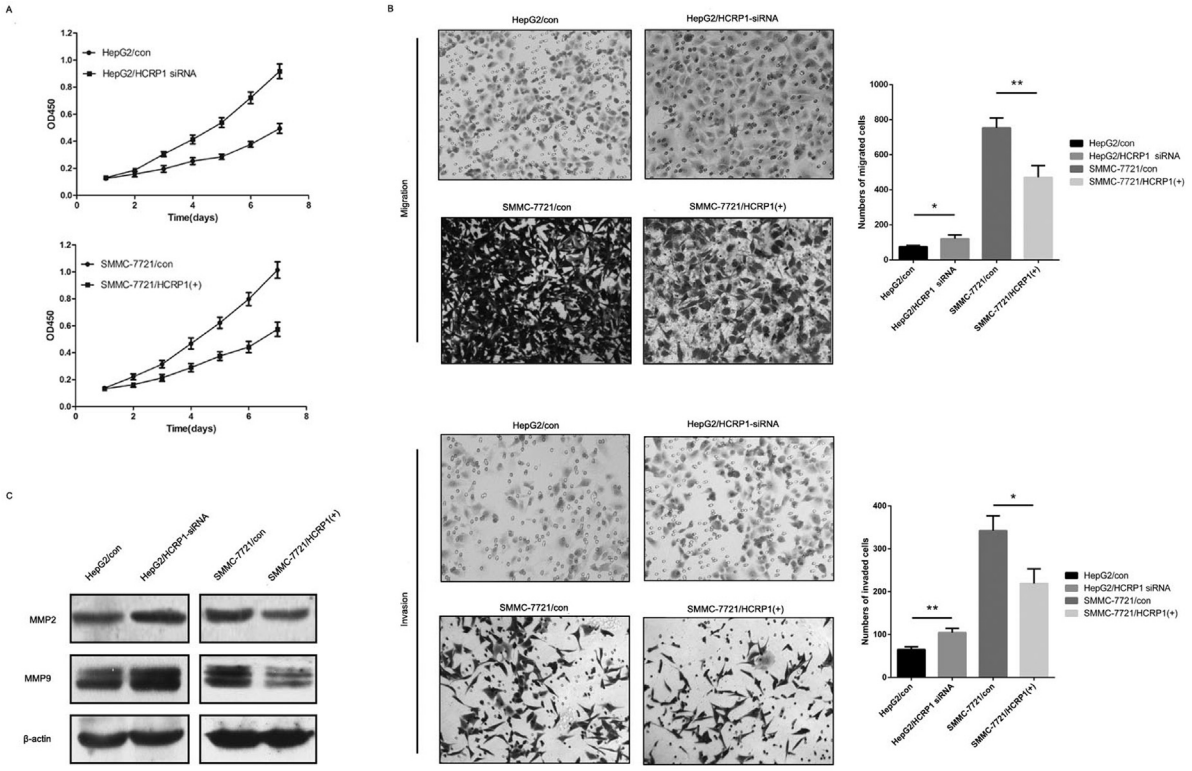

Fig. 3. HCRP1 inhibits *in vitro* cell growth and migration/invasion in HCC. HepG2 and SMMC-7721 cells were transfected with HCRP1 siRNA and infected with HCRP1 lentiviral vector, respectively. (A). Cell growth curve measured by CCK8 assay. (B). Effects of HCRP1 on migration and invasion in HepG2 and SMMC-7721 cells were evaluated by using transwell chamber assays as described before. The bar graphs show the number of cells migrated or invaded in five fields for each experimental group as an average, corresponding to the upper fiures. Statistical analysis was carried out with Dunnett's-test. Each point indicates the mean of spectrometric absorbance ± SEM of three independent experiments.* $P < 0.05$, ** $P < 0.01$. (C) MMP2 and MMP9 protein levels assessed by Western blot analysis. β-actin served as control for sample loading.

3.5. Decreased HCRP1 expression promotes EMT by activating p-Akt in HCC

EMT is a key step in tumor progression that particularly involves changes in cell–cell and cell–matrix interactions. To verify whether EMT participated in promoting malignant behavior in HCRP1-mediated HCC cell progression, we observed the mRNA and protein levels of several representative markers of EMT, E-cadherin, vimentin, Snail and Twist1 after intervention of HCRP1 expression in HCC cell lines. As shown in Fig. 5A, B, E-

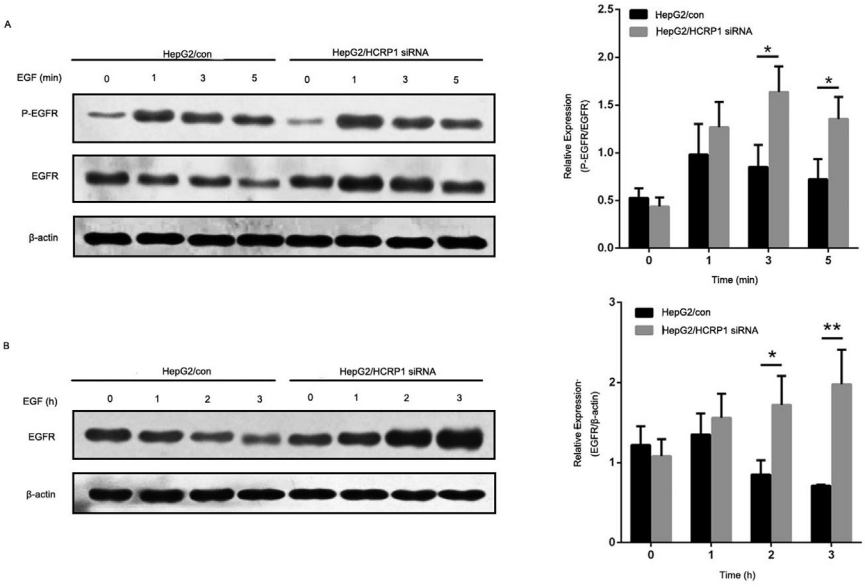

Fig. 4. HCRP1 knockdown promotes EGF-induced EGFR activation and suppresses EGFR degradation. (A) HepG2 and HepG2 HCRP1 siRNA cells were treated with 100 ng/mL EGF for 0, 1, 3, 5 min, the relative expression of p-EGFR/EGFR was significantly higher in HepG2 HCRP1 siRNA cells than control cells after 3 and 5 min. (B) HepG2 and HepG2 HCRP1 siRNA cells were stimulated with EGF for 0–3 h. EGFR expression was higher in HepG2 HCRP1-siRNA cells than control cells after 2 and 3 h. Data are showed as mean ± SEM. * $P < 0.05$; ** $P < 0.01$.

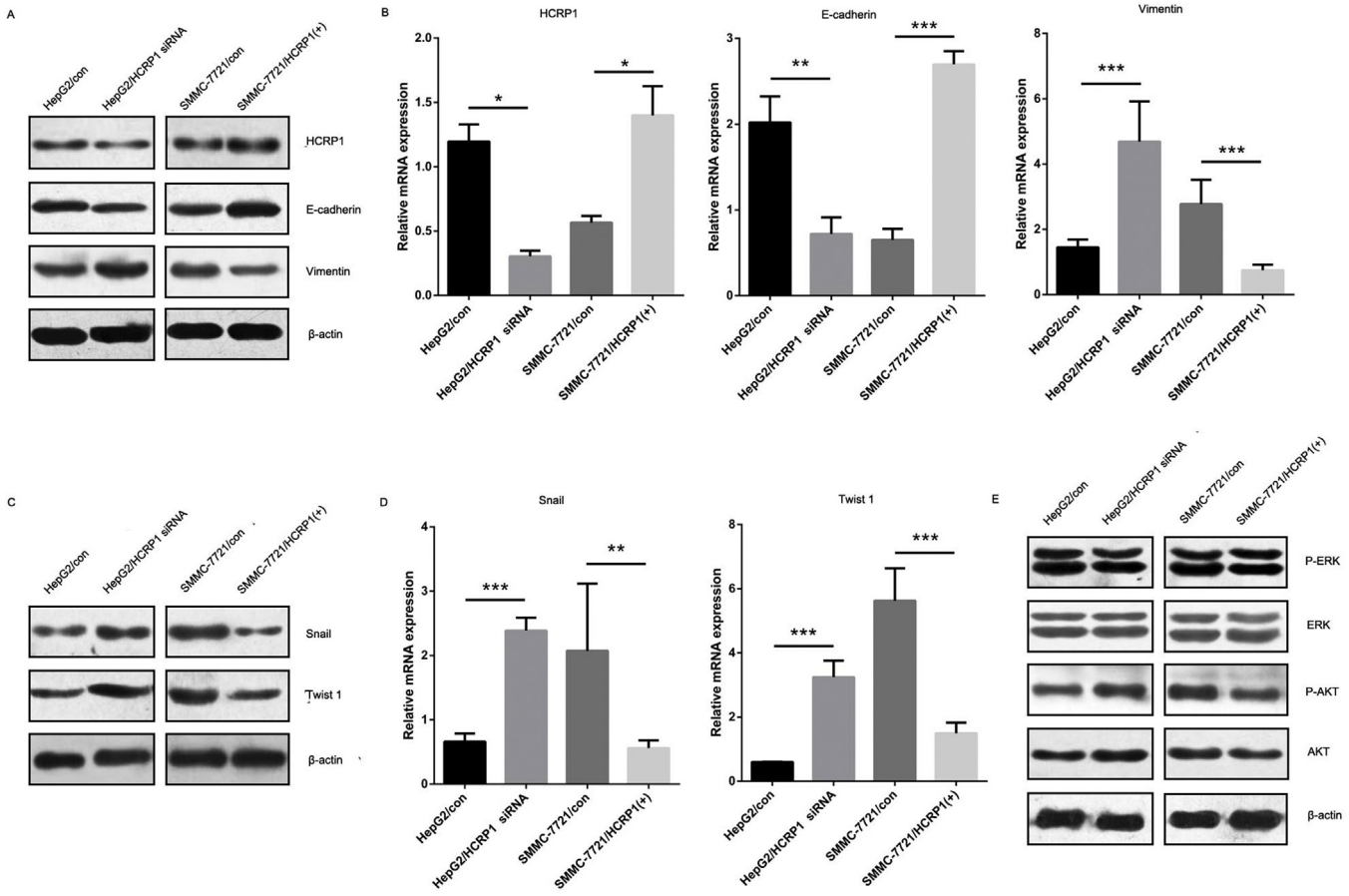

Fig. 5. HepG2 and SMMC-7721 cells were transfected with HCRP1 siRNA and infected with HCRP1 lentiviral vector, respectively. HCRP1, EMT phenotype marker E-cadherin and Vimentin protein levels and mRNA levels assessed by Western blot (A) and qPCR (B) analysis, respectively. EMT key transcription factors Snail and Twist1 protein levels and mRNA levels assessed by Western blot (C) and qPCR (D) analysis, respectively. (E) An increased phosphorylation of Akt in HepG2-HCRP1 siRNA cells and a decreased phosphorylation of Akt in SMMC-7721-HCRP1-infected cell were shown by Western blot analysis. β-actin served as control for sample loading.

cadherin expression was enhanced, whereas vimentin was downregulated in SMMC-7721 HCRP1(+) cells. Significantly reduced levels of Snail and Twist1 were also found in SMMC-7721 HCRP1(+) cells, while the opposite effect was observed in HepG2 HCRP1 siRNA cells. These results indicated that the loss of HCRP1 efficiently induced EMT progression in HCC *in vitro* and may regulate EMT of ICC cells through two transcriptional factors Twist1 and Snail. Moreover, increased phosphorylation of Akt in HepG2-HCRP1 siRNA cells and decreased phosphorylation of Akt in SMMC-7721 HCRP1(+) cells were observed (Fig. 5C), indicating that the PI3K-Akt pathway might be involved in this EMT, whereas the ERK pathway was inactivated during this process.

4. Discussion

HCRP1, located in a high-frequency LOH region of chromosome 8p22-23, is known to be downregulated in several tumors and predicts worse outcome [3–5]. We provided previously the fact that decreased expression of HCRP1 was significantly correlated with increased short-term survival of breast cancer patients by enhancing EGFR phosphorylation [6,7]. Although two studies have reported that HCRP1 may be involved in inhibiting HCC cell growth [3,8], whether its protein level is a predictor remains unknown and the underlying mechanism has not yet been explored.

In this study, we detected the protein level of HCRP1 in 101 HCC cases of which 59 presented decreased expression, which is consistent with the results of Lai [8]. Furthermore, we first observed the HCRP1 protein level in other common benign liver mass lesions, such as HCA, FNH, and large regenerative nodules arising in cirrhotic livers. Surprisingly, all the benign cases were strongly positive for HCRP1 by immunostaining and none demonstrated HCRP1 expression depletion, indicating that low expression of HCRP1 protein could be used as a surrogate marker in differentiating HCC from benign liver lesions in routine clinical practice, especially in the fine-needle aspiration samples. In accordance with the tissue findings, the mRNA and protein levels were significantly reduced in HCC cell lines as compared to L02 cells, and the most aggressive cell line (SMMC-7721) exhibited the lowest expression level. Downregulation of HCRP1 is an independent prognostic factor in oropharyngeal and breast cancers [4,6]. In the present study, patients with decreased HCRP1 expression had significantly shorter RFS and OS. In addition, both univariate and multivariate survival analyses strongly indicated that reduced expression of HCRP1 in HCC was a prognostic factor for reduced survival. Moreover, HCRP1 expression was significantly related to young age as well as high pathological grade, capsule invasion, recurrence, and death. Notably, we found that the low expression rates of HCRP1 were 35.3%, 54.8%, and 81.8% for histological grades I, II, and III, respectively. The expression of HCRP1 tends to be significantly lost as tumor differentiation becomes increasingly

poor. The findings also suggest that downregulation of HCRP1 may play an important role in promoting malignant progression of liver cells.

Cell viability is a vital predictor of survival in HCC patients [13]. To verify whether the proliferation of HCC cells can be regulated by HCRP1, we selected SMMC-7721 and HepG2 (with endogenous low and high expression of HCRP1 respectively) cells as counterparts, and then upregulated and downregulated HCRP1 expression, respectively. The results of the CCK8 assay demonstrated that a reduction in HCRP1 expression in HepG2 cells significantly promoted cell viability, whereas the upregulation of HCRP1 expression efficiently inhibited cell proliferation in SMMC-7721 cells. The results were consistent with the finding of Xu [3], who determined that upregulating the expression of HCRP1 in SMMC-7721 cells could significantly reduce the number of colonies through an anchorage-independent growth assay. Our study also confirmed that HCRP1 was essentially a growth-inhibitory protein.

Hepatocellular carcinoma (HCC) is known to frequently metastasize to various organs. Therefore, the capabilities of HCRP1 in HCC invasion and migration were detected. We knocked down HCRP1 expression in HepG2 cells *via* siRNA transfection and found that the migration/invasiveness of transfected cells increased significantly *via* transwell chamber assays, whereas elevated HCRP1 expression in SMMC-7721 cells exhibited the opposite effect. MMP2 and MMP9 are the most important secreted matrix metalloproteinases (MMPs) for enhancing tumor metastasis in breast, gastric and colorectal cancers [14–16]. We observed the expressions of MMP2 and MMP9 after silencing HCRP1 expression in HepG2 cells, and found that both proteins were highly upregulated. In contrast, atopic HCRP1 expression in SMMC-7721 cells exhibited the opposite result. This finding indicated that HCRP1 deletion might play a role in enhancing MMP expression, and consequently facilitate HCC cell migration/invasion.

It has been shown that EGFR is involved in regulating cell growth in a variety of solid tumor types and the activated form p-EGFR is correlated with increased tumor growth and invasion as well as poor prognosis [17] To the best of our knowledge, EGFR is activated in 40–70% of HCC and correlates with pathogenesis and worse outcome of HCC [18]. HCRP1, a subunit of mammalian ESCRT-I which is essential for lysosomal sorting of EGFR for degradation, as we observed before, its depletion could effectively inhibit EGFR degradation in breast cancer, whereas, it has not yet been fully investigated in HCC. In the present work, we found that knockdown of HCRP1 could efficiently enhance EGF induced EGFR activation and counteract EGFR degradation in HepG2 cells, indicating HCRP1 maybe a key factor in regulating EGFR degradation and might be considered as a potential target modulator in EGFR therapeutically area.

EMT, which is crucial for enhancing the invasiveness and metastasis of cancer cells, can be triggered by variations in tumor microenvironment, inflammatory mediators, and protein products produced by the tumor cells [19,20]. HCRP1 has not been reported to be involved in regulating EMT and function in human cancers. In this study, after the downregulation of HCRP1 in HepG2 cells, the epithelial marker E-cadherin was significantly decreased, whereas the mesenchymal marker vimentin was increased. Snail and Twist1, which are well-known for their ability to trigger EMT through repression of E-cadherin, were also increased after depletion of HCRP1 expression. Recent studies have reported that Ras/ERK and PI3 K/AKT signaling could be involved in the regulation of EMT programs [21]. In our work, knockdown HCRP1 efficiently increased phosphorylation of Akt in HepG2 cells whereas the phosphorylation level of ERK remained unchanged. Herein, our data suggested that HCRP1 may be involved in Akt activation pathways in EMT, however, this still needs further investigation.

In summary, our data indicated that HCRP1 was significantly associated with the recurrence and metastasis of HCC, and was an independent predictor of DFS and OS. Moreover, downregulation of HCRP1 could promote EGFR activation and EMT in HCC, and also migratory and invasive abilities, by activating the Akt signaling pathway. These results may provide novel prognostic and predictive factors for HCC as well as guidelines for designing HCRP1-targeted therapeutic strategies against HCC.

Conflict of interest

None.

Acknowledgements

This work was supported by grants from the Shandong Province Key Research and Development Projects (No.2015GSF118015), China Postdoctoral Science Foundation (No.2015M572053), National Natural Science Foundation of China (No.81202092), and the Natural Science Foundation of Shandong Province (No.2012HM079, No.2012HM033).

References

[1] W. Chen, R. Zheng, P.D. Baade, et al., Cancer statistics in China, CA: Cancer J. Clin. 115–132 (66) (2015) 2016.
[2] Abstracts of the 64th Annual Meeting of the American Association for the Study of Liver Diseases: the Liver Meeting 2013. November 1–2, 2013. Washington, D.C., USA.H epatology 58 Suppl 1(2013) 208A-1376A.
[3] Z. Xu, L. Liang, H. Wang, T. Li, M. Zhao, HCRP1, a novel gene that is downregulated in hepatocellular carcinoma, encodes a growth-inhibitory protein, Biochem. Biophys. Res. Commun. 311 (2003) 1057–1066.
[4] C. Perisanidis, B. Savarese-Brenner, T. Wurger, HCRP1 expression status is a significant prognostic marker in oral and oropharyngeal cancer, Oral Dis. 19 (2013) 206–211.
[5] M. Wittinger, P. Vanhara, A. El-Gazzar, hVps37A Status affects prognosis and cetuximab sensitivity in ovarian cancer, Clin. Cancer Res. 17 (2011) 7816–7827.
[6] J. Xu, W. Yang, Q. Wang, Decreased HCRP1 expression is associated with poor prognosis in breast cancer patients, Int. J. Clin. Exp. Pathol. 7 (2014) 7915–7922.
[7] W. Yang, J.G. Wang, Q. Wang, Decreased HCRP1 promotes breast cancer metastasis by enhancing EGFR phosphorylation, Biochem. Biophys. Res. Commun. 477 (2016) 222–228.
[8] M.W. Lai, S.F. Huang, S.M. Lin, Expression of the HCRP1 mRNA in HCC as an independent predictor of disease-free survival after surgical resection, Hepatol. Res. 39 (2009) 164–176.
[9] Y.D. Jung, J.W. Shim, S.J. Park, Downregulation of UHRF1 promotes EMT via inducing CXCR4 in human cancer cells, Int. J. Oncol. 46 (2015) 1232–1242.
[10] I. Ota, T. Masui, M. Kurihara, E.M.T. Snail-induced, promotes cancer stem cell-like properties in head and neck cancer cells, Oncol. Rep. 35 (2016) 261–266.
[11] S. Ingthorsson, K. Andersen, B. Hilmarsdottir, G.M. Maelandsmo, M.K. Magnusson, T. Gudjonsson, HER2 induced EMT and tumorigenicity in breast epithelial progenitor cells is inhibited by coexpression of EGFR, Oncogene 35 (32) (2015) 4244.
[12] S.B. Edge, C.C. Compton, The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM, Ann. Surg. Oncol. 17 (2010) 1471–1474.
[13] Y. Zhang, Y. Wang, Y. Wei, Molecular chaperone CCT3 supports proper mitotic progression and cell proliferation in hepatocellular carcinoma cells, Cancer Lett. 372 (2016) 101–109.
[14] J.W. Xu, Q.Q. Li, L.L. Tao, Involvement of EGFR in the promotion of malignant properties in multidrug resistant breast cancer cells, Int. J. Oncol. 39 (2011) 1501–1509.
[15] S.X. Chen, J.F. Yin, B.C. Lin, Upregulated expression of long noncoding RNA SNHG15 promotes cell proliferation and invasion through regulates MMP2/MMP9 in patients with GC, Tumour Biol. 37 (5) (2016) 6801.
[16] H. Niu, Z. Hu, H. Liu, Long non-coding RNA AK027294 involves in the process of proliferation, migration, and apoptosis of colorectal cancer cells, Tumour Biol. 37 (8) (2016) 1–9.
[17] C. Magkou, L. Nakopoulou, C. Zoubouli, Expression of the epidermal growth factor receptor (EGFR) and the phosphorylated EGFR in invasive breast carcinomas, Breast Cancer Res.: BCR 10 (2008) R49.
[18] S.X. Li, L.J. Liu, L.W. Dong, CKAP4 inhibited growth and metastasis of hepatocellular carcinoma through regulating EGFR signaling, Tumour Biol. 35 (2014) 7999–8005.

[19] X. Long, Y. Ye, L. Zhang, IL-8 a novel messenger to cross-link inflammation and tumor EMT via autocrine and paracrine pathways (Review), Int. J. Oncol. 48 (2016) 5–12.
[20] D.N. Debruyne, N. Bhatnagar, B. Sharma, ALK inhibitor resistance in ALK-driven neuroblastoma is associated with AXL activation and induction of EMT, Oncogene 35 (28) (2016) 3681.
[21] B. Shan, H. Man, J. Liu, TIM-3 promotes the metastasis of esophageal squamous cell carcinoma by targeting epithelial-mesenchymal transition via the Akt/GSK-3beta/Snail signaling pathway, Oncol. Rep. 36 (2016) 1551–1561.