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Original Article

HLJ1 is a novel biomarker for colorectal carcinoma progression and overall patient survival

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Abstract: The implication of HLJ1, a member of the heat shock protein-40 chaperone family, in colorectal carcinoma (CRC) remains unclear. The aim of this study was to determine the dynamic changes of HLJ1 in CRC both *in vitro* and *in vivo*, and the relationship between its level and the survival rate of CRC patients. Both real-time RT-PCR and Western blot were used to detect the expression of HLJ1 in CRC cells, while the distribution of HLJ1 in CRC and its adjacent normal mucosa tissues from CRC patients was determined with immunohistochemistry. Moreover, MTT and *in vitro* invasive assays were performed to determine the effect of HLJ1 overexpression on cell proliferation and invasion of CRC cells. The results indicated that in highly metastatic CRC cells, the HLJ1 expression was lower than that in lowly metastatic ones, and that the overexpression of HLJ1 significantly inhibited CRC cell proliferation and invasion *in vitro*. Interestingly, the HLJ1 expression was significantly down-regulated in CRC or lymphatic metastatic tissues from patient, compared to that in the normal mucosa ($P<0.05$), and the HLJ1 expression was correlated strongly with lymph metastasis, Dukes' stage, and remote metastasis ($P<0.05$). Most surprisingly, patients with a higher HLJ1 level had a better overall survival rate, compared to that in patients with lower HLJ1 level ($P<0.05$). Based on all these findings, we conclude that HLJ1 is a strong tumor suppressor for CRC, and thus the down-regulation of the HLJ1 expression may be used as a biomarker to predict clinical outcome of patients with CRC.

Keywords: HLJ1, colorectal carcinoma (CRC), biomarker, metastasis, survival rate

Introduction

Colorectal carcinoma (CRC) is currently one of the leading causes of cancer death worldwide. The high mortality of this disease is partially due to the lack of an efficient method for an early diagnosis and a well-established prognostic criterion [1, 2]. Thus, to identify biomarkers related to invasion, metastasis, recurrence, and survival of CRC is of a significant importance for us to fight against this devastating disease [3-5].

Heat shock proteins (HSPs) have been classified into six major family members based on their molecular size including Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small HSPs [6, 7]. HSP40/DnaJ (heat shock protein 40) family, detectable in prokaryotes and eukaryotes, is characterized by the presence of the J domain [8]. Among the most extensively studied members of this family is defined as a group of proteins that are orthologs of E. coli HSP40 (DnaJ) protein [7, 9].

HLJ1, first identified from the human liver cDNA library, is a DnaJ-like HSP belonging to the HSP 40 family. It contains the J and G/F domain sequence of the Hsp40 family members [6] To date, many studies of HLJ1 focused on its function and molecular mechanism in lung cancer and hepatocellular cancer [8, 9]. It was reported that transcriptional factor YY1 expression could increase the expression of HLJ1, thus reducing lung cancer and hepatocellular cancer cell invasive capability [8-10]. HLJ1 has been implicated in the inhibition of cell proliferation, anchorage-independent growth, tumorigenesis,

motility, and invasion of NSCLC (non-small-cell lung cancer) [11]. Most importantly, the reduction of the HLJ1 expression in lung cancer cell leads to a down regulation of E-cadherin expression, a central step of the epithelial-mesenchymal transition (EMT) process, in which epithelial cells are converted into mesenchymal cells, and are associated with cancer invasion and metastasis [8, 12]. In addition, in hepatocyte cells, HBV up-regulated the HLJ1 expression via the transcription factor YY1 by modulating its promoter activity [9].

However, the function of HLJ1 in CRC remains unclear. In this study, we evaluated the expressions of HLJ1 in CRC cell lines and tumor tissues from patients, elucidated the prognostic value by correlating HLJ1 expressions with clinical pathologic features and CRC patients' survival, and determined the effect of HLJ1 overexpression on CRC cell proliferation and invasion *in vitro*.

Materials and methods

Patients

A total of 120 patients who underwent surgery at the Affiliated Hospital of Luzhou Medical College during 2007-2010 were selected in this study. All the patients were follow-up visited till Oct. 2013 (median, 35 months). This study was approved by the Ethics Committee of the Affiliated Hospital of Luzhou Medical College. A written-informed consent was signed by study participants and/or their legal guardians.

Real-time RT-PCR

The total RNA was extracted by using Trizol reagent (Invitrogen, Foster City, USA), and cDNA was synthesized using an access RT system (Promega, Madison, WI). RT was carried on 20 min at 42°C. Real-time RT-PCR was performed by using Mx3000P Real-time PCR System (Stratagene, La Jolla, CA), with the following reaction conditions: 95°C for 30 s, followed by 40 cycles of amplification (95°C for 5 s, 60°C for 34 s, and 72°C for 34 s). The relative quantity of the target transcripts in each sample was expressed as fold difference relative to the control, and according to the equation: $\Delta\Delta Ct$ = [Ct(target gene) - Ct(GAPDH)] experimental group - [Ct(target gene)- Ct(GAPDH)] control group. Primer sequence of HLJ1 was as follows: QHLJ1-F, 5-CCAGCAGACATTG-TTTTTATCATT-3; reverse primer QHLJ1-R, 5-CCATCCAGTGTTGG-TACATTAATT-3. Human GAPDH gene was amplified as an internal control. Each sample was tested three times.

Western blot analysis

Cells were lysed on ice in RIPA buffer with protease inhibitors and quantified by BCA method. Protein lysates, 50 μg, were resolved on 10% SDS polyacrylamide gel, electrotransferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, USA) and blocked in 5% nonfat dry milk in Tris-buffered saline. Membranes were immunoblotted overnight at 4°C with anti-HLJ1 polyclonal antibody (Proteintech Group, Inc, USA), anti-Actin (Santa Cruz Biotechnology, CA, USA), followed by their respective secondary antibodies. Signals were detected using an enhanced chemiluminescence reaction by MultiImage II (Alpha Innotech, USA).

Immunohistochemistry

Sections were de-paraffined and rehydrated, and the endogenous peroxidase was inhibited with 0.3% H_2O_2 methanol. For antigen retrieval, slides were boiled in 0.01 M, pH 6.0 sodium citrate buffer for 15 min in a microwave oven. After blocked with the 5% normal goat serum, primary anti-HLJ1 polyclonal antibody (1:100, Proteintech Group, Inc, USA) in blocking buffer (1:50) was applied and the slides were incubated at 4°C overnight. The slides were developed with the DAB method, and the sections were counterstained with hematoxylin. All the stained sections were checked and scored separately by two pathologists with a blind fashion. The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The amount of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%), according to the percentage of the positive staining areas. The sum of the intensity and amount scores was used as the final staining score (0-7). The staining of HLJ1 was assessed as follows: (-) means a final staining score of <3; (+) a final staining score of 3; (++) a final staining score of 4; and (+++) a final staining score of ≥5. Tissues that had a final staining score of 3 or higher were considered to be positive. This relatively simple, reproducible scoring method gave highly concordant results between independent evaluators and was used in previous study [1, 2]. An optimal cut off value was identified: tumors with a final staining score 0~+ were classified as tumors with low expression of HLJ1, and tumors with a final staining

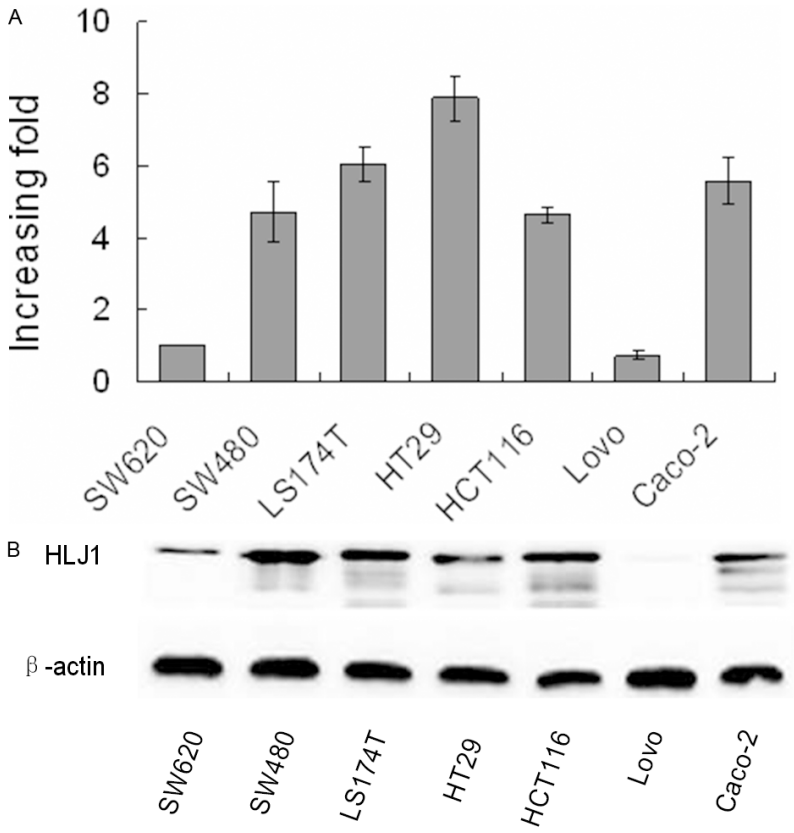

Figure 1. Expression of HLJ1 in six CRC cell lines with different metastatic abilities. A: Real-time RT-PCR analysis showed that the expressions of HLJ1 in SW620 and Lovo cell lines with high metastatic abilities were obviously down-regulated compared with those in other four low metastatic cell lines, and gradually increased in HT29, LS174T, Caco-2, SW480, and HCT116 cell lines. B: Western blot results confirmed the similar changes of down-regulation of HLJ1 protein in high metastatic CRC cell lines.

score ++~+++ were classified as tumors with high expression of HLJ1.

Construct of the HLJ1 Expression Plasmid and transfection

Human HLJ1 cDNA was amplified by PCR using the primers: 5'-GTG TAT GAG CTC ACC ATG GGG AAA GAC TAT TAT TGC-3' and 5'-GAA TAG GGC CCA TTC TATG AGG CAG GAA GAT GTT-3'. PCR products (1014 bp) were cloned into Pegfp-C1 vector containing GFP (Invitrogen). Cells were all transfected with 3 μg of plasmids using Lipofectamine 2000, according to the instructions (Invitrogen). The independent clones resistant to neomycin were picked up and subcultured for 14 days with the G418 selection.

MTT method

The cells were seeded in 96-well plates (1×10^4 cells/mL) with 100 mL cell suspension buffer in each well, and were incubated for 7 days. MTT assay was performed by adding 20 mL of MTT (5 mg/mL; Promega) for 4 h until a purple precipitate was visible. Precipitates were dissolved in 150 mL of dimethylsulfoxide (DMSO). The absorbance value of each well was measured with a microplate reader set at 570 nm. Each experiment was repeated three times.

Plate colony formation test

About 2×10^2 cells were added to each well of a 6-well culture plate. The cells were incubated at 37°C for 14 days, then washed twice with PBS and stained with Giemsa solution. The number of colonies containing 50 cells or more was counted under a microscope (plate clone formation efficiency = [number of colonies/number of cells inoculated] ×100%).

In vitro invasive assay

Invasion Boyden Chamber was inserted with 8 mm-pores in the polyethylene terephthalate membrane (PET), which was coated by matrigel (BD Biosciences, Foster City, CA, USA). First, the invasion chambers were rehydrated with RPMI 1640 (serum-free) for 2 h at 37°C with 50 mL/L CO_2 atmosphere. RPMI 1640 with 100 mL/L fetal bovine serum was added to the lower compartment as the chemotactic factor. Then 1.5×10^5 tumor cells in serum-free RPMI 1640 were added to the upper compartment of the chamber. Each cell group was plated in three duplicate wells. After incubation for 48 h, the noninvasive cells were removed with a cotton swab. Cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed with methanol and stained with hematoxylin. Finally, the cells in a lower compartment of the chamber that had invaded the lower sides of the PET membrane

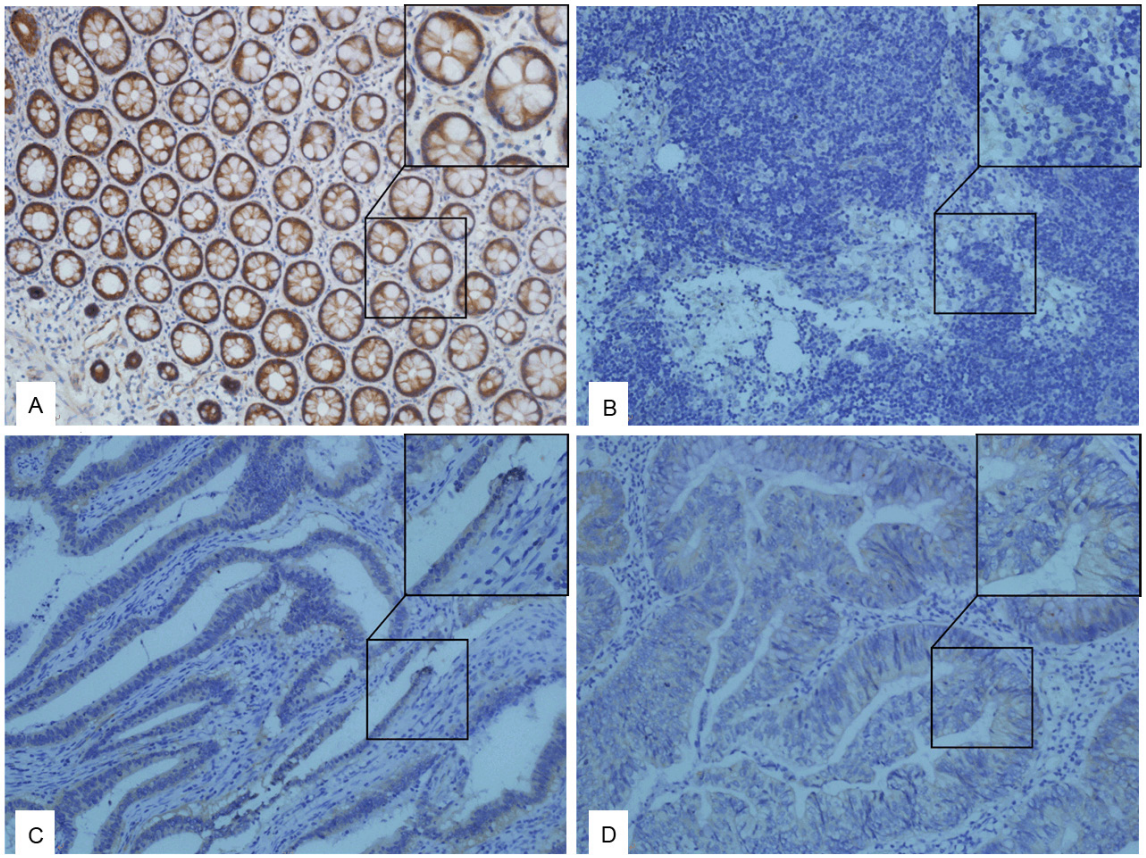

Figure 2. Immunohistochemical staining of HLJ1 in CRC archived tissues, adjacent normal mucosa, and lymphatic metastatic tissues. A: Strong positive staining signal of HLJ1 was observed in adjacent normal mucosa; ×200. B: Weak positive signal of HLJ1 was detected in metastatic CRC tissues in lymph node; ×200. C, D: Weak positive signal of HLJ1 was detected in CRC tissues.

were counted under a light microscope in five random visual fields (200×).

Statistical analysis

All *in vitro* experiments were performed at least in triplicate to confirm reproducibility. Groups were compared with Student's test. The overall survival rate of patients with low versus high expression of HLJ1 was analyzed using the log-rank test. Multivariable Cox proportional hazards regression was performed, with overall survival as the response variable. $P<0.05$ was considered to be statistically significant.

Results

HLJ1 expression in CRC cell lines

Real-time RT-PCR and western blotting analyses were done in CRC cell lines. The results showed that HLJ1 was up-regulated in lowly metastatic CRC cell lines (SW480, LS174T, HT29, HCT116, Caco-2) compared with highly metastatic ones (SW620, LOVO) (**Figure 1**). Although all cell lines expressed HLJ1, the expression levels were fourfold higher in the lowly metastatic CRC cell lines than in the highly metastatic cells, as measured by real-time PCR (**Figure 1A**). HLJ1 protein levels were also markedly higher in the lowly metastatic CRC cell lines than that in the highly metastatic cells, as shown by western blot analysis (**Figure 1B**).

HLJ1 expression in CRC tissues

The HLJ1 expression was determined by immunohistochemistry (IHC) in the 120 surgical specimens of CRC. The expressions of HLJ1 were significantly lower in CRC tissues or lymphatic metastatic tissues than in adjacent normal mucosa respectively (*Z=-9.735, P=0.000; Z=-7.664, P=0.000*; **Figure 2**, **Table 1**). HLJ1

Table 1. Expressions of HLJ1 in CRC tissues, lymphatic metastatic tissues and the adjacent normal mucosa by IHC

Group	HLJ1 expression				Total (*n*)	*P* value
	-	+	++	+++		
Normal mucosa	2	13	39	66	120	
CRC tissues	22	59	33	6	120	0.000†
CRC tissues with lymphatic metastasis	13	30	6	2	51	0.000‡
CRC tissues without lymphatic metastasis	9	29	27	4	69	0.003§
Lymphatic metastatic tissues	11	27	11	2	51	0.437¶

†CRC tissues verse adjacent normal mucosa. *P*=0.000, Z=-9.735. ‡Lymphatic metastatic tissues verse adjacent normal mucosa. *P*=0.000, Z=-7.664. §CRC tissues without lymphatic metastasis verse those with lymphatic metastasis. *P*=0.003, Z=-2.978. ¶Lymphatic metastatic tissues verse CRC tissues with lymphatic metastasis. *P*=0.437, Z=-0.777.

Table 2. Relationship between HLJ1 expressions and clinicopathologic features of colorectal adenocarcinoma patients

Features	N	High expression	Low expression	*P*	x^2
All case (120 cases)	120	39	81		
Age					
<55	46	16	30	0.674	1.777
≥55	74	23	51		
Gender					
Male	69	26	43	0.159	1.987
Female	51	13	38		
Tumor size					
<5 cm	63	19	44	0.565	0.331
≥5 cm	57	20	37		
Differentiation					
Well	29	7	22	0.357	2.059
Moderate	54	21	33		
Poor	37	11	26		
Serosal Invasion					
N	20	7	13	0.794	0.068
Y	100	32	68		
Lymph Metastasis					
N	69	31	38	*0.001*	11.430
Y	51	8	43		
LNR					
N	107	36	71	0.442	0.590
Y	13	3	10		
Dukes' stage					
A+B	62	30	32	*0.006*	14.759
C+D	58	9	49		
Remote Metastasis					
N	94	35	59	*0.035*	4.432
Y	26	4	22		

was obviously down-regulated in CRC tissues with lymphatic metastasis compared with those without lymphatic metastasis (*Z=-2.978, P<0.01*; **Figure 2**, **Table 1**). There was no significant difference of HLJ1 expression between CRC tissues with lymphatic metastasis and lymphatic metastatic tissues (*Z=-0.777, P=0.437*; **Figure 2**, **Table 1**).

HLJ1 expression and clinicopathologic features of patients with CRC

The relationship between clinicopathologic features and HLJ1 expression in CRC was summarized in **Table 2**. No significant associations were found between HLJ1 expression and age, gender, tumor size, differentiation, serosal invasion and LNR. Interestingly, we observed that HLJ1 expression was positively correlated with lymph metastasis (*P*< 0.001), Dukes' stage (*P*<0.01), and remote metastasis (*P*<0.05).

HLJ1 expression and survival of patients with CRC

The prognostic effect of HLJ1 on CRC patients' overall survival was compared between patients with high and low HLJ1 protein levels. By Kaplan–Meier curve assessment, patients with high HLJ1 protein level had a significantly higher 5-year survival rate than those with low HLJ1 protein level (*P*=0.02; **Figure 3**). From univariate analysis, the significant prognostic factors were HLJ1 expression

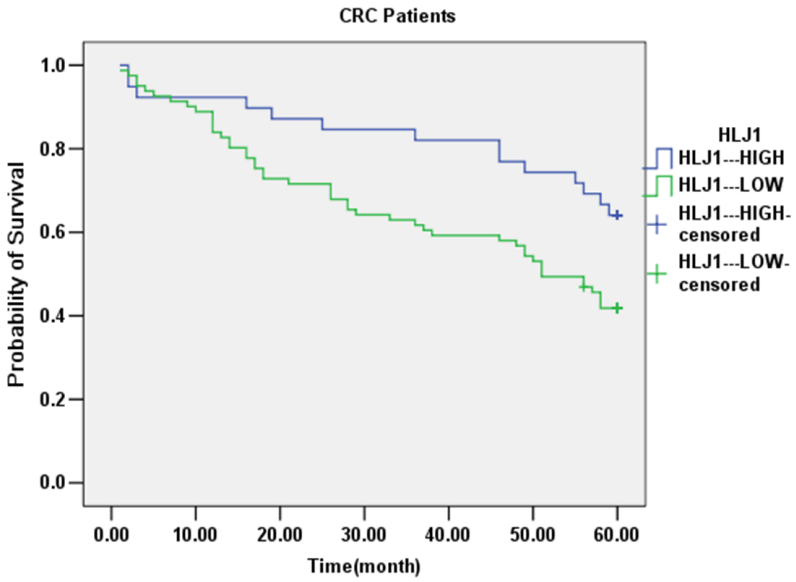

Figure 3. Kaplan–Meier survival analysis of CRC patients. The survival rate for CRC patients in the HLJ1-high expression group was significantly higher than that for patients in the HLJ1-low group (P=0.02).

Table 3. Univariate and multivariate analyses of individual parameters for correlations with overall survival rate: Cox proportional hazards model

variables	Univariate		*P* value
	HR	CI (95%)	
HLJ1	0.502	0.276-0.912	0.024
Age	0.879	0.521-1.483	0.629
Gender	0.807	0.487-1.339	0.407
Tumor Size	0.736	0.443-1.224	0.238
Tumor Grade	0.655	0.463-0.925	0.016
Serosal Invasion	1.771	0.806-3.893	0.155
Lymph Metastasis	3.91	2.062-7.416	0.003
LNR	2.714	1.605-4.591	0
Dukes' stage	2.714	1.605-4.591	0
Remote Metastasis	3.061	1.797-5.214	0
variables	Multivariate		*P* value
	HR	CI (95%)	
Lymph Metastasis	0.393	0.181-0.854	0.018
HLJ1	0.545	0.134-0.452	0.038

(P<0.05), Tumor Grade (P<0.05), lymph metastasis (P<0.01), LNR (P=0.000), Duke's stage (P=0.000), and remote metastasis (P=0.000) (**Table 3**). Multivariate analysis results showed that HLJ1 expression and lymphatic metastasis might play a role in predicting the overall survival in CRC patients (P<0.05; **Table 3**). These results suggest that HLJ1 expression may be an independent prognostic marker for survival of CRC patients.

HLJ1 expression and inhibition of CRC cell proliferation, motility, and invasion in vitro

To further determine the role of HLJ1 in CRC, we performed a series of assays to detect the effect of HLJ1 overexpression on cancer cell proliferation, motility, and invasion *in vitro* (**Figure 4**). MTT assay showed that forced expression of HLJ1 detected by Western blotting (**Figure 4A**) caused a significant decrease of the proliferation rate in SW480 (P<0.05, **Figure 4B**) and HCT116 (P<0.05, **Figure 4C**) cell line. Overexpression of HLJ1 resulted in a more dramatic decrease of the proliferation rate in SW480 or HCT116 cells than in control cells (P<0.05, **Figure 4D**). Boyden Chamber assay showed that the cells penetrating the artificial basement membrane in HLJ1-overexpressing cells were less than in mock cells (P<0.05, **Figure 4E** and **4F**). The above results suggest that HLJ1 negatively regulates proliferation, motility and invasion of CRC cells *in vitro.*

Discussion

HLJ1 has been identified as a member of HSP40/DnaJ (heat shock protein 40) family, which plays important roles in proliferation, anchorage-independent growth, motility, invasion, tumorigenesis, and cell cycle progression [11] Expression of HLJ1 and its prognostic value have been well documented in lung cancer and hepatocarcinoma cells [13, 14]. A lower HLJ1 protein level is a significant prognostic factor

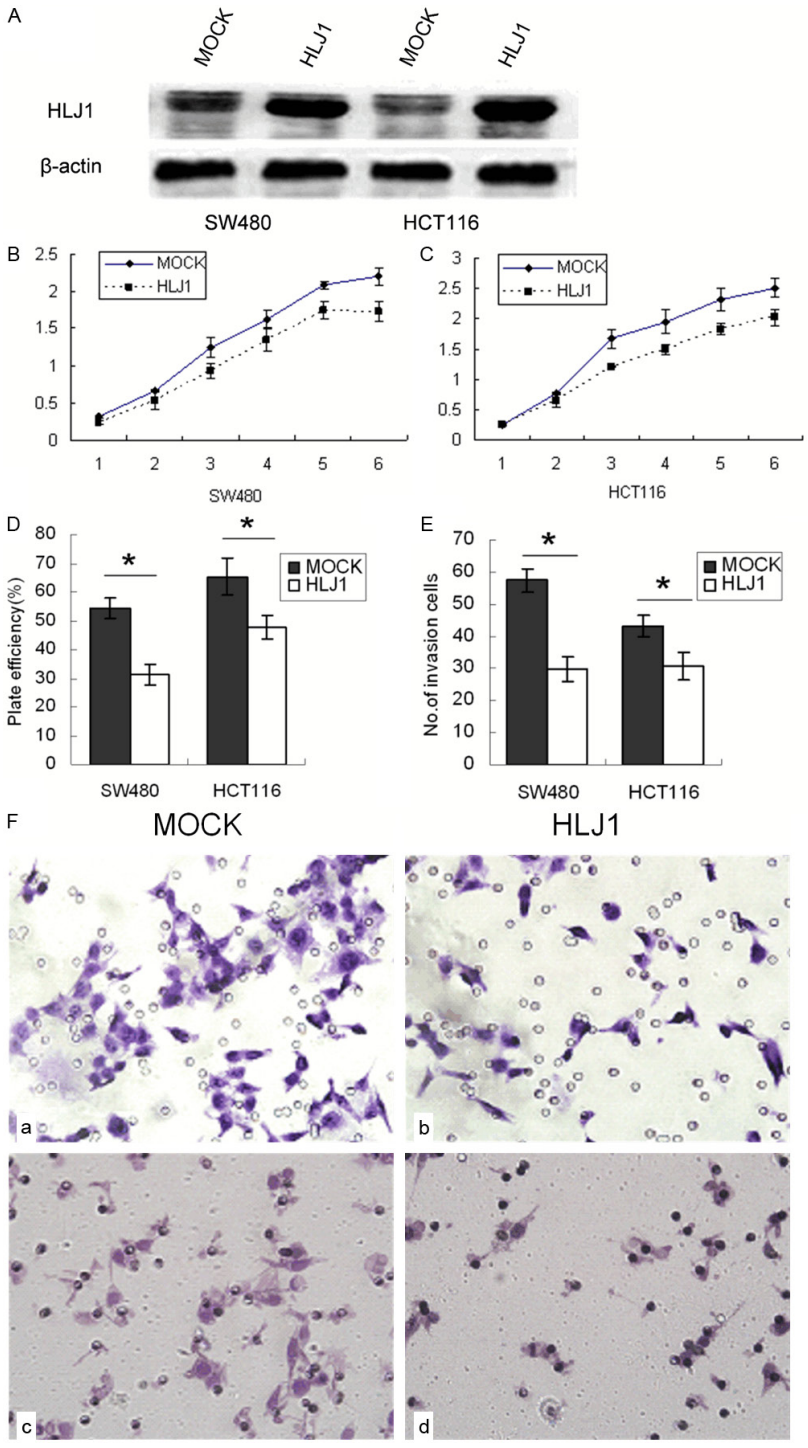

Figure 4. Overexpression of HLJ1 promotes CRC cell proliferation, motility, and invasion in *vitro*. (A) Upregulation of HLJ1 by the HLJ1 Expression Plasmid in SW480 and HCT116 cell lines were analyzed by Western blot. (B, C) Overexpression of HLJ1 inhibited SW480 (B) and HCT116 (C) cell growth as determined by MTT assay. Each bar represents the average ± SD of three independent experiments. (D) Effect of HLJ1 overexpression on the colonic formation abilities of SW480 and HCT116 (*: $P<0.05$). Soft agar colonic formation by the indicated engineered cell lines after 14 days of culture. (E, F) Effect of HLJ1 overexpression on the invasive abilities of SW480 (a, b) and HCT116 (c, d) cells by Boyden chamber after 20 h (*: $P<0.05$; a, b: SW480; c, d: HCT116).

for a poor overall survival in NSCLC patients [15]. In addition, the enhancer-binding AP-1 and promoter-binding YY1 through DNA bending up-regulates the expression of HLJ1, and a correlation of the increase in HLJ1 with E-cadherin expression was detected [8]. In non-small cell lung carcinoma, HLJ1 is a novel substrate of caspase-3 during the UV-induced apoptotic process [16]. Furthermore, HBV could induce the expression of transcription factor YY1, which resulted in the activation of the HLJ1 promoter and subsequently up-regulation of the HLJ1 expression [9]. Thus, the role of HLJ1 in tumor progression remains controversial. Until now, the expression pattern and role of HLJ1 in the progression of CRC have not been illustrated.

In this study, we first detected the expressions of HLJ1 in 6 CRC cell lines and 120 cases of clinical paraffin-embedded CRC tissues with long-term follow-up. The results showed that HLJ1 was down-regulated in highly metastatic CRC cell lines compared with lowly metastatic ones. IHC results showed that HLJ1 expression was significantly lower in CRC tissues, lymphatic metastatic tissues than in adjacent normal mucosa, and positively correlated with lymph metastasis, Duke's stage and Remote Metastasis. Moreover, we demonstrated that low HLJ1 protein level was a significant prognostic factor for poor overall survival in CRC

patients. Our Kaplan–Meier survival analysis revealed that patients with low HLJ1 protein level had a significantly lower 5-year survival rate than those with high HLJ1 protein level. Low HLJ1 protein level was a significant prognostic factor for poor overall survival in CRC patients. By univariate analysis of Cox proportional hazard model, HLJ1 expression and metastasis were associated with an increased risk of death from CRC. Moreover, HLJ1 expression was singled out as one significant and independent prognostic factor relative to overall survival on multivariate analysis.

Since HLJ1 was reported to be involved in invasion and metastasis of liver [9, 10] and lung cancer [8, 10, 11], we speculated HLJ1 might play a role in CRC by suppressing proliferation and invasion of CRC cells. So we performed *in vitro* proliferation and invasion assays to investigate the effect of HLJ1 silencing on CRC cell behaviors. Our results showed that the overexpression of HLJ1 resulted in a more dramatic decrease of the proliferation rate and invasive ability in SW480 or HCT116 cells than in control cells ($P<0.05$). HLJ1 could suppress cell proliferation and invasion of CRC cells *in vitro*.

Overexpression of the HLJ1 gene could reduce lung cancer cell invasive capability with positively regulating E-cadherin expression [8]. Consistent with this notion, recent studies have shown that the expression of HLJ1 correlates inversely with cancer cell invasion ability through a novel STAT1/P21 (WAF1) pathway, accompanied by a decrease in cyclin D1 expression [11]. Lai YH, et al. identified several herbal compounds with the capacity to enhance HLJ1 promoter activity and suppress tumorigenesis and invasion of non-small-cell lung both *in vitro* and *in vivo*, via up-regulating Jun-B activation, which modulates AP-2α binding at the MMP-2 promoter and thus, represses the expression of MMP-2 [13].

In summary, our study shows that the down-expression of HLJ1 is related to the CRC progression. Moreover, we report the role of HLJ1 in predicting the prognosis of CRC patients. HLJ1 suppresses CRC cell proliferation, motility, and invasion *in vitro*. To reveal the exact role played by HLJ1 in CRC progression not only increases our understanding of the biology of CRC but may also provide a novel therapeutic target for clinical CRC patients.

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Disclosure of conflict of interest

None.

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