Epigenetic Identification of Mitogen-activated Protein Kinase 10 as a Functional Tumor Suppressor and Clinical Significance for Hepatocellular Carcinoma

Liping Tang [1] , **Shasha Zhu** [2] , **Weiyan Peng** [3] , **Xuedong Yin** [3] , **Cui Tan** [3] , **Yaying Yang** [Corresp. 4]

[1] Department of Gastroenterology, the First Affiliated Hospital of Chongqing Medical University, Chongqing Medical University, Chongqing, China

[2] The Center for Clinical Molecular Medical Detection, the First Affiliated Hospital of Chongqing Medical University, Chongqing, Chongqing, China

[3] Chongqing Key Laboratory of Molecular Oncology and Epigenetics, the First Affiliated Hospital of Chongqing Medical University, Chongqing Medical University, Chongqing, Chongqing, China

[4] Department of Pathology, Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing, China

Corresponding Author: Yaying Yang
Email address: 100500@cqmu.edu.cn

Background: Mitogen-activated protein kinase 10 (*Mapk10*) is a member of the c-jun N-terminal kinases (*jnk*) subgroup in the MAPK superfamily, and was proposed as a tumor suppressor inactivated epigenetically. Its role in hepatocellular carcinoma(HCC) has not yet been illustrated. We aimed to investigate the expression and epigenetic regulation of *mapk10* as well as its clinical significance in HCC. **Results:** *Mapk10* was expressed in almost all the normal tissues including liver, while we found that the protein expression of MAPK10 was significantly downregulated in clinical samples of HCC patients compared with these levels in adjacent normal tissues (29/46, p<0.0001). Clinical significance of MAPK10 expression was then assessed in a cohort of 59 HCC cases, which indicated its negative expression was significantly correlated with advanced tumor stage (p=0.001), more microsatellite nodules(p=0.025), higher serum AFP(p=0.001) and shorter overall survival time of HCC patients. Methylation was further detected in 58% of the HCC cell lines we tested and in 66% of primary HCC tissues by methylation-specific PCR (MSP), which was proved to be correlated with the silenced or downregulated expression of *mapk10*. To get the mechanisms more clear, the transcriptional silencing of *mapk10* was reversed by pharmacological demethylation, and ectopic expression of *mapk10* in silenced HCC cell lines significantly inhibited the colony formation ability, induced apoptosis, or enhanced the chemosensitivity of HCC cells to 5-fluorouracil. **Conclusion:** *Mapk10* appears to be a functional tumor suppressor gene frequently methylated in HCC, which could be a valuable biomarker or a new diagnosis and therapy target in a clinical setting.

1 **Epigenetic Identification of Mitogen-activated Protein Kinase 10 as a Functional Tumor**
2 **Suppressor and Clinical Significance for Hepatocellular Carcinoma**
3

5 Liping Tang[1], Shasha Zhu[2], Weiyan Peng[3], Xuedong Yin[3], Cui Tan[3] Yaying Yang[4*]
6 [1]Department of Gastroenterology, the First Affiliated Hospital of Chongqing Medical University,
7 Chongqing Medical University, Chongqing, 400016, P.R. China.
8 [2]The Center for Clinical Molecular Medical Detection,the First Affiliated Hospital of Chongqing
9 Medical University, Chongqing, 400016, P.R., China.
10 [3]Chongqing Key Laboratory of Molecular Oncology and Epigenetics, the First Affiliated
11 Hospital of Chongqing Medical University, Chongqing Medical University, Chongqing,400016,
12 P.R. China.
13 [4]Department of Pathology, Molecular Medicine and Cancer Research Center, Chongqing
14 Medical University, Chongqing,400016, P.R. China
15
16
17 **E-maill:**
18 Liping Tang, 547053591@qq.com
19 Shasha Zhu,shasha0310@foxmail.com
20 Weiyan Peng,pwy16@sina.com
21 Xuedong Yin, 13753950@qq.com
22 Cui Tan,tancui0803@163.com
23 Yaying Yang,100500@cqmu.edu.cn
24
25 [*]**Corresponding Author:**
26 Yaying Yang
27 Department of Pathology, Molecular Medicine and Cancer Research Center, Chongqing Medical
28 University, Chongqing,400016, P.R. China
29 Tel: +86- 23-68485789
30 E-mail: 100500@cqmu.edu.cn

32 **Abstract**

33 **Background:** Mitogen-activated protein kinase 10 (*Mapk10*) is a member of the c-jun N-
34 terminal kinases (*Jnk*) subgroup in the MAPK superfamily, and was proposed as a tumor
35 suppressor inactivated epigenetically. Its role in hepatocellular carcinoma (HCC) has not yet
36 been illustrated. We aimed to investigate the expression and epigenetic regulation of *mapk10* as
37 well as its clinical significance in HCC.

38 **Results:** *Mapk10* was expressed in almost all the normal tissues including liver, while we found
39 that the protein expression of MAPK10 was significantly downregulated in clinical samples of
40 HCC patients compared with these levels in adjacent normal tissues (29/46, p<0.0001). Clinical
41 significance of MAPK10 expression was then assessed in a cohort of 59 HCC cases, which
42 indicated its negative expression was significantly correlated with advanced tumor stage
43 (p=0.001), more microsatellite nodules(p=0.025), higher serum AFP(p=0.001) and shorter
44 overall survival time of HCC patients.   Methylation was further detected in 58% of the HCC
45 cell lines we tested and in 66% of primary HCC tissues by methylation-specific PCR (MSP),
46 which was proved to be correlated with the silenced or downregulated expression of *mapk10*. To
47 get the mechanisms more clear, the transcriptional silencing of *mapk10* was reversed by
48 pharmacological demethylation, and ectopic expression of *mapk10* in silenced HCC cell lines
49 significantly inhibited the colony formation ability, induced apoptosis, or enhanced the
50 chemosensitivity of HCC cells to 5-fluorouracil.

51 **Conclusion:** *Mapk10* appears to be a functional tumor suppressor gene frequently methylated in
52 HCC, which could be a valuable biomarker or a new diagnosis and therapy target in a clinical
53 setting.

54 Keywords: hepatocellular carcinoma, *mapk10,* tumor suppressor gene, methylation

Background

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies in humans. It is the sixth most common cancer worldwide and the leading cause of deaths among patients with cirrhosis in Europe and the USA accompanied about 782000 deaths per year[1, 2]. Since the patients with HCC are always diagnosed at the advanced stage and the efficacy of curative surgery or adjuvant therapy including systemic chemotherapy on advanced HCC remains poor[3-5], it is particularly important to find reliable biomarkers in HCC early diagnosis and potential therapeutic targets .

Genetic and epigenetic alterations in regulatory genes are critical in the development of HCC, resulting in the imbalance between oncogenes and tumor suppressor genes (TSG) [6, 7]. For example, promoter CpG methylation, one of the epigenetic alterations, which occurs frequently during tumor development and progression, causes the loss of TSG functions. Accumulating evidence demonstrated that the promoter of CpG methylation was essential in initiating carcinogenesis and the epigenomic changes, and thus, could be applied as a new biomarker for the diagnosis, in clinical trials of epigenetic drugs, and as an add-on to the drugs such as multikinases[8-11]. Furthermore, it was identified some valuable TSGs that were frequently methylated in HCC and established a specific methylation profile that would provide novel reliable biomarkers for HCC management[12] .

MAPK10 (JNK3) encoded by the *mapk10* gene is a member of JNKs, which were implicated in multiple signaling pathways including apoptosis, differentiation, and proliferation[13, 14]. Previous studies showed that JNK contributed to the proliferation or survival of tumors; nevertheless, it might also act as a suppressor in some tumor cell types, indicating various roles of JNK in tumor progression[15]. Hitherto, the genetic and mechanistic basis for the complex role of JNK in tumors is yet to be elucidated.

Loss of heterozygosity (LOH) at specific chromosomal regions in human tumors suggests the presence of candidate TSGs in the affected region. A deletion at 4q21.3 locus was identified in two esophageal carcinoma cell lines by high-resolution (1 Mb) array-comparative genomic hybridization (CGH); as a result, *mapk10* was found to be localized within this deleted region[16] . Other reports demonstrated that the frequent loss of expression of *mapk10* in 10/19 human cell lines originated in brain tumors and the JNK3 signaling pathway mediated cell apoptosis in the central nervous system in *jnk*3-deficient mice[17, 18]. Moreover, the effect of *Jnk* deficiency on Ras-stimulated transformation resulted in a substantial increase in the number and growth of tumor nodules in vivo[19] , which was consistent with the conclusion that *mapk10* plays a role in tumor suppression. Furthermore, previous studies showed that the expression of *mapk10* was frequently downregulated or silenced in several tumor cell lines such as lymphoma, breast cancer, gastric cancer, in which, the methylation of *mapk10* promoter is correlated with its expression[20]. Also, the ectopic expression of *mapk10* markedly suppresses proliferation of breast cancer cells [16].In conclusion, these results suggested that *mapk10* might function as a TSG to suppress the tumorigenesis with respect to epigenetics.

94   As far as we know, expression and role of *mapk10* in HCC have not been illustrated.
95 In our experiment, the expression of *mapk10* was observed downregulated in HCC
96 tissues, which dramatically correlated with the clinical characteristics and the overall survival

97 (OS) of HCC patients. Furthermore, the experiments showed that promoter methylation was

98 responsible for the *mapk10* downregulation in HCC cell lines and tumor tissues, and the
99 pharmacological demethylation and ectopic expression of *mapk10* in silenced HCC cells could
100 suppress the clonogenicity, induce the cell apoptosis, or enhance the sensitivity of HCC to 5-
101 fluorouracil (5-FU). Overall our finding indicated that *mapk10* be a functional tumor suppressor
102 gene frequently methylated in HCC, suggesting that new therapy targeting *mapk10* holds
103 promise for HCC patients.

104

Materials and methods

105 **Materials and methods**
106 **Tumor cell lines**
107   A series of HCC cell lines (Hep3B, HepG2, huH1, huH4, huH6, huH7, PLC/PRF/5,
108 SNU387, SNU398, SNU423, SNU449, and SNU475) were maintained in Cancer Epigenetics
109 laboratory, Department of Clinical Oncology, PWH, The Chinese University of Hong Kong,
110 Shatin, Hong Kong and cultured in RPMI or DMEM (Gibco, USA) supplemented with 10%
111 fetal bovine serum (FBS) (Gibco, USA) and incubated at 37 °C in a humidified chamber
112 containing 5% CO_2.

113

114 **Tissues of HCC**
115   A total of 18 fresh primary HCC tissues were collected from patients who underwent
116 hepatectomy for HCC in 2009 at the First Affiliated Hospital of Chongqing Medical University
117 (Chongqing, China). Among them,10 has the matched non-tumor liver samples, obtained from at
118 least 5 cm distant from the tumor. All of the fresh tissues were processed routinely for
119 histopathological assessment and immediately immersed in liquid nitrogen after surgical
120 resection.
121   Along with the paraffin-embedded tissues, a retrospective cohort of 59 patients with HCC
122 was enrolled in the current study. These patients underwent hepatectomy at the First affiliated
123 hospital of Chongqing Medical University (Chongqing, China) between June 2005 and
124 December 2009. All patients were diagnosed with primary HCC by two pathological doctors,
125 and there was no previous radio- or chemotherapy before surgery. The study was carried out in
126 accordance with the Declaration of Helsinki and the protocol was approved by Ethics Committee
127 of Chongqing Medical University (Chongqing, China) and written informed consent was
128 obtained from each patient involved in the study.
129

130 **Immunohistochemistry (IHC)**
131 IHC staining was carried out by using Ultrasensitive TMS-P (MXB, China). The primary

132 antibody against MAPK10 was obtained from Abcam (Abcam, USA) and incubated the sections
133 at a dilution 1:100. The staining intensity was scored as 0 (absent expression), 1(weak
134 expression), 2 (moderate expression), and 3(strong expression) and the percentage of positive
135 staining as 0 (0%-9%), 1 (10%-25%), 2 (26%-50%) or 3 (51%-100%)[21]. The final score was

136 obtained by multiplying as the following: the staining intensity score $\times$ the percentage of

137 positive staining and   ranked from 0 to 9. So, the *Mapk10* expression of HCC was defined as "-
138 ","+","++"and"+++"corresponding to score 0, 1-3, 4-6 and 7-9 respectively. For further clinical
139 analysis, we divided the HCC into two subgroups: the "*Mapk10*-negative" subgroup ("-") or
140 "*Mapk10*-positive" subgroup"+-+++". The IHC staining score was performed in a double-blind
141 manner by two pathologists to minimize the observational bias.
142
143 **Semiquantitative RT-PCR**
144   RT-PCR was conducted to detect the expression of *Mapk10* in HCC cell lines. RT-PCR was
145 performed for 32 cycles with hot-start Go-Taq (Promega, USA)[22]. The primers are listed in
146 Supplementary Table 1.
147
148 **Western blot**
149   Primary antibodies against Mapk10 (1:000, Abcam, USA) and PARP ((1:000, Abcam, USA)
150 were used. At 48 h post-transfection, the cells were collected and lysed in RIPA lysis buffer
151 (Beyotime, China). Total protein was dissociated by SDS-PAGE, transferred onto PVDF
152 membrane, and immunoblotted. The immunoreactive bands were exposed by the ECL detection
153 system.
154
155 **Methylation-specific PCR (MSP)**
156   After DNA bisulfite treatment, MSP was performed in the HCC cell lines as well as in the
157 primary HCC tumors and adjacent non-tumor tissues as described previously[22]. The MSP primers
158 detecting the methylated or unmethylated alleles are MAPK10m3/MAPK10m5 and
159 MAPK10u3/MAPK10u5, respectively (Supplementary Table 1)[23]. These primer pairs have been
160 tested previously that they did not amplify any unbisulfited DNA. MSP was performed for 40
161 cycles with AmpliTaq-Gold (Applied Biosystems, USA)[22]. YccB1, a cell line of breast
162 carcinoma with hypermethylated MAPK10 which has been demonstrated in the previous
163 literature was used as the positive control of MAPK10 methylation[23].
164
165 **5-aza-2'-deoxycytidine (Aza) and Trichostatin A (TSA) treatment**
166   A density of 1×10^5 HCC cells/mL were allowed to grow overnight. Then, fresh medium
167 containing Aza at a final concentration of 10 mM (Sigma-Aldrich, USA) was used to replace the
168 culture medium. The cells were treated with Aza for 72h, followed by the histone deacetylase
169 inhibitor TSA at 100nM for another 24h. Finally, the cells were collected for methylation-
170 specific PCR (MSP) and the gray value of bands was analyzed by Image J software.
171

Gene cloning and plasmid construction

The *Mapk10* recombinant plasmids were purchased from GeneCopoeia (USA). These were termed as OmicsLink™ expression clone (EX-A1150-M29) and pcDNA3.0(+)-Flag-*MAPK* with the accurately confirmed sequence and orientation.

Subcellular localization

A density of 5×10^4 of Hep3B or HepG2 cells were planted on coverslips in a 6-well plate. Then, the cells were transfected with pcDNA3.0(+)-Flag-*Mapk10* using Lipofectamine 2000 (Invitrogen, USA). After 24h, the cells were fixed with 4% paraformaldehyde phosphate buffer solution and incubated with anti-Flag M2 monoclonal antibody (Sigma, USA), followed by FITC-conjugated rabbit anti-mouse IgG F(ab)2 antibody (Sigma, USA). Hereafter, cell nuclei were stained with DAPI (Thermo, USA) and images were captured by Leica TCS SP2 AOBS confocal laser-scanning microscope (Mannheim, Germany).

Colony formation assay

1×10^5 Hep3B and HepG2 cells/well were seeded in a 12-well plate and transfected with *Mapk10* plasmid or control vector, using Lipofectamine 2000. After 48 h, the cells were seeded into a 6-well plate, and subjected to G418 (0.4 mg/mL) for 10-14 days, with the medium refreshed every 2 days. The surviving colonies were enumerated after Giemsa staining. All the experiments were conducted three times in triplicate wells.

Apoptosis assay

The cell apoptosis and viability were evaluated using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences,USA) by flow cytometry. Apoptosis was always graded as viable, early apoptotic, necrotic and late apoptotic, which corresponds to Annexin V-/7-AAD-,Annexin V+/ 7-AAD-,Annexin V-/7-AAD+ and AnnexinV+/7-AAD+.   Hep3B and HepG2 cells were transfected with *Mapk10* plasmid or the control vector and harvested after 48 h. The GFP-positive cells were selected, and the apoptotic status was assessed by Annexin V-PE and 7-AAD staining. Both early and late apoptotic cells were considered for relative apoptotic alterations. Apoptosis was also depended on the terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay. At 48h post-transfection, the apoptotic cells were detected using the In Situ Cell Death Detection Kit POD (Roche, USA), wherein the DNA fragmentation was determined and visualized by a fluorescence microscope. All the experiments were repeated 3 times.

XTT proliferation assay

HCC is resistant to systemic chemotherapy and 5-FU is one of the most common drugs for HCC. Herein, we assessed whether *Mapk10* could enhance the chemotherapeutic effects of 5-FU on HCC cells. XTT Cell Proliferation Assay kit (Beyotime, China) was used to determine the chemotherapy-induced cytotoxicity on HCC cells. *Mapk10* or control vector was transfected into HCC cells, respectively and treated with 5-FU at different concentrations (0, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) for 72h. Subsequently, the relative number of viable cells between

213   *Mapk10*-transfected group and control vector-transfected group was compared and expressed as
214   a percentage using the following formula: cell viability (%) = A450 of cells treated with 5-
215   FU/A450 of cells without 5-FU treatment. Three independent experiments were performed, each
216   in duplicate.
217

218   **Statistical analysis**
219   The clinicopathological features in MAPK10-positive patients and *MAPK10*-negative
220   patients were compared using the Pearson's chi-squared test. Kaplan–Meier plots and log-rank
221   tests were used for the survival analysis. Student's t-test was used to compare the differences in
222   the effect of MAPK10 expression on colony formation, cell apoptosis, and cell proliferation.
223   SPSS 19.0 was used for data analysis. A p-value <0.05 was deemed as statistically significant.
224

225   **Results**
226   **The protein expression of MAPK10 in clinical samples of HCC and its clinical significance**
227   **in HCC patients**
228   The cohort of 59 patients (Table 1) consisted of 77.9% males with a median age of 49.94
229   (range, 21-77) years. Chronic HBV carriers accounted for the majority (66.2%). The median
230   AFP (α-fetoprotein) value was 5181.8169 (range 2.8-96401) ng/ml. Among all the samples, 46
231   pairs were eligible for the comparison of the expression of *mapk10* between HCC and adjacent
232   non-tumor tissues by IHC. All of the 59 patients with follow-up data were well qualified for the
233   clinical correlation analysis and OS analysis, with the median follow-up for 13.0 (range 2-46)
234   months.
235   As for 46 paired paraffin-embedded tissues, all of the adjacent non-tumor tissues show the
236   positive expression of MAPK10, whereas 29 HCC tissues show the negative expression, the
237   negative expression rate of MAPK10 between the HCC and the adjacent non-tumor tissues was
238   significant (*P*<0.0001) (Figure 1). The clinicopathological correlation analysis showed that the
239   negative expression of MAPK10 in HCC was significantly associated with higher serum AFP
240   (p=0.05), more microsatellite nodules (*P*=0.025) and advanced tumor stage (*P*=0.001) (table 1).
241

242   **Downregulated MAPK10 expression may indicate poor prognosis in HCC patients**
243   Prognostic significance of MAPK10 in HCC was also studied in this cohort of 59 patients
244   with follow-up data, which indicated that the expression of MAPK10 was extremely correlative
245   with OS of patients (log rank=7.123, *P*=0.008): the median OS time in MAPK10-negative
246   subgroup (n=23) and MAPK10-positive subgroup (n=36) were 10.86 months (95% CI 5.927 to
247   15.792) and 24.040 months (95% CI 17.511 to 30.568) respectively (Figure 2). These data
248   suggested that MAPK10 could be an indicator for the prognosis of HCC patients.
249

250   **Expression profiling of *Mapk10* in HCC cell lines**
251   Previous studies found a deletion at 4q21.3 in two esophageal carcinoma cell lines and found
252   that *Mapk10* was located in this deleted region with a 633bp bidirectional promoter, which was a
253   typical CpG island, as well as that *Mapk10* was expressed in all normal adult tissues[23]. However,

254 semi-quantitative RT-PCR in our experiments revealed a frequently reduced or silenced
255 expression of *Mapk10* in HCC cell lines (67%, 8/12) (Figure 3). Together, the above results
256 showed that *Mapk10* is a widely expressed gene but frequently disrupted in multiple HCC cell
257 lines.
258
259 Frequent inactivation of *Mapk10* by promoter CpG methylation
260   In fresh primary tumors, the *Mapk10* promoter was frequently methylated in 12/18 (66%)
261 HCC tissues, while reduced or no methylation was detected in the adjacent non-tumor tissues
262 (Figure 4A), suggesting that the methylation of *Mapk10* is tumor-specific. Furthermore, we
263 detected *Mapk10* methylation in HCC cell lines using MSP and found that the methylation of
264 *Mapk10* is associated with the dysregulated or silenced expression of the gene shown in RT-PCR
265 (Figure 4B).
266
267 Pharmacological and genetic demethylation restores *Mapk10* expression
268   Two HCC cell lines, Hep3B and HepG2, were subjected with DNA methyltransferase
269 inhibitor Aza combined with histone deacetylase inhibitor Trichostatin A (TSA). The treatment
270 restored the *Mapk10* expression, accompanied by decreased methylated promoter alleles (HepG2)
271 or increased unmethylated alleles (Hep3B) (Figure 5). The above results indicated that promoter
272 methylation directly mediated the transcriptional silencing of *Mapk10.*
273   The frequent silencing of *Mapk10* by hypermethylation in HCC cell lines rendered *Mapk10*
274 as a putative tumor suppressor. Furthermore, we evaluated the growth characteristics of the cells
275 overexpressing *Mapk10* by colony formation assay. HepG2 and Hep3B cell lines with silenced
276 *Mapk10* were transfected with *Mapk10* recombinant plasmids, and the number of colonies was
277 enumerated after 10-14-day culture under G418 selection. The subcellular localization of
278 MAPK10 was examined by indirect immunofluorescence staining, which indicated that Flag-
279 tagged MAPK10 was localized in the nucleus after the transfection into HCC cells (Figure 6A)
280 and the western blot detection of MAPK10 protein expression also confirmed the success of
281 transfection (Figure 6B and 6C). The ectopic expression of *Mapk10* markedly decreased the
282 colony formation efficiency of Hep3B and HepG2 cells (34% and 40% respectively, **p<0.01),
283 indicating the growth inhibitory activity of *Mapk10* (Figure 6D and 6E).
284
285 Ectopic expression of *Mapk10* induces apoptosis
286   To explore the mechanism of the tumor suppression by *Mapk10*, we performed apoptosis
287 assay using Annexin V-PE and 7-AAD double staining. The percentage of Annexin V (+/-) and
288 7-AAD (+/-) cells in GFP-positive cells by flow cytometry was determined. In Hep3B cells and
289 HepG2 cells, the ectopic *Mapk10* expression resulted in a significant increase in the number of
290 apoptotic cells as compared to the control (*P*<0.05) (Figure 7A). Furthermore, the apoptotic
291 induction was assessed by TUNEL assay at the individual cell level. Green fluorescent signals,
292 characteristic of cell apoptosis, were presented in *Mapk10*-transfected cells but less in control
293 vector- transfected cells (Figure 7B). Increased protein level of cleaved-PARP was also shown in
294 HepG2 and Hep3B cells after *MAPK10* transduction (Figure 7C).

295

296 ***Mapk10* enhances the chemosensitivity of HCC cells to 5-FU**

297   The efficacy of chemotherapeutic drugs is dependent on their ability to trigger apoptosis [21].
298 Since our studies showed that *Mapk10* induces apoptosis in HCC cells, we speculated the
299 enhanced chemotherapy effects of 5-FU on HCC cells. Notably, the cell viability in *Mapk10*-
300 transfected group was significantly inhibited as compared to that in the control group (Figure
301 8A). Furthermore, the IC50 of HepG2 cells for 5-FU was significantly reduced after *Mapk10*
302 transfection, which was 16.253 μg/mL in the *Mapk10*-transfected group as compared to 251.356
303 μg/mL for the control group (Figure 8B).

304

305 **Figure 8** *MAPK10* enhances the chemosensitivity of HCC cells to 5-FU. **A.** Cell viability assay of Hep3B after
306 transfected with *Mapk10*. **B.** Cell viability assay of HepG2 after transfected with *Mapk10*. (*P<0.01, **P<0.01)

307

308 **Discussion**

309   In the present study, we found that the expression of *Mapk10* is frequently silenced or
310 downregulated in most of the paraffin-embedded HCC tissues (63%) when compared with the
311 adjacent non-tumor tissues. The clinical analysis of the paraffin-embedded tissues also revealed
312 that the negative expression of *Mapk10* was significantly associated with higher serum AFP,
313 more tumor microsatellite nodules, advanced tumor stage, and the shorter OS of HCC patients.
314 Moreover, hypermethylation of *Mapk10* promoter was further detected in most of these primary
315 HCC tumors (66%), and it was also demonstrated that the hypermethylation is correlated with
316 the silenced gene expression of *Mapk10* in several HCC cell lines. Conversely, *Mapk10*
317 hypermethylation was reduced in paired non-tumor tissues, suggesting a role of *Mapk10* as a
318 candidate TSG in the pathogenesis of HCC. The expression of reactivated *Mapk10* could be
319 observed after treatment with the demethylating reagent in silenced HCC cells, indicating that
320 DNA hypermethylation directly mediated the inactivation of *Mapk10*. Furthermore, the ectopic
321 expression of *Mapk10* in silenced HCC cells dramatically inhibited the tumor cell colony
322 formation, induced the cell apoptosis, and enhanced the chemosensitivity of HCC cells to 5-FU.
323 Thus, *Mapk10* was suggested to function as a TSG inactivated epigenetically for HCC and might
324 act as a clinical biomarker for early diagnosis and therapy target.

325   The epigenetic inactivation of TSGs by the hypermethylation of promoter has been
326 recognized as an alternative and critical mechanism in tumorigenesis. Rather than changing the
327 genetic information, DNA methylation only changes the readability of the DNA and results in
328 the inactivation of genes by subsequent suppression of mRNA transcription. As we know, TSG
329 protects the cells from malignant transformation by mechanisms such as apoptosis or cell cycle

330 regulation[24]. Thus, the aberrant hypermethylation of the promoter and epigenetic silencing of

331 TSG affect every step of tumor progression[25–27]. Currently, the transcriptional silencing of TSGs

332 associated with DNA methylation has been shown to be a common epigenetic event in several

333 tumors, including hematologic malignancies, nasopharyngeal carcinomas, gastroenterological

334 neoplasia, esophageal carcinomas, and breast cancers[25, 28–31] . The identification of potential

335 TSGs and specific methylation profiles of TSGs for every specific tumor might be valuable for

336 the diagnosis of tumor and prediction of the prognosis[32].

337   So far, the studies in HCC found some epigenetic silenced TSGs, such as p16, E-cadherin,

338 hMLH1, SOCS1, SOCS, and RASSF1A, which play a role in proliferation, apoptosis, cell

339 adhesion, invasion, and DNA repair[7] . Compared to several other tumors, the prognosis of HCC

340 is poor, and the underlying molecular pathogenesis, including the epigenetic mechanism, is less

341 understood. Most of the HCCs arise on the background of chronic liver disease and chronic

342 hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin, alcohol consumption, and

343 hemochromatosis constitute the major factors associated within HCC initiation[33]. Reportedly, the

344 aberrant methylation of TSG in HCC occurs in a genetic- and disease-specific manner and could

345 be observed not only in advanced tumors but also in premalignant conditions of HCC[7, 12] . Also,

346 studies revealed that HCC and precancerous lesions might present epigenetic signals correlated

347 with the specific risk factors and tumor progression stage, indicating that the epigenetic changes

348 in HCC might be potential targets for biomarker discovery or future therapeutic strategies[34].

349 *Mapk10 (JNK3)* is a third member of JNKs, which belongs to MAP kinases, implicated in

350 critical physiological processes, including proliferation, apoptosis, and differentiation[14, 15].

351 Numerous studies have confirmed that JNK acts as a suppressor of Ras transformation and

352 contributes towards apoptosis in tumors. For example, the activation of the mitochondrial

353 apoptotic pathway in many cell types and the interferon-alpha (IFN-α)-induced apoptosis in B-

354 cell lymphoma were reported[35] . Nevertheless, it might also enhance the proliferation and

355 survival responses of some tumors[15], thereby rendering it as a putative TSG. Thus, the strategies

356 of tumor prevention and therapy necessitate further in-depth investigation. Unlike *JNK1* and

357 *JNK2*, *Mapk10 (JNK3)* is not expressed ubiquitously and performs non-redundant functions[18] .

358 Frequent loss of *Mapk10* expression has been detected in a panel of tumor cell lines[16, 17], together

359 with the proapoptotic functions observed[18, 19], suggesting its potential role as a TSG. Previously,

360 it was identified that *Mapk10 (JNK3)* could be inactivated in multiple lymphomas and

361 carcinomas by epigenetic methylation, albeit only few studies have addressed this concern[16] . In

362 the present study, we focused on HCC. *Mapk10 (JNK3)* was shown to be silenced or

363 downregulated in a majority of the HCC cell lines or primary HCC tumor samples examined,

364 while the demethylating reagent could restore its expression in silenced HCC cells. Further steps

365 indicated that the ectopic expression of *Mapk10* in silenced HCC cells significantly inhibited the

366 tumor cell growth or induced the apoptosis. So our results demonstrated that *Mapk10* served as a
367 TSG in HCC and could be inactivated by promoter methylation in tumorigenesis, which might
368 be one of the mechanisms in HCC progression. However, due to the complexity of gene
369 expression regulation, the additional mechanisms beside methylation may be involved in the
370 regulation of Mapk10 expression and needed for further explored.

371

372   As we know, chemoresistance was facing challenge with respect to the treatment of HCC
373 patients[36].The efficacy of chemotherapeutic drugs has been speculated to depend on the ability to
374 trigger apoptosis[37]. In our research, Since MAPK10 could induce apoptosis in these HCC cells;
375 we speculated that it might also enhance the efficacy of the chemotherapeutic drugs on HCC.
376 Herein, the chemotoxic effects of 5-FU on HCC cells were compared between the *Mapk10*-
377 transfected group and the control vector-transfected group. Consequently, significant
378 enhancement of chemosensitivity of HCC cells to 5-FU was observed in *Mapk10*-transfected
379 group, indicating ectopic expression of *Mapk10* as a novel therapeutic assistant for drug
380 resistance in HCC. Finally, the clinical correlation analysis found that the silenced expression of
381 *Mapk10* in HCC was dramatically correlative with the advanced tumor stage and poor prognosis
382 of HCC patients. This phenomenon supported that *Mapk10* expression might be a valuable
383 molecular biomarker for HCC in a clinical setting.

384

385 **Conclusion**
386   In summary, *Mapk10* is functions as a tumor suppressor and frequently inactivated by
387 promoter methylation in HCC. The downregulated expression of *Mapk10* caused by
388 hypermethylation in HCC tumors is correlated with the advanced tumor stages and   poor
389 prognosis of patients, which hinted that the epigenetic inactivation of *Mapk10* is one of the major
390 mechanisms in tumorigenesis of HCC, along with the evidence that *Mapk10* is a general TSG for
391 HCC. The high incidence of epigenetic inactivation of *Mapk10* in HCC rendered
392 hypermethylation as a reliable molecular biomarker and a potential epigenetic therapeutic target
393 in HCC management.

394

395

396 **Declaration**
397 **Ethics approval and consent to participate**
398 Not applicable.
399 **Consent for publication**
400 Not applicable.
401 **Competing interests**
402 The authors have declared that there were no competing interests.
403 **Funding**
404 This work was supported by the National Natural Science Foundation Committee of China under
405 contract No. 81000907.
406 **Authors' contributions**

407   Liping Tang and Shasha Zhu carried out the studies, participated in collecting data, and drafted
408   the manuscript. Weiyan Peng and Xuedong Yin performed the statistical analysis and
409   participated in its design. Cui Tang and Yaying Yang participated in acquisition, analysis, or
410   interpretation of data and draft the manuscript. All authors read and approved the final
411   manuscript.

412   **Acknowledgements**

413   We greatly thank Professor Qian Tao (Cancer Center, Department of Clinical Oncology, PWH,
414   The Chinese University of Hong Kong, Shatin, Hong Kong) for his kindly help and guidance in
415   the experimental design and methodology.

References:

1. Lombardi A, Grimaldi A, Zappavigna S, Misso G, Caraglia M. Hepatocarcinoma: genetic and epigenetic features. *Minerva Gastroenterol Dietol.* 2018;64(1):14-27.

2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394-424.

3. J Z, A V, JC N, JM L. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. *Gastroenterology.* 2015;149(5):1226-1239.

4. J C, R J, J Z, et al. Potential molecular, cellular and microenvironmental mechanism of sorafenib resistance in hepatocellular carcinoma. *Cancer Lett.* 2015;367(1):1-11.

5. Kudo M, Trevisani F, Abou-Alfa GK, Rimassa L. Hepatocellular Carcinoma: Therapeutic Guidelines and Medical Treatment. *Liver Cancer.* 2017:16-26.

6. Lee SM, Kim-Ha J, Choi WY, et al. Interplay of genetic and epigenetic alterations in hepatocellular carcinoma. *Epigenomics-Uk.* 2016;8(7):993-1005.

7. Tischoff I, Tannapfe A. DNA methylation in hepatocellular carcinoma. *World J Gastroenterol.* 2008;14(11):1741-1748.

8. Nakamura M, Chiba T, Kanayama K, et al. Epigenetic dysregulation in hepatocellular carcinoma: an up-to-date review. *Hepatol Res.* 2019;49(1):3-13.

9. Hardy T, Mann DA. Epigenetics in liver disease: from biology to therapeutics. *Gut.* 2016;65(11):1895-1905.

10. Ozen C, Yildiz G, Dagcan AT, et al. Genetics and epigenetics of liver cancer. *N Biotechnol.* 2013;30(4):381-384.

11. Sceusi EL, Loose DS, Wray CJ. Clinical implications of DNA methylation in hepatocellular carcinoma. *HPB (Oxford).* 2011;13(6):369-376.

12. Nishida N, Nagasaka T, Nishimura T, Ikai I, Boland CR, Goel A. Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. *Hepatology.* 2008;47(3):908-918.

13. Zhang Y, Dong C. Regulatory mechanisms of mitogen-activated kinase signaling. *Cell Mol Life Sci.* 2007;64(21):2771-2789.

14. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell.* 2000;103(2):239-252.

15. Kennedy NJ, Davis RJ. Role of JNK in tumor development. *Cell Cycle.* 2003;2(3):199-201.

16. Ying J, Li H, Cui Y, Wong AH, Langford C, Tao Q. Epigenetic disruption of two proapoptotic genes MAPK10/JNK3 and PTPN13/FAP-1 in multiple lymphomas and carcinomas through hypermethylation of a common bidirectional promoter. *Leukemia.* 2006;20(6):1173-1175.

17. Yoshida S, Fukino K, Harada H, et al. The c-Jun NH2-terminal kinase3 (JNK3) gene: genomic structure, chromosomal assignment, and loss of expression in brain tumors. *J Hum Genet.* 2001;46(4):182-187.

18. Yang DD, Kuan CY, Whitmarsh AJ, et al. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature.* 1997;389(6653):865-870.

19. Kennedy NJ, Sluss HK, Jones SN, Bar-Sagi D, Flavell RA, Davis RJ. Suppression of Ras-stimulated transformation by the JNK signal transduction pathway. *Genes Dev.* 2003;17(5):629-637.

20. Ying J, Li H, Cui Y, Wong AHY, Langford C, Tao Q. Epigenetic disruption of two proapoptotic genes MAPK10/JNK3 and PTPN13/FAP-1 in multiple lymphomas and carcinomas through hypermethylation of a common bidirectional promoter. *Leukemia.* 2006;20(6):1173-1175.

457   **21.** H J, J D, X H, et al. Genetic variants in de novo lipogenic pathway genes predict the prognosis of surgically-
458   treated hepatocellular carcinoma. *Sci Rep-Uk.* 2015;5:9536.

459   **22.** Tao Q, Huang H, Geiman TM, et al. Defective de novo methylation of viral and cellular DNA sequences in
460   ICF syndrome cells. *Hum Mol Genet.* 2002;11(18):2091-2102.

461   **23.** Ying J, Li H, Cui Y, Wong AHY, Langford C, Tao Q. Epigenetic disruption of two proapoptotic genes
462   MAPK10/JNK3 and PTPN13/FAP-1 in multiple lymphomas and carcinomas through hypermethylation of a
463   common bidirectional promoter. *Leukemia.* 2006;20(6):1173-1175.

464   **24.** Hayslip J, Montero A. Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review.
465   *Mol Cancer.* 2006;5:44.

466   **25.** Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet.* 2002;3(6):415-428.

467   **26.** Luo C, Hajkova P, Ecker JR. Dynamic DNA methylation: In the right place at the right time. *Science.*
468   2018;361(6409):1336-1340.

469   **27.** Dor Y, Cedar H. Principles of DNA methylation and their implications for biology and medicine. *Lancet.*
470   2018;392(10149):777-786.

471   **28.** Tao Q, Chan AT. Nasopharyngeal carcinoma: molecular pathogenesis and therapeutic developments. *Expert*
472   *Rev Mol Med.* 2007;9(12):1-24.

473   **29.** Rashid A, Issa JP. CpG island methylation in gastroenterologic neoplasia: a maturing field. *Gastroenterology.*
474   2004;127(5):1578-1588.

475   **30.** Xiang TX, Yuan Y, Li LL, et al. Aberrant promoter CpG methylation and its translational applications in breast
476   cancer. *Chin J Cancer.* 2013;32(1):12-20.

477   **31.** Barbano R, Muscarella LA, Pasculli B, et al. Aberrant Keap1 methylation in breast cancer and association with
478   clinicopathological features. *Epigenetics-Us.* 2013;8(1):105-112.

479   **32.** Kazanets A, Shorstova T, Hilmi K, Marques M, Witcher M. Epigenetic silencing of tumor suppressor genes:
480   Paradigms, puzzles, and potential. *Biochim Biophys Acta.* 2016;1865(2):275-288.

481   **33.** Herath NI, Leggett BA, MacDonald GA. Review of genetic and epigenetic alterations in hepatocarcinogenesis.
482   *J Gastroenterol Hepatol.* 2006;21(1 Pt 1):15-21.

483   **34.** Herceg Z, Paliwal A. Epigenetic mechanisms in hepatocellular carcinoma: how environmental factors
484   influence the epigenome. *Mutat Res.* 2011;727(3):55-61.

485   **35.** Yanase N, Hata K, Shimo K, Hayashida M, Evers BM, Mizuguchi J. Requirement of c-Jun NH2-terminal
486   kinase activation in interferon-alpha-induced apoptosis through upregulation of tumor necrosis factor-related
487   apoptosis-inducing ligand (TRAIL) in Daudi B lymphoma cells. *Exp Cell Res.* 2005;310(1):10-21.

488   **36.** Lohitesh K, Chowdhury R, Mukherjee S. Resistance a major hindrance to chemotherapy in hepatocellular
489   carcinoma: an insight. *Cancer Cell Int.* 2018;18(1):44.

490   **37.** Chen L, Chan HM, Guan XY. Clinical significance of CHD1L in hepatocellular carcinoma and therapeutic
491   potentials of virus-mediated CHD1L depletion. 2011.

492

494 **Figure legends**

495 **Figure 1** Mapk10 protein expression in HCC specimens shown by immunohistochemistry.

496 Positive *Mapk10* staining was observed in adjacent non-tumor tissue. Negative Mapk10 staining

497 in HCC tissue.

498 **Figure 2** Kaplan-Meier survival curves of HCC (n =59) after gastrectomy.

499 The survival rate of patients in the group of negative MAPK10 expression was significantly

500 lower than that of patients in the group of positive expression (log-rank test *P*=0.008).

501 **Figure 3** *Mapk10* mRNA expression in HCC cell lines.

502 **Figure 4** Promoter CpG methylation detection of *Mapk10*. A. *Mapk10* methylation in HCC and

503 adjacent non-tumor tissues. B. *Mapk10* methylation in HCC cell lines. C. *Mapk10* gene

504 expression in HCC cell lines. (MSP: methylation-specific PCR; M: methylated alleles; U:

505 unmethylated alleles)

506 **Figure 5** *Mapk10* expression of HCC cell lines after treatment with TSA and 5-Aza. (MSP:

507 Methylation-specific PCR; M: methylated alleles; U: unmethylated alleles)

508 **Figure 6** MAPK10 inhibits the colony formation of HCC cells. **A.** The subcellular localization of

509 MAPK10 was observed after HCC cell lines were transfected with *Mapk10* recombinant

510 plasmids. **B.** MAPK10 protein expression detected by WB. **C.** Analysis of MAPK10 protein

511 expression. **D.** Colony formation detection. **E.** Colony formation efficiency. (**$P<0.01$)

512 **Figure 7** Ectopic expression of MAPK10 induces apoptosis of HCC cell lines.

513 **A.** Apoptosis assay using Annexin V-PE and 7-AAD double staining. **B.** TUNEL assay. **C.**

514 Protein level of cleaved-PARP detected by WB after the transduction of *Mapk10*.

515 **Figure 8** *MAPK10* enhances the chemosensitivity of HCC cells to 5-FU. **A.** Cell viability assay

516 of Hep3B after transfected with *Mapk10*. **B.** Cell viability assay of HepG2 after transfected with

517 *Mapk10*. (*$P<0.01$, **$P<0.01$)

518

Figure 1

Figure 1 Mapk10 protein expression in HCC specimens shown by immunohistochemistry.

Positive *Mapk10* staining was observed in adjacent non-tumor tissue. Negative Mapk10 staining in HCC tissue.

Figure 2

Figure 2 Kaplan-Meier survival curves of HCC (n =59) after gastrectomy.

The survival rate of patients in the group of negative MAPK10 expression was significantly lower than that of patients in the group of positive expression (log-rank test *P*=0.008).
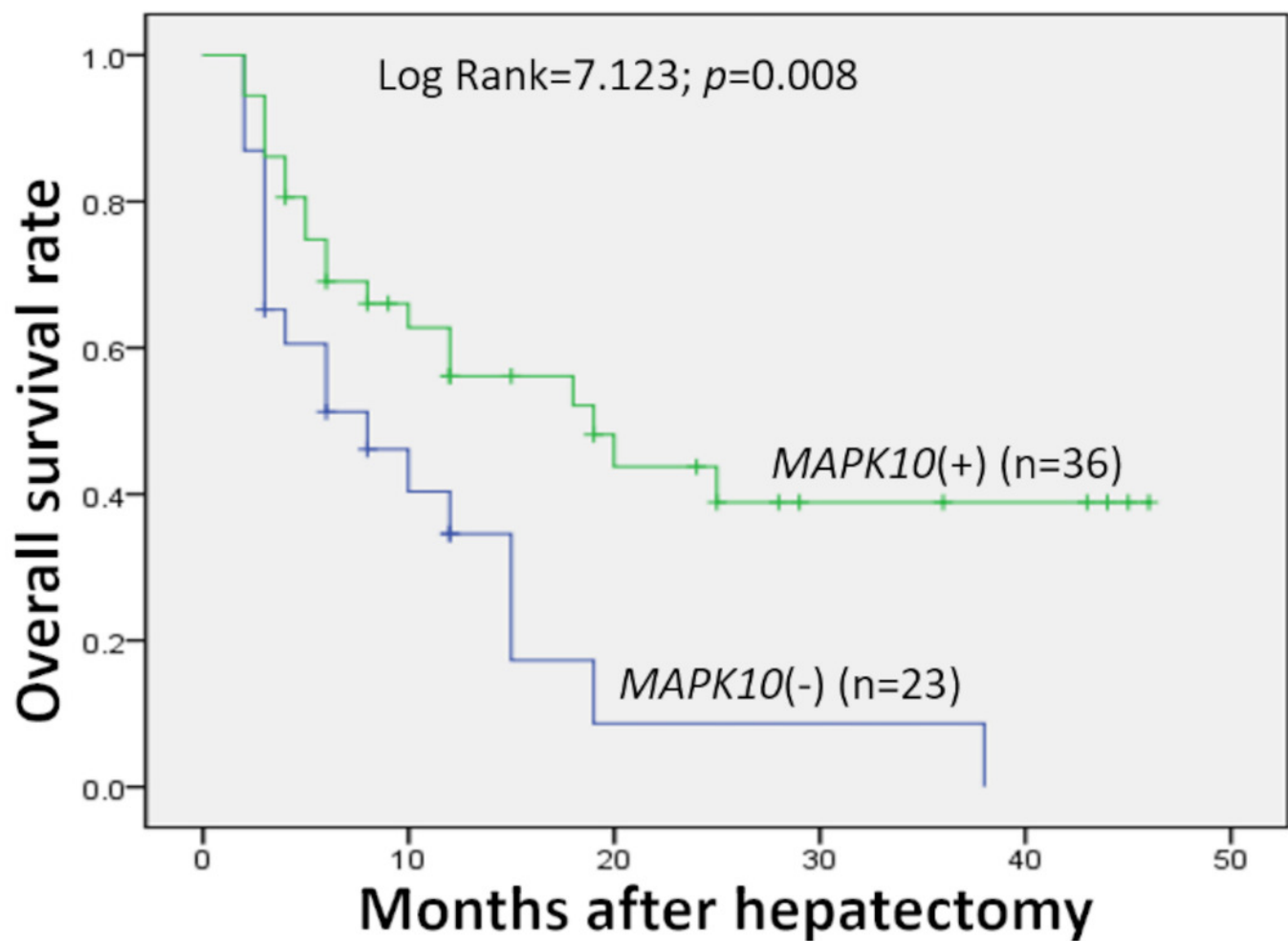
Figure 3

Figure 3 *Mapk10* mRNA expression in HCC cell lines.
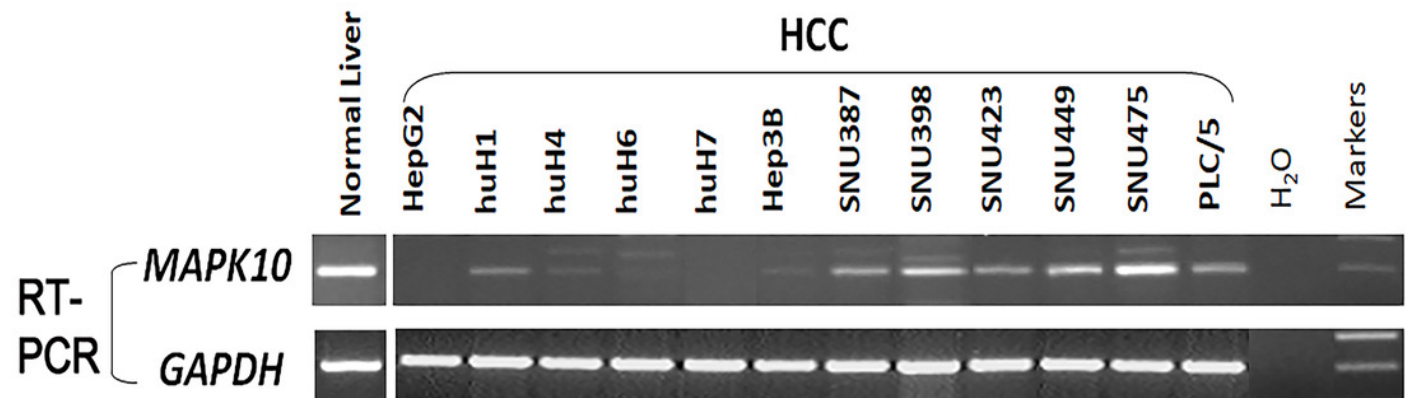
Figure 4

Figure 4 Promoter CpG methylation detection of *Mapk10*.

A. *Mapk10* methylation in HCC and adjacent non-tumor tissues. B. *Mapk10* methylation in HCC cell lines. C. *Mapk10* gene expression in HCC cell lines. (MSP: methylation-specific PCR; M: methylated alleles; U: unmethylated alleles)
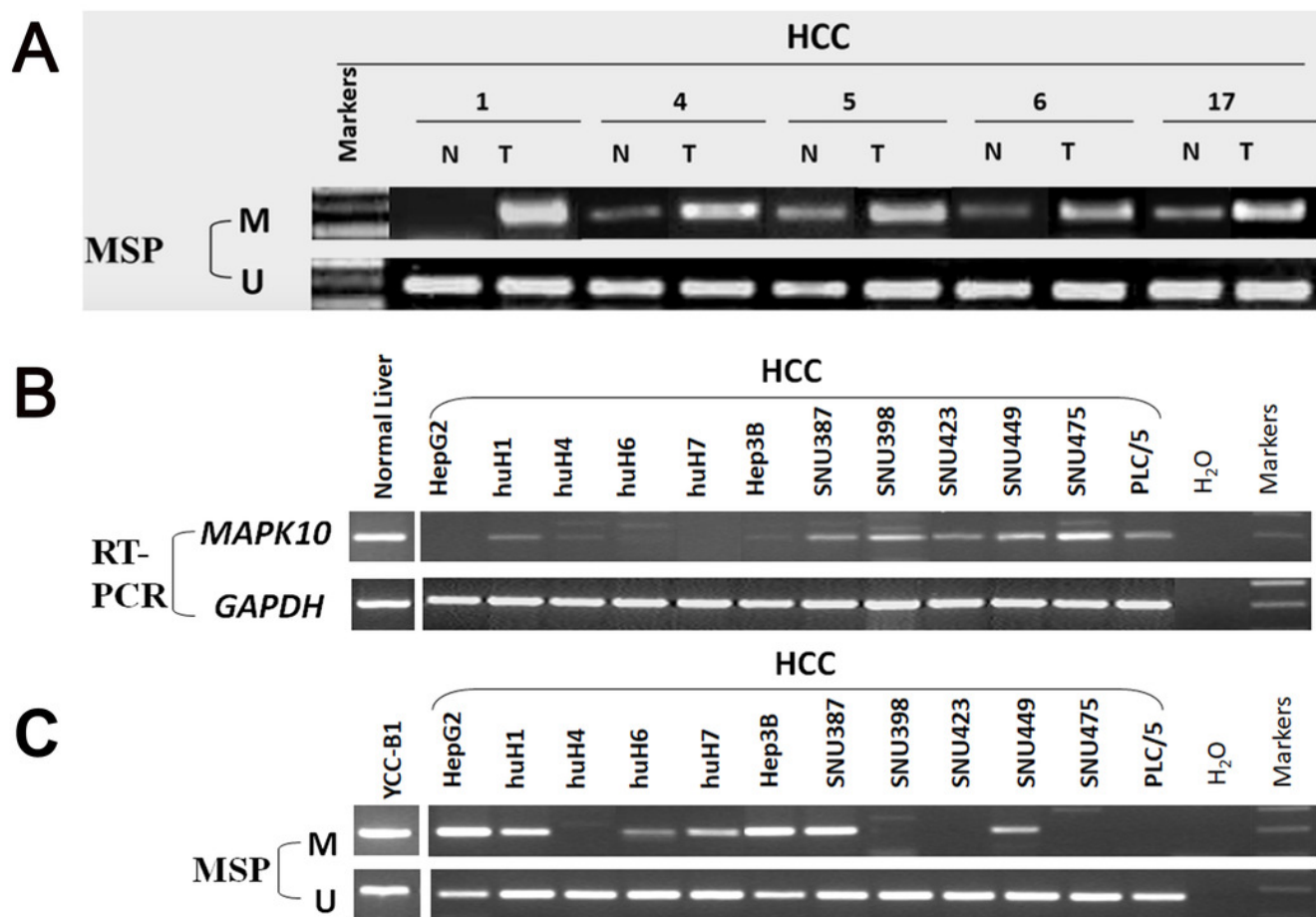
Figure 5

Figure 5 *Mapk10* expression of HCC cell lines after treatment with TSA and 5-Aza. (MSP: Methylation-specific PCR; M: methylated alleles; U: unmethylated alleles)
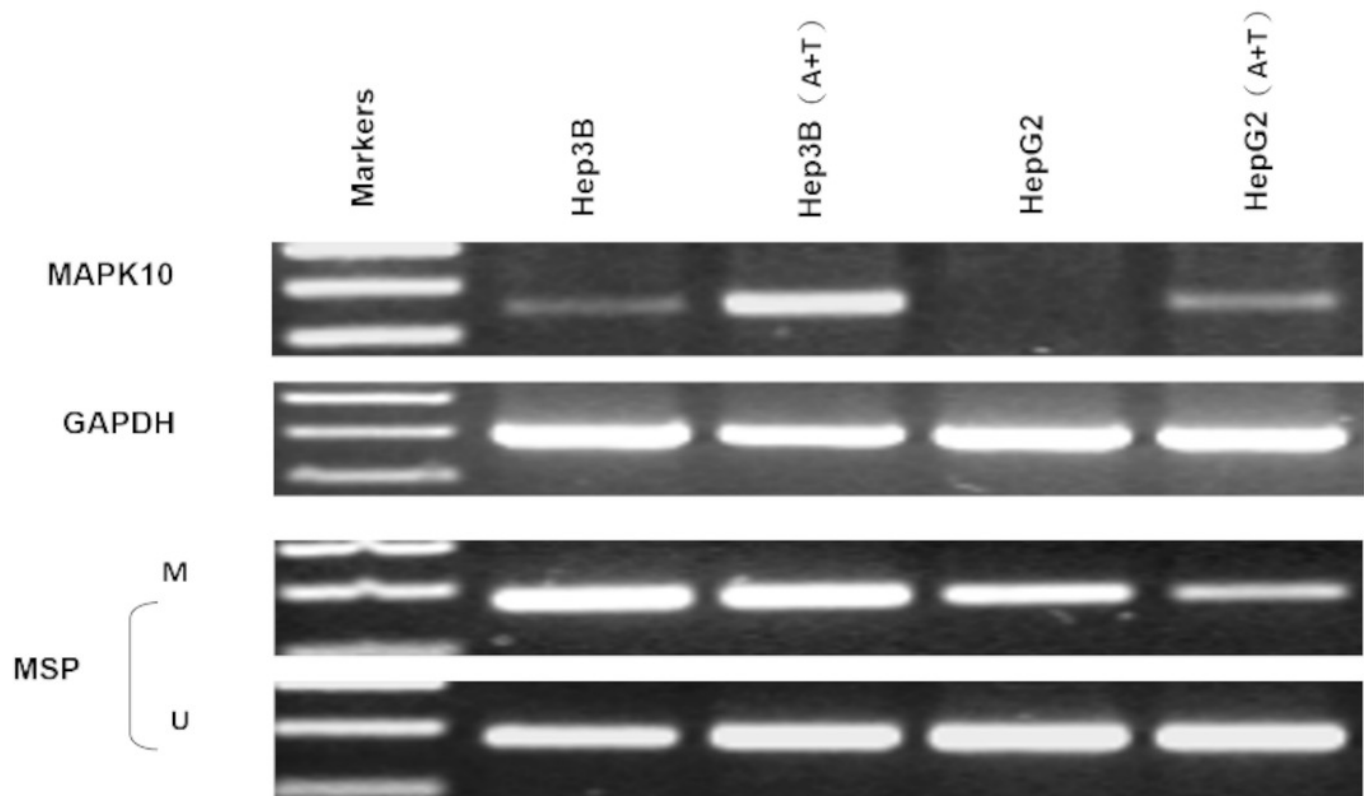
Figure 6

Figure 6 MAPK10 inhibits the colony formation of HCC cells.

A. The subcellular localization of MAPK10 was observed after HCC cell lines were transfected with *Mapk10* recombinant plasmids. **B.** MAPK10 protein expression detected by WB. **C.** Analysis of MAPK10 protein expression. **D.** Colony formation detection. **E.** Colony formation efficiency. (*******P*<0.01)

A

Flag-MAPK10   DAPI   Merged

Hep3B

HepG2

B

	Vector	MAPK10		Vector	MAPK10

MAPK10

GAPDH

Hep3B   HepG2

C

Relative MAPK10 protein expression

□ Control   ■ MAPK10

2.0
1.5
1.0
0.5
0.0

Hep3B   HepG2

*   **

D

Vector   MAPK10

Hep3B

HepG2

E

Relative colony formation rate (%)

□ Vector   ■ MAPK10

150
100
50
0

Hep3B   HepG2

**   **
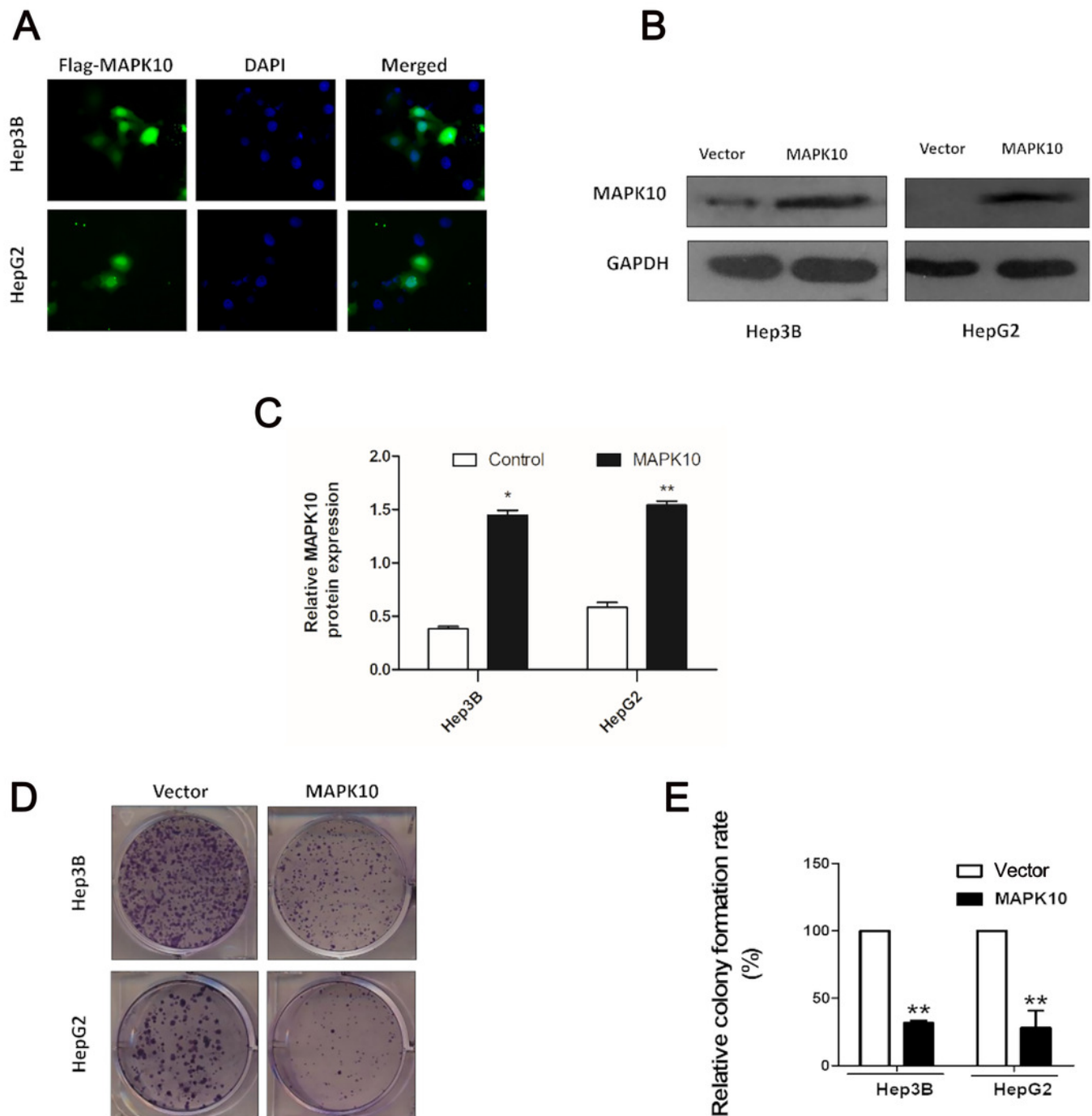
Figure 7

Figure 7 Ectopic expression of MAPK10 induces apoptosis of HCC cell lines.

A. Apoptosis assay using Annexin V-PE and 7-AAD double staining. B. TUNEL assay. C. Protein level of cleaved-PARP detected by WB after the transduction of *Mapk10*.
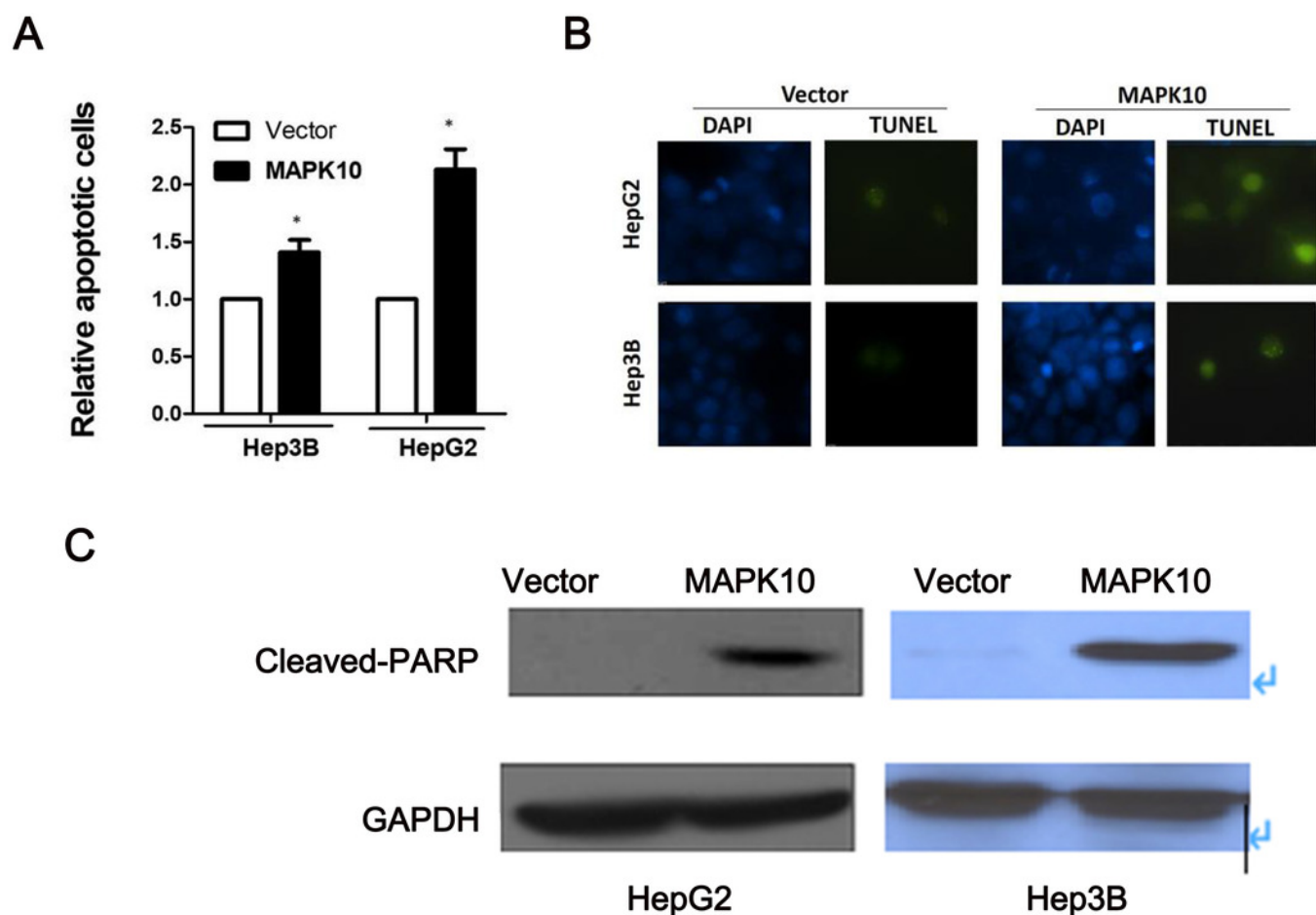
Figure 8

Figure 8 *MAPK10* enhances the chemosensitivity of HCC cells to 5-FU.

A. Cell viability assay of Hep3B after transfected with *Mapk10.* B. Cell viability assay of HepG2 after transfected with *Mapk10*. (*$P<0.01$, **$P<0.01$)

A

B
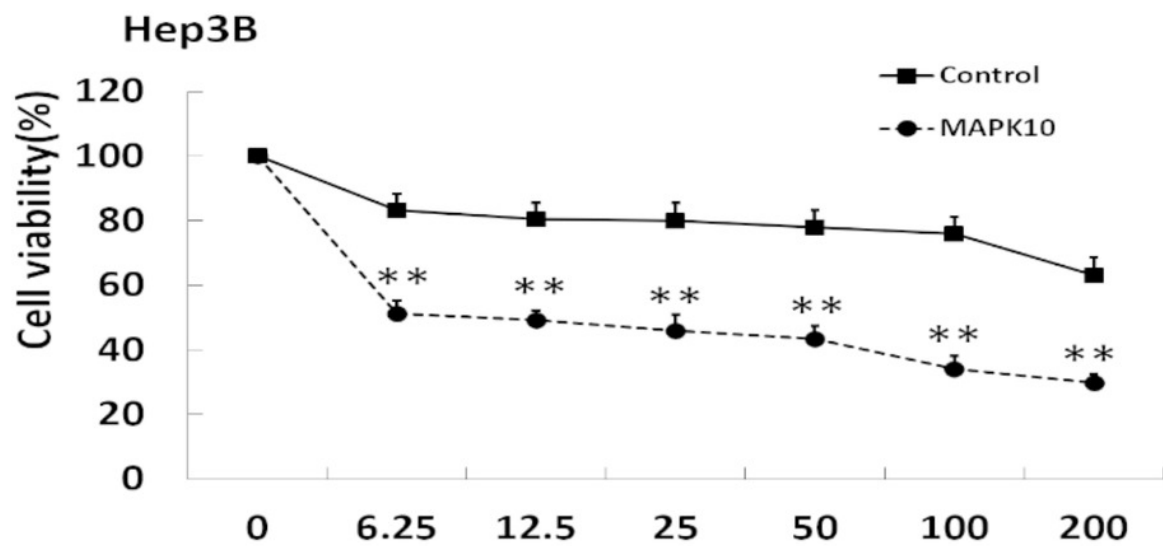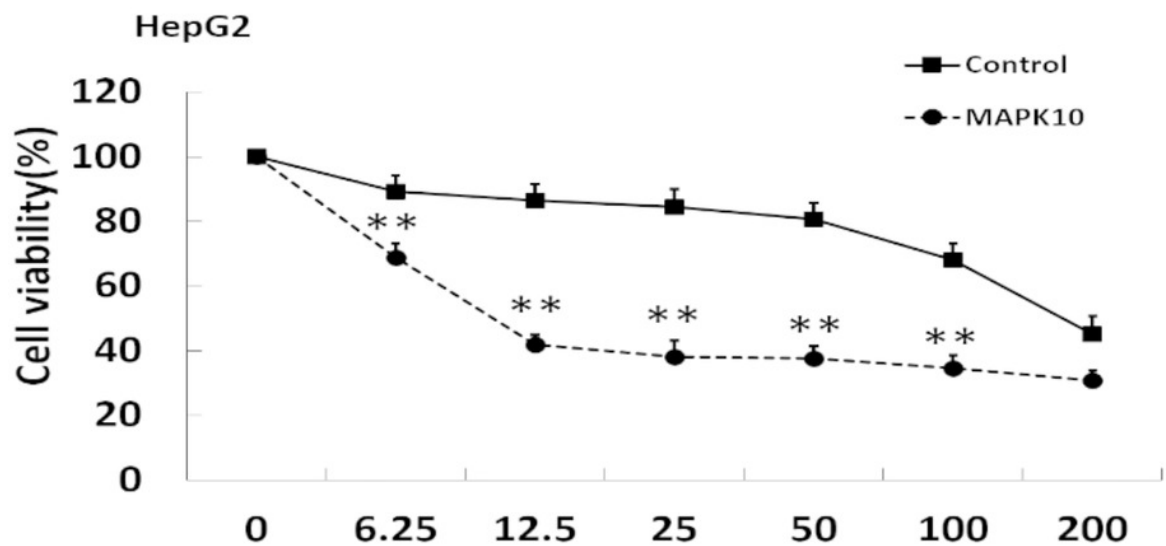
Table 1. PCR primer sequences and reaction conditions

1   Table 1. PCR primer sequences and reaction conditions

PCR	Primers	Size (bp)	TA (℃)	PCR cycles
RT-PCR				
MAPK10	F3: 5'-cagctctctaaattgactcag-3'			
	RR3: 5'-ccaatgttggttcactgcag-3'	248	55	32
GAPDH	333: 5'-gatgaccttgcccacagcct-3'			
	355: 5'-atctctgcccctctgctga-3'	304	55	23
Methylation-specific PCR (MSP)				
Methylated	m3: 5'-cgagtagttttagcggttac-3'	160	60	40
	m5: 5'-aaaaccttctaacgcgaacga-3'			
Unmethylated	u3: 5'-tgtgagtagttttagtggttat-3'	163	58	40
	u5: 5'-caaaaccttctaacacaaacaa-3'			

2   TA, annealing temperature.

Table 2. Clinicopathological correlation of *Mapk10* protein expression in HCC

1 **Table 2. Clinicopathological correlation of *Mapk10* protein expression in HCC**

Clinicopathological parameters	number	*Mapk10* expression		p value
Features	(n=59)	negative	positive	
Age				
≤60	49	18	31	0.442
>60	10	5	5	
Sex				
Male	47	17	30	0.389
Female	12	6	6	
HBsAg				
Negative	17	6	11	0.717
Positive	42	17	25	
Serum AFP(ng/ml)				
≤400	30	8	22	0.05*
>400	29	15	14	
Tumor size(cm) ^:				
≤5	20	5	15	0.119
>5	39	18	21	
Microsatellite nodules				
Absent	51	17	34	0.025*

Present	8	6	2	
Cirhosis				
Absent	29	10	19	0.494
Present	30	13	17	
PVTT				
Absent	47	18	29	0.834
Present	12	5	7	
Tumor stage(AJCC)				
Stage I	23	3	20	0.001**
Stage II	7	3	4	
Stage III	28	17	12	

2 ^: tumor size was measured by the length of the largest tumor nodule

3 *: Statistically significant (*P*<0.05).

4 **: Statistically significant (*P*<0.01).

5 PVTT: Portal vein tumor thrombus

6 AJCC: American Joint Committee on Cancer