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ORIGINAL ARTICLE

SMAD4 exerts a tumor-promoting role in hepatocellular carcinoma

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Further understanding of the molecular biology and pathogenesis of hepatocellular carcinoma (HCC) is crucial for future therapeutic development. SMAD4, recognized as an important tumor suppressor, is a central mediator of transforming growth factor beta (TGFB) and bone morphogenetic protein (BMP) signaling. This study investigated the role of SMAD4 in HCC. Nuclear localization of SMAD4 was observed in a cohort of 140 HCC patients using tissue microarray. HCC cell lines were used for functional assay *in vitro* and in immune-deficient mice. Nuclear SMAD4 levels were significantly increased in patient HCC tumors as compared with adjacent tissues. Knockdown of SMAD4 significantly reduced the efficiency of colony formation and migratory capacity of HCC cells *in vitro* and was incompatible with HCC tumor initiation and growth in mice. Knockdown of SMAD4 partially conferred resistance to the anti-growth effects of BMP ligand in HCC cells. Importantly, simultaneous elevation of SMAD4 and phosphorylated SMAD2/3 is significantly associated with poor patient outcome after surgery. Although high levels of SMAD4 can also mediate an antitumor function by coupling with phosphorylated SMAD1/5/8, this signaling, however, is absent in majority of our HCC patients. In conclusion, this study revealed a highly non-canonical tumor-promoting function of SMAD4 in HCC. The drastic elevation of nuclear SMAD4 in sub-population of HCC tumors highlights its potential as an outcome predictor for patient stratification and a target for personalized therapeutic development.

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INTRODUCTION

SMAD proteins are recognized as central mediators of transforming growth factor beta (TGFB) and/or bone morphogenetic protein (BMP) signaling pathways, which regulate a plethora of physiological processes including cell growth and differentiation.[1] Accordingly, deregulation of TGFB/BMP pathways almost invariably leads to developmental defects and/or diseases, in particular cancer.[2] These two pathways signal through the family of SMAD proteins to exert their effects. In mammals, there are eight SMADs that are subdivided into three distinct classes: receptor-regulated SMADs (R-SMADs) comprising SMAD2 and SMAD3 (transduce TGFB signaling) and SMAD1, SMAD5 and SMAD8 (transduce BMP signaling); a common SMAD called SMAD4; and two inhibitory SMADs, namely, SMAD6 and SMAD7.[3] SMAD proteins are highly conserved within their family and across species, with SMAD4 representing a somewhat divergent subtype, which still retains about 40% identity with other family members.[4] SMAD4 binds to R-SMADs and forms heteromeric complexes and facilitating the translocation of these heteromeric complexes into the nucleus. In the nucleus, the heteromeric complex binds to promoters and interacts with transcriptional activators[2,5] and the presence of nuclear SMAD4 protein has profound consequences for gene expression.

Originally identified as a candidate tumor-suppressor gene at 18q21.1 decades ago,[6] the tumor-suppressive function of SMAD4 has now almost achieved dogmatic status and loss of its activity has been implicated in the initiation and progression of a multitude of cancer types.[2,7–10] Loss or inactivation of both normal gene copies is associated with carcinoma in several organ systems, including approximately 55% of pancreatic adenocarcinomas,[6] 15–55% of extrahepatic cholangiocarcinomas[11] and a smaller percentage of gastrointestinal and other carcinomas.[12,13] Strikingly, loss of SMAD4 expression in hepatocellular carcinoma (HCC) has not been observed, prompting investigations into role and importance of this tumor suppressor in this disease. Hence, we endeavored to establish the role of SMAD4 in HCC, which we uncovered a non-conventional function of SMAD4 in HCC as a tumor promoter.

RESULTS

SMAD4 gene mutation is rare in HCC patients but its mRNA expression is significantly upregulated in the tumor tissue

As *SMAD4* genomic alterations have been reported for several cancers, we have attempted to analyze its genomic abnormalities

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in patient HCC tissues. We have searched the database of the cBioPortal for Cancer Genomics. We identified three cohorts of total 457 HCC patients with genomics data of *SMAD4* gene (Figure 1a). There are only two patients identified to harbor mutations (R87W; A462T), suggesting that *SMAD4* gene mutation in HCC is rather rare, in contrast to pancreatic or colorectal cancers (up to 20–30%) (Figure 1a). In addition, one HCC patient was found to have *SMAD4* gene amplification and another one has *SMAD4* deletion (Figure 1a).

We further searched the Oncomine microarray database to analyze mRNA expression of SMAD4 in patient HCC. In total, we have identified five cohorts of 424 HCC tissue samples compared with 344 liver tissues. SMAD4 mRNA was upregulated in all the cohorts. By polling all the cohorts, there is a significant increase of SMAD4 mRNA expression in the HCC tumor compared with liver tissue ($P < 0.05$) (Figure 1b). These data indicate that genomic alteration is rare but elevation of mRNA expression is common in patient HCC tumor.

Drastic elevation of nuclear SMAD4 expression in the tumors of sub-population of HCC patients

The paucity of data surrounding the functionality of SMAD4 in HCC prompted us to analyze SMAD4 expression and activation in a panel of resected HCC from 140 individual patients and compare the results with adjacent non-transformed tissue. In these patients, nuclear SMAD4 protein (Figure 2a) was taken as measure of SMAD4 signaling activity, as it is generally assumed that this fraction of the SMAD4 pool represents the transcriptionally active form of the protein. The staining was scored by two independent investigators with a Kappa test of 0.773, suggesting that there was an excellent agreement in scoring between the two investigators. The levels of SMAD4 protein positivity range from low (score: 0– < 2), moderate (score: 2– < 3) to high (score: 3–4) both in the HCC tumors and their adjacent sites (Figures 2a and b). Interestingly, nuclear SMAD4 levels were considerably higher in human HCC tissue as compared with normal adjacent liver tissue ($n = 140$, $P < 0.01$) (Figures 2a and c), which is consistent with the upregulation of mRNA expression in HCC (Figure 1b). Subsequent subgroup analysis according to the nuclear SMAD4 score in the tumor showed that there was no difference of SMAD4 levels between tumor and adjacent tissue in patients displaying low-to-moderate nuclear SMAD4 scores ($n = 97$, data not shown); whereas a drastic elevation was observed in tumor compared with adjacent tissue in the high SMAD4 expression group, (3.47 ± 0.45 vs 2.27 ± 0.92, mean ± s.e.m., $n = 43$, $P < 0.001$) (Figure 2c).

Analysis focusing on clinical behavior of the cancer (Supplementary Table S1) revealed that high levels of nuclear SMAD4 were not significantly associated with tumor size ($n = 98$ analyzable patients), number of tumor lesions ($n = 129$ analyzable patients) and vascular invasion ($n = 78$ analyzable patients), but significantly associated with higher levels of alpha-fetoprotein pre-resection ($n = 135$ analyzable patients, $P < 0.01$). Serum alpha-fetoprotein has been suggested as an independent indicator for HCC prognosis and patients with high alpha-fetoprotein levels have been reported to have shorter survival.[14] In addition, SMAD4 is significantly associated with fibrosis ($P < 0.01$) (Supplementary Figure S1). Liver fibrosis is in turn strongly correlated with HCC development.[15] Furthermore, nuclear SMAD4 level was significantly higher in undifferentiated tumor than in well-differentiated tumor of HCC (2.53 ± 0.23 vs 1.94 ± 0.11, mean ± s.e.m., $n = 127$, $P < 0.05$) (Figure 2d).

Importantly although, apparently high SMAD4 positivity in surgically resected HCC ($n = 130$ analyzable patients) tend to have higher risk of fast recurrence (hazard ratio (HR) = 1.420, 95% confidence interval (CI): 0.711–2.836 in the high-level group) and higher risk of poor survival (HR = 1.844, 95%CI: 0.894–3.803 in the high-level group) (Figures 2e and f). Kaplan–Meier analysis ($n = 130$ analyzable patients) also indicated a trend of shorter time to recurrence and lower cumulative survival in high SMAD4 level patients, although not statistically significant (Figures 2e and f). We interpreted that higher nuclear SMAD4 levels may be associated with more aggressive types of tumors in HCC patients.

Silencing of SMAD4 expression reduced colony formation in human hepatoma cell lines

In order to obtain an insight into the mechanisms possibly mediating the negative relation between SMAD4 signaling and HCC clinical behavior, we used lentiviral RNA interference vectors expressing short hairpin RNA (sh-SMAD4) to stably knockdown SMAD4 expression in human HCC cell lines and subsequently characterized the cellular consequences thereof. Supplementary Figure S2 showed the efficacy of gene silencing using this strategy. A vector expressing short hairpin RNA targeting green fluorescent protein served as control (CTR). The success of this approach was confirmed by western blot and probing for SMAD4 protein (Figure 3a), which showed almost absence of the protein in the knockdown cell lines, whereas the control cell lines remain SMAD4 proficient. Using immunofluorescent staining, it has confirmed the efficiency of SMAD4 knockdown in Huh7, Huh6 and PLC cell lines (Figure 3b).

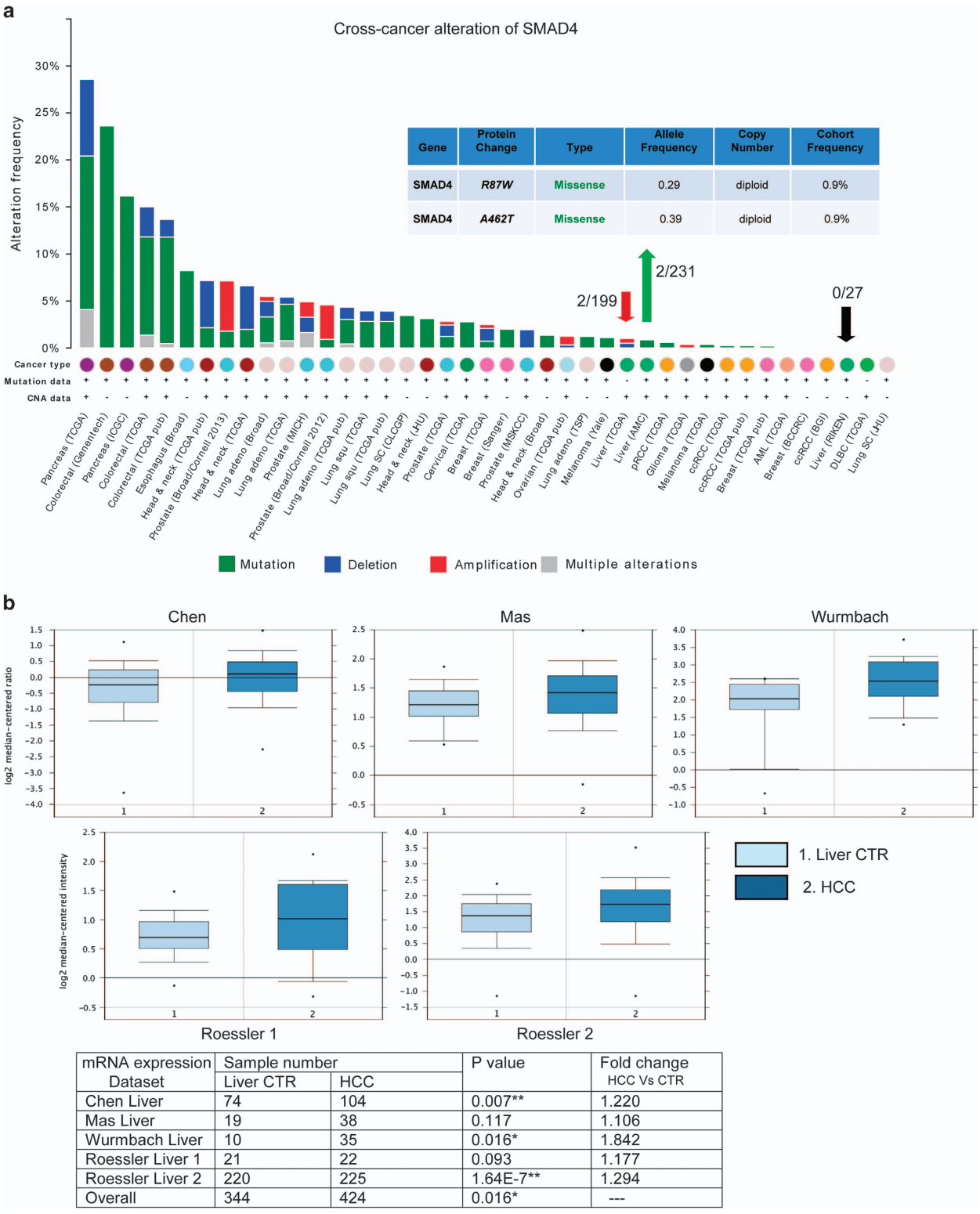
Colony formation assay is a robust tool to evaluate the ability of a single cell to support proliferation. Using this assay, we observed a significant decrease in the numbers of formed colonies in Huh7 cells with SMAD4 knockdown compared with the mock cells (CTR vs sh-SMAD4: 270.8 ± 25.82 vs 144.8 ± 32.11 colonies per 1000 cells, mean ± s.d., $n = 4$, $P < 0.05$) (Figure 3c). Similar results were observed in Huh6 and PLC cells (Figure 3c). Thus, in contrast to most other cell types where SMAD4 expression is associated with reduced cancer cell growth, SMAD4 expression supports proliferation of HCC cells.

Knockdown of SMAD4 attenuated the ability of HCC cell migration

Cell migration is a fundamental function underlying cellular processes including invasion or metastasis of cancer cells. We thus investigated the role of SMAD4 in migration of HCC cells using a ring-barrier system (Figure 4a). Silencing of SMAD4 expression resulted in attenuated migratory capacity toward the cell-free area in Huh7 cells. In Huh7 cells with SMAD4 knockdown, quantification revealed a significant reduction in total migration (CTR vs sh-SMAD4: 174.1 ± 54.3 μm vs 128.7 ± 42.1 μm, mean ± s.d., $n = 30$, $P < 0.01$), effective migration (CTR vs sh-SMAD4: 109.1 ± 33.2 μm vs 55.4 ± 22.4 μm, mean ± s.d., $n = 30$, $P < 0.001$), migration efficiency (CTR vs sh-SMAD4: $63.60 \pm 9.60\%$ vs $43.95 \pm 16.62\%$ mean ± s.d., $n = 30$, $P < 0.0001$) and migration velocity (CTR vs sh-SMAD4: 7.3 ± 2.3 vs 5.4 ± 1.8 μm/h, mean ± s.d., $n = 30$, $P < 0.001$) (Figure 4b). These results indicate that SMAD4 in HCC cells support migration and in conjunction with the colony formation data support the notion of a non-canonical pro-oncogenic function of SMAD4 in HCC.

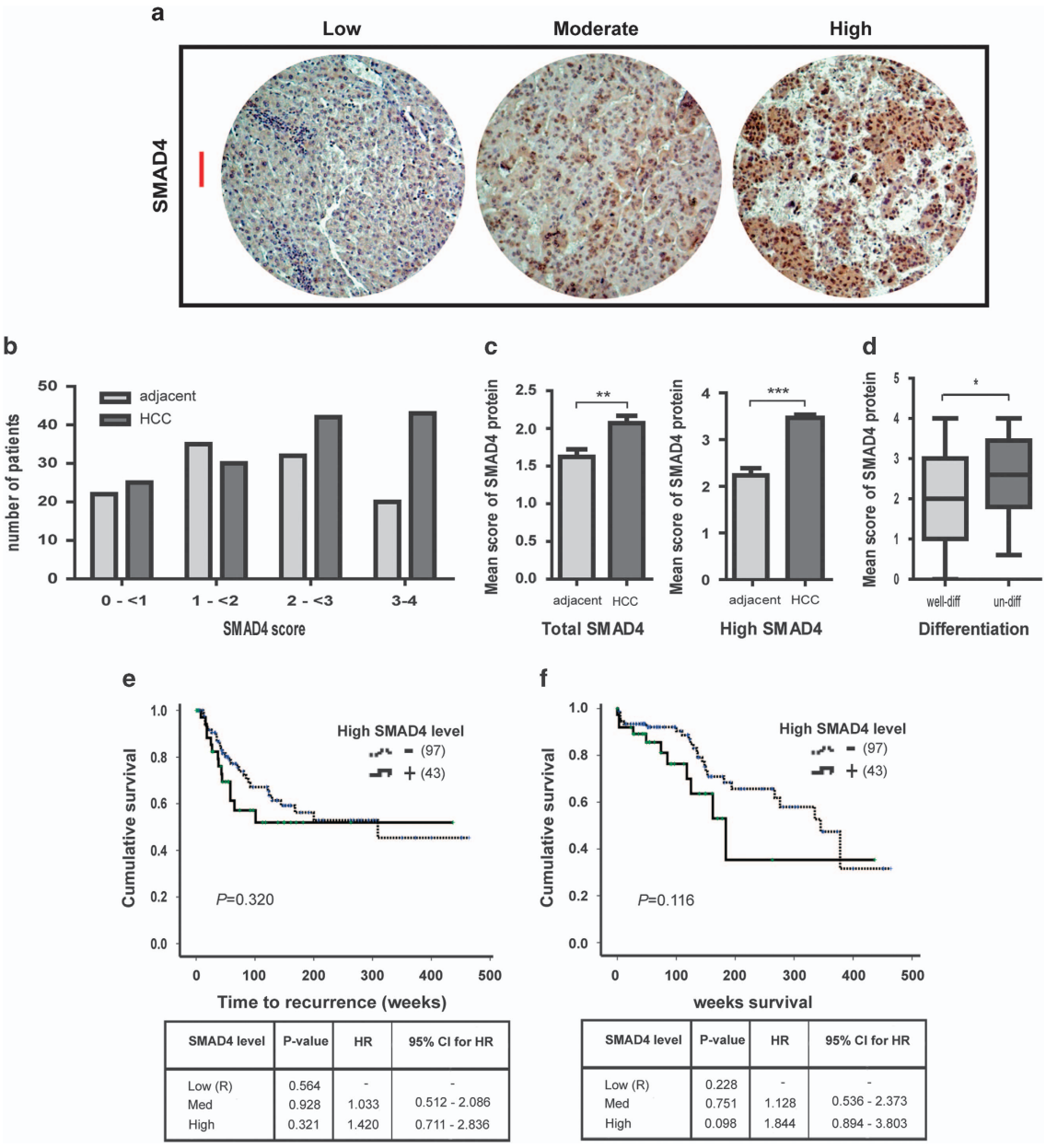
Silencing of SMAD4 limited hepatoma initiation and growth in mice

To finally ensure the tumor-promoting effects of SMAD4, we evaluated the impact of SMAD4 loss on tumor initiation and growth in nude mice. One million CTR and SMAD4 knockdown cells were subcutaneously injected into the left or right side of the mice, respectively. As shown in Figure 5, impressively, knockdown of SMAD4 in Huh7 cells resulted in complete abolishment of tumor formation, whereas 7 out of 10 mice in the CTR group formed tumor (weight: 0.59 ± 0.15 g, mean ± s.e.m., $n = 7$). Collectively, this result is in line with the outcomes of our *in vitro*



mRNA expression Dataset	Sample number		P value	Fold change HCC Vs CTR
	Liver CTR	HCC		
Chen Liver	74	104	0.007**	1.220
Mas Liver	19	38	0.117	1.106
Wurmbach Liver	10	35	0.016*	1.842
Roessler Liver 1	21	22	0.093	1.177
Roessler Liver 2	220	225	1.64E-7**	1.294
Overall	344	424	0.016*	---

Figure 1. *SMAD4* gene mutation is rare but upregulation of its mRNA expression is common in patient HCC. (**a**) In the database of the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/), three cohorts of total 457 HCC patients with genomics data of *SMAD4* gene were identified. There are only two HCC patients harboring mutations (R87W; A462T). In addition, one HCC patient has *SMAD4* gene amplification and another one has *SMAD4* deletion. Green arrow indicated the cohort identified *SMAD4* mutation (2 of 231 HCC patients); red arrow indicated the cohort identified *SMAD4* copy number variation (2 of 199 patients); and dark arrow indicated the cohort withour genomic alteration identified (27 patients). (**b**) The Oncomine microarray database (https://www.oncomine.org) was searched to analyze mRNA expression of SMAD4 in patient HCC. In total, five cohorts of 424 HCC tissue samples compared with 344 liver tissues were identified. SMAD4 mRNA was upregulated in all the cohorts. There is a statistically significant increase of SMAD4 mRNA expression in the HCC tumor compared with liver tissue by polling all the cohorts. *T*-test was used for individual cohort. Meta-analysis of the five cohorts indicated its *P*-value for the median-ranked analysis. $*P < 0.05$, $**P < 0.01$.



SMAD4 level	P-value	HR	95% CI for HR
Low (R)	0.564	-	-
Med	0.928	1.033	0.512 - 2.086
High	0.321	1.420	0.711 - 2.836

SMAD4 level	P-value	HR	95% CI for HR
Low (R)	0.228	-	-
Med	0.751	1.128	0.536 - 2.373
High	0.098	1.844	0.894 - 3.803

Figure 2. Strong elevation of nuclear SMAD4 expression in the tumors of sub-population of HCC patients. (**a**) The levels of SMAD4 protein positivity range from low (score: 0– < 2), moderate (score: 2– < 3) to high (score: 3–4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (**b**) The distribution of SMAD4 score among HCC patients. (**c**) Overall, SMAD4 expression levels were significantly higher in human HCC tissues compared with normal adjacent liver tissues. Error bars represents mean ± s.e.m. from $n = 140$, paired *t*-test, $^{**}P < 0.01$. A significant increase was also observed in tumors compared with adjacent tissues in the high-grade patients ($n = 43$, paired *t*-test, $^{***}P < 0.001$). (**d**) Nuclear SMAD4 level was significantly higher in undifferentiated tumor than in well-differentiated tumor of HCC $^{*}P < 0.05$. From Cox regression analysis ($n = 130$ analyzable patients), high SMAD4 level in surgical resected HCC tumor tend to have higher risk of fast recurrence (HR = 1.420) (**e**) and higher risk of poor survival (HR = 1.844) (**f**). Kaplan–Meier analysis ($n = 130$) also indicated a trend of faster disease recurrence (**e**) and lower cumulative survival (**f**), although not statistically significant.

experimentation and the observation that high SMAD4 expression in human HCC tissue is associated with worse prognoses firmly demonstrates that SMAD4 exerts a tumor-promoting role in HCC.

Simultaneous elevation of SMAD4 and phosphorylated SMAD2/3 is significantly associated with poor patient outcome

Upon binding of the cognate ligands to the TGFB receptor, phosphorylated SMAD2/3 (p-SMAD2/3) binds to SMAD4 to form heteromeric complex, translocate to the nucleus and activate TGFB signaling.[16] The signaling receptors phosphorylate R-SMAD proteins at the carboxy-terminal (C-terminal) and the linker region.[17] Recent studies uncover a role for agonist-induced phosphorylation of the R-SMAD linker region[18] that may modulate downstream cellular responses to the TGFB family of ligands. We thus performed immunohistochemistry staining of p-SMAD2/3 both at the C-terminal phosphorylation (p-SMAD2/3C, Ser423/425) and the linker phosphorylation region (p-SMAD2/3L, Thr220/179) in the tissue microarray (TMA) that was used for SMAD4 staining

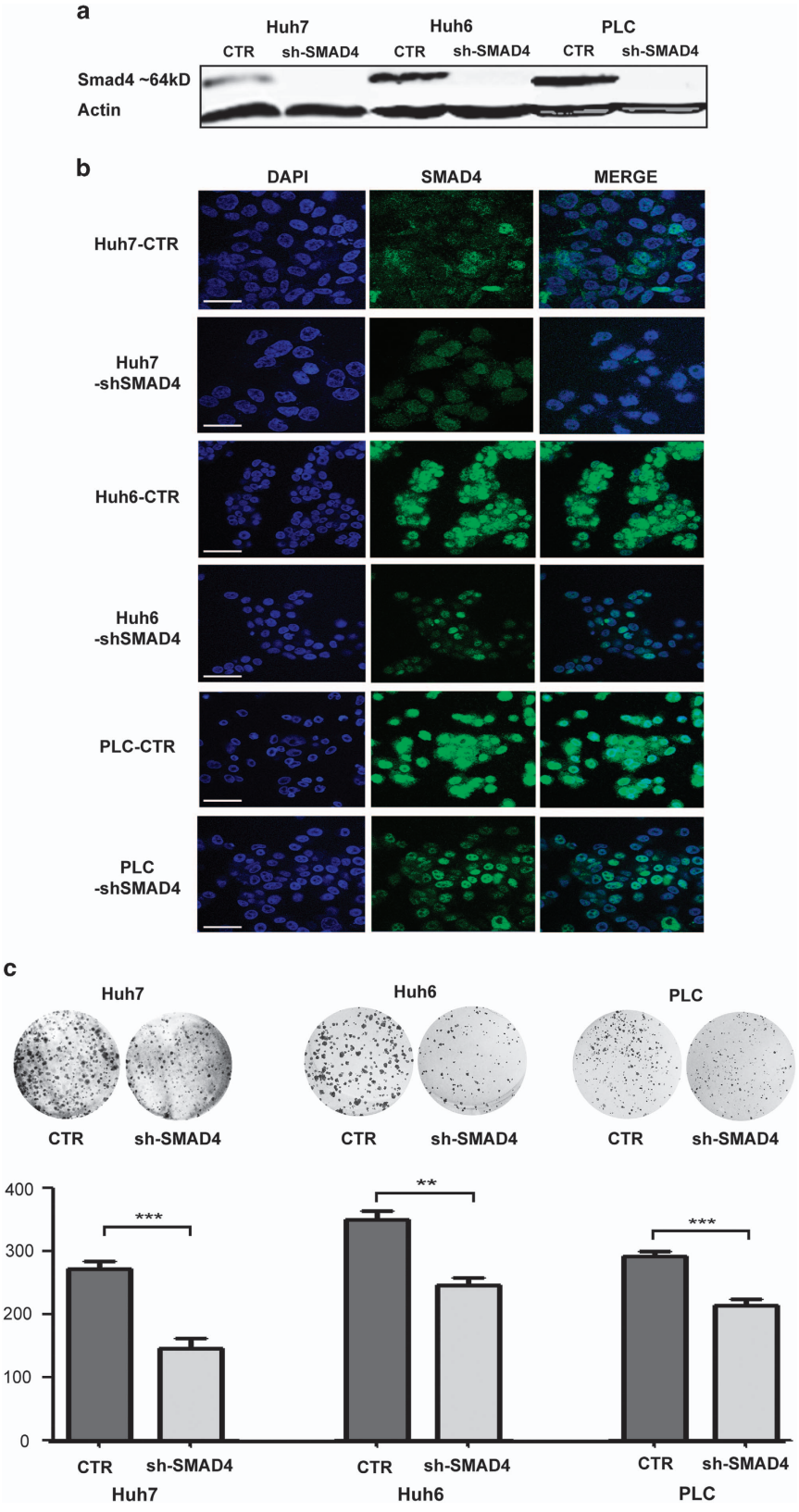


Figure 3. Decreased efficiency of colony formation in SMAD4 knockdown HCC cells. (**a**) Successful knockdown of SMAD4 in Huh7, Huh6 and PLC cell lines was first confirmed on protein levels by western blot. (**b**) Immunofluorescent staining confirmed the efficacy of SMAD4 knockdown. Scale bar, 50 um. (**c**) The control cells (CTR) are significantly more efficient in forming colony than the sh-SMAD4 cells. A significant decrease in the numbers of formed colonies was observed in three HCC cells with SMAD4 knockdown (sh-SMAD4), compared with mock knockdown (CTR). Error bars represent mean ± s.d., $n = 4$, *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$.

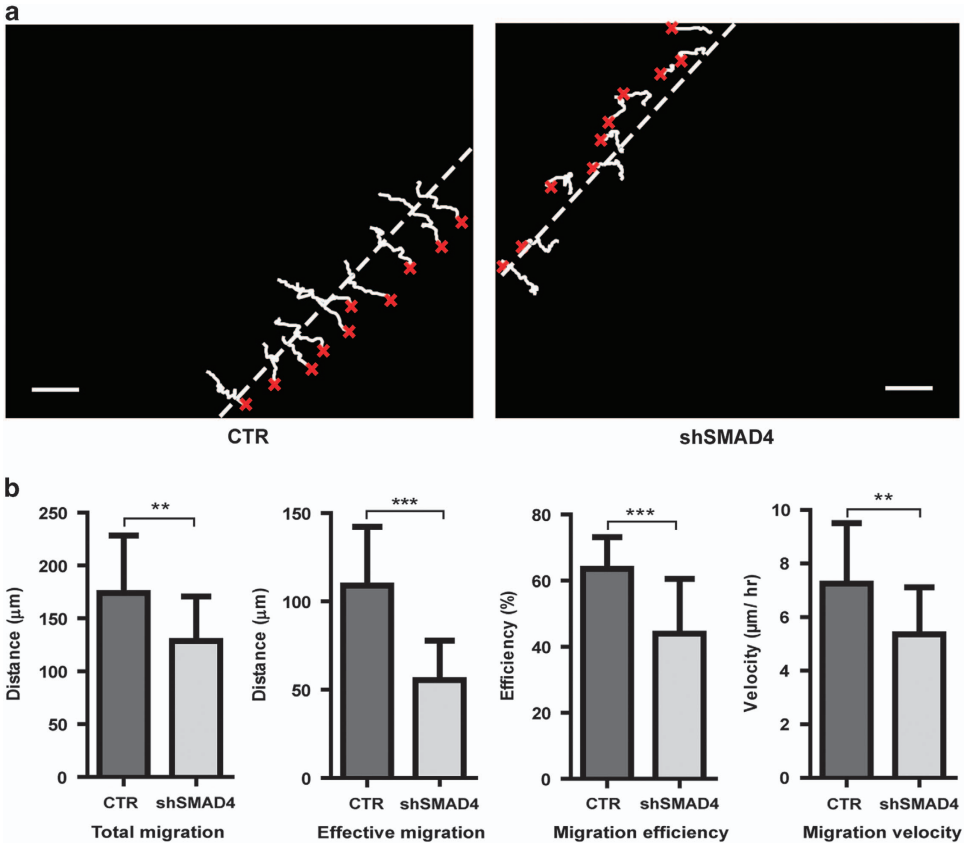


Figure 4. Silencing of SMAD4 inhibited HCC cell migration. (**a**) Migration assay of Huh7 cells (CTR) and its sh-SMAD4 cells using ring-barrier method system. (**b**) Quantification revealed a significant reduce in total migration, effective migration, migration efficiency and migration velocity in 24 h in SMAD4 knockdown cells compared with the control cells. Error bars represent mean ± s.d. from $n = 30$, Mann–Whitney test, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not significant. Scale bar, 100 μm.

($n = 140$). The levels of p-SMAD2/3-C/L protein positivity range from low (score: 0– < 2), moderate (score: 2– < 3) to high (score: 3-4) both in the HCC tumors and their adjacent sites (Figures 6a and 7a). The patient groups (low, moderate or high) is categorized according to expression levels in the tumors. Although no significant difference overall ($n = 140$), p-SMAD2/3C levels were significantly lower in HCC tissue as compared with normal adjacent liver tissue in patients with low-to-moderate scores ($n = 86$, $P < 0.001$, data not shown); whereas it is significantly higher in the tumor of in patients with high scores ($n = 54$, $P < 0.001$) (Figure 6b). Moreover, overall p-SMAD2/3L expression was significantly higher in the HCC tumor than in the adjacent area ($P < 0.001$) (Figure 7b). High p-SMAD2/3C expression in tumor is significantly associated with high recurrence rate ($n = 47$, $P < 0.05$) and patient death rate ($n = 44$, $P < 0.05$) (Supplementary Table 2). Cox regression and Kaplan–Meier analysis ($n = 140$) also revealed a tendency of shorter time to recurrence and a trend to less cumulative survival in patients with high levels of p-SMAD2/3-C/L in the tumor HCC (Figures 6c and d and 7c).

As a phosphorylated protein, moderate levels of p-SMAD2/3-C/L would be expected to be already sufficient to trigger the downstream signaling transduction in the presence of SMAD4. A sub-population of HCC patients have a simultaneous elevation of SMAD4 and p-SMAD2/3C ($n = 22$) and p-SMAD2/3L ($n = 34$), which represents as a hallmark for the activation of the downstream signaling of TGFB (Supplementary Figure S3 and S4; Figures 6f and 7c). Cox regression and Kaplan–Meier analysis also confirmed that these patients are significantly faster to disease recurrence and worse survival ($P < 0.05$) (Figures 6f and 7c).

Nuclear p-SMAD3L (Ser213) binds to SMAD-binding element in the promoter with high affinity and specificity[19] and transmits fibrogenic/carcinogenic (fibro-carcinogenic) signaling.[20] Both pSMAD3C(Ser425) and p-SMAD3L(Ser213) form hetero-complexes with SMAD4, and move to nucleus.[21] Chronic inflammation and hepatitis viral additively shift hepatocytic SMAD3 signaling from tumor-suppressive pSMAD3C to fibro-carcinogenic p-SMAD3L.[22,23] Therefore, we also investigated the expression of this particular form in our cohort. p-SMAD3L levels were significantly higher in HCC tissue as compared with normal adjacent liver tissue. ($P < 0.001$, Figure 7e). Cox regression analysis and Kaplan–Meier analysis ($n = 131$) indicated that patients with high level of p-SMAD3L have worse survival (HR = 1.155, 95% CI: 1.117–5.371 in the high-level group) and these features were also seen in co-expression of SMAD4 and p-SMAD3L (Figure 7f). Taken together (Figures 6 and 7; Supplementary Tables S2–S7), these results indicate that SMAD4 together with p-SMAD2/3, both C-terminal (C) and linker phosphorylation (L), with Ser or Thr residues, exert a tumor-promoting function in HCC patients.

An antitumor signaling mediated by phosphorylated SMAD1/5/8 and SMAD4 is inactivated in majority of HCC patients

Upon binding of BMP ligands, phosphorylated SMAD1/5/8 (p-SMAD1/5/8) binds to SMAD4 to form heteromeric complex, translocate to the nucleus and activate BMP signaling.[16] Although

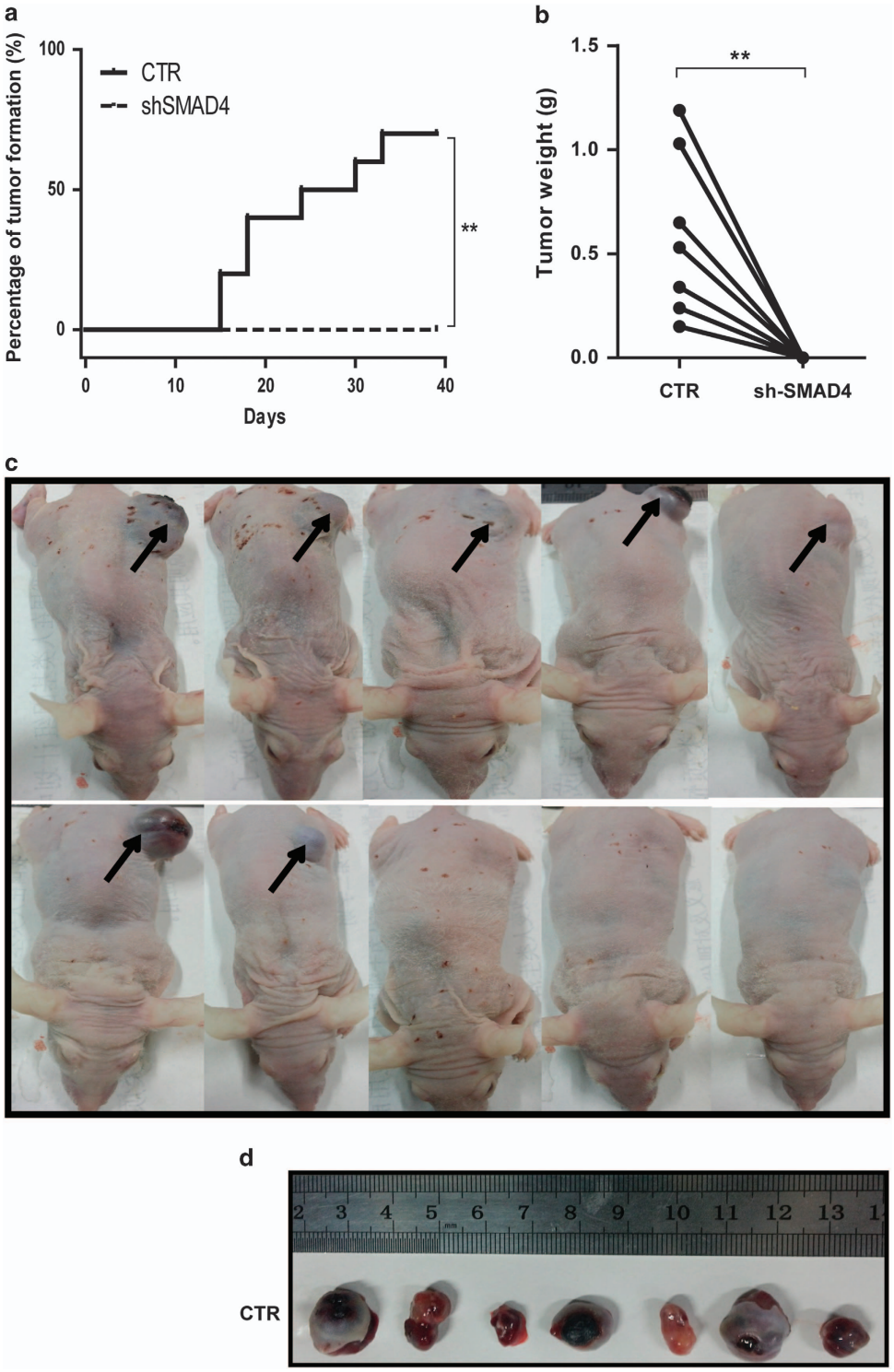
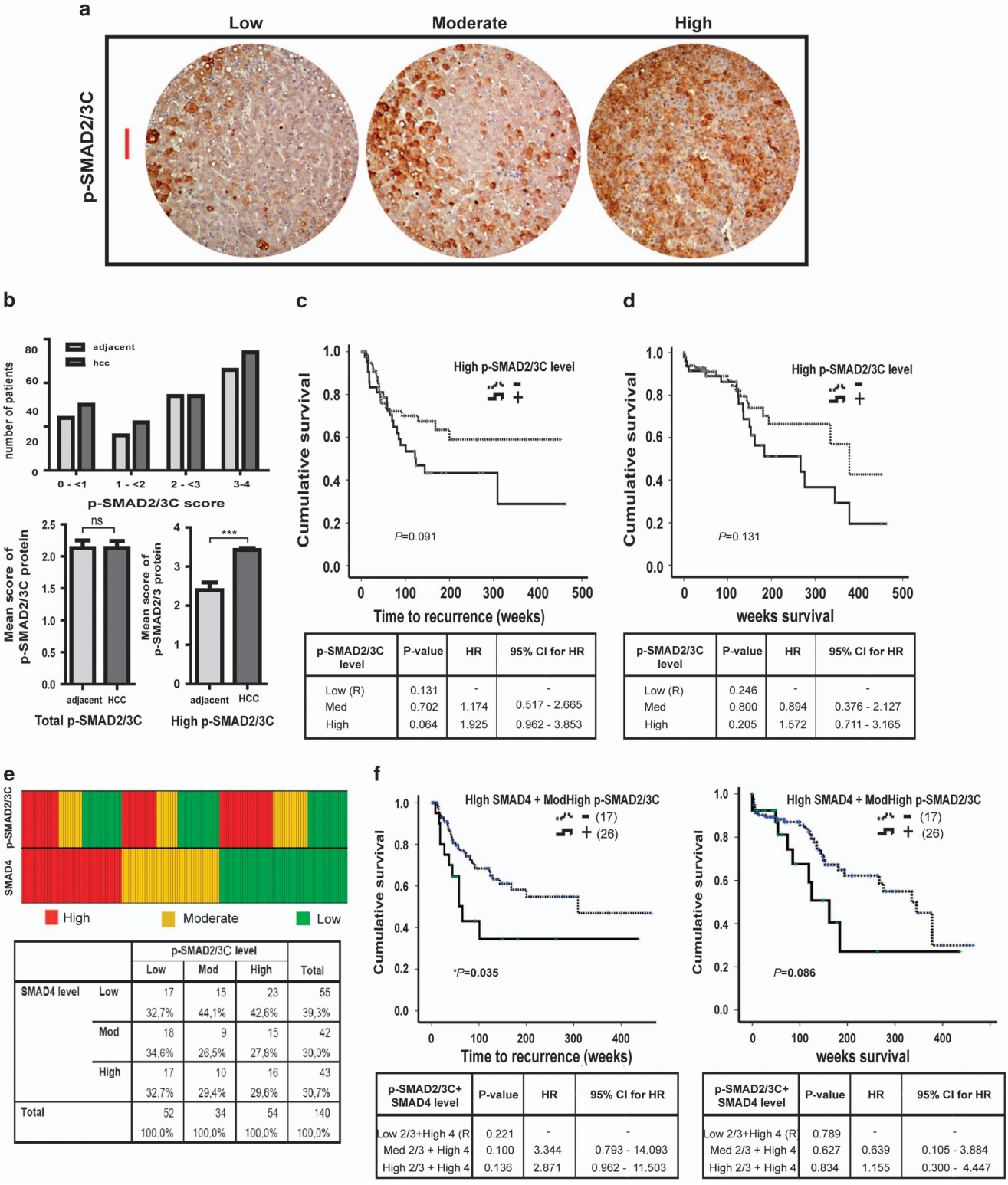


Figure 5. Knockdown of SMAD4 in Huh7 cells failed to initiate tumor in nude mice. (**a**) Knockdown of SMAD in Huh7 significantly abolished the tumor formation, whereas 7 out of 10 mice in the control group formed tumors (paired *t*-test, $^{**}P < 0.01$). (**b**) The weight of formed tumor. (**c**) The solid arrows indicate Huh7 in control group formed tumors in mice. (**d**) The appearance of formed tumors in centimeter length.

the exact role of BMP signaling in cancer is highly context dependent, a recent study demonstrated that BMP4, a BMP ligand, inhibited the tumorigenic capacity of HCC cells.[24] We further examined the effects of BMP4 on HCC cells. In Huh7 cells, BMP4 significantly reduces colony formation ability of Huh7 cells and knockdown of SMAD4 attenuated the effects of BMP4. The efficiency of colony formation was reduced by BMP4 treatment in CTR cells by $47.02 \pm 6.5\%$ but only by $25.3 \pm 6.4\%$ in SMAD4 knockdown Huh7 cells (mean $\pm$ s.d., $n = 4$, $P < 0.01$) (Figure 8a). Consistently, adding BMP inhibitor Noggin appears to increase the



p-SMAD2/3C level	P-value	HR	95% CI for HR
Low (R)	0.131	-	-
Med	0.702	1.174	0.517 - 2.665
High	0.064	1.925	0.962 - 3.853

p-SMAD2/3C level	P-value	HR	95% CI for HR
Low (R)	0.246	-	-
Med	0.800	0.894	0.376 - 2.127
High	0.205	1.572	0.711 - 3.165

		p-SMAD2/3C level			
		Low	Mod	High	Total
SMAD4 level	Low	17	15	23	55
		32,7%	44,1%	42,6%	39,3%
	Mod	18	9	15	42
		34,6%	26,5%	27,8%	30,0%
	High	17	10	16	43
		32,7%	29,4%	29,6%	30,7%
Total		52	34	54	140
		100,0%	100,0%	100,0%	100,0%

p-SMAD2/3C+ SMAD4 level	P-value	HR	95% CI for HR
Low 2/3+High 4 (R	0.221	-	-
Med 2/3 + High 4	0.100	3.344	0.793 - 14.093
High 2/3 + High 4	0.136	2.871	0.962 - 11.503

p-SMAD2/3C+ SMAD4 level	P-value	HR	95% CI for HR
Low 2/3+High 4 (R	0.789	-	-
Med 2/3 + High 4	0.627	0.639	0.105 - 3.884
High 2/3 + High 4	0.834	1.155	0.300 - 4.447

Figure 6. Simultaneous elevation of p-SMAD2/3C and SMAD4 is significantly associated with poor clinical outcome in HCC patients. (**a**) The levels of p-SMAD2/3C protein positivity range from low (score: 0– < 2), moderate (score: 2– < 3) to high (score: 3–4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (**b**) There were more patients with higher p-SMAD2/3C score both in tumor and adjacent sites. No significant overall difference of p-SMAD2/3C expression between HCC tissue and normal adjacent liver tissue. Nevertheless, in the high-grade patients group, p-SMAD2/3C expression was significantly higher in HCC tissues compared with adjacent sites ($n = 54$). Error bars represents mean ± s.e.m., paired *t*-test, $^{***}P < 0.001$. From Cox regression and Kaplan–Meier analysis ($n = 130$), high levels of p-SMAD2/3C tend to have higher risk of fast recurrence (HR = 1.649) (**c**) and tend to have higher risk of poor survival (HR = 1.633) (**d**). (**e**) Twenty-two out of 140 patients have simultaneously sufficient levels of both p-SMAD2/3C ($n = 16$ high; $n = 6$ moderate levels) and SMAD4. (**f**) These patients have significantly poor clinical outcome as shown by both Cox regression and Kaplan–Meier analysis. $^{*}P < 0.05$. NS, not significant.

efficiency of colony formation in CTR cells (124.5 ± 19.1%, mean ± s.d., $n = 4$) but has much less effect (109.6 ± 9.5%, mean ± s.d., $n = 4$) in SMAD4 knockdown Huh7 cells (Figure 8b). Thus, BMP4 significantly reduced the colony formation ability of hepatoma cells, which was consistent with previous reports in other cancer,[25–27] and knockdown of SMAD4 attenuated the effects of BMP4. Western blot analysis showed the effects of BMP4 and Noggin on the protein levels of SMAD4, p-SMAD2/3 and p-SMAD1/5/8. This was in broad agreement with the efficacy of the experimental strategy but also suggested the existence of SMAD4-dependent feedback loops on BMP signaling elements (Figures 8c and d). Our results confirm that activation of BMP signaling, which involves both SMAD4 and p-SMAD1/5/8, exerts anti-HCC effects.

Next, we further explored the role of this pathway in our HCC cohort. Immunohistochemistry staining of p-SMAD1/5/8 was performed in the TMA ($n = 140$), and was scored and categorized as described before (Figures 9a and b). Although p-SMAD1/5/8 is significantly higher in the tumor tissue compared with adjacent liver tissue (Figure 9c), only a small subset of patients have high levels of p-SMAD1/5/8 in the tumor (17 out of 140, see Supplementary Table S8). No significant relation was observed regarding to the size ($n = 98$) and the number of tumor foci ($n = 129$) (Supplementary Table S8). Interestingly, a Bonferroni-corrected clinical parameter analysis revealed a negative correlation between tumor p-SMAD1/5/8 level and age (Supplementary Table S8). Patients with high levels of p-SMAD1/5/8 appear to have lower risk of fast recurrence (HR = 0.542, 95% CI: 0.191–1.538 in the high-level group) and lower risk to poor survival (HR = 0.596, 95% CI: 0.210–1.697 in the high-level group) (Figure 9d). Kaplan–Meier analysis also revealed a trend of longer time to recurrence and higher cumulative survival in these patients (Figure 9d).

As a phosphorylated protein, p-SMAD1/5/8 could sensitively control the downstream signaling transduction. As the anti-tumor function of this signaling requires both SMAD4 and p-SMAD1/5/8, we further categorized the expression levels of both proteins in the same patients. As shown in Figure 9e, there are only eight patients having simultaneously sufficient levels of both SMAD4 and p-SMAD1/5/8 ($n = 2$ high; $n = 6$ moderate levels). These results suggest that SMAD4 and p-SMAD1/5/8-mediated antitumor signaling is inactivated in majority of our HCC patients.

DISCUSSION

In this study, we reported a drastic elevation of nuclear SMAD4 localization in tumors of subset of HCC patients. High expression of SMAD4 was further demonstrated to be functionally important for hepatoma formation and progression. Importantly, simultaneous elevation of SMAD4 and p-SMAD2/3 in sub-population of HCC patients significantly associated with poor outcome after surgery. Although SMAD4 coupled with p-SMAD1-/5/8 can also mediate an antitumor effect, this signaling, however, is silent in majority of our HCC patients. Thus, we conclude that high nuclear SMAD4 expression has been screwed toward a tumor-promoting signaling in HCC (Supplementary Figure S5). This is unexpected in view of the dogma that SMAD4 is a potent tumor suppressor.

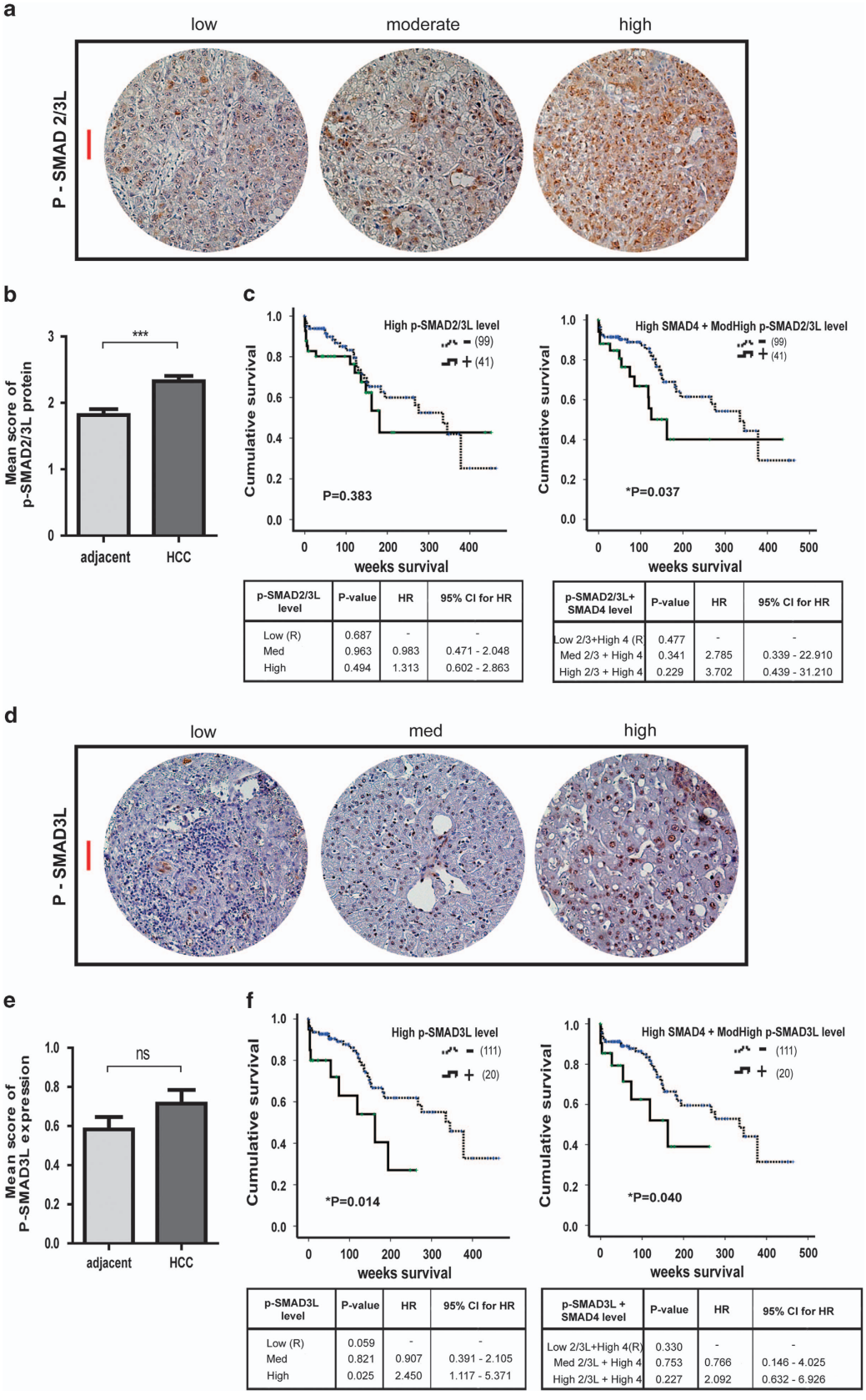
SMAD4 was initially described in pancreatic cancer, named DPC4 (deleted in pancreatic carcinoma, locus 4), and appears critical in pancreatic cancer progression.[28,29] *SMAD4* loss occurs in 40–50% of colon cancers,[30] which is associated with metastasis, advanced disease and reduced survival. Similarly, its loss in cholangiocarcinoma[31] or prostate cancer[8] is also related to more progressive disease. The tumor-suppressor function of SMAD4 is often closely linked to its capacity to mediate TGFB and BMP signals. However, we question whether activation or silencing of TGFB/BMP downstream components, including SMADs, is always ligand dependent in cancer? As in xenografts of human hepatoma cell lines in mice, which are thus unlikely to encounter their (human) ligands, we observed that high expression of SMAD4 is even required for tumor formation and growth. In contrast to our observation, a previous study has reported a lower protein level of SMAD4 in HCC tissue compared with adjacent liver tissue in an Asian cohort.[32] A possible explanation could be that the etiologies of HCC may influence the expression of SMAD4. In Asia, viral hepatitis is the main cause of HCC; whereas only $< 30\%$ of patients in our European cohort have viral hepatitis history, although high expression of SMAD4 was also reported in another Asian HCC cohort.[33] In addition, technical differences, including the source of antibody and the protocol of immunohistochemical staining, may also result in discrepancy. In this study, we have used a robust staining protocol for SMAD4 (see Materials and methods section) that was optimized and established in our previous studies.[34,35]

The essential role of TGFB/BMP signaling in cancer is certainly well documented, whereas its exact functions are also context dependent.[36] TGFB1 was also well recognized for its dual role in carcinogenesis.[37] It acts as a tumor suppressor in early stages of hepatocarcinogenesis by inducing apoptosis[38] and at a later stage, however, liver tumor cells often become resistant to its pro-apoptotic effect, and produce large amounts of TGFB themselves.[39] This was in line with our result that the different levels of phosphorylated R-SMADs is associated with distinct patient outcome. We speculate that dysregulation of key R-SMADs may lead to the opposite effect of the canonical TGFB and BMP signaling. In our HCC cohort, a sub-population of HCC patients have a simultaneous elevation of SMAD4 and p-SMAD2/3C ($n = 22$) or p-SMAD2/3L ($n = 34$), indicating the activation of TGFB down-stream signaling. Both high expression of the C-terminal (C) and linker phosphorylation (L) region of p-SMAD2/3 are associated with worse outcome after surgical resection in our HCC cohort, which is in line with previous report in colorectal cancer,[40] confirming a tumor-promoting function of TGFB signaling in these HCC patients.

Several distinct BMP ligands were reported to act together to promote the migratory and invasive potential of cancer cells,[41] including in HCC.[42,43] In contrast, a recent study demonstrated that BMP4 induced differentiation of HCC cancer stem cells and inhibited their tumorigenic capacity.[24] Our *in vitro* study indicated activating BMP signaling by adding BMP4 ligand in HCC was able to effectively suppress colony formation of HCC cells, which was consistent with previous reports in other cancer.[25–27] However, silencing of *SMAD4* gene attenuated this effect, confirming that these anti-oncogenic actions require basal levels of SMAD4. Despite an antitumor effects of BMP pathway, this signaling, however, is silent in majority of our HCC patients, by losing the key components, either SMAD4 or p-SMAD1/5/8, or both of them. The obvious implication of this observation is that HCC cells should prove exquisitely sensitive to stimulation with BMP ligands mediating such signaling. In conjunction with the recent Food and Drug Administration approval of BMP2 and BMP7 as treatment for certain bone pathologies.[44] However, we have to be cautious that there are also studies reporting pro-oncogenic roles of BMP ligands in particular settings. For instance, BMP7 and BMP9 have been shown to have tumor-promoting functions in some experimental cancer (including HCC) models.[42,45,46] Nevertheless, our results call for further study exploiting this Achilles' heel of HCC.

In summary, this study reports a significant elevation of nuclear SMAD4 localization in patient HCC tumors. High nuclear SMAD4 has been screwed toward tumor-promoting effects because of simultaneous elevation of p-SMAD2/3 in subset of patients. SMAD4 can also mediate an antitumor signaling by coupling

p-SMAD1/5/8; this complex, however, is absent in majority of patients because of lack of either SMAD4 or p-SMAD1/5/8, or both of them. These results have certainly shed new light on the molecular biology of HCC and more importantly SMAD-based molecules may have potential as outcome predictors for patient stratification.



p-SMAD2/3L level	P-value	HR	95% CI for HR
Low (R)	0.687	-	-
Med	0.963	0.983	0.471 - 2.048
High	0.494	1.313	0.602 - 2.863

p-SMAD2/3L+ SMAD4 level	P-value	HR	95% CI for HR
Low 2/3+High 4 (R	0.477	-	-
Med 2/3 + High 4	0.341	2.785	0.339 - 22.910
High 2/3 + High 4	0.229	3.702	0.439 - 31.210

p-SMAD3L level	P-value	HR	95% CI for HR
Low (R)	0.059	-	-
Med	0.821	0.907	0.391 - 2.105
High	0.025	2.450	1.117 - 5.371

p-SMAD3L + SMAD4 level	P-value	HR	95% CI for HR
Low 2/3L+High 4(R)	0.330	-	-
Med 2/3L + High 4	0.753	0.766	0.146 - 4.025
High 2/3L + High 4	0.227	2.092	0.632 - 6.926

MATERIALS AND METHODS

Tissue microarray

To make TMA, paraffin-embedded HCC patient tissues ($n = 140$, between 2004 and 2013) were collected from the Pathology Department of Erasmus Medical Centre (Erasmus MC) Rotterdam. The use of patient materials was approved by the medical ethical committee of Erasmus MC (Medisch Ethische Toetsings Commissie Erasmus MC).[47,48]

Bioinformatics analysis of genomics and mRNA assay data sets

To analyze the prevalence of genomic alterations of *SMAD4* gene in patient HCC tissues, the database of the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/) was searched. Both copy number variation and gene mutation data were analyzed across cancer types with focusing on HCC.

To analyze mRNA expression of SMAD4 in HCC, the Oncomine microarray database (https://www.oncomine.org) was searched. SMAD4 mRNA expression was analyzed in identified cohorts by comparing expression levels in HCC tumors with liver tissues.

Immunohistochemistry

Paraffin-embedded liver tumor tissue in TMA slides were deparaffinized in xylene, rehydrated in graded alcohols. For antigen retrieval, slides were boiled in Tris/EDTA pH 9.0 for 30 min (for SMAD4 antibody) and 10 min for other antibodies; 3% H_2O_2 was used to block endogenous peroxidase for 10 min at room temperature. The slides were incubated in 5% milk blocking solution followed by overnight incubation in mouse SMAD4 antibody (1:100 dilution, Santa Cruz Biotechnology, Inc., Huissen, The Netherlands), goat p-SMAD2/3C (Ser423/425) antibody (1:250 dilution, Santa Cruz Biotechnology, Inc.), mouse p-SMAD2/3L (Thr220/179) (1:250 dilution, Takara Bio, Shiga, Japan), mouse p-SMAD3L (Ser213) (1:250 dilution, Takara Bio), rabbit p-SMAD1/5/8 (1:500 dilution, Cell Signaling, Leiden, The Netherlands) and p-Histone H3 (1:1000 dilution, Merck Millipore, Amsterdam, The Netherlands), and then counterstained with hematoxylin. The SMAD4 scoring was based on the nuclear staining and the p-SMAD2/3 and p-SMAD1/5/8 scoring were based on cytoplasm and/or nuclear staining. The following scores were applied: score 0 for 0–10% positive staining, score 1 for 10–30% positive staining, score 2 for 30–70% positive staining, score 3 for $>70\%$ positive staining and

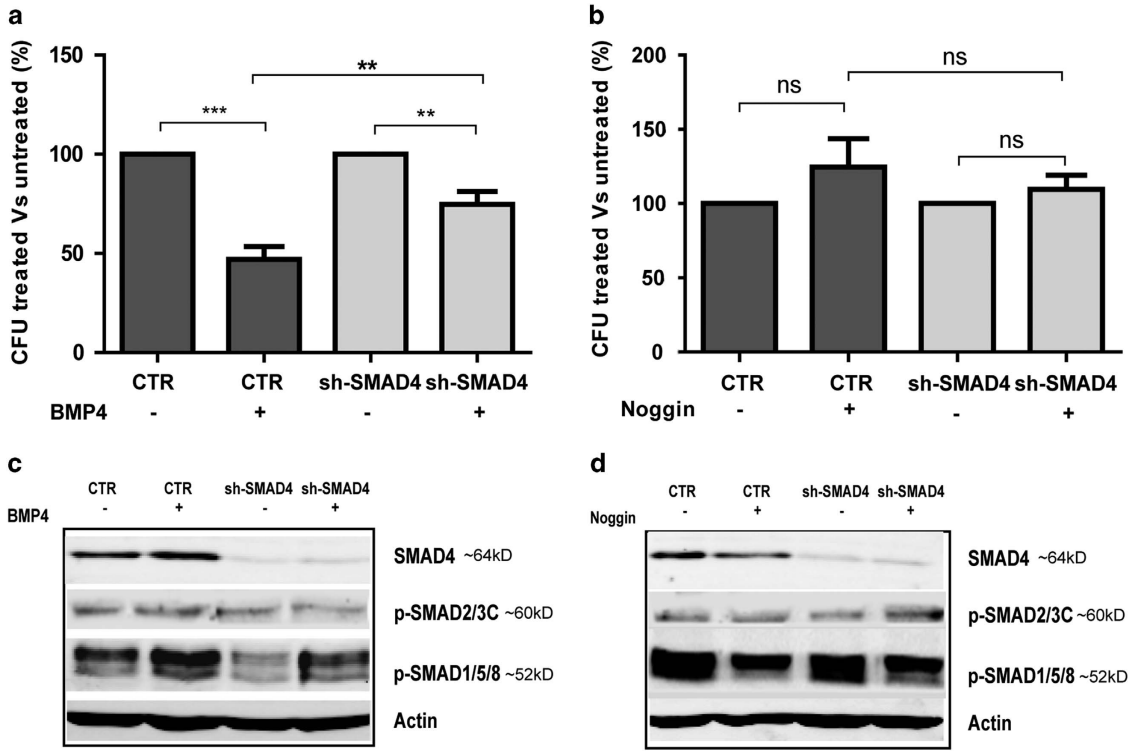
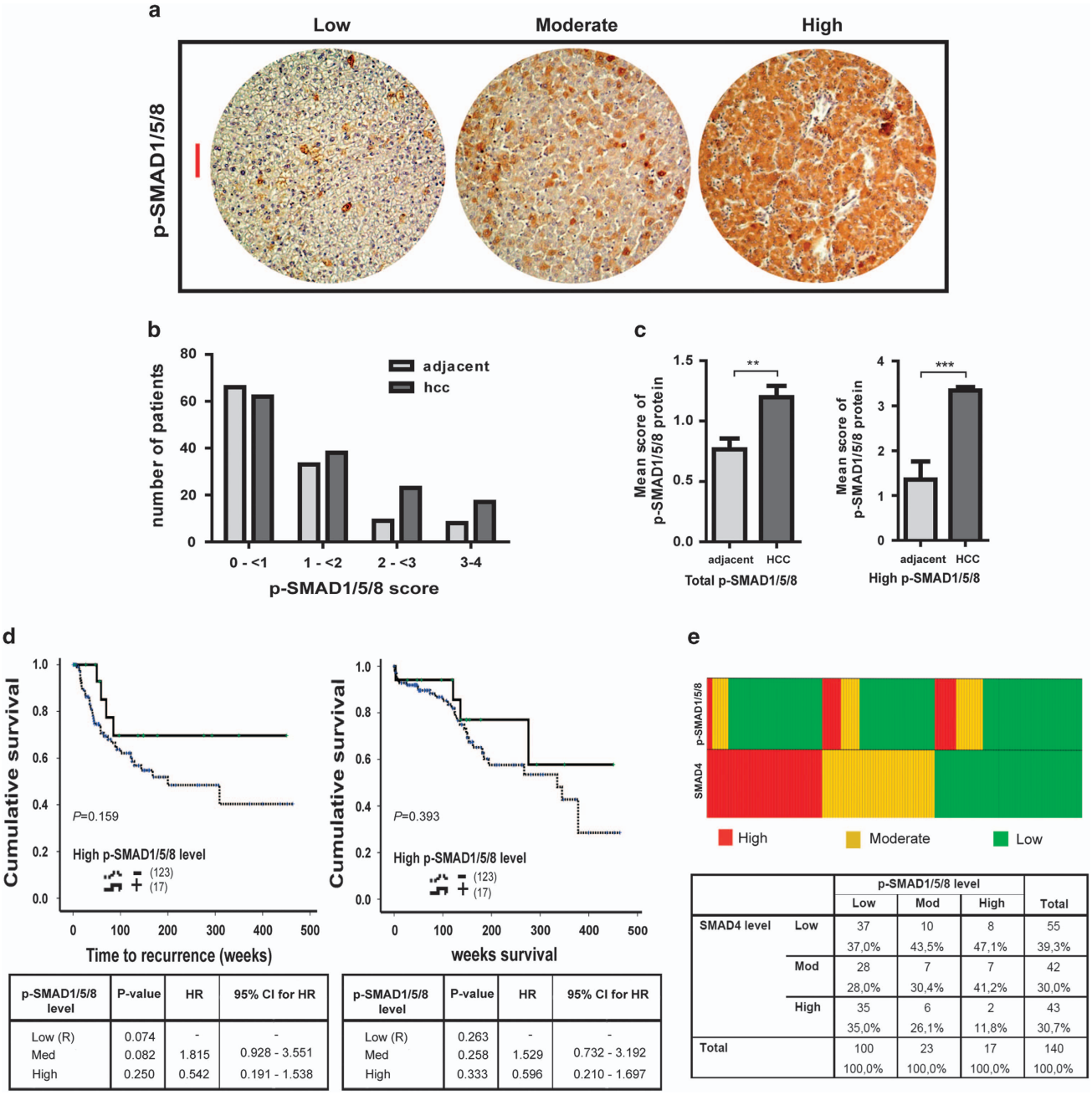


Figure 8. BMP4 significantly reduced the colony formation ability of HCC cells and its ability was attenuated by silencing SMAD4. (**a**) The efficiency of decreasing colony formation by BMP4 treatment was significantly reduced in Huh7 cells with SMAD4 knockdown. Error bars represent mean ± s.d. from $n = 4$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**b**) Although the difference was not statistically significant, adding BMP inhibitor Noggin appeared to increase the efficiency of colony formation in control cells and to a lesser extend in SMAD4 knockdown Huh7 cells. Error bars represent mean ± s.d. from $n = 4$, NS, not significant. (**c**) Protein levels of SMAD4, phospho-SMAD2/3 and phospho-SMAD1/5/8 after BMP4 treatment and (**d**) protein levels of SMAD4, phospho-SMAD2/3 and phospho-SMAD1/5/8 after Noggin treatment.

Figure 7. Simultaneous elevation of p-SMAD2/3L and SMAD4 is significantly associated with poor clinical outcome in HCC patients. The levels of p-SMAD2/3L (**a**) and p-SMAD3L (**d**) protein range from low (score: 0– < 2), moderate (score: 2– < 3) to high (score: 3–4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (**b**) P-SMAD2/3L expression was significantly higher in HCC tissues compared with adjacent sites. Error bars represents mean ± s.e.m., paired *t*-test, $^{***}P < 0.001$. (**c**) High levels of p-SMAD2/3L tend to have higher risk of poor survival (HR = 1.313) and 34 out of 140 patients have simultaneously sufficient levels of both p-SMAD2/3L and SMAD4. These patients have significantly poor clinical outcome as shown by both Cox regression and Kaplan–Meier analysis, $^{*}P < 0.05$. (**e**) The p-SMAD3L expression had no difference in HCC tissues compared with adjacent sites. Error bars represents mean ± s.e.m., paired *t*-test, NS, not significant. (**f**) High levels of p-SMAD3L tend to have higher risk of poor survival (HR = 2.450) and 21 out of 140 patients have simultaneously sufficient levels of both p-SMAD3L and SMAD4. These patients have significantly poor clinical outcome as shown by both Cox regression and Kaplan–Meier analysis. $^{*}P < 0.05$.



p-SMAD1/5/8 level	P-value	HR	95% CI for HR
Low (R)	0.074	-	-
Med	0.082	1.815	0.928 - 3.551
High	0.250	0.542	0.191 - 1.538

p-SMAD1/5/8 level	P-value	HR	95% CI for HR
Low (R)	0.263	-	-
Med	0.258	1.529	0.732 - 3.192
High	0.333	0.596	0.210 - 1.697

		p-SMAD1/5/8 level			
		Low	Mod	High	Total
SMAD4 level	Low	37	10	8	55
		37,0%	43,5%	47,1%	39,3%
	Mod	28	7	7	42
		28,0%	30,4%	41,2%	30,0%
	High	35	6	2	43
		35,0%	26,1%	11,8%	30,7%
Total		100	23	17	140
		100,0%	100,0%	100,0%	100,0%

Figure 9. The antitumor signaling mediated by p-SMAD1/5/8 and SMAD is inactivated in most of the HCC patients. (**a**) The levels of p-SMAD1/5/8 protein positivity range from low (score: 0– < 2), moderate (score: 2– < 3) to high (score: 3–4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (**b**) Different to SMAD4 or p-SMAD2/3, there were less patients with high p-SMAD1/5/8 score both in tumor and adjacent sites. (**c**) Overall p-SMAD1/5/8 expression was significantly higher in HCC tissue compared with adjacent liver tissue. The p-SMAD1/5/8 levels were also significantly higher in HCC tissue compared with adjacent tissue in the high-grade ($n = 17$) group. Error bars represents mean ± s.e.m., paired *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$. (**d**) From Cox regression analysis ($n = 130$), patients with high level of p-SMAD15/8 tend to have less risk of fast recurrence (HR = 0.542) and less risk to poor survival (HR = 0.596). Kaplan–Meier analysis ($n = 130$) showed similar trends. (**e**) However, there are only eight patients who have simultaneously sufficient levels of both SMAD4 and p-SMAD1/5/8 ($n = 2$ high; $n = 6$ moderate levels), suggesting that this signaling is inactivated in most of the HCC patients.

score 4 for >70% positive staining+high intensity. The scorings were done by two investigators and the difference of scoring was valued by Kappa test.

Lentiviral short hairpin RNA vectors

Lentiviral backbone vectors for *SMAD4* knockdown and non-targeting control were obtained from the Erasmus Center for Biomics (the Sigma–Aldrich TRC library, Zwijndrecht, The Netherlands). A vectors expressing short hairpin RNA targeting green fluorescent protein (not expressed in HCC cell lines) served as control (CTR). Lentiviral viral particles were generated as described previously.[49]

Cell culture and reagents

Human hepatoma cell lines (Huh7, Huh6 and PLC) were cultured in Dulbecco's modified Eagle's medium (Lonza, Breda, The Netherlands) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and 1% penicillin/streptomycin (Gibco, Bleiswijk, The Netherlands). SMAD4 knockdown cells and control cells were generated by inoculation of lentiviral

vectors and subsequently selected and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin/streptomycin and 2 μg/ml puromycin (Sigma–Aldrich). Recombinant human BMP4 protein (100 μg/ml, Merck Millipore) and recombinant human Noggin (50 μg/ml, R&D System, Oxon, UK) were used to treat cells, respectively.

Colony forming assay

Colony formation was performed in Huh7 cells as described previously.[48] After trypsinizing, 1000 cells were added to each well of a six-well plate and were cultured in Dulbecco's modified Eagle's medium as previously described. The colonies formed are counterstained with hematoxylin and eosin after 2 weeks.

Western blotting

Subconfluent cells were lysed in Laemmli sample buffer containing 0.1 M dithiothreitol and incubated for 5 min at 96 °C. Immunoblotting was performed using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE, USA). Antibodies used were mouse SMAD4 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc.), goat p-SMAD2/3 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc.) and rabbit p-SMAD1/5/8 (1:500 dilution, Cell Signaling). Quantification was performed using Odyssey LI-COR software.

Ring-barrier migration assay

Ring-barrier-based migration assays were performed as previously described.[50,51] Huh7 and its sh-SMAD4 cells, 3×10^5 cells were seeded in the ring in Dulbecco's modified Eagle's medium+10% fetal bovine serum +1% penicillin/streptomycin. After 24 h, the migration barrier was removed and the cells were washed twice followed by the addition of fresh medium. All cell tracking measurements were conducted using AxioVision 4.9.1 (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). *P*-values were calculated using the two-tailed Mann–Whitney test. Track diagram images were processed in Adobe Illustrator CS6 (Adobe Systems Inc., San Jose, CA, USA).

HCC xenograft tumor in nude mice

HCC xenograft tumor model in nude mice was established as previously described.[52] Ten mice for each cell line (Huh7), aged 6–8 weeks, were subcutaneously engrafted with 1 million control (CTR) and SMAD4 knockdown cells into the lower left or right flank, respectively. Tumor initiation in the mice was monitored. At the end of experiment, mice were killed and tumors were harvested and weighed. The use of animals was approved by the Animal Care and Ethics Committee at Hangzhou Normal University, Hangzhou, China.

Statistical analysis

Statistical analysis was performed by using X^2 test, nonparametric Mann–Whitney test, Cox regression analysis and Kaplan–Meier survival analysis in IBM SPSS Statistical (IBM Corporation, Armonk, NY, USA). *T*-test was also used using GraphPad InStat software (GraphPad Software Inc., San Diego, CA, USA). *P*-values < 0.05 were considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Supplementary Information

Supplementary Figure 1

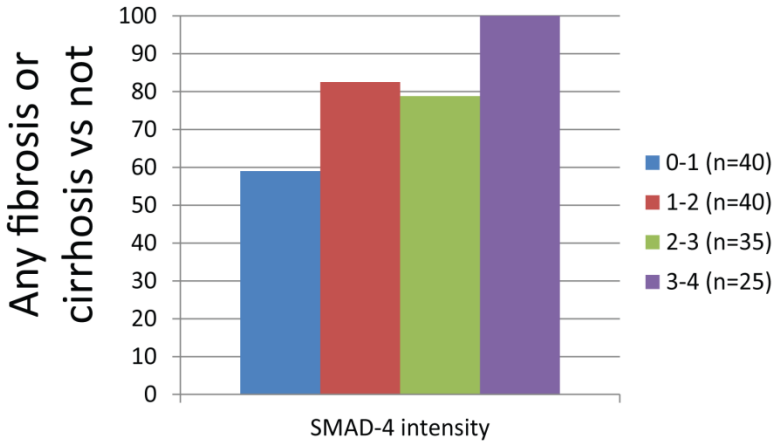


Figure S1. SMAD4 is significantly associated with liver fibrosis. Score 0 for 0-10% positive staining, score 1 for 10-30% positive staining, score 2 for 30-70% positive staining, score 3 for >70% positive staining, and score 4 for >70% positive staining. 0-1 represents any score from 0 to (including) 1; 1-2 represents any score higher than 1 and up to 2; 2-3 represents any score higher than 2 and up to 3; 3-4 represents any score higher than 3 and up to 4. $P < 0.01$; Chi-Square test.

Supplementary Figure 2

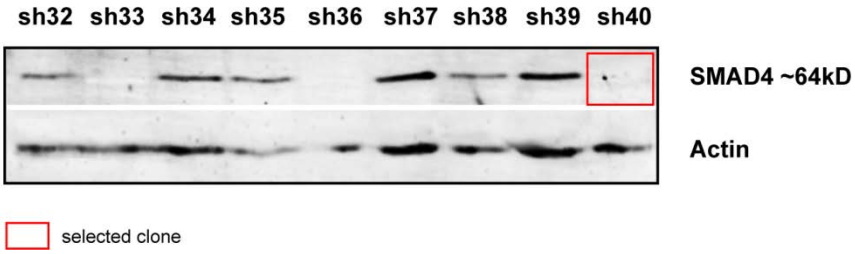
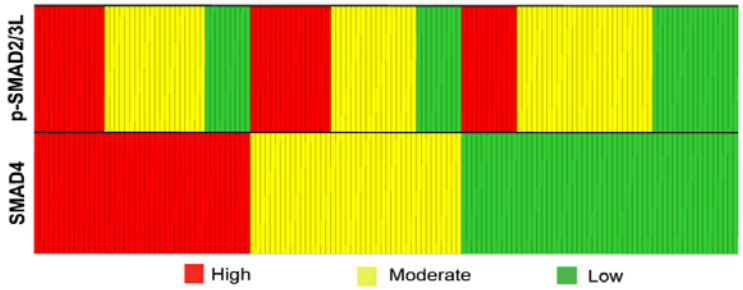


Figure S2. Selection of optimal lentiviral shRNA vectors for targeting SMAD4. Western blotting was used to evaluated the efficacy of SMAD4 knockdown in Huh7, Huh6 and PLC cells. Sh40 was selected for follow-up experimentation.

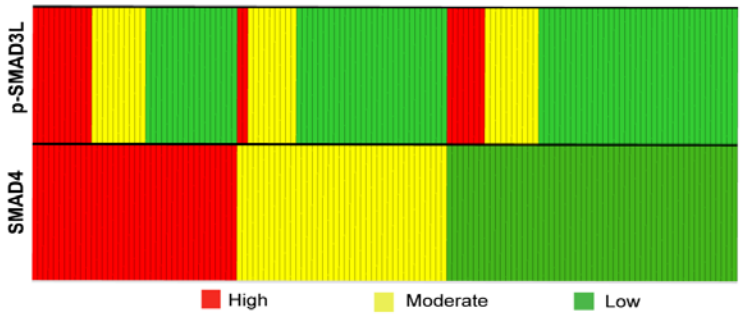
Supplementary Figure 3

A



		P-SMAD2/3L Level			
		Low	Moderate	High	Total
SMAD4 Level	Low	17	27	11	55
		48,6%	42,2%	26,8%	39,3%
	Moderate	9	17	16	42
		25,7%	26,6%	39,0%	30,0%
	High	9	20	14	43
		25,7%	31,2%	34,1%	30,7%
Total		35	64	41	140
		100,0%	100,0%	100,0%	100,0%

B



		P-SMAD3L Level			
		Low	Moderate	High	Total
SMAD4 Level	Low	37	10	7	54
		45,1%	34,5%	35,0%	41,2%
	Moderate	28	9	2	39
		34,1%	31,0%	10,0%	29,8%
	High	17	10	11	38
		20,7%	34,5%	55,0%	29,0%
Total		82	29	20	131
		100,0%	100,0%	100,0%	100,0%

Figure S3. The expression level of SMAD4 together with p-SMAD2/3L (A) and p-SMAD3L (B) in our HCC cohort and its cross tabulation.

Supplementary Figure 4

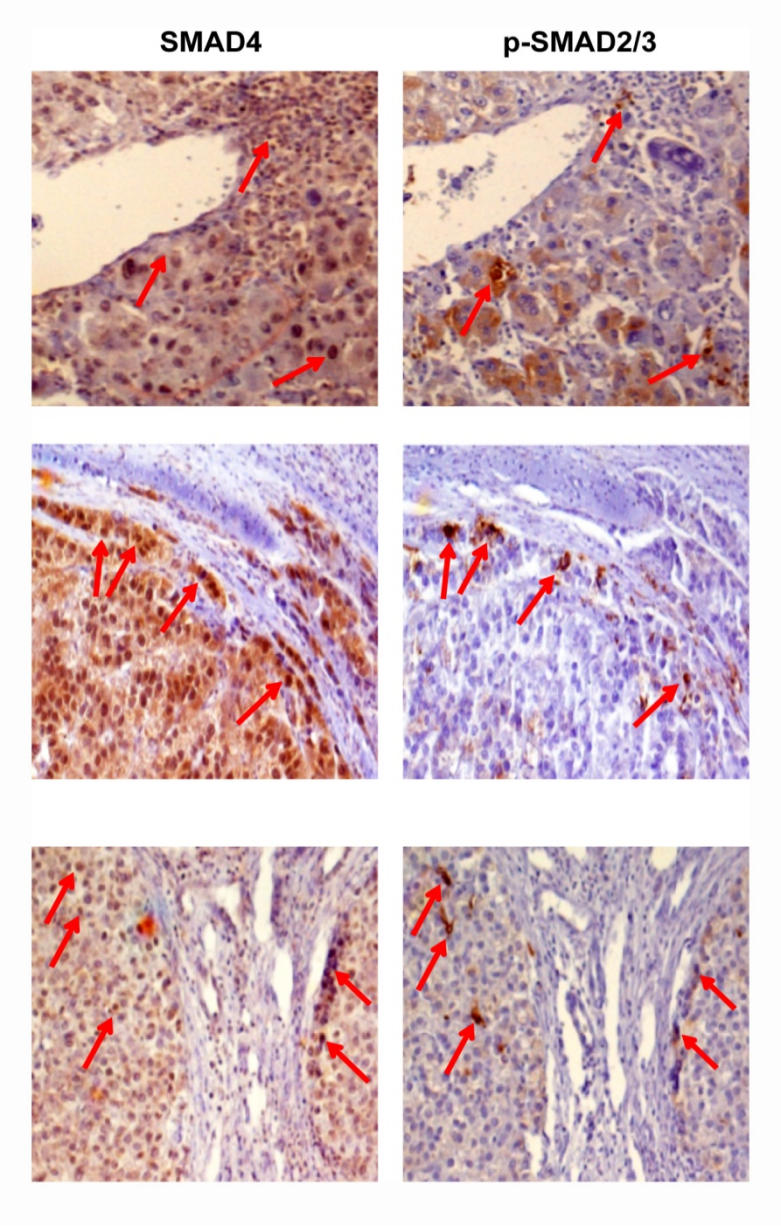


Figure S4. Immunohistochemistry staining of SMAD4 and p-SMAD2/3C in consecutive tissue slices of HCC patient

Supplementary Figure 5

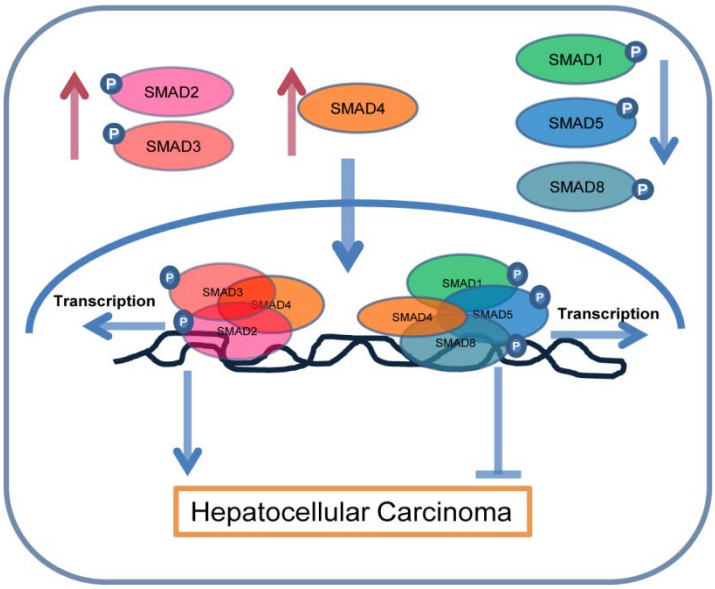


Figure S5. The model of action of SMADs in HCC. SMAD4 and p-SMAD2/3 are elevated in subset of patients that mediate a tumor promoting effect. Although high SMAD4 together with high p-SMAD1/5/8 can also exert an anti-tumor effect, this complex however is absent in majority patients, due to missing of either SMAD4 or p-SMAD1/5/8, or both of them.

Supplementary Table S1 Patient characteristics according to SMAD4 expression level.

No	Characteristics	SMAD4 expression		Total patients	*P*-value[a]
		Low-mod	High		
1	Age	60.70 ± 15.62	59.88 ± 12.61	140/140	0.763
2	Sex (% male)	68/97 (70.1%)	29/43 (67.4%)	97/140	0.753
3	Recurrence	32/97 (33,0%)	15/43 (34.9%)	47/140	0.827
4	Death	30/97 (30,9%)	14/43 (32.6%)	44/140	0.848
5	Size of tumor	6.88 ± 0.63	6.55 ± 0.99	98/140	0.777
6	Number of lesions	1.56 ± 0.11	1.39 ± 0.17	129/140	0.413
7	Vascular invasion	54/85 (63.5%)	24/36 (66.7%)	78/140	0.742
8	AFP before resection**	5.00	16.00	135/140	0.005

***P* value < 0.01

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table S2 Patient characteristics according to p-SMAD2/3C expression level.

No	Characteristics	p-SMAD2/3C expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age	58.71 ± 15.76	63.22 ± 12.56	140/140	0.077
2	Sex (% male)	57/86 (66.3%)	40/54 (74.1%)	97/140	0.330
3	Recurrence*	23/86 (26.7%)	24/54 (44.4%)	47/140	0.031
4	Death*	21/86 (24.4%)	23/54 (42.6%)	44/140	0.024
5	Size of tumor	6.68 ± 5.38	6.97 ± 5.13	98/140	0.796
6	Number of lesions	1.44 ± 1.00	1.6 ± 1.20	129/140	0.376
7	Vascular invasion	51/74 (68.9%)	27/47 (57.4%)	78/140	0.199
8	AFP before resection	10.00	7.00	135/140	0.077

**P* value < 0.05

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table S3 Patient characteristics according to p-SMAD2/3L expression level.

No	Characteristics	p-SMAD2/3L expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age	61.42 ± 13.12	58.10 ± 17.98	140/140	0.225
2	Sex (% male)	72/99 (72.7%)	25/41 (61.0%)	97/140	0.170
3	Recurrence	34/99 (34.3%)	13/41 (31.7%)	47/140	0.764
4	Death	30/99 (30.3%)	14/41 (34.1%)	44/140	0.656
5	Size of tumor	6.74 ± 4.89	6.90 ± 6.14	98/140	0.893
6	Number of lesions	1.54 ± 1.09	1.41 ± 1.07	129/140	0.520
7	Vascular invasion	56/84 (66.7%)	22/37 (59.5%)	78/140	0.445
8	AFP before resection	11.00	7.00	135/140	0.343

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table S4 Patient characteristics according to p-SMAD3L expression level.

No	Characteristics	p-SMAD3L level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	60.77 ± 15.00	60.25 ± 14.46	140/140	0.885
2	Sex (% male)	80/111 (72.1%)	11/20 (55.0%)	97/140	0.127
3	Recurrence	37/111 (33.3%)	8/20 (40.0%)	47/140	0.563
4	Death	32/111 (28.8%)	9/20 (45%)	44/140	0.151
5	Size of tumor	6.92 ± 5.34	6.83 ± 5.27	98/140	0.957
6	Number of lesions	1.55 ± 1.15	1.24 ± 0.56	129/140	0.080
7	Vascular invasion	62/95 (65.3%)	10/17 (58.8%)	78/140	0.610
8	AFP before resection	7.00	16.00	135/140	0.658

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table S5 Patient characteristics according to high SMAD4 expression and moderate-high p-SMAD2/3C expression level.

No	Characteristics	High SMAD4 + HighMod p-SMAD2/3C level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	61.04 ± 15.45	57.85 ± 10.82	140/140	0.319
2	Sex (% male)	80/114 (70.2%)	17/26 (65.4%)	97/140	0.633
3	Recurrence	35/114 (30.7%)	12/26 (46.2%)	47/140	0.132
4	Death	34/114 (29.8%)	10/26 (38.5%)	44/140	0.392
5	Size of tumor	6.85 ± 5.23	6.50 ± 5.53	98/140	0.800
6	Number of lesions	1.52 ± 1.08	1.44 ± 1.12	129/140	0.744
7	Vascular invasion	67/100 (67%)	11/21 (52.4%)	78/140	0.203
8	AFP before resection	6.50	14.00	135/140	0.136

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table S6 Patient characteristics according to high SMAD4 expression and moderate-high p-SMAD2/3L expression level.

No	Characteristics	High SMAD4 + ModHigh p-SMAD2/3L level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	60.92 ± 15.23	58.96 ± 13.18	140/140	0.503
2	Sex (% male)	76/106 (71.7%)	21/34 (61.8%)	97/140	0.275
3	Recurrence	36/106 (34.0%)	11/34 (32.4%)	47/140	0.863
4	Death	31/106 (29.2%)	13/34 (38.2%)	44/140	0.392
5	Size of tumor	6.89 ± 5.32	6.48 ± 5.12	98/140	0.738
6	Number of lesions	1.52 ± 1.04	1.47 ± 1.22	129/140	0.831
7	Vascular invasion	58/91 (63.7%)	20/30 (66.7%)	78/140	0.771
8	AFP before resection**	5.50	27.00	135/140	0.004

***P* value < 0.01
[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table S7 Patient characteristics according to high SMAD4 expression and moderate-high p-SMAD3C expression level.

No	Characteristics	High SMAD4 + ModHigh p-SMAD3C level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	60.54 ± 15.00	59.67 ± 14.06	140/140	0.804
2	Sex (% male)	81/114 (71.1%)	12/21 (57.1%)	97/140	0.206
3	Recurrence	38/114 (33.3%)	7/21 (33.3%)	47/140	1.000
4	Death	35/114 (30.7%)	8/21 (38.1%)	44/140	0.504
5	Size of tumor	6.58 ± 5.12	8.14 ± 6.26	98/140	0.313
6	Number of lesions	1.59 ± 1.16	1.06 ± 0.23	129/140	0.054
7	Vascular invasion	63/98 (64.3%)	12/18 (66.7%)	78/140	0.846
8	AFP before resection	6.50	13.50	135/140	0.154

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table S8 Patient characteristics according to p-SMAD1/5/8 expression level.

No	Characteristics	p-SMAD1/5/8 expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age***	62.24 ± 11.96	47.53 ± 24.29	140/140	0.000
2	Sex (% male)	88/123 (71.5%)	9/17 (52.9%)	97/140	0.119
3	Recurrence	43/123 (35,0%)	4/17 (23.5%)	47/140	0.350
4	Death	40/123 (32.5%)	4/17 (23.5%)	44/140	0.454
5	Size of tumor	6.70 ± 5.14	7.45 ± 6.38	98/140	0.657
6	Number of lesions	1.50 ± 1.07	1.41 ± 1.23	129/140	0.708
7	Vascular invasion	70/105 (66.7%)	78/121 (64.5%)	78/140	0.194
8	AFP before resection	8.00	7.50	135/140	0.664

****P* value < 0.001
[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test