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Sonic hedgehog signaling pathway induces cell migration and invasion through focal adhesion kinase/AKT signaling-mediated activation of matrix metalloproteinase (MMP)-2 and MMP-9 in liver cancer

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The aberrant activation of sonic hedgehog (SHH) pathway contributes to initiation and progression of various malignancies. However, the roles and underlying mechanisms of SHH signaling pathway in invasion and metastasis of liver cancer have not been well understood. In this study, we found that SHH signaling was activated and correlated with invasion and metastasis in hepatocellular carcinoma (HCC). Enhanced SHH signaling by recombinant human SHH N-terminal peptide (rSHH-N) promoted hepatoma cell adhesion, migration and invasion, whereas blockade of SHH signaling with SHH neutralizing antibody or cyclopamine suppressed hepatoma cell adhesion, migration and invasion. Furthermore, matrix metalloproteinase (MMP)-2 and MMP-9 expressions and activities were upregulated and downregulated by rSHH-N and SHH signaling inhibitor, respectively. The rSHH-N-mediated hepatoma cell migration and invasion was blocked by MMP-specific inhibitors or neutralizing antibodies to MMP-2 and MMP-9. In addition, phosphorylations of AKT and focal adhesion kinase (FAK) were increased and decreased by rSHH-N and SHH signaling inhibitor, respectively. Further investigations showed that activation of AKT and FAK were required for rSHH-N-mediated upregulation of MMP-2 and MMP-9, cell migration and invasion. Finally, we found that SHH protein expression was positively correlated with phosphorylatd FAK Tyr397, phosphorylatd AKT Ser473, MMP-2 and MMP-9 protein expressions in HCC samples. Taken together, our findings suggest that SHH pathway induces cell migration and invasion through FAK/AKT signaling-mediated MMP-2 and MMP-9 production and activation in liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies and the third most common cause of death from cancer in the world (1). Despite great advances in the diagnosis and treatment of HCC, its prognosis is dismal. Postoperative recurrence or metastasis is quite common and the main factor related to poor prognosis in patients with HCC. Further insight into the molecular mechanisms underlying HCC recurrence and metastasis might help identify novel therapeutic targets and consequently improve the prognosis.

Abbreviations: FAK, focal adhesion kinase; Gli-1, glioma-associated oncogene-1; HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; MTT, 3-[4,5-dimethylthiazol-2- yl]-2,5-diphenyltetrazolium bromide; PI3K, phosphoinositide 3-kinase; PTCH1, patched1; rSHH-N, recombinant human SHH N-terminal peptide; SD, standard deviation; SHH, sonic hedgehog; siRNA, small interfering RNA; SMO, smoothened.

Hedgehog (HH) signaling pathway plays critical roles in embryonic patterning and maintenance of adult tissue homeostasis (2,3). There are three known mammalian HH ligands: Sonic hedgehog (SHH), Indian hedgehog and Desert hedgehog. Among them, SHH is the most widely expressed and the most potent (4). In the absence of the HH ligand, patched1 (PTCH1), a 12-transmembrane protein, represses the activity of smoothened (SMO), a seven-transmembrane protein (2,4). HH signaling is initiated by the binding of HH ligands to PTCH1 that relieves PTCH1-mediated repression of SMO. Upon activation, SMO triggers downstream signaling cascades that result in nuclear translocation of glioma-associated oncogene (Gli), a zinc-finger transcription factor, and consequently activates HH target gene transcription (2,4). Aberrant activation of HH signaling pathway has been implicated in tumorigenesis and development of a variety of tumors including basal cell carcinoma, lung, breast, ovarian, pancreatic, prostatic, gastrointestinal cancers, leukemia and medulloblastoma (2,5–8). Recently, constitutive activation of HH signaling pathway has been found in HCC (9–11). Furthermore, some studies suggest that SHH signaling pathway is involved in invasion and metastasis of prostatic (12), gastric (13), esophageal (14), pancreatic (15,16) and ovarian (17) carcinomas. However, the role of SHH signaling pathway in invasion and metastasis of HCC remains unclear. In addition, the mechanisms by which SHH signaling pathway promotes tumor invasion and metastasis need to be further elucidated.

Cancer invasion and metastasis is a complex multistep process involving multiple genetic alterations. The degradation of extracellular matrix is an essential step in cancer invasion and metastasis. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that degrade almost all extracellular matrix components, play important roles in cancer invasion and metastasis (18,19). Our previous study has suggested that MMP-2 and MMP-9 are closely associated with invasion and metastasis in HCC (20). Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase, is a downstream target of several growth factor receptors. FAK has been suggested to be critical for invasion and metastasis through regulating the expressions and activities of MMP-2 and MMP-9 in HCC (21). Phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is one of downstream effectors of FAK (22–24) and involved in invasion and metastasis of cancer (25). Accumulated data have shown that PI3K/AKT signaling pathway promotes invasion and metastasis via upregulating MMP-2 or MMP-9 in several cancers (26–28).

In this study, we analyzed the effects of SHH signaling pathway on invasion and metastasis of liver cancer. In addition, the mechanisms behind SHH signaling pathway regulation in liver cancer cell invasion were investigated. Our results indicate that SHH signaling pathway induces liver cancer cell invasion by regulating FAK/AKT signaling pathway-mediated activation and expression of MMP-2 and MMP-9.

Materials and methods

Patients and samples

Two hundred patients who underwent surgery for HCC in the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China) between July 2004 and March 2007 were enrolled in the study. The clinicopathological features of these patients have already been described (20,21). Paired HCC and adjacent non-cancerous liver tissue (at least 20 mm beyond HCC) samples from all patients were obtained immediately after resection and formalin-fixed and then paraffin-embedded. All HCCs were graded using World Health Organization grading system and staged according to the sixth edition of the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system. In addition, RNA was obtained from 36 (9 at stage I, 12 at stage II and 15 at stage III–IV; 19 metastatic HCCs and 17 non-metastatic HCCs) of these 200 patients with HCC as described previously (20,21). Informed consent was obtained from all patients. This study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University.

Tissue microarray construction and immunohistochemistry

We constructed tissue microarray blocks consisting of three representative 1.0 mm cores of each tumor and matched adjacent non-cancerous liver tissue from 200 patients with HCC as described previously (20). Immunohistochemistry was performed on 4 μm thick tissue microarray sections as described previously (20). The primary antibodies against SHH (sc-1194), PTCH1 (sc-6149), SMO (sc-13943) and Gli-1 (sc-20687) (Santa Cruz Biotechnology, Santa Cruz, CA) were applied at 1:100 dilution. Immunoreactivity of these antibodies was evaluated and scored as negative (–), weak (1+), moderate (2+) or strong (3+) as described previously (20). Immunohistochemical analysis of Ki-67 was described previously (20).

Real-time PCR

Total RNA was extracted from the tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was synthesized and real-time PCR was performed as described previously (21). The primers for human SHH (forward primer: 5′CCCAATTACAACCCCGACATC 3′, reverse primer: 5′ TCACCCGCAGTTTCACTCCT 3′), PTCH1 (forward primer: 5′ TGAGACTGACCACGGCCTG 3′, reverse primer: 5′ACCCTCAGTTGGAGCTGCTTC 3′), SMO (forward primer: 5′ GAGACTCTGTCCTGCGTCATCA 3′, reverse primer: 5′ AGGCATAGGT GAGGACCACAA 3′) and Gli-1 (forward primer: 5′ AGGGCTGCAG TAAAGCCTTCA 3′, reverse primer: 5′ CTTGACATGTTTTCGCAGCG 3′) were used in the real-time PCR. The primers specific for human MMP-2, MMP-9 and β-actin were reported previously (20).

Cell culture and reagents

Human hepatoma cell lines, SMMC-7721, SK-Hep1 and human hepatocyte cell line L02, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin–streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Recombinant human SHH N-terminal peptide (rSHH-N) (Catalog no. 1314-SH) and human/mouse SHH N-terminal peptide monoclonal antibody (Anti-SHH) (Catalog no. MAB464) were purchased from R&D Systems (Minneapolis, MN). Cyclopamine (Catalog no. BML-GR334), a specific antagonist of SMO, and GM6001 (Catalog no. BML-EI300), a MMP inhibitor, were obtained from Biomol (Plymouth, PA). Tomatidine (Catalog no. T2909), an inactive analogue of cyclopamine, and Ly294002 (Catalog no. L9908), a selective inhibitor of PI3K were from Sigma (St Louis, MO). Anti-phospho-Akt (Ser473), anti-AKT and anti-FAK antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-FAK (Tyr 397) antibody was from Abcam (Cambridge, MA). Anti-GAPDH, anti-TIMP-1, anti-TIMP-2, monoclonal MMP-2 and MMP-9 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell adhesion assay

Cell adhesion assay was performed as described previously (21). Briefly, the pretreated cells (1×10^5/well) suspended in medium were plated into 96-well plates coated with fibronectin (Becton–Dickinson, Bedford, MA). After incubation in 5% CO_2 at 37°C for 1 h, the cells were washed thrice with phosphate-buffered saline to remove the non-adherent cells. The attached cells were analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell migration and invasion assay

The cells (1×10^5/well) were suspended in 100 μl of serum-free medium with or without different concentrations of reagents and seeded into the upper chamber of the 24-well transwell inserts with 8 μm pores (Corning, NY). Dulbecco's modified Eagle's medium with 20% fetal bovine serum was added in the lower chamber. Following 24 h of incubation at 37°C, the cells on the upper surface of the filter were mechanically removed with a cotton swab, and the migrated cells on the lower surface were fixed with methanol and stained with 0.5% crystal violet. The stained cells were counted in five randomly selected fields per filter under a microscope (100× magnification). The cell invasion potential was assessed as described above, except that the transwell inserts were coated with Matrigel (50 μl/well; BD Bioscience, Bedford, MA). Each experiment was performed in triplicate wells and repeated three times.

Cell viability assay

Cell viability was assessed by MTT assay. Cells (5×10^3/well) were seeded in 96-well plates and cultured overnight. The cells were then treated with different concentrations of reagents. Twenty hours later, 20 μl MTT (5 mg/ml in phosphate-buffered saline; Sigma) was added to each well and incubated for a further 4 h. Next, the supernatant was then aspirated and 100 μl dimethyl sulfoxide was added into each well. The optical density at 570 nm was measured with an enzyme-linked immunity implement. Each experiment was repeated three times in quadruplicate wells.

Western blot

Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk for 1 h and then incubated overnight at 4°C with the primary antibodies against SHH (1:200), PTCH1 (1:200), SMO (1:200), Gli-1 (1:200), MMP-2 (1:500), MMP-9 (1:400), TIMP-1 (1:500), TIMP-2 (1:500), AKT(1:1000), phospho-AKT (Ser473) (1:500), FAK (1:1000), phospho-FAK Tyr 397 (1:800) or GAPDH (1:2000). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature and finally visualized using SuperSignal® West Pico chemiluminescent substrate kit (Pierce, Rockford, IL).

MMP gelatinase activity assay

Cells were incubated in serum-free medium with or without different concentrations of reagents for 24 h. The activities of MMP-2 and MMP-9 in the conditioned medium were determined with CHEMICON Gelatinase Activity Assay Kit (Chemicon, Temecula, CA) as described previously (21).

Small interfering RNA transfection

The small interfering RNA (siRNA) specific to FAK and control siRNA were reported previously (21). siRNA transfection was done as described previously (21,29).

Statistical analysis

Chi-square (χ^2) or Fisher's exact test was used to assess the associations between expression of SHH signaling pathway components and clinicopathological parameters. Spearman's correlation test was used to determine the correlation between various protein expressions. Quantitative variables were expressed as means ± standard deviation (SD) and analyzed by Student's *t*-test. All analyses were performed using SPSS 13.0 (SPSS, Chicago, IL). A $P < 0.05$ was considered statistically significant.

Results

Overexpression of SHH signaling pathway components was correlated with invasion and metastasis in HCC

To determine expression of main components of SHH signaling pathway in HCC tissues, we performed immunohistochemistry on tissue microarrays including 200 pairs of HCC and adjacent non-cancerous liver tissues. The representative immunostaining profile of SHH, PTCH1, SMO and Gli-1 in HCC was shown in Figure 1A. SHH protein was detected in 66% (132/200) of HCC, whereas it was stained in 18.5% (37/200) of matched adjacent non-cancerous liver tissues ($P < 0.001$). SHH overexpression was correlated with capsular invasion ($P = 0.046$), higher tumor stage ($P = 0.002$), vascular invasion ($P = 0.010$) and intrahepatic metastasis ($P = 0.041$) in HCC (Table I). The immunoreactivity of PTCH1 was increased in HCC (48.5%, 97/200) compared with matched adjacent non-cancerous liver tissues (36%, 72/200) ($P = 0.011$). There was a significant association between PTCH1 expression and tumor grade ($P = 0.006$) (Table I). Positive staining of SMO was present in 63.5% (127/200) of HCC, whereas its expression was observed in 11.5% (23/200) of matched adjacent non-cancerous liver tissues ($P < 0.0001$). Elevated SMO expression was associated with higer TNM stage ($P = 0.038$), vascular invasion ($P = 0.035$) and intrahepatic metastasis ($P = 0.005$) in HCC (Table I). Gli-1 protein expression was detected more frequently in HCC (74.5%, 149/200) than in matched adjacent non-cancerous liver tissues (9.5%, 19/200) ($P < 0.0001$). High expression of Gli-1 was significantly correlated with capsular invasion ($P = 0.026$), advanced tumor stage ($P = 0.027$), vascular invasion ($P = 0.011$) and intrahepatic metastasis ($P = 0.022$) in HCC (Table I).

To confirm our immunohistochemical results, we analyzed the mRNA levels of SHH pathway components in 36 of the 200 pairs of HCC and adjacent non-cancerous tissues. Our results showed that the mRNA levels of SHH, PTCH1, SMO and Gli-1 were significantly higher in HCC tissues than in the matched adjacent non-cancerous liver tissues ($P < 0.01$) (Figure 1B). Furthermore, SHH, SMO and Gli-1 mRNA levels were markedly increased in advanced TNM stage of HCC ($P < 0.01$) (Figure 1C) and in metastatic HCC tissues compared with non-metastatic HCC tissues ($P < 0.01$) (Figure 1D). These results were consistent with the immunohistochemical findings.

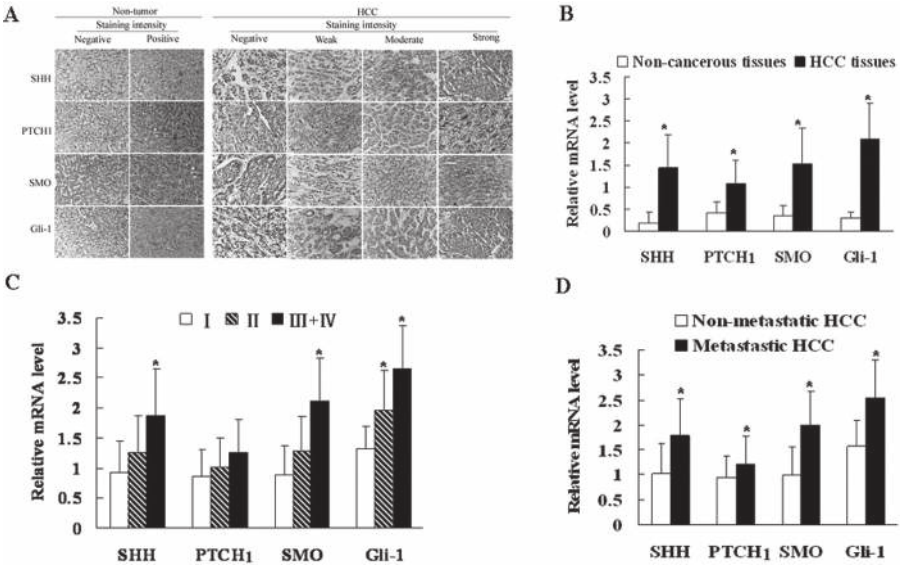

Fig. 1. Expression of SHH signaling pathway components in HCC tissues. (**A**) Representative immunostaining of SHH, PTCH1, SMO and Gli-1 in HCC and adjacent non-tumorous liver tissues (×200 magnification). (**B**) The expression levels of SHH, PTCH1, SMO and Gli-1 mRNA in HCC tissues and paired adjacent non-cancerous liver tissues via real-time PCR. The mRNA expression levels were normalized against β-actin. Columns, mean of three independent experiments; bars, SD. **P* < 0.001, compared with adjacent non-cancerous liver tissues. (**C**) Transcript levels of SHH, PTCH1, SMO and Gli-1 in various stage HCC tissues via real-time PCR. The transcript levels were normalized to that of β-actin. Data are presented as the mean ± SD of three independent experiments. **P* < 0.001 compared with stage I. (**D**) Real-time PCR analysis of SHH, PTCH1, SMO and Gli-1 mRNA expression in metastatic and non-metastatic HCC tissues. **P* < 0.001, compared with non-metastatic HCC tissues.

SHH signaling pathway was involved in hepatoma cell adhesion, migration and invasion

Cell adhesion, migration and invasion are critical events in tumor metastasis, therefore the effect of SHH signaling pathway on adhesion, migration and invasion of liver cancer cells was explored *in vitro*. First, we detected SHH, PTCH1, SMO and Gli-1 mRNA and protein levels in LO2, SMMC-7721 and SK-Hep1 cells. Our results showed that SHH, PTCH1, SMO and Gli-1 mRNA and protein levels were significantly higher in both SMMC-7721 and SK-Hep1 cells than LO2 cells (Supplementary Figure 1, available at *Carcinogenesis* Online). Next, we found that rSHH-N induced cell adhesion, migration and invasion of SMMC-7721 and SK-Hep1 cells in a dose-dependent manner, whereas Anti-SHH significantly suppressed cell adhesion, migration and invasion (Figure 2A–C). Furthermore, our results demonstrated that cyclopamine, a specific antagonist of SMO, dose-dependently inhibited cell adhesion, migration and invasion; whereas tomatidine (5 μM), an inactive analogue of cyclopamine, had not significant effect (Figure 2D–F). However, we found that Anti-SHH or cyclopamine did not affect cell adhesion, migration and invasion in LO2 cells (Supplementary Figure 2, available at *Carcinogenesis* Online), in which hedgehog signaling is not activation, indicating that the effects of Anti-SHH and cyclopamine are specific to SHH signaling in these cells. In addition, we determined whether the effect of SHH pathway on cell migration and invasion was dependent on its cellular cytotoxicity. The results of MTT assay showed that rSHH-N, Anti-SHH or cyclopamine did not affect cell viability at the indicated concentrations (Supplementary Figure 3, available at *Carcinogenesis* Online). These results suggest that the effect of SHH pathway on hepatoma cell migration and invasion is independent of its cellular cytotoxicity.

SHH signaling promoted cell migration and invasion by increasing expressions and activities of MMP-2 and MMP-9 in hepatoma cells

MMPs, especially MMP-2 and MMP-9, have been implicated in tumor invasion and metastasis (18,30). Therefore, we next determined whether SHH pathway-induced cell invasion was correlated with the expression and/or activity of MMP-2 and MMP-9. Our results showed that rSHH-N increased MMP-2 and MMP-9 mRNA and protein levels and activities in SMMC-7721 cells in a dose-dependent manner. However, both Anti-SHH and cyclopamine dose-dependently decreased MMP-2 and MMP-9 mRNA and protein expression and activities, whereas IgG antibody and tomatidine showed negligible effect (Figure 3A–C).

The activity of MMP is regulated by their endogenous tissue inhibitors, TIMPs (31). However, our western blot analysis showed that there was no change in TIMP-1 and TIMP-2 protein expressions after rSHH-N, Anti-SHH or cyclopamine treatment in SMMC-7721 cells (Figure 3D). These results indicate that TIMP-1 and TIMP-2 are not directly involved in SHH signaling-mediated induction of MMP-2 and MMP-9.

To assess the effect of MMP-2 and MMP-9 in the rSHH-N-enhanced migration and invasion of HCC cells, we blocked the gelatinase activities with MMP inhibitor GM6001, MMP-2 or MMP-9 neutralizing antibody in the presence of 0.5 μg/ml rSHH-N. Our results demonstrated that the stimulatory effects of rSHH-N on cell migration and invasion were significantly reversed by specific inhibition of MMP-2 and MMP-9 (Figure 3E,3F), suggesting that rSHH-N-induced MMP-2 and MMP-9 play a critical role in rSHH-N-increased cell migration and invasion.

SHH signaling induced cell migration and invasion through PI3K/AKT pathway-mediated expressions and activation of MMP-2 and MMP-9 in hepatoma cells

Studies have shown that the PI3K/AKT signaling pathway plays an important role in invasion and metastasis of liver cancer through regulating MMP-2 and MMP-9 (26,27,32). Moreover, PI3K/AKT signaling pathway is crucial for SHH signaling (33,34). Therefore, we studied if PI3K/AKT signaling pathway plays a role in SHH signaling-induced cell invasion and MMP-2 and MMP-9 activation in hepatoma cells. We first examined the potential effect of SHH signaling on PI3K/AKT pathway. Our results showed that rSHH-N significantly increased phosphorylated AKT (Ser 473) protein expression, whereas both Anti-SHH and cyclopamine decreased phosphorylated AKT (Ser 473) protein expression in SMMC-7721 cells. However, they did not affect the total protein expression level of AKT (Figure 4A). These results indicate that SHH signaling induces activation of PI3K/AKT pathway.

Table I. Correlations of SHH, PTCH1, Smo and Gli-1 expression with clinicopathologic variables in patients with hepatocellular carcinoma

Variable	*n*	SHH				*P*	PTCH1				*P*	Smo				P	Gli-1				*P*
		—	1+	2+	3+		—	1+	2+	3+		—	1+	2+	3+		—	1+	2+	3+	
Age						0.421					0.914					0.845					0.252
≤50	95	29	17	24	25		50	25	12	8		33	23	25	14		21	13	31	30	
>50	105	39	12	31	23		53	29	16	7		40	27	22	16		30	10	24	41	
Sex						0.739					0.053					0.714					0.468
Male	183	61	27	52	43		96	49	27	11		69	45	42	27		45	21	53	64	
Female	17	7	2	3	5		7	5	1	4		4	5	5	3		6	2	2	7	
Etiology						0.448					0.468					0.941					0.580
Non-infection	42	13	5	10	14		26	7	7	2		18	9	8	7		8	3	12	19	
Hepatitis B	145	53	22	39	31		70	44	20	11		51	38	35	21		38	18	41	48	
Hepatitis C and other	13	2	2	6	3		7	3	1	2		4	3	4	2		5	2	2	4	
Liver cirrhosis						0.780					0.864					0.187					0.562
Absence	79	25	11	21	22		42	22	9	6		23	22	18	16		16	9	23	31	
Presence	121	43	18	34	26		61	32	19	9		50	28	29	14		35	14	32	40	
Tumor size (cm)						0.175					0.531					0.835					0.249
≤5	58	25	10	11	12		28	16	11	3		22	12	15	9		20	6	16	16	
>5	142	43	19	44	36		75	38	17	12		51	38	32	21		31	17	39	55	
Serum AFP (μg/l)						0.906					0.398					0.650					0.892
≤20	62	21	8	19	14		35	12	9	6		25	13	13	11		16	6	16	24	
>20	138	47	21	36	34		68	42	19	9		48	37	34	19		35	17	39	47	
Capsular invasion						0.046					0.864					0.304					0.026
Absence	118	47	20	28	23		58	33	18	9		49	29	24	16		35	14	37	32	
Presence	82	21	9	27	25		45	21	10	6		24	21	23	14		16	9	18	39	
Tumor grade						0.212					0.006					0.100					0.237
I	29	14	3	5	7		10	9	3	7		13	5	5	6		11	3	8	7	
II	131	43	16	38	34		72	37	16	6		41	32	36	22		32	15	31	53	
III	40	11	10	12	7		21	8	9	2		19	13	6	2		8	5	16	11	
TNM stage						0.002					0.060					0.038					0.027
I	61	31	8	9	13		30	11	14	6		26	18	12	5		23	5	19	14	
II	56	20	11	16	9		26	22	4	4		25	7	16	8		10	4	16	26	
III	83	17	10	30	26		47	21	10	5		22	25	19	17		18	14	20	31	
Vascular invasion						0.01					0.107					0.035					0.011
Absence	140	55	21	39	25		68	36	25	11		56	33	36	15		41	10	41	48	
Presence	60	13	8	16	23		35	18	3	4		17	17	11	15		10	13	14	23	
Intrahepatic metastasis						0.041					0.512					0.005					0.022
Absence	127	52	18	29	28		65	36	19	7		54	35	26	12		41	15	33	38	
Presence	73	16	11	26	20		38	18	9	8		19	15	21	18		10	8	22	33	
Ki-67						0.412					0.997					0.734					0.815
≤50%	132	43	22	33	34		68	36	18	10		50	33	28	21		33	14	39	46	
>50%	68	25	7	22	14		35	18	10	5		23	17	19	9		18	9	16	25	

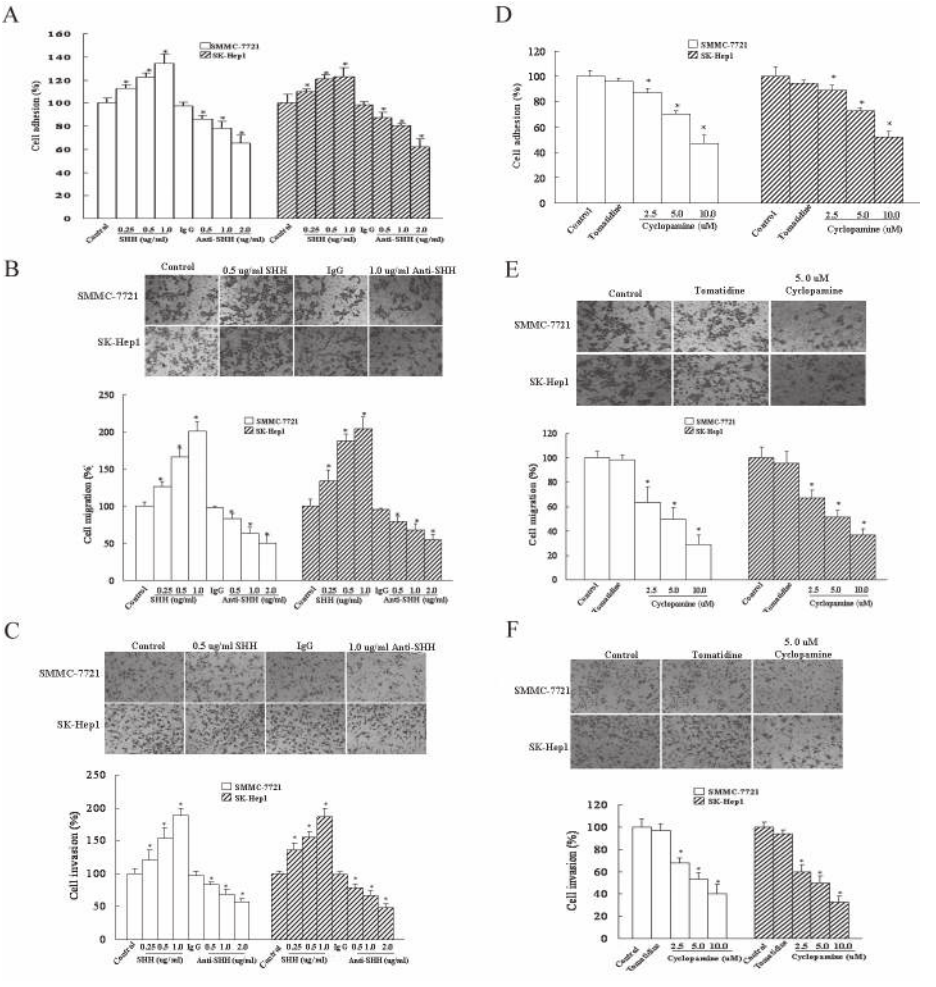

Fig. 2. SHH signaling pathway promotes hepatoma cell adhesion, migration and invasion. (**A**) and (**D**) SMMC-7721 and SK-Hep1 cells were pretreated with various concentrations of rSHH-N, Anti-SHH or cyclopamine for 24 h. The pretreated cells (1×10^5/well) were then seeded into fibronectin-coated 96-well plates and incubated for 1 h. The adhered cells were analyzed by MTT assay. The data were presented as the mean of three independent experiments ± SD and expressed as the percentage of control. $^*P < 0.001$, compared with controls. (**B**) and (**E**) SMMC-7721 and SK-Hep1 cells (1×10^5/well) were seeded into the upper chambers of the Transwell and incubated for 24 h in the presence or absence of various concentrations of rSHH-N, Anti-SHH or cyclopamine. The migrated cells were fixed, stained and counted. The data were expressed as percentage relative to control. $^*P < 0.001$, compared with controls. (**C**) and (**F**) SMMC-7721 and SK-Hep1 cells (1×10^5/well) were seeded onto the Matrigel-coated filter of Transwell chambers. After 24 h of incubation with or without various concentrations of rSHH-N, Anti-SHH or cyclopamine, the invaded cells were stained with 0.5% crystal violet and counted under the microscope. The data were presented as the percentage of control. Top, representative pictures. Columns, mean of three independent experiments; bars, SD. $^*P < 0.001$, compared with controls.

Next, to investigate whether activation of the PI3K/AKT pathway is involved in SHH-mediated upregulation of MMP-2 and MMP-9 expressions and activities, cell migration and invasion in hepatoma cells, we blocked PI3K/AKT pathway using PI3K inhibitor, Ly294002. The results showed that Ly294002 significantly decreased rSHH-N-dependent MMP-2 and MMP-9 mRNA and protein expressions and activities (Figure 4B–D) and phosphorylation of AKT (Figure 4C). Furthermore, rSHH-N-mediated hepatoma cell migration and invasion were markedly inhibited by Ly294002 (Figure 4E, 4F). These data suggest that SHH signaling induces hepatoma cell migration and invasion, expressions and activation of MMP-2 and MMP-9 through activation of PI3K/AKT pathway.

FAK was required for SHH signaling-induced cellular migration and invasion in hepatoma cells

FAK plays a critical role in HCC invasion and metastasis by modulating MMP-2 and MMP-9 (21). Furthermore, FAK mediates the activation of PI3K/AKT signaling pathway (22–24). Therefore, we asked whether FAK is involved in cellular migration and invasion, activation of PI3K/AKT pathway, and MMP-2 and MMP-9 activation induced by SHH. We first investigated the effect of SHH signaling on FAK and phosphorylation of FAK at tyrosine (Tyr) 397, which is critical for its function and used as a marker of FAK activity (35,36). The results of western blot analysis demonstrated that phosphorylated FAK Tyr397 (phospho-FAK Tyr 397) expression was increased in a dose-dependent manner in response to rSHH-N stimulation, whereas phospho-FAK Tyr 397 expression was reduced by Anti-SHH or cyclopamine in SMMC-7721 cells. However, they had little effect on the expression of total FAK (Figure 5A).

We next asked whether FAK phosphorylation is required for SHH-induced activation of PI3K/AKT pathway, enhanced MMP-2 and MMP-9 expressions and activities, cell migration and invasion in hepatoma cells. To that end, we knocked down FAK using FAK-specific siRNA in hepatoma cells. The effectiveness of the siRNA to inhibit FAK expression was confirmed previously (21). We found that knock down of FAK expression by siRNA significantly inhibited the rSHH-N-mediated MMP-2 and MMP-9 expressions and activation and AKT phosphorylation, whereas control siRNA showed no effect (Figure 5B–D). Furthermore, deletion of FAK markedly suppressed rSHH-N-dependent migration and invasion of hepatoma cells (Figure 5E and 5F). Taken together, these findings indicate that SHH

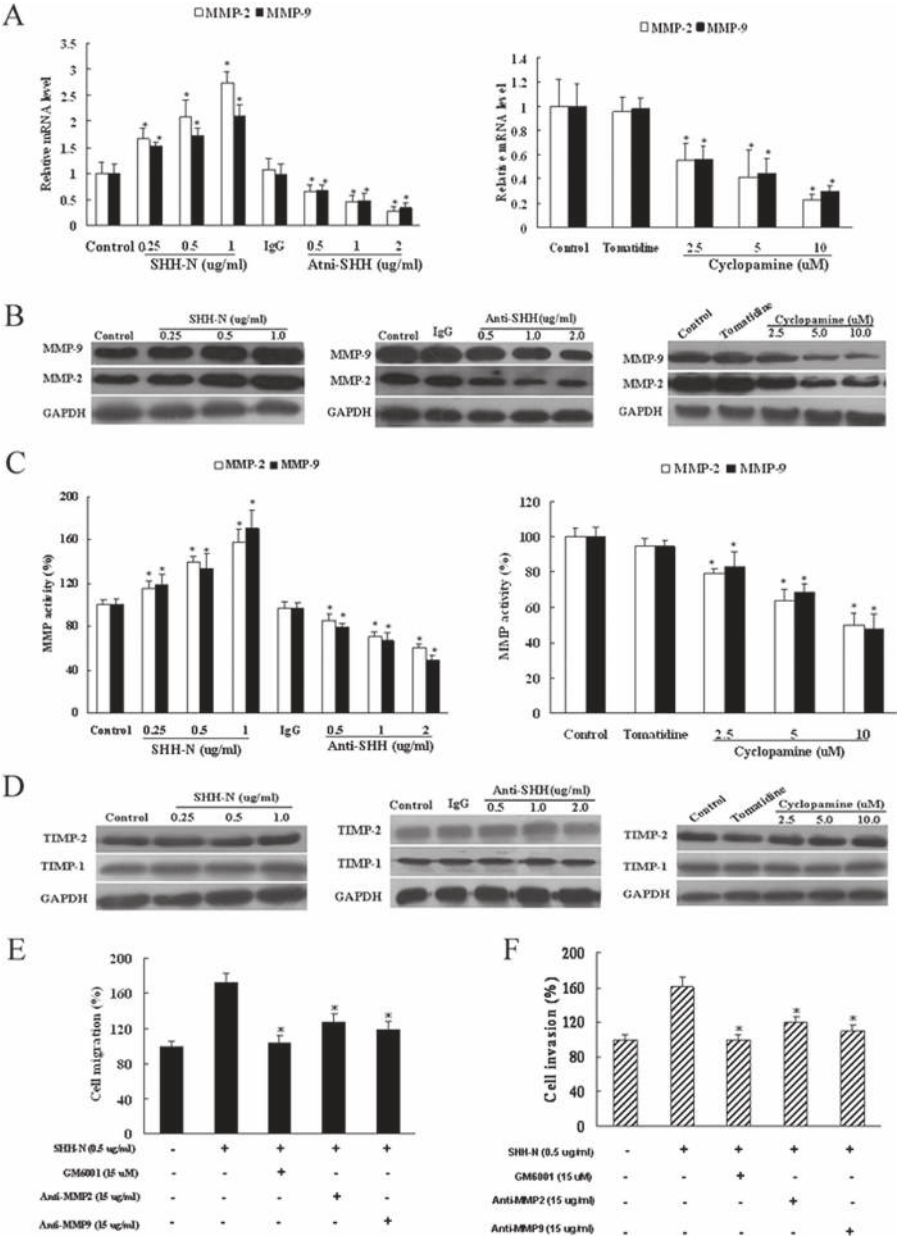

Fig. 3. Effect of SHH signaling on expressions and activities of MMP-2 and MMP-9 in SMMC-7721 cells. (**A**) SMMC-7721 cells were treated with increasing concentrations of rSHH-N, Anti-SHH (left) or cyclopamine (right) for 24 h, then expression of MMP-2 and MMP-9 mRNA was detected by real-time PCR analysis. *$P < 0.001$, compared with controls. (**B**) Western blot analysis of MMP-2 and MMP-9 protein expressions in SMMC-7721 cells treated with various concentrations of rSHH-N (left), Anti-SHH (middle) or cyclopamine (right) for 24 h. (**C**) Conditioned medium from SMMC-7721 cells treated with the indicated concentrations of rSHH-N, Anti-SHH (left) or cyclopamine (right) for 24 h was collected and determined by the MMP gelatinase activity assay. *$P < 0.001$, compared with controls. (**D**) SMMC-7721 cells were incubated with increasing concentrations of rSHH-N (left), Anti-SHH (middle) or cyclopamine (right) for 24 h, cell lysates were analyzed by western blot to detect TIMP-1 and TIMP-2. (**E**) and (**F**) SMMC-7721 cells were either left untreated (control) or pretreated with MMP inhibitor GM6001, MMP-2 neutralizing antibody (Anti-MMP2) or MMP-9 neutralizing antibody (Anti-MMP9) for 30 min followed by incubation with 0.5 μg/ml rSHH-N for 24 h, cell migration and invasion were then evaluated by migration (E) and invasion(F) assay. *$P < 0.001$, compared with rSHH-N-treated group.

signaling induces hepatoma cell migration and invasion through FAK/AKT signaling-mediated activation of MMP-2 and MMP-9.

Correlation between SHH protein and FAK/AKT pathway components, MMP-2 and MMP-9

We further investigated the correlation between SHH protein and both key components of FAK/AKT pathway and MMP-2 and MMP-9 in HCC samples. The immunohistochemical analysis of MMP-2, MMP-9, AKT, phosphorylated AKT, FAK, phosphorylated FAK in the 200 pairs of HCC and adjacent non-cancerous liver tissues was described in our previous study (20,21). We found that SHH protein expression was positively correlated with p-FAK Tyr397 ($r = 0.184$, $P = 0.009$), p-AKT (Ser473) ($r = 0.148$, $P = 0.036$), MMP-2 ($r = 0.186$, $P = 0.008$) and MMP-9 ($r = 0.161$, $P = 0.023$) protein expressions. These results were consistent with our *in vitro* results.

Discussion

SHH signaling has been shown to play a crucial role in carcinogenesis and progression of a variety of human cancers (2,7,8,37). In this

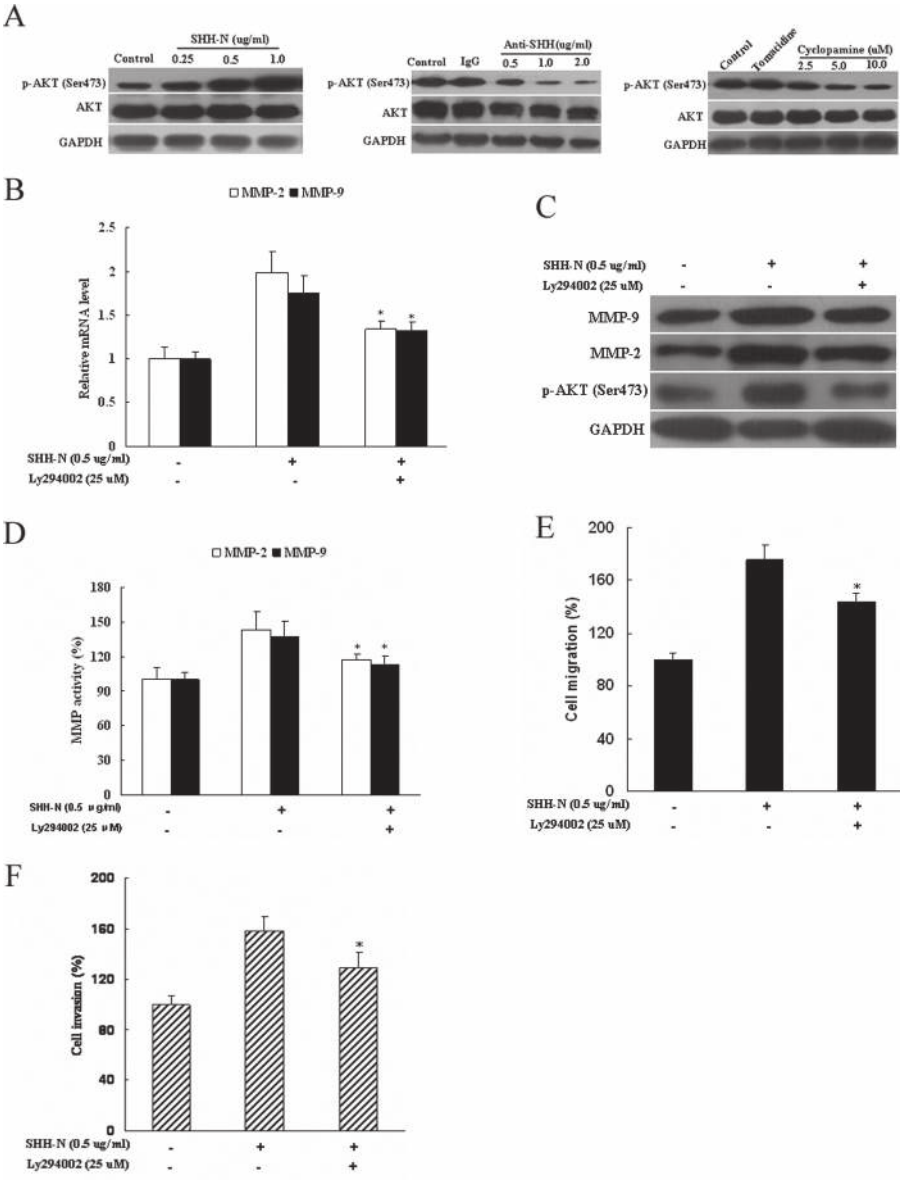

Fig. 4. Involvement of PI3K/AKT pathway in SHH signaling-induced MMP-2 and MMP-9 production and activation, cell migration and invasion. (**A**) Protein expressions of total AKT and phosphorylated AKT (p-AKT) (Ser473) were detected via western blot in SMMC-7721 cells treated with or without indicated concentrations of rSHH-N (left), Anti-SHH (middle) or cyclopamine (right) for 24 h. (**B–F**) SMMC-7721 cells were either left untreated (control) or pretreated with the PI3K inhibitor Ly294002 (25 μM) for 30 min and then treated with 0.5 μg/ml rSHH-N for 24 h. Expression of MMP-2 and MMP-9 mRNA was examined via real-time PCR analysis (B). p-AKT (Ser473), MMP-2 and MMP-9 protein expression was analyzed by western blot analysis (C). The activities of MMP-2 and MMP-9 in the conditioned media were detected by the MMP gelatinase activity assay (D). Cell migration and invasion were determined by migration (E) and invasion (F) assay. *$P < 0.001$, compared with rSHH-N-treated group.

study, we provide evidence for the first time that SHH signaling pathway promotes liver cancer invasion and metastasis through FAK/AKT signaling-mediated activation of MMP-2 and MMP-9.

SHH signaling is required for liver development during embryogenesis (38). Recent evidence has demonstrated that SHH signaling plays a critical role in HCC (9–11). However, the roles of SHH signaling in liver cancer invasion and metastasis are not well understood. In this study, we focused on the effect of SHH on invasion and metastasis of liver cancer. Our findings indicate that SHH signaling is activated and correlated with invasion/metastasis in HCC. Moreover, the data *in vitro* demonstrated that SHH signaling induced HCC cell adhesion, migration and invasion independently of its cellular cytotoxicity. Collectively, these data suggest that SHH signaling is an important mediator of HCC invasion and metastasis and might be a potential therapeutic target for HCC.

Cancer invasion and metastasis are complex processes involving multiple genetic alterations. Proteolytic degradation of extracellular matrix by MMP is a critical step during cancer invasion and metastasis. MMPs, especially MMP-2 and MMP-9, have been implicated in HCC invasion and metastasis (20,39–41). Recent studies suggest that SHH signaling plays a critical role in regulating activities and/or expression of MMP-2 and/or MMP-9 (13,16). Our data showed that MMP-2 and MMP-9 expressions and activities were enhanced by rSHH-N and inhibited by blocking SHH signaling with SHH neutralizing antibody or cyclopamine. Furthermore, inhibition of MMP-2 or MMP-9 reversed the stimulatory effect of SHH-N on cell migration and invasion. In addition, we found that SHH protein expression was closely associated with MMP-2 and MMP-9 in HCC samples. Taken together, these results suggest that SHH signaling induced hepatoma cell migration and invasion through elevated expressions and activities of MMP-2 and MMP-9.

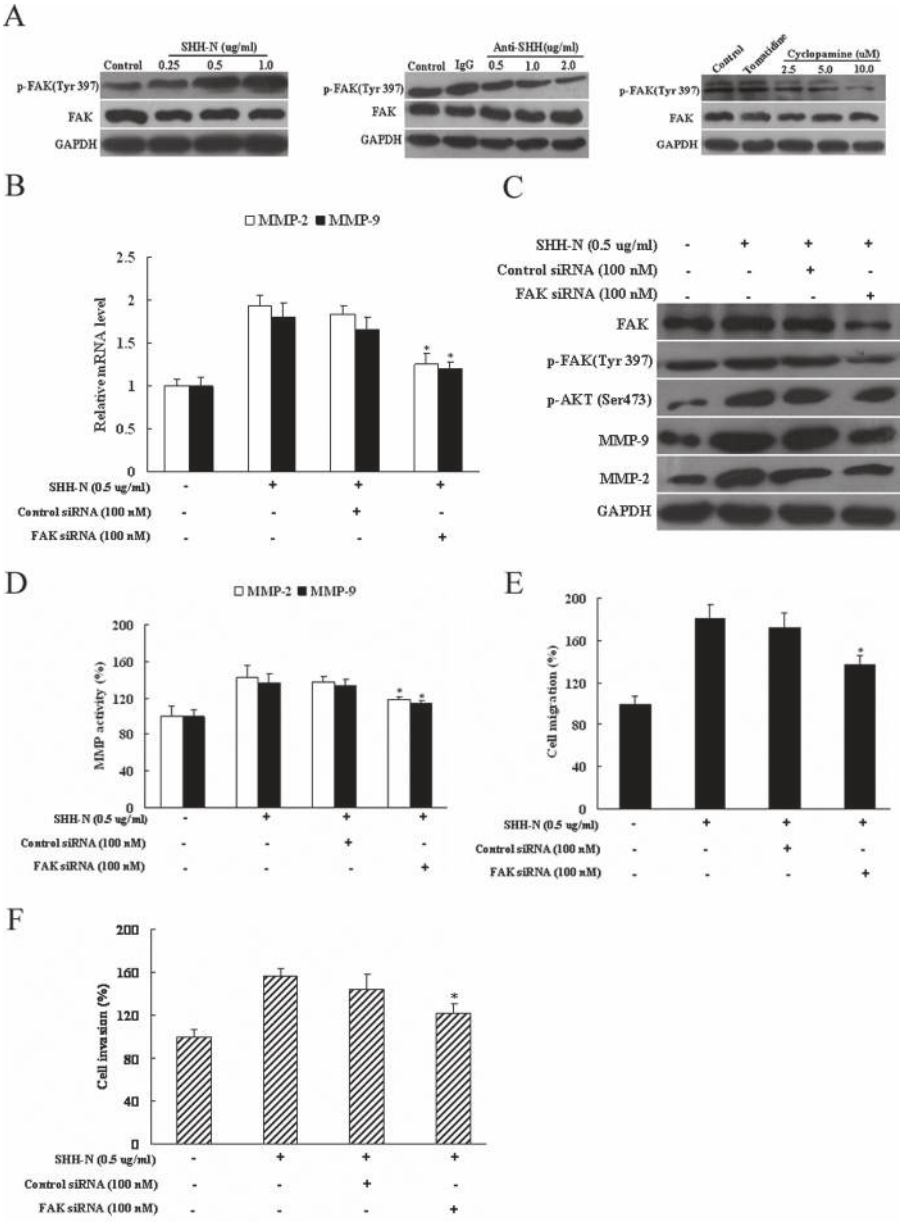

Fig. 5. Involvement of FAK/AKT pathway in SHH signaling-induced MMP-2 and MMP-9 production and activation, cell migration and invasion. (**A**) SMMC-7721 cells treated with or without indicated concentrations of rSHH-N (left), Anti-SHH (middle) or cyclopamine (right) for 24 h, total FAK and phosphorylated FAK (p-FAK) Tyr397 protein expressions were examined via western blot. (**B–F**) SMMC-7721 cells were either left untreated (control) or transfected with control siRNA or FAK siRNA for 24 h followed by stimulation with 0.5 μg/ml rSHH-N for another 24 h. Expression of MMP-2 and MMP-9 mRNA was detected via real-time PCR analysis (**B**). p-AKT (Ser473), MMP-2, MMP-9, p-FAK Tyr397 and total FAK protein expression was determined by western blot analysis (**C**). The activities of MMP-2 and MMP-9 in the conditioned media were assayed by the MMP gelatinase activity assay (**D**). Cell migration and invasion were measured by migration (**E**) and invasion (**F**) assay. *$P < 0.001$, compared with rSHH-N-treated group.

Accumulating evidence indicates that PI3K/AKT signaling pathway plays an important role in the genesis and progression of some human cancers, including HCC (20,25,42,43). Moreover, PI3K/AKT signaling pathway has been shown to contribute to HCC cell invasion by regulating MMP-2 and/or MMP-9 (20,26). Furthermore, recent studies have shown that the PI3K/AKT signaling pathway is modulated by SHH signaling (33,34,44). In this study, we found that the expression of phosphorylated AKT, a constitutively active form of AKT, was increased by rSHH-N and decreased by SHH neutralizing antibody or cyclopamine in hepatoma cells. In addition, rSHH-N-stimulated expressions and activation of MMP-2, MMP-9, hepatoma cell migration and invasion were attenuated by Ly294002, a selective inhibitor of PI3K. Thus, these results suggest that SHH signaling promotes hepatoma cell migration and invasion by PI3K/AKT pathway-mediated expressions and activation of MMP-2 and MMP-9.

FAK is a non-receptor protein tyrosine kinase that is overexpressed in many human tumors (45–47). Activation of FAK by both integrin and growth factors plays a vital role in a variety of biological processes, including cell survival, proliferation, attachment, migration and invasion (45,47,48). Our previous study has shown that elevated expression levels of both FAK and phosphorylated FAK Tyr397 are correlated with HCC invasion and metastasis and that FAK silencing inhibits HCC cell migration and invasion through downregulating MMP-2 and MMP-9 expressions and activities (21). Furthermore, activation of FAK has been shown to mediate several signal transduction pathways (49). Phosphorylation of FAK at Tyr 397 creates a binding site for the SH2 domains of p85 subunit of PI3K

(22,23). Subsequent phosphorylation of the p85 subunit of PI3K by FAK activates PI3K/AKT signaling pathway (22,23). Therefore, we investigated whether FAK was required for SHH-mediated activation of PI3K/AKT signaling pathway. We found that rSHH-N stimulation induced expression of phosphorylated FAK Tyr397, whereas SHH blocking antibody or cyclopamine downregulated expression of phosphorylated FAK Tyr397. We further demonstrated that rSHH-N-induced AKT activation, MMP-2 and MMP-9 expressions and activation, cell migration and invasion were abolished by siRNA-mediated depletion of FAK. Therefore, our data indicate that activation of FAK/AKT signaling pathway is required for SHH-stimulated MMP-2, MMP-9 activation, migration and invasion of hepatoma cells. However, other molecular mechanisms underlying SHH-induced migration and invasion of hepatoma cells remain to be elucidated.

In conclusion, we show that SHH signaling pathway contributes to invasion and metastasis of liver cancer. Moreover, our findings emphasize the potential role of FAK/AKT signaling-mediated MMP-2 and MMP-9 production and activation in SHH-induced hepatoma cell migration and invasion. Thus, these data provide a novel molecular mechanism responsible for the SHH signaling-mediated hepatoma cell migration and invasion and a potential valid therapeutic target for liver cancer.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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