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 phosphorylated AKT Tyr397, phosphorylated 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 activation of 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; MT, 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 glial-cell-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 tumorogenesis 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 patients. 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.

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Western blot

Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk for 1h and then incubated overnight at 4°C with the primary antibodies against SHH (1:200), PTCH1 (1:200), SM0 (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 (Yr397) (1:800) and GAPDH (1:2000). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1h 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 24h. 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 ($\chi^2$) 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 its expression was observed in 44% (36/81) of adjacent non-cancerous tissues ($P = 0.001$). SHH overexpression was correlated with capsular invasion ($P = 0.046$), higher tumor stage ($P = 0.022$) and 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 ($P < 0.001$). Elevated SMO expression was associated with higher 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 tissues than in the matched adjacent non-cancerous liver tissues (36%, 72/200) ($P = 0.011$). A high Gli-1 expression was significantly correlated with capsular invasion ($P = 0.026$), 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.
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 no 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-induced 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>SHH</th>
<th>P</th>
<th>PTCH1</th>
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|                           |    | ![
| 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 |       | 30 10 24 41 |   |
| >50                       | 105| 39 12 31 23 | 0.739 | 53 29 16 7 | 40 27 22 16 | 30 10 24 41 |       | 0.468     |   |
| Sex                       |    | 0.053     |       | 0.714     | 0.468 |
| Male                      | 183| 61 27 52 43 |       | 96 49 27 11 | 69 45 42 27 | 45 21 53 64 |       | 6 2 2 7 |   |
| Female                    | 17 | 7 2 3 5   | 0.448 | 7 5 1 4    | 4 5 5 3    | 0.580     |       | 0.562     |   |
| Etiology                  |    | 0.468     |       | 0.941     | 0.580 |
| Non-infection             | 42 | 13 5 10 14 | 0.175 | 26 7 7 2   | 18 9 8 7   | 8 3 12 19 |       | 0.249     |   |
| Hepatitis B               | 145| 53 22 39 31|       | 70 44 20 11| 51 38 35 21| 38 18 41 48|       | 0.448     |   |
| Hepatitis C and other     | 13 | 2 2 6 3   |       | 0.780     |       | 0.864     |       | 0.187     |   |
| Liver cirrhosis           | 79 | 25 11 21 22|       | 42 22 9 6  | 23 22 18 16 | 16 9 23 31 |       | 0.562     |   |
| Absence                   | 121| 43 18 34 26|       | 61 32 19 9 | 50 28 29 14 | 35 14 32 40|       | 0.249     |   |
| Presence                  | 142| 43 19 44 36| 0.175 | 28 16 11 3 | 22 12 15 9 | 20 6 16 16 |       | 0.448     |   |
| Tumor size (cm)           |    | 0.531     |       | 0.835     | 0.249 |
| ≤5                        | 58 | 25 10 11 12| 0.175 | 28 16 11 3 | 22 12 15 9 | 20 6 16 16 |       | 0.249     |   |
| >5                        | 142| 43 19 44 36|       | 75 38 17 12| 51 38 32 21| 31 17 39 55|       | 0.249     |   |
| Serum AFP (μg/l)          |    | 0.906     |       | 0.398     | 0.650 |
| ≤20                       | 62 | 21 8 19 14 | 0.046 | 35 12 9 6  | 25 13 13 11| 16 6 16 24|       | 0.892     |   |
| >20                       | 138| 47 21 36 34|       | 68 42 19 9 | 48 37 34 19| 35 17 39 47|       | 0.026     |   |
| Capsular invasion         |    | 0.864     |       | 0.304     | 0.026 |
| Absence                   | 118| 47 20 28 23| 0.046 | 58 33 18 9 | 49 29 24 16| 35 14 37 32|       | 0.237     |   |
| Presence                  | 82 | 21 9 27 25|       | 45 21 10 6 | 24 21 23 14| 16 9 18 39|       | 0.237     |   |
| Tumor grade               |    | 0.006     |       | 0.100     | 0.237 |
| I                         | 29 | 14 3 5 7  | 0.212 | 10 9 3 7   | 13 5 5 6   | 11 3 8 7 |       | 0.237     |   |
| II                        | 131| 43 16 38 34|       | 72 37 16 2 | 41 32 36 22| 32 15 31 53|       | 0.237     |   |
| III                       | 40 | 11 10 12 7 | 0.002 | 21 8 9 2   | 19 13 6 2 | 8 5 16 11 |       | 0.237     |   |
| TNM stage                 |    | 0.060     |       | 0.038     | 0.027 |
| I                         | 61 | 31 8 9 13 | 0.01  | 30 11 14 6 | 26 18 12 5 | 23 5 19 14 |       | 0.011     |   |
| II                        | 56 | 20 11 16 9 | 0.107 | 26 22 4 4  | 25 7 16 8 | 10 4 16 26 |       | 0.011     |   |
| III                       | 83 | 17 10 30 26| 0.01  | 47 21 10 5 | 22 25 19 17 | 18 14 20 31 |       | 0.027     |   |
| Vascular invasion         |    | 0.035     |       | 0.011     | 0.011 |
| Absence                   | 140| 55 21 39 25| 0.041 | 68 36 25 11| 56 33 36 15| 41 10 41 48|       | 0.011     |   |
| Presence                  | 60 | 13 8 16 23| 0.512 | 35 18 3 4  | 17 17 11 15| 10 13 14 23|       | 0.022     |   |
| Intrahepatic metastasis   |    | 0.005     |       | 0.022     | 0.022 |
| Absence                   | 127| 52 18 29 28| 0.412 | 65 36 19 7 | 54 35 26 12| 41 15 33 38|       | 0.022     |   |
| Presence                  | 73 | 16 11 26 20| 0.997 | 38 18 9 8  | 19 15 21 18| 10 8 22 33 |       | 0.022     |   |
| Ki-67                     |    | 0.734     |       | 0.815     | 0.022 |
| ≤50%                      | 132| 43 22 33 34| 0.412 | 68 36 18 10| 50 33 28 21| 33 14 39 46|       | 0.022     |   |
| >50%                      | 68 | 25 7 22 14 | 0.977 | 35 18 10 5 | 23 17 19 9 | 18 9 16 25|       | 0.022     |   |
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
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, ACT, phosphorylated ACT, 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. 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 24h, 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 24h. (C) Conditioned medium from SMMC-7721 cells treated with the indicated concentrations of rSHH-N, Anti-SHH (left) or cyclopamine (right) for 24h 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 24h, 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 24h, cell migration and invasion were then evaluated 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.
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.

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.
Supplementary material

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

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References


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