Original Article
Partial hepatectomy promotes implanted mouse hepatic tumor growth by activating hedgehog signaling

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Abstract: Objective: To investigate the role hedgehog signaling (Hh) in the growth of implanted hepatic tumors after partial hepatectomy (PH) in mice. Methods: H22 cells were implanted to the scapula of 2 BALB/c (nu/nu) nude mice and tumor developed in 2 weeks. 40 nude mice were randomized into 4 groups: non-hepatectomy group (Sham operation group), 30% hepatectomy group, 70% hepatectomy group, and 70% hepatectomy with cyclopamine (Hh inhibitor). The hepatectomy model of nude mice was established. After hepatectomy, the tumor tissues incised from the scapula were implanted to the rest of the livers of the 4 groups. After 2 weeks, the tumor formation rates and the volumes of the implanted tumors were compared. Hh related proteins and downstream cytokine VEGF were tested by Western blot and Immunohistochemistry. All the data were analyzed to explore the role of Hh in the growth of tumor after PH. Results: The volumes of the implanted tumors after liver resection were significantly higher in the 70% PH group than those in 0% and 30% PH groups; meanwhile, we also found that expression of the Hh ligand Indian Hh, its downstream transcription factor protein Gli-1, and its target VEGF were remarkably increased after PH, especially in the 70% PH group. Additionally, applying the Hh inhibitor cyclopamine to mice that underwent 70% PH significantly inhibited the growth of implanted tumors. Conclusions: The Hh signaling pathway was activated after PH and promoted liver regeneration. The growth of implanted hepatic tumors was also accelerated after PH via paracrine signaling.

Keywords: Hepatocellular carcinoma, hedgehog signaling pathway, partial hepatectomy, tumor growth, Balb/c nu, Hcc model

Introduction

Worldwide, hepatocellular carcinoma (HCC) is the fourth most common malignant disease and the third leading cause of cancer-related deaths. Currently, a multidisciplinary treatment is the optimal management strategy for HCC [1], and radical hepatectomy is the treatment of choice for early and intermediate-stage liver cancer with sufficiently reserved hepatic function [2]. However, tumors recur within 5 years of surgery in over 80% of patients that undergo radical hepatectomy, which seriously affects long-term survival [3]. Recurrence following radical hepatectomy is closely related to multicentric growth and micro hepatic tumor thrombus [4].

When the apoptosis rate of tumor cells is balanced with the proliferation rate, micrometastases will be dormant. During radical hepatectomy for HCC, especially during liver regeneration after major hepatectomy, the liver releases many growth factors including hepatocyte growth factor (HGF), epidermal growth factor (EGF), and basic-fibroblast growth factor (bFGF), and breaks down and reconstructs its extracellular matrix. These mechanisms likely activate dormant micrometastases or promote the growth and metastasis of residual multicentric tumor
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Additionally, the activation of coagulation factor chains following hepatectomy, postoperative temporary local or systemic immune suppression, and the activation of epithelial progenitor cells and other bone marrow-derived hepatic progenitor cells may also promote tumor recurrence and metastasis [5, 6].

The hedgehog (Hh) signaling pathway is an important intercellular communication system in animal development. It promotes cell proliferation and differentiation in a tissue-specific manner during embryogenesis and determines the formation of embryonic patterns. The Hh signaling pathway regulates the self-renewal and proliferation of stem cells in many tissues and organs and maintains the normal morphologies and functions of tissues and organs. Hh pathway activation promotes the differentiation and proliferation of tissue stem cells to repair damaged tissues and organs. However, abnormal Hh pathway activation in adult tissues and mature organs can lead to the occurrence of multiple tumors such as liver cancer, gastric cancer, colon cancer, and lung cancer [7].

The Hh signaling pathway consists of Hh ligand, two transmembrane protein receptors (Patched [Ptch] and Smoothened [Smo]), and the downstream transcription factor Gli. In mammals, there are three distinct Hh ligands: Sonic (Shh), Indian (Ihh), and Desert (Dhh) Hedgehog. Ptch and Smo are transmembrane proteins located on the cell membrane, and Ptch is the Hh receptor that binds to the three Hh ligands. When Hh ligand is absent, Ptch inhibits Smo activity, which leads to the inhibition of downstream gene expression. When the Hh ligand binds to Ptch, the inhibitory effect on Smo is relieved, which activates the downstream transcriptional regulator Gli, which can induce target gene expression including cyclin, c-myc, and vascular endothelial cell growth factor (VEGF) [8].

Hh signaling is closely related to the development and metastasis of liver cancer. In HCC tissues, one study found the rate of positive Shh expression was 60% (69/115), and the rates of positive Gli and Smo expression exceeded 50% [9]. Hh pathway suppression can inhibit the proliferation of some HCC cell lines, promote their apoptosis, and remarkably affect the metastasis and invasion capabilities of tumor cells [9-11]; it can also significantly inhibit the growth and metastasis of HCC xenografts in nude mice [12].

The Hh pathway is activated during liver regeneration and repair after partial hepatectomy (PH) or severe liver injury. In our studies [13, 14] as well as in studies performed by Ochoa et al. [15] and Cai et al. [16], the Hh pathway was activated following 70% PH in rats and mice, and strongly promoted liver regeneration. While the Hh pathway can be activated by PH, and thus promotes liver regeneration, whether it facilitates liver cancer recurrence and metastasis following resection remains unclear. This study investigated the role and mechanism of Hh signaling in the growth of implanted tumors in nude mice after PH.

Materials and methods

HCC cell line and experimental animals and grouping

The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee at The Third Affiliated Hospital of Sun Yat-sen University. The H22 mouse hepatoma cell line was used in this study to intraperitoneally inoculate 4-week-old male C57BL/6J mice. Also, 4-week-old male BALB/c nude mice weighing 20-25 g were used to prepare models of subscapular implanted tumors and models of hepatic implanted tumors. Mice were housed in plastic cages under a 12:12 light:dark cycle with free access to food and water. Cell lines and mice were purchased from the Animal Experimental Center of the East Campus of Sun Yat-sen University. All experiments were carried out at the Experimental Animal Center of the North Campus of Sun Yat-sen University.

Reagents

RPMI1640 low-sugar medium and 10% dimethyl sulfoxide (DMSO) were purchased from Gibco (Waltham, MA, USA). Fetal bovine serum, Phosphate Buffer Saline (PBS, pH=7.4), and MATRIGEL were purchased from Corning Inc. (Corning, NY, USA). Cyclopamine (50 mg) was purchased from Selleck Chemicals (Houston, TX, USA). Ihh and Gli-1 antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA, USA).
Establishing mouse models of implanted liver tumors and grouping

Culturing H22 mouse hepatoma cells: H22 cells were cultured in ascites. H22 cells (2×10⁶) and PBS were prepared into a 1 ml cell suspension at a ratio of 1:1, and then intraperitoneally injected into C57BL/6J mice. After one week, the ascites was collected and centrifuged. After repeated rinsing with PBS, a purified mouse H22 hepatoma cell suspension was obtained.

Establishing mouse models with subscapular implanted tumors: All mice operations were conducted in the morning (9:00-12:00 am). Animal surgeries were performed using midline laparotomy after the mice were anaesthetized by intraperitoneal injection of 1.5% pentobarbital (30 mg/kg). H22 cells (2×10⁶) and 0.2 ml of PBS were prepared into a cell suspension, which was injected into the subscapular region of nude mice. After two weeks, tumors were visible in the scapular region and were pathologically confirmed to be HCC tissues by two pathologists from the Third Affiliated Hospital of Sun Yat-sen University.

Establishing the 0%, 30%, and 70% PH models: A left lateral lobe hepatectomy was performed to establish the 30% PH model, and combined middle lobe and left lateral lobe hepatectomies were performed to establish the 70% PH model [17].

Establishing mouse models of implanted liver tumors: The implanted tumor tissue in the subscapular region of nude mice was cut into tissue blocks, each sized 1 mm³. After being washed in a sterile PBS solution, the tissue blocks were implanted into the right liver lobe.

Grouping: Forty BALB/c nude mice were divided into four groups using a random number table: group A (Sham operation group); the tumor tissue blocks were implanted into the right liver lobe, but neither liver resection nor ligature were performed; group B (30% PH group); tumor tissue blocks were implanted following 30% PH; group C (70% PH group); tumor tissue blocks were implanted following 70% PH; and group D (70% PH + cyclopamine group); tumor tissue blocks were implanted following 70% PH, and then the Hh inhibitor cyclopamine (30 mg/ kg/d) was intraperitoneally injected on a daily basis for 14 days.

Specimen collection and analysis

Mice in each group were sacrificed on day 14 after the operation, then the implanted tumors and para-tumor liver tissues were collected. The tumor formation rate, tumor volumes (V=L×W²×0.52) were measured, and the character of the implanted tumors were pathologically confirmed (by two pathologists from the Third Affiliated Hospital of Sun Yat-sen University). Thereafter, a part of each sample was frozen with liquid nitrogen, then stored at -80°C. The left tissues were fixed with 4% paraformaldehyde, then manufactured into paraffin sections. The expression of the Hh pathway ligand Ihh, downstream protein Gli-1, target gene VEGF, and the proliferation marker proliferating cell nuclear antigen (PCNA) in the implanted tumors and para-tumor samples were detected using Western blotting and Immunohistochemical staining.

Immunohistological staining and Western blotting analysis

Sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide successively as previously described (cited from Molecular expression of caprine estrogen receptor gene 1 in reproductive and non-reproductive tissues). Subsequently, antigen retrieval was performed by boiling the sections in an Ethylene Diamine Tetraacetic Acid (EDTA) antigen retrieval solution (pH 9.0) for 2 min in an autoclave. Non-specific antibody binding was blocked by incubating with 15% goat serum for 30 min at 37°C. Then the slides were incubated with primary antibodies: Ihh (ab39634, 1:1000, Abcam, USA), Gli-1 (ab151796, 1:1000, Abcam, USA) and PCNA (ab29, 1:1000, Abcam, USA) at 4°C overnight. For Immunohistochemistry (IHC) staining, the target protein was detected using secondary antibodies and the ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were then dehydrated and mounted after being counterstained with hematoxylin. Two investigators evaluated and scored the slides independently. The scores of staining intensities were assigned as: 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). The extensity of staining, based on percentage of positively stained tumor cells, was scored as: 0 (0-25%), 1 (26%-50%), 2 (51%-75%) and 3 (76%-100%). A final score was calculated by multiplying the...
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extensity score and intensity score (0, 1, 2, 3, 4, 6, 9). Scores < 4 were defined as low expression; scores ≥ 4 were assigned as high expression.

Western blotting using whole cell lysates from implanted tumor and para-tumor liver tissue were performed as previously described [18]. The protein concentration was quantified using the BCA Protein Assay (Kaiji, Jiangsu, China). Protein lysates (30 μg/sample) were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). After transferring, membranes were blocked with 5% nonfat-milk for 1 hour at room temperature and then incubated using the following antibodies at 4°C for overnight: Ihh (ab39634, 1:1000, Abcam, USA), Gli-1 (ab151796, 1:1000, Abcam, USA), PCNA (ab29, 1:1000, Abcam, USA), and VEGF (ab32152, 1:1000, Abcam, USA), and β-actin (#4970, 1:1000, Cell Signal Technology, USA). Then the membranes were washed using (Tris Buffered Saline Tween, TBST) followed by incubation with an anti-rabbit or anti-mouse IgG antibody (#7074, 1:5000, Cell Signal Technology, USA) for 1 hour at room temperature. The specific bands were detected using Chemidoc™ MP Imaging System after visualizing with Enhanced Chemiluminescence, ECL, (Advansta, USA). The protein expressions were calculated by using β-actin (#4970, 1:1000, Cell Signal Technology, USA) antibody as a control.

Statistical analysis

All data were analyzed using GraphPad Prism 7.0 software (IBM, Armonk, NY, USA), and the density of western blotting bands were ana-
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A

PCNA

actin

B

PCNA IHC (200×):

0% PH

30% PH

70% PH

C

Tumor:

05PH

305PH

705PH

Ihh

actin

D

Ihh IHC (200×):

0% PH

30% PH

70% PH

E

Para-Tumor:

05PH

305PH

705PH

Ihh

actin

F

Gli-1 IHC (200×):

0% PH

30% PH

70% PH

G

Gli-1

actin

Para-Tumor:

05PH

305PH

705PH

Gli-1

actin

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Figure 2. PH enhanced the proliferation of liver cells and activated the Hh signaling. PCNA, Ihh and Gli-1 expression in para-tumor liver tissue in mice after partial hepatectomy increased, but Ihh and Gli-1 expression was not shown in the implanted tumor. All the protein levels were detected by western blotting and the band density quantitative analyzed. All the data are expressed as mean ± S.D. (n=10). A. PCNA protein level in Group B and C was higher than that in Group A. Data were analyzed by One-way ANOVA. (*P < 0.05 for B vs A; *P < 0.05 for C vs A; **P < 0.05 for C vs B). B, G. The Immunohistochemical staining of Gli-1 in para-tumor liver tissue (*×200). Data were analyzed by One-way ANOVA. (*P < 0.05 for B vs A; *P < 0.05 for C vs A; **P < 0.05 for C vs B). C. Ihh protein level in para-tumor liver tissue and implanted tumor. Data were analyzed by One-way ANOVA. (*P < 0.05 for C vs A; *P < 0.05 for C vs B). D, G. The Immunohistochemical staining of Ihh in para-tumor liver tissue (*×200). Data were analyzed by One-way ANOVA. (*P < 0.05 for C vs A; *P < 0.05 for C vs B). E. Gli-1 protein level in para-tumor liver tissue and implanted tumor. Data were analyzed by One-way ANOVA. (*P < 0.05 for B vs A; *P < 0.05 for B vs C). F, G. The Immunohistochemical staining of Gli-1 in para-tumor liver tissue (*×200). Data were analyzed by One-way ANOVA. (*P < 0.05 for B, C vs A; *P < 0.05 for C vs B).

Results

Mouse models of implanted liver tumors after PH were successfully established

Implanted tumors were visible two weeks after inoculating H22 hepatoma cells into the scapular region. After hematoxylin and eosin staining, it was pathologically confirmed to be HCC. Microscopy of the tumor tissue revealed that the cells had large and deeply stained nuclei, and some nuclei were lobulated (Figure 1A).

Two weeks after inoculating the tumor tissue blocks, the liver mass in each group was harvested. The mass was white, with an irregular shape and clear boundaries. Pathology revealed that there was a clear boundary between the liver and tumor tissue; the nuclei of the tumor cells were large and deeply stained, and some were multinucleated or lobulated (Figure 1B). The tumor formation rates of groups A, B and C were 90%, 90% and 80%, respectively, showing no significant difference (P=0.075).

PH promoted the growth of implanted liver tumors

The implanted tumors were harvested 2 weeks after their establishment in nude mice. The volumes of the implanted tumors in groups A, B, and C were 24.0±8.1 mm³, 30.8±8.3 mm³, and 129.9±42.6 mm³, respectively, showing significant differences (P < 0.01). However, the implanted tumor volumes were not significantly different between groups A and B (P > 0.05). Furthermore, group C had significantly larger tumor volumes than groups A and B (A vs C and B vs C, P < 0.05) (Figure 1C). It was found that the volumes of implanted tumors in group C were larger than those in groups A and B. Thus, larger resections significantly promoted the growth of implanted liver tumors in nude mice.

Enhanced proliferation of liver cells after PH

Western blotting revealed significantly different levels of PCNA expression in the para-tumor liver tissues among the three groups (P < 0.01); PCNA expression was significantly higher in group B and group C (P < 0.05) and highest in group C (P < 0.05) (Figure 2A). Immunohistochemical staining showed that PCNA was expressed in the nucleus, and there was a significant difference in the number of PCNA-positive cells among the three groups (P < 0.01); the number of PCNA-positive cells was significantly higher in group C than in groups A or B (P < 0.05), and significantly higher in group B than group A (P < 0.05) (Figure 2B, 2G).

These results showed that PCNA expression was increased in para-tumor liver tissues following PH, especially in the 70% PH group, suggesting the proliferation capability of liver tissue cells was enhanced after PH. Furthermore, this capability was associated with the size of the removed liver tissue; liver cell proliferation was stronger in mice with larger resection sizes.

Activated Hh signaling in liver tissues after PH

Western blotting revealed that the level of Ihh protein expression in para-tumor liver tissues...
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Significantly differed among the three groups ($P < 0.01$); Ihh was significantly higher in groups B and C than in group A ($P < 0.05$), and higher in group C than group B ($P < 0.05$) (Figure 2C).

Figure 3. Suppressing Hh signaling inhibited the growth of implanted tumors after PH. Cyclopamine inhibited the growth of hepatic implanted tumor, and the expression of Ihh, Gli-1 and PCNA in para-tumor liver tissue in mice after 70% partial hepatectomy. All the protein levels were detected by western blotting and were quantitative analyzed of the band density. All the immunohistochemical staining positive cells were quantitative analyzed. All the data are expressed as mean ± S.D. (n=10). A, E. Naked eye and microscopic morphology of hepatic implanted tumor in mice after partial hepatectomy and volume comparison of the hepatic implanted tumors in mice of C and D group. Data were analyzed by student’s-t test. (*$P < 0.05$ for C vs D). B. Ihh protein level in para-tumor liver tissue of C and D. Data were analyzed by student’s-t test. (*$P < 0.05$ for C vs D). B, E. Immunohistochemical staining of Ihh in para-tumor liver tissue of C and D group. Data were analyzed using Student’s-t test. (*$P < 0.05$ for C vs D). C. Gli-1 protein level in para-tumor liver tissue of C and D. Data were analyzed using Student’s-t test. (*$P < 0.05$ for C vs D). C, E. Immunohistochemical staining of Gli-1 in para-tumor liver tissue of C and D group. Data were analyzed Using Student’s-t test. (*$P < 0.05$ for C vs D). D. PCNA protein level in para-tumor liver tissue of C and D. Data were analyzed using Student’s-t test. (*$P < 0.05$ for C vs D). D, E. Immunohistochemical staining of PCNA in para-tumor liver tissue of C and D group. Data were analyzed using Student’s-t test. (*$P < 0.05$ for C vs D).
However, no obvious Ihh expression was detected in the implanted tumor tissue in each group (Figure 2G). Immunohistochemical staining showed that Ihh was expressed on the cell membrane, and the number of Ihh-positive cells in para-tumor liver tissues was significantly different among the three groups \((P < 0.05)\); additionally, it was significantly higher in groups B and C than in group A \((P < 0.05)\), and significantly higher in group C than group B \((P < 0.05)\) (Figure 2D, 2G).

Western blotting revealed that the level of Gli-1 protein expression in the para-tumor liver tissues significantly differed among the three groups \((P < 0.01)\); it was significantly higher in groups B and C than group A \((P < 0.05)\), whereas there was no significant difference between groups C and B \((P > 0.05)\) (Figure 2F). Additionally, no obvious Gli-1 protein expression was detected in the implanted tumor tissue in any group (Figure 2F). Immunohistochemical staining showed that Gli-1 was mainly expressed in the nucleus, although some cytoplasms had a positive signal. The number of Gli-1-positive cells in para-tumor liver tissues was significantly different among the three groups \((P < 0.05)\); additionally, it was significantly higher in groups B and C than in group A \((P < 0.05)\), and significantly higher in group C than in group B \((P < 0.05)\) (Figure 2E, 2G).

Therefore, the expression of the Hh pathway ligand Ihh and its downstream transcription factor Gli-1 were remarkably increased after PH, especially in the 70% PH group. Hh signaling was activated in liver tissues after PH, and the degree of activation was related to the size of PH. However, Hh signaling was not activated in the implanted tumors themselves.

Suppressing Hh signaling inhibited the growth of implanted tumors after PH

Hh signaling was blocked by cyclopamine in group D, and the tumor formation rate was 90%, which was not significantly different from group C (80%; \(P > 0.05\)); additionally, the volume of implanted tumors was 18.0±5.4 mm\(^3\) in group D, which was significantly smaller than that of group C (129.9±42.6 mm\(^3\); \(P < 0.05\)) (Figure 3A, 3E). Therefore, Hh suppression inhibited the growth of implanted tumors after PH.

Western blotting and immunohistochemical staining showed that the expression of Ihh and Gli-1 were lower in group D than in group C (Figure 3A, 3E). Therefore, cyclopamine inhibited the expression of Hh signaling components in liver tissues after PH. Western blotting and immunohistochemical staining also showed that PCNA expression was significantly lower in group D than group C (Figure 3D, 3E) \((P < 0.05)\), suggesting that suppressing Hh signaling might inhibit liver regeneration after PH.

Hh signaling promoted tumor growth by increasing VEGF expression in para-tumor tissues

Hh signaling promotes tumor growth by activating downstream VEGF and other target genes [8]. Therefore, we detected VEGF protein expression in para-tumor liver tissues after PH. Western blotting showed that VEGF expression significantly differed among the four groups \((P < 0.01)\); in particular, VEGF expression in the para-tumor liver tissues was significantly higher in groups B and C than in group A (both \(P < 0.05\), and significantly higher in group C than in group B \((P < 0.05)\). VEGF expression was signifi-
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Significantly inhibited in group D ($P < 0.05$). Therefore, Hh pathway activation promoted tumor growth by increasing VEGF expression in para-tumor liver tissues (Figure 4).

Discussion

The growth rate of metastatic lesions can be 8 times that of normal liver tissues after PH. Additionally, PH of different proportions may also have different effects on liver tumor growth [18]. After 70% PH, the number and volume of residual liver tumors were significantly higher than after 30% PH [19]. After partial liver transplantation, grafts can regenerate to restore the normal liver mass; however, after an HCC patient receives a partial liver transplantation, the recurrence rate of HCC is higher than that of patients who receive a full liver transplantation [20]. In this study, the growth rate of implanted tumors in the liver of mice was higher in the 70% PH group than those in the 30% PH group or 0% PH group, suggesting major PH promoted the growth of implanted liver tumors, further indicating that hepatectomy promotes tumor recurrence.

Recent studies have suggested that liver regeneration following liver resection, especially after major hepatectomy or partial liver transplantation, may promote tumor recurrence and metastasis, although the exact mechanism is not fully understood. When the apoptosis rate of tumor cells is balanced with their proliferation rate, micrometastases are dormant. After partial liver resection (especially after major hepatectomy), some liver regeneration-related signaling pathways and growth factors, such as HGF, EGF, b-FGF, VEGF, TGF-β, and MMPS, can activate dormant micrometastases, promoting tumor recurrence and metastasis.

Hh signaling is markedly activated during liver regeneration after PH. Abnormal Hh activation can cause the development of a variety of tumors, including HCC [7]; however, few studies have explored whether Hh signaling, which promotes liver regeneration after PH, can also promote tumor recurrence and metastasis after HCC resection.

In this study, the growth rate of implanted tumors after resection was significantly higher in the 70% PH group than in the 0% and 30% PH groups; meanwhile, we also found that the expression of Hh ligand Ihh and its downstream transcription factor Gli-1 were remarkably increased after PH, especially in the 70% PH group. Additionally, application of the Hh inhibitor cyclopamine in mice after 70% PH significantly inhibited the growth of implanted tumors. Therefore, the Hh signaling pathway that promotes liver regeneration after hepatectomy may also promote the growth of implanted liver tumors.

Hh signaling promotes tumor growth via the following three mechanisms: a) non-Hh ligand-dependent pathways: mainly by inhibiting the PTCH1 or SUFU gene by mutation or by activating the Hh signaling pathway by stimulating Smo gene mutations, which are mainly seen in basal cell carcinoma or neural tube tumors [21, 22]; b) Hh ligand-dependent autocrine pathway: tumor cells secrete Hh ligands that act in an autocrine fashion to activate Hh signaling and promote tumor cell proliferation; this mechanism is mainly seen in lung cancer, prostate cancer, and colon cancer [23, 24]; and c) The Hh ligand-dependent paracrine pathway: tumor cells or para-tumor cells secrete the Hh ligand, which binds to stromal cells, promoting Hh signaling in para-tumor cells to facilitate the secretion of tumor growth-promoting factors such as VEGF and IGF, thereby promoting tumor growth; this mechanism is mainly seen in lymphoma and plasma cell tumors [25]. In this study, the Hh pathway-related proteins Ihh and Gli-1 were detected in para-tumor tissues but not in the implanted tumors. Therefore, liver tissue-secreted Ihh following PH, which activated Hh signaling in liver tissue, thereby promoting the expression of downstream tumor growth-related genes and cytokines (e.g., Gli-1, HGF, and VEGF) and ultimately contributing to tumor growth. After Hh signaling in liver tissues was blocked, the growth of implanted tumors was remarkably inhibited. Detection of VEGF, one of the downstream target genes of Hh signaling, in each group showed that the VEGF expression increased in the para-tumor tissue in both the 30% and 70% PH groups, whereas suppression of Hh signaling inhibited VEGF expression. It was therefore speculated that activated Hh signaling after PH may promote the growth of liver tumors by promoting VEGF expression through Hh-dependent paracrine signaling. Further studies are needed to confirm this conclusion, and the secretion of other
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downstream cytokines warrants further experiments.

Currently, three categories of Hh pathway inhibitors are under development: a) Smo inhibitors including cyclopamine, vitamin D3, vismodegib, SANT, BMS-833923, LDE225, and LEQ506, among which, the role of vismodegib in rectal cancer and ovarian cancer has been studied in phase II clinical trials [23]; SANT has been manufactured by several drug companies as a promising anti-cancer reagent [15]; b) The Shh ligand antagonists, i.e. robotnikinin, which bind to the N-terminal fragments of Shh, blocking translation of Hh target genes [21]; c) Gli inhibitors, including GANT58, GANT61, and HPI-1, -2, -3 and -4, which block Gli-mediated gene translation. Both GANT58 and GANT61 can inhibit the growth of implanted prostate cancer [25]. Additionally, both itraconazole (a commonly used antifungal drug that can act on Smo) and arsenic trioxide (an anti-cancer drug that inhibits Gli) can block Hh signaling. In some clinical trials, itraconazole has been used to treat basal cell carcinoma [26]. Our study revealed that Hh signaling promotes liver regeneration after hepatectomy and also promotes the growth of liver implanted tumors, suggesting Hh signaling may be a new target for the prevention and treatment of HCC recurrences and metastases. Some commonly used drugs (e.g. vitamin D3 and itraconazole) have been shown to exert anti-cancer effects by blocking Hh signaling [27, 28]. Therefore, the prevention and treatment of HCC recurrence by blocking Hh signaling is worthy of further study. Our findings may thus provide new evidence for the prevention and treatment of HCC recurrence following hepatectomy.

In summary, Hh signaling, which is activated after PH and promotes liver regeneration, can promote the growth of implanted hepatic tumors. The possible mechanism may be that Hh signaling promotes the growth of liver tumors by promoting VEGF expression through a Hh ligand-dependent paracrine pathway. However, further research is warranted regarding Hh signaling-related genes and their target genes in HCC.

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Disclosure of conflict of interest

None.

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References

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