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Hedgehog signaling pathway is a potential therapeutic target for gallbladder cancer

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Key words

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Gallbladder cancer (GBC) is a particularly deadly type of cancer with a 5-year survival rate of only 10%. New effective therapeutic strategies are greatly needed. Recently, we have shown that Hedgehog (Hh) signaling is reactivated in various types of cancer and is a potential therapeutic target. However, little is known about the biological significance of Hh signaling in human GBC. In this study, we determined whether Hh signaling could be a therapeutic target in GBC. The Hh transcription factor Gli1 was detected in the nucleus of GBC cells but not in the nucleus of normal gallbladder cells. The expression levels of Sonic Hh (Shh) and Smoothened (Smo) in human GBC specimens ($n = 37$) were higher than those in normal gallbladder tissue. The addition of exogenous Shh ligand augmented the anchor-dependent and anchor-independent proliferation and invasiveness of GBC cells *in vitro*. In contrast, inhibiting the effector Smo decreased the anchor-dependent and anchor-independent proliferation. Furthermore, the suppression of Smo decreased GBC cell invasiveness through the inhibition of MMP-2 and MMP-9 expression and inhibited the epithelial–mesenchymal transition. In a xenograft model, tumor volume in *Smo* siRNA-transfected GBC cells was significantly lower than in control tumors. These results suggest that Hh signaling is elevated in GBC and may be involved in the acquisition of malignant phenotypes, and that Hh signaling may be a potential therapeutic target for GBC.

The prognosis of gallbladder cancer (GBC) is still poor despite advances in diagnostic tools and surgical techniques. The 5-year survival rate in patients with GBC is approximately 10%.[1] Gallbladder cancer is usually detected at an advanced stage when symptoms such as abdominal pain and jaundice have emerged. This diagnostic delay reduces the possibility of curative surgery. Indeed, early-stage GBC is often found only incidentally during cholecystectomy for cholecystolithiasis and accounts for only 1% of GBCs.[2,3] Curative surgical resection is the best therapy for long-term survival. However, because many patients cannot undergo curative surgery, they must rely on chemotherapy and radiation therapy, which are limited in their efficacy. This is likely due to the aggressive phenotype of GBC including proliferation and invasiveness. Therefore, new therapeutic strategies for GBC are needed.

The relationship between developmental signaling and cancer initiation and progression has been recently acknowledged. Hedgehog (Hh) signaling is a key developmental pathway. There are three Hh ligands in vertebrates, Sonic (Shh), Indian, and Desert.[4] In the absence of a Hh ligand, Patched, the 12-transmembrane receptor, prevents Smoothened (Smo) activity. Smoothened is a 7-transmembrane effector of the pathway. In the presence of a Hh ligand, Patched becomes inactive allowing Smo activation. Smoothened activates the downstream Gli transcription factors Gli1, Gli2, and Gli3. Gli1 is a strong activator of downstream target genes and itself. Therefore, Gli1 localization within the nucleus is a biomarker of Hh activity. Previously we have shown that Hh signaling is activated in various kinds of cancer and that it contributes to the induction of malignant phenotypes. For example, it promotes high proliferative and invasive ability in pancreatic cancer, colorectal cancer, gastric cancer, and breast cancer.[5–8] In addition, a link between cancer stem cells and Hh signaling activation has been reported.[9–12] Currently, the Smo inhibitors erismodegib and saridegib are in clinical trials[13,14] and the Smo inhibitor vismodegib was approved by the US Food and Drug Administration for the treatment of unresectable or metastatic basal cell carcinomas of the skin in 2012.[15] Thus, the Hh signaling pathway is a therapeutic target in some cancers. However, in GBC, the status of Hh signaling is still unknown. In the present study, we analyzed Hh signaling in GBC and determined if the Hh pathway might be a therapeutic target in GBC.

Materials and Methods

Cell lines. We used GBC cell lines GBd15[16] and TGBC2TKB.[17] GBd15 and TGBC2TKB cells were cultured

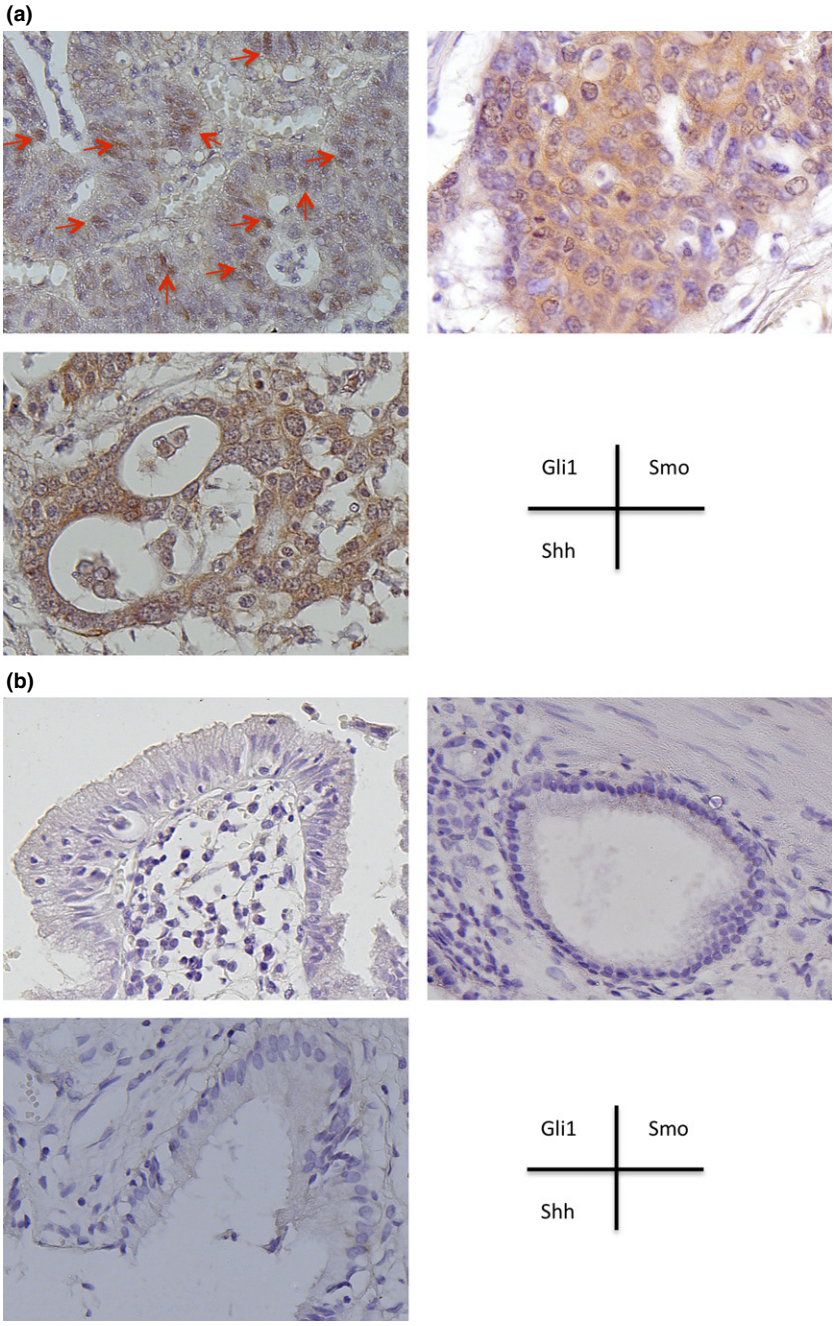

Fig. 1. Expression of Hedgehog (Hh) components in gallbladder cancer is significantly higher than in normal gallbladder tissue. Immunohistochemical staining for Hh components in gallbladder cancer (a) or normal gallbladder (b) specimens. Clockwise (from upper left) are antibodies to Gli1, Smoothened (Smo), and Sonic Hh (Shh). A positive reaction is brown in color. Red arrows indicate Gli1 expression in the nucleus of cancer cells. Original magnification, ×400.

in DMEM supplemented with 10% FBS (Sigma-Aldrich, Hokkaido, Japan) at 37°C in a 5% CO_2 humidified incubator.

Immunohistochemistry. Tissue samples were obtained from patients with GBC who underwent resection at Kyushu University Hospitals (Fukuoka, Japan) from 2001 to 2012. The approval for the use of tissues was obtained from patients in accordance with the Ethical Committees of Clinical Study at Kyushu University. Paraffin sections of GBC were deparaffinized and rehydrated. The sections were bleached in 3% H_2O_2 for 30 min and then incubated in 10% rabbit serum with primary antibody for Gli1 (SC-6153, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Shh (SC-1194, 1:100, Santa Cruz Biotechnology), or Smo (SC-6366, 1:100; Santa Cruz Biotechnology) at 4°C overnight. The samples were incubated with Histofine Simple Stain MAX PO (G) (Nichirei, Tokyo, Japan) and 3,3′-diaminobenzidine, and visualized using a hematoxylin counterstain. To estimate the number of positive cells, 10 independent areas were selected and total positive cell numbers in that area were counted. If more than 50% of the tumor cells were stained, protein expression was considered to be positive. The cut-off line for determination of positive cells was determined previously.[8]

Isolation of RNA and conventional and real-time RT-PCR. Total RNA isolation and cDNA synthesis were carried out as previously reported.[5] The conventional and real-time RT-PCR reactions were carried out using primers listed in Table S1. MasterMix Kit (Qiagen, Hilden, Germany) and iQSYBER Green Supermix (Bio-Rad Laboratories, Philadelphia, PA, USA) were used. In the conventional RT-PCR experiments, conditions were 95°C for 120 s and 35 cycles were completed.

Western blot analysis. Protein samples were extracted using M-PER reagent (Thermo Fisher Scientific, Chicago, IL, USA).

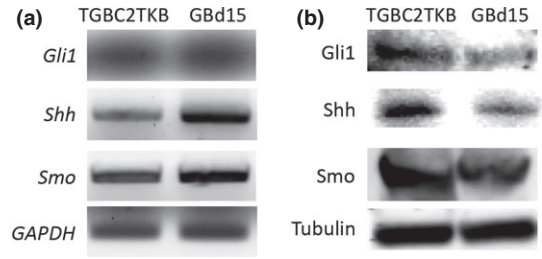

Fig. 2. Expression of Hedgehog (Hh) components in gallbladder cancer (GBC) cells is also evident at mRNA and protein levels. (a) Total RNA from GBC cells (GBd15, TGBC2TKB) was reverse transcribed and the cDNA was subjected to conventional RT-PCR for the indicated mRNAs. GAPDH was used as control. (b) Protein extracted from GBC cells (GBd15, TGBC2TKB) was subjected to Western blot analysis for Hh components. Tubulin was used as the loading control. Shh, Sonic Hh; Smo, Smoothened.

Immunoblotting was carried out with primary antibodies against Gli1 (SC-6153, 1:200), Shh (SC-1194, 1:200), Smo (SC-6366, 1:200), MMP-2 (SC-10736, 1:200), MMP-9 (SC-6840, 1:100), E-cadherin (SC-7870, 1:200), vimentin (SC-5565, 1:500) (all Santa Cruz Biotechnology), or α-tubulin (T6199, 1:1000; Sigma-Aldrich). Blots were developed with the ECL plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA).

Small interfering RNA transfection. Small interfering RNA targeting of Smo (Lot#130731), MMP-2 (Lot#121127), or MMP-9 (Lot#120606) were purchased from Dharmacon RNA Technologies (Lafayette, CO, USA). The indicated siRNAs were transfected into GBd15 and TGBC2TKB cells at a final concentration of 50 μM using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Non-targeting siRNA (Dharmacon) was used as the negative control.

Proliferation assay. GBd15 and TGBC2TKB cells were seeded onto 96-well plates at a density of 5000 cells/well and incubated with recombinant human Shh (rhShh; R&D Systems, Minneapolis, MN, USA) or the Smo inhibitor cyclopamine (Toronto Research Chemicals, North York, Canada) for 24, 48, or 72 h. Cell proliferation was determined by the absorbance at 492 nm (ref. 620 nm) using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan). Forty-eight hours after *Smo*, *MMP-2*, and *MMP-9* siRNA transfection, the cells were reseeded onto 96-well plates and the proliferation rates were measured.

Cell invasion assay. The invasiveness of the GBC cells was assessed by an invasion assay as described previously.[5] In brief *Smo*, *MMP-2*, or *MMP-9* siRNA were transfected into cells. After 48 h, 5×10^3 cells were added to the chambers and incubated for 16 h. The total number of cells that had migrated to the lower side of the filter were fixed and stained with Diff-Quik reagent (Sysmex, Kobe, Japan) and then counted under a light microscope.

Gelatin zymography. The enzyme activity of MMP-2 and MMP-9 was assessed by gelatin zymography as described previously.[26] In brief, *Smo* siRNA was transfected into cells. After 48 h, protein samples were extracted and used in the gelatin zymography kit (Cosmobio, Tokyo, Japan) according to the manufacturer's instructions.

Immunocytochemistry. Forty-eight hours after *Smo* siRNA transfection, the cells were immunostained with primary antibodies to E-cadherin (SC-7870, 1:500; Santa Cruz Biotechnology) followed by Alexa 488-labeled secondary antibodies (Life Technologies), as previously described.[19]

Soft agar colony formation assay. Soft agar colony formation assays were carried out as previously described.[20] In brief, the cells were mixed into DMEM containing 0.3% agar and 10% FBS. Where indicated, each well was then covered with DMEM containing 1.0 μg/mL rhShh or 10 μM cyclopamine. Two weeks later, the colonies were stained with crystal violet (Sigma-Aldrich).

***In vivo* xenograft tumor model.** Five-week-old female athymic nude mice (BALB/c nu/nu) were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and acclimated for 2 weeks. All animal procedures were approved by the Animal

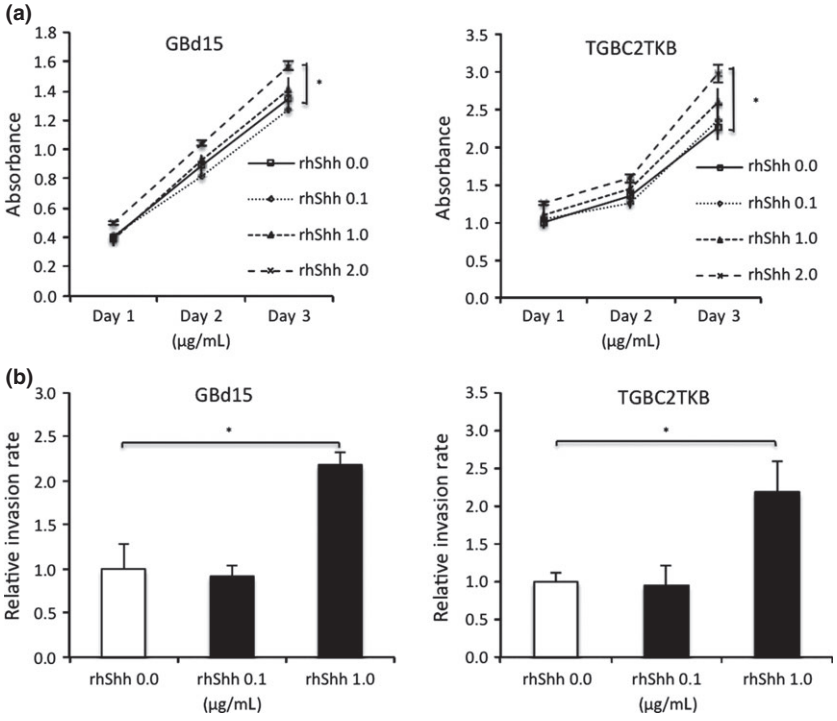

Fig. 3. Recombinant human Sonic Hedgehog (rhShh) augments the proliferative and invasive phenotypes of gallbladder cancer cells. (a) GBd15 and TGBC2TKB cells were seeded onto 96-well plates at a density of 5000 cells/well and incubated with rhShh (at 0.1, 1.0, or 2.0 μg/mL) for 24, 48, or 72 h as indicated. (b) GBd15 and TGBC2TKB cells were incubated for 16 h in the presence or absence of rhShh (at 0.1 or 1.0 μg/mL). Migrated cells were quantified by bright-field microscopy. Error bars represent standard deviations. $^*P < 0.05$.

Care and Use Committee at Kyushu University (A25-027-0). TGBC2TKB cells transfected with *Smo* siRNA or non-targeting control siRNA were s.c. implanted into the flank (1×10^6 cells in PBS per mouse) of nude mice ($n = 5$ in each treatment group). Tumor size was measured twice a week and the volume was calculated as follows: length $\times$ (width)^2 $\times$ 0.5 mm^3.

Statistical analysis. All data are presented as the mean $\pm$ SD. Student's *t*-test was used to compare the variability between the two groups. $P < 0.05$ was considered significant.

Results

Expression of Hh components in GBC is significantly higher than that in normal gallbladder tissue. First, we evaluated the expression of Hh pathway components in GBC tissues from patients by immunohistochemistry. The ratio of female to male patients was 1.2:1.0 (20 women, 17 men). Distribution according to the stage of the UICC was: IA, $n = 8$; IB, $n = 18$; IIA, $n = 2$; IIB, $n = 8$; III, $n = 1$. Median age at time of diagnosis was 67 years; 68 years (range, 53–83 years) for men and 66 years (range, 42–85 years) for women (Table S2). The expression of Gli1 was detected in the nucleus of cancer cells in 19/37 (51.3%) GBCs (Fig. 1a, red arrows), which suggests that Hh signaling is activated in GBC. Both Shh and Smo expression were also detected in 17/37 (45.9%) and 24/37 (64.9%) of the GBCs, respectively. The subcellular localization of Shh and Smo were mainly cytoplasmic in cancer cells (Fig. 1a). Gli1 was not detected in the nuclei of normal gallbladder cells. The expression of Shh and Smo was notably elevated in GBC tissues versus normal gallbladder tissues where

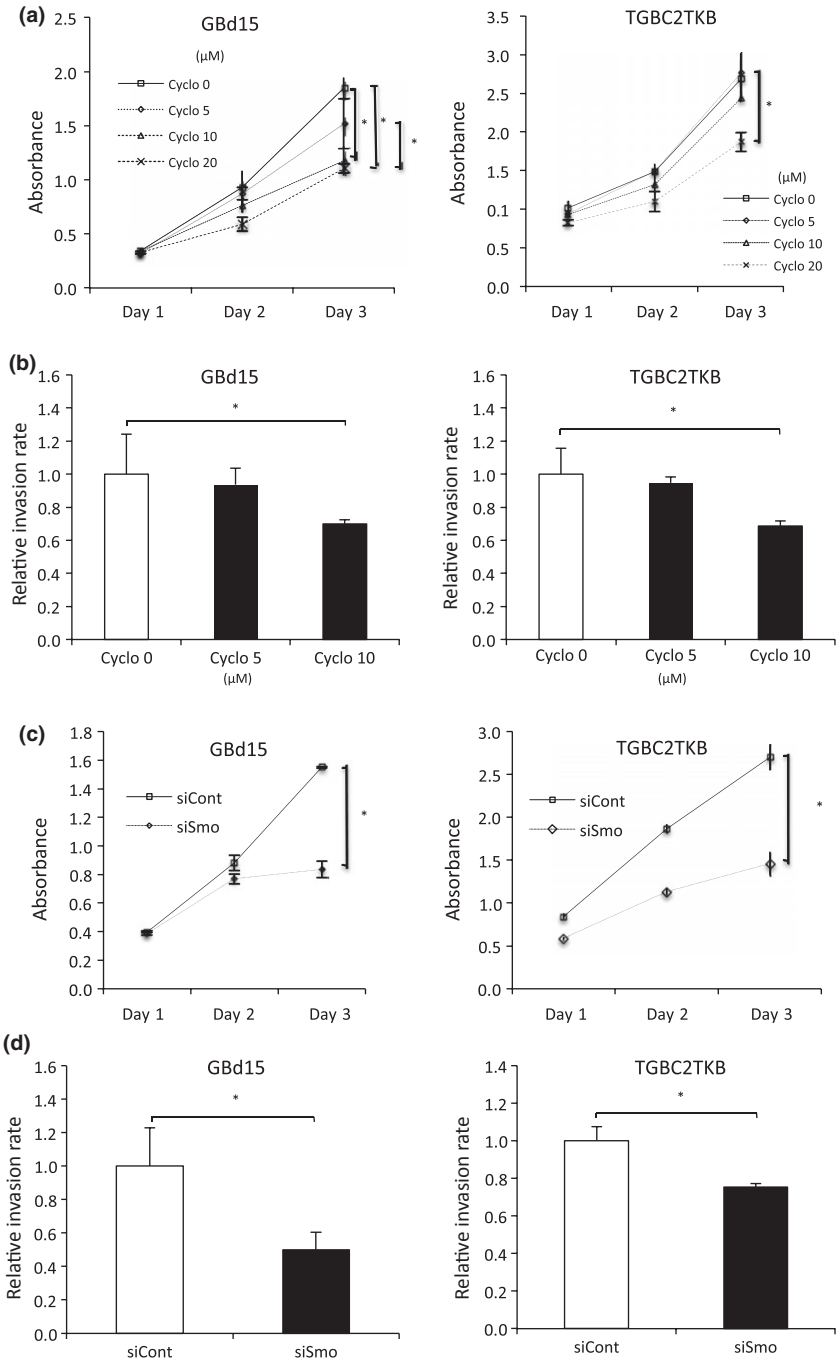

Fig. 4. Cyclopamine (Cyclo) and *Smo* siRNA suppress proliferative and invasive phenotypes of gallbladder cancer cells. (a) GBd15 and TGBC2TKB cells were seeded onto 96-well plates and incubated with cyclopamine (at 5, 10, or 20 μM) for 24, 48, or 72 h. (b) GBd15 and TGBC2TKB cells were incubated for 16 h in the presence or absence of cyclopamine (at 5 or 10 μM). Migrated cells were quantified by bright-field microscopy. (c) GBd15 and TGBC2TKB cells were transfected with *Smo* siRNA (siSmo) and incubated for 24, 48, or 72 h. (d) GBd15 and TGBC2TKB cells were transfected with *Smo* siRNA and incubated for 16 h. Migrated cells were quantified by bright-field microscopy. Error bars represent standard deviations. *$P < 0.05$. siCont, control siRNA.

they were virtually undetectable (Fig. 1b). These results suggest that Hh signaling is active in GBC and may play an important role in disease progression and severity.

Expression of Hh components in GBC cells also detected. We used GBC cell lines to characterize Hh signaling *in vitro*. As shown in Figure 2, mRNA and protein products for of Gli1, Shh, and Smo were detected in GBd15 and TGBC2TKB cells.

Recombinant human Shh augments proliferative and invasive phenotypes of GBC cells. We hypothesized that Hh signaling may contribute to the malignancy of GBC. We examined the impact of Hh activation on proliferation and invasiveness. The addition of rhShh augmented the proliferation rate (Fig. 3a) and invasiveness (Fig. 3b) of GBd15 and TGBC2TKB cells. These results indicate that activation of Hh signaling further enhances the proliferative and invasive phenotypes of GBC cells.

Cyclopamine and *Smo* siRNA suppress proliferative and invasive phenotypes of GBC cells. To verify the link between Shh/Smo signaling and the phenotypic alterations in GBCs, we used cyclopamine, an inhibitor of Smo. In contrast to rhShh, cyclopamine suppressed proliferation (Fig. 4a) and invasiveness (Fig. 4b) in GBd15 and TGBC2TKB cells. To exclude the possibility of a non-specific effect of cyclopamine, we also used *Smo*-targeting siRNA. *Smo* siRNA transfection decreased *Smo* expression by 90% and *Gli1* expression by 70% (Fig. S1). *Smo* siRNA also significantly suppressed the proliferation (Fig. 4c), and invasiveness (Fig. 4d) of GBd15 and TGBC2TKB cells. These results suggest that Hh signaling can modulate the proliferative and invasive phenotypes of GBC cells and that *Smo* inhibition ameliorates the phenotype.

Smooothened-regulated invasion mediated through MMP-2 and MMP-9. To explore how Hh signaling was inducing invasion in GBC cells, we examined whether MMP-2 and MMP-9 were altered in GBd15 and TGBC2TKB cells. The expression and enzyme activity of MMP-2 and MMP-9 in *Smo* siRNA transfected GBC cells significantly decreased compared with siRNA control cells as measured by quantitative RT-PCR (Fig. 5a), Western blotting (Fig. 5b), and gelatin zymography (Fig. 5c). We then investigated the effect of MMP-2 and MMP-9 knockdown on the invasiveness of GBC cells. *MMP-2* siRNA and *MMP-9* siRNA transfection inhibited the expression by 80% for both enzymes (Fig. S2). Their knockdown did not affect proliferation (Fig. S3) but it significantly inhibited their invasiveness (Fig. 5d). *MMP-2* siRNA and *MMP-9*

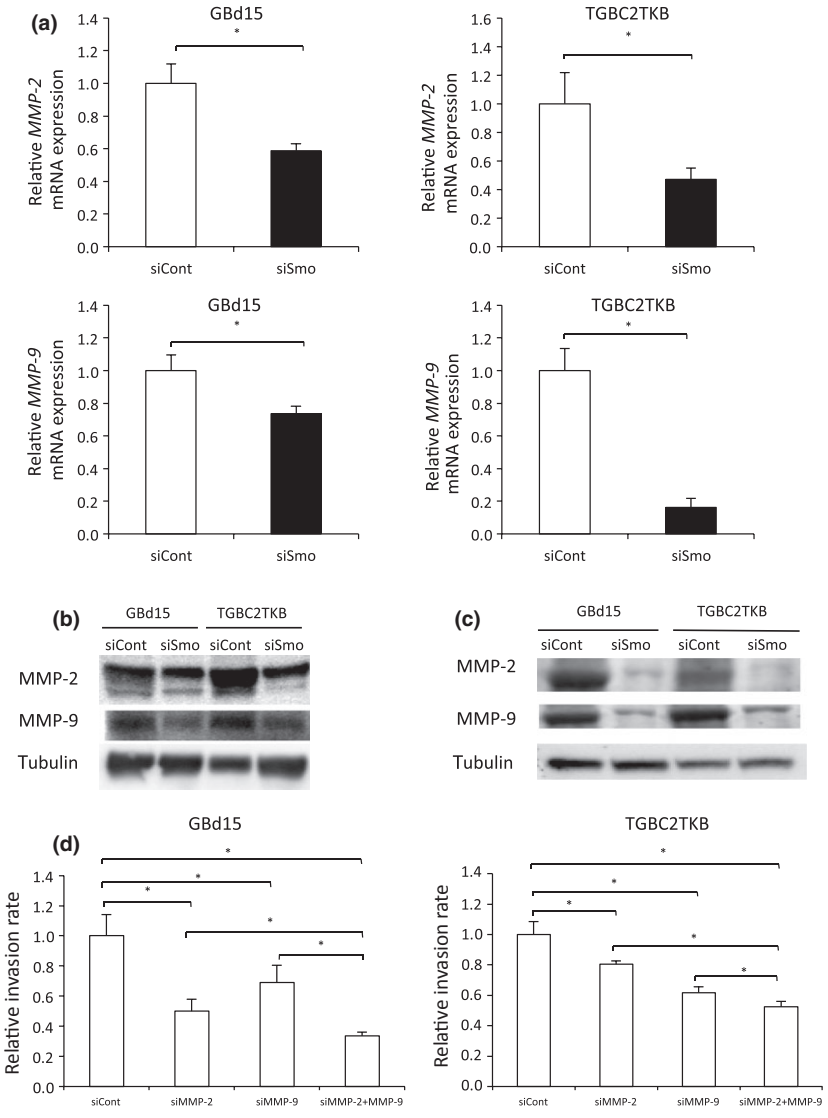

Fig. 5. Smoothened (Smo)-regulated invasion is induced through MMP-2 and MMP-9. (a) Quantitative RT-PCR, (b) Western blotting, and (c) gelatin zymography assay using *Smo* siRNA (siSmo) transfected GBd15 and TGBC2 cells. (d) Cell invasion assay using *MMP-2* siRNA (siMMP-2) and *MMP-9* siRNA (siMMP-9) transfected GBd15 and TGBC2TKB cells. siCont, control siRNA. $*P < 0.05$.

siRNA cotransfection inhibited invasion more than either *MMP-2* siRNA or *MMP-9* siRNA transfection alone (Fig. 5d). These results indicate that decreased cell invasion when Smo is inhibited is likely because MMP-2 and MMP-9 are not activated.

Smoothened-regulated invasion mediated through epithelial–mesenchymal transition. Epithelial–mesenchymal transition (EMT) is another important factor in invasiveness.[21] Therefore, we also examined the effect of Smo knockdown on EMT in GBd15 and TGBC2TKB cells. An increase in the expression of E-cadherin and a decrease in the expression of vimentin were seen by quantitative RT-PCR (Fig. 6a) and Western blotting (Fig. 6b) after *Smo* siRNA treatment in GBd15 and TGBC2TKB cells. Furthermore, E-cadherin accumulated in the membrane of *Smo* siRNA transfected cells (Fig. 6c, red arrow). As shown in Figure 6(d), *Smo* siRNA transfection also substantially reduced the percentage of spindle-shaped cells and the cells tended to aggregate. These results suggest that decreased cell invasion following Smo knockdown is because of a lack of EMT in GBCs.

Hedgehog signaling enhances colony formation *in vitro* and inhibition of Smo suppresses tumor growth *in vivo* for GBC cells. We next investigated the role of Hh signaling on anchorage-independent growth of GBC cells. The addition of rhShh significantly enhanced colony formation, an anchorage-independent phenotype, in GBd15 and TGBC2TKB cells (Fig. 7a). Cyclopamine and *Smo* siRNA also significantly suppressed colony formation in GBd15 and TGBC2TKB cells (Fig. 7b,c). These results indicate that Hh signaling affects anchorage-independent growth in GBC cells. To determine whether the observed changes induced by Hh signaling in cells is reflected *in vivo*, we investigated the tumorigenicity of GBC cells transfected with *Smo* siRNA in athymic nude mice. Subcutaneous tumors developed in three out of five mice injected with cells transfected with *Smo* siRNA. In contrast, all of the five mice injected with control siRNA transfected cells showed tumor development (Fig. 7d). There were significant differences in tumor growth/size between *Smo* siRNA and control siRNA groups as well (Fig. 7e). Furthermore, we confirmed that the expression of Gli1 and Smo in

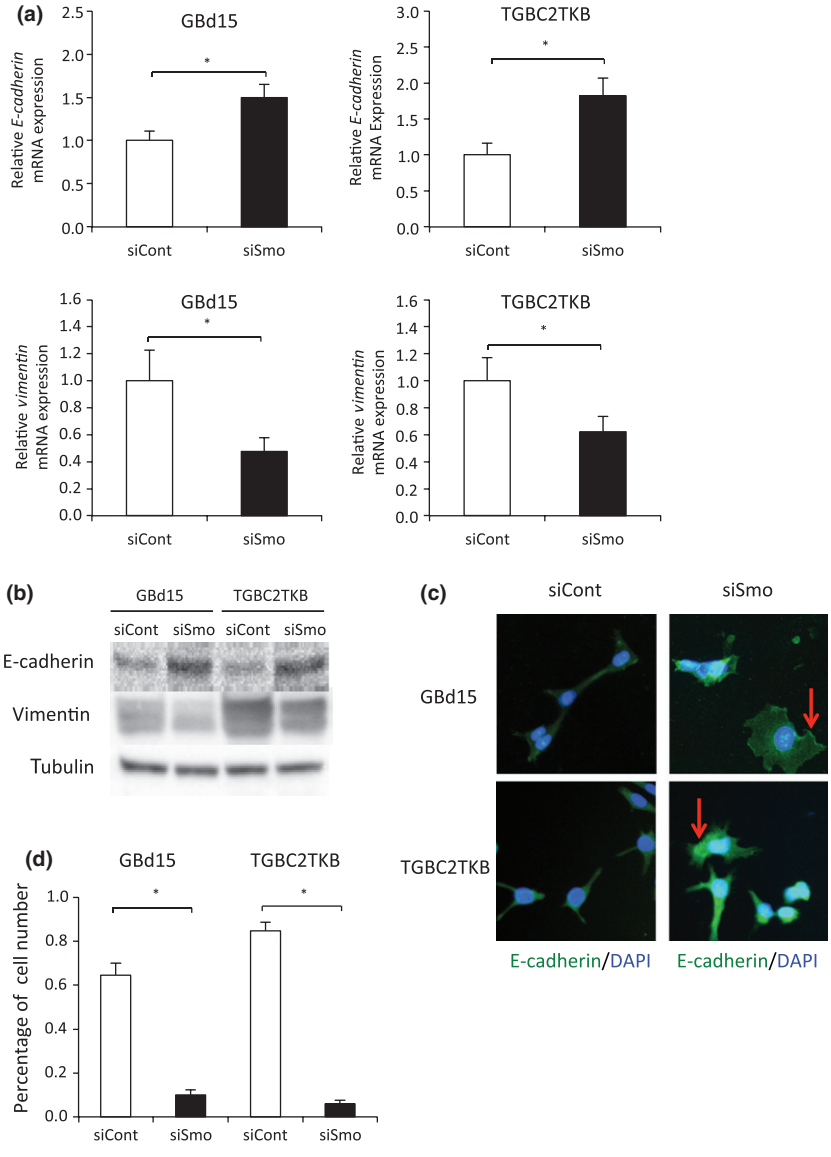

Fig. 6. Smoothened (Smo)-regulated invasion is mediated through epithelial–mesenchymal transition. (a) Quantitative RT-PCR (b) and Western blotting of E-cadherin and vimentin in *Smo*-siRNA transfected GBd15 and TGBC2TKB cells. (c) GBd15 and TGBC2TKB cells transfected with control siRNA (siCont) or *Smo* siRNA (siSmo) were immunostained with E-cadherin antibody (green) and DAPI (blue). Red arrows, E-cadherin accumulated in the membrane of *Smo* siRNA transfected cells. Original magnification, ×200. (d) GBd15 and TGBC2TKB cells transfected with *Smo* siRNA for 48 h before the number of spindle-shaped cells were counted using bright-field microscopy. Error bars represent standard deviations. $*P < 0.05$.

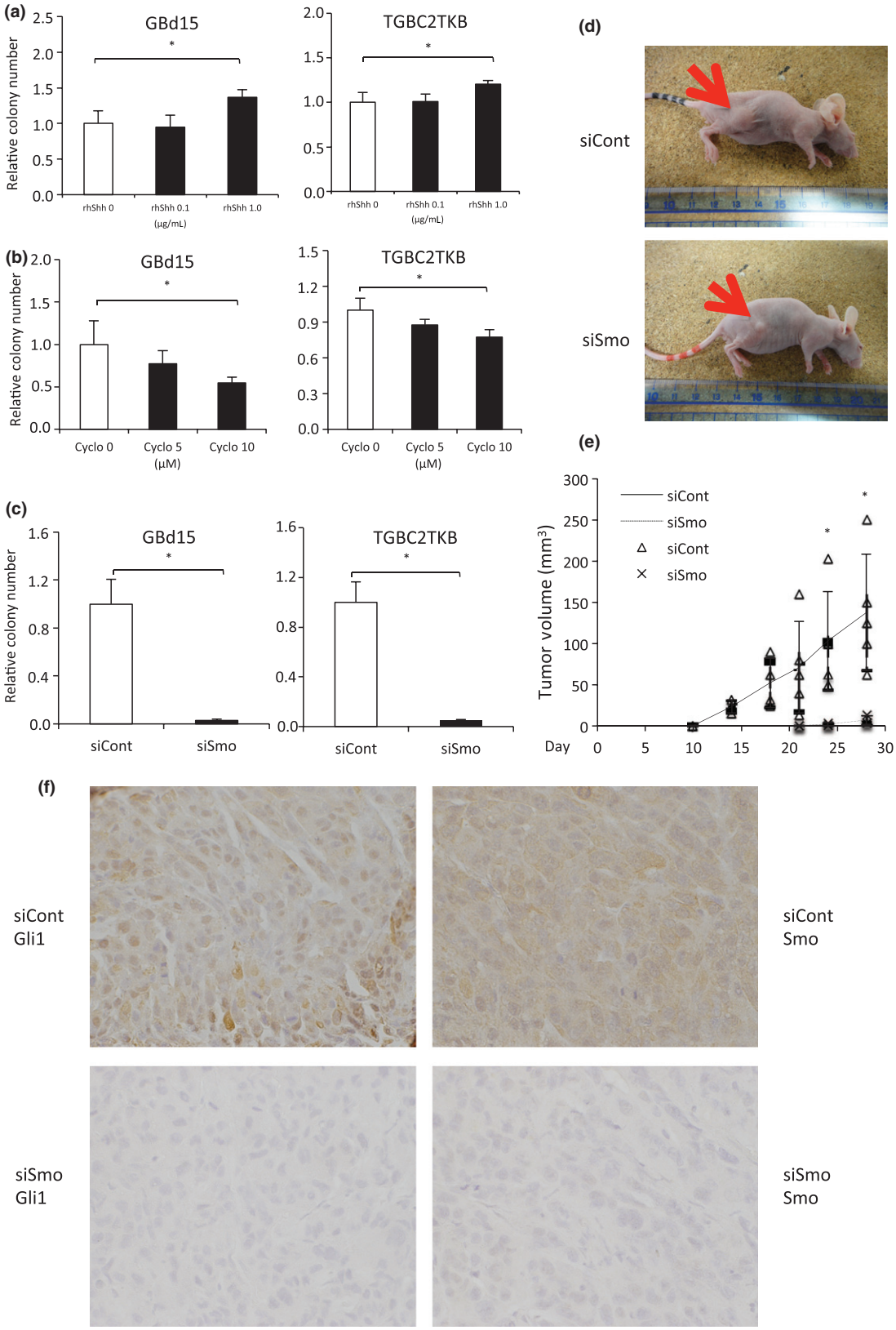

Fig. 7. Hedgehog (Hh) signaling enhances gallbladder cancer colony formation *in vitro* and inhibition of Smoothened (Smo) suppresses tumor growth *in vivo*. (a, b) GBd15 and TGBC2TKB cells were incubated with recombinant human Sonic Hh (rhShh) (a, at 0.1 or 1.0 μg/mL) or cyclopamine (Cyclo) (b, at 5 or 10 μM) for 14 days before colony formation was assessed. (c) GBd15 and TGBC2TKB cells were transfected with *Smo* siRNA (siSmo) and incubated for 14 days before colony formation was assessed. (d, e) TGBC2TKB cells were transfected with control siRNA (siCont) ($n = 5$) or *Smo* siRNA ($n = 5$), and then implanted into the flanks of athymic nude mice. The data represent the volume of each tumor (red arrows). Student's *t*-test was carried out between the tumor volumes of the two groups. (f) Gli1 and Smo expression were confirmed by immunohistochemistry in tumor tissue explants from the nude mice. Original magnification, ×400. Error bars represent standard deviations. $*P < 0.05$.

Smo siRNA transfected GBC cells were lower than in the controls (Fig. 7f).

Discussion

Hedgehog signaling is reactivated in many types of cancer.[5–8] Recently, Hh inhibitors have drawn much attention as potential therapies against refractory cancers.[22,23] Previously, Li *et al.*[24] has shown that Hh pathway components are expressed in GBC. In the present study we focused on whether Hh signaling affects the function of GBC, especially the induction of the malignant phenotype including proliferation and invasiveness. We first examined the expression of Hh components in GBC specimens and established the expression of Gli1, Shh, and Smo. We followed this analysis with phenotypic assays in which we inhibited Hh signaling and determined the metastatic capability of treated versus untreated cells. Our findings using cyclopamine and *Smo* siRNA suggest that Smo inhibition may be useful for treating GBC.

Metastasis is responsible for cancer-related mortality. It is a multistep process that begins when cancer cells escape through the basement membrane. The gallbladder wall is thin and there are rich lymphatic and perivascular tissues around the gallbladder, facilitating cancer cell invasion into the surrounding tissues. Therefore, we investigated the significance of Hh signaling in GBC progression with respect to invasion. Degradation of type IV collagen in the basement membrane is required for invasion into adjacent blood or lymphatic vessels. Matrix metalloproteinases play critical roles in the degradation of the ECM.[25] Both MMP-2 and MMP-9 are type IV collagenases that have the ability to degrade type IV collagen.[26] Therefore, we focused on MMP-2 and MMP-9, which have been shown to correlate with Hh signaling in previous studies.[5,18] In the present study, Hh signaling enhanced the invasive phenotype of gallbladder cells through upregulation of MMP-2 and MMP-9.

Epithelial–mesenchymal transition is defined as the loss of polarized epithelial character and the acquisition of a migratory mesenchymal phenotype. The association between Hh signaling and EMT has been previously reported in several types of cancer.[27–29] E-cadherin is one of the typical molecules facilitating cell adherence, such that the inhibition of E-cadherin expression is triggered by EMT, and this is used as index of EMT. In the present study, Smo inhibition enhanced E-cadherin expression. Snail, a zinc finger transcriptional factor, is an EMT-inducing transcription factor.[30] However, we did not find a correlation between E-cadherin and Snail expression (data not shown). Inhibition of the key metastasis suppressor E-cadherin can occur through a variety of mechanisms, including promoter hypermethylation of EMT-inducing factors,[31] or the action of microRNAs.[32] The exact mechanisms of EMT in GBC is still unknown and will require further investigation, but it is likely that Hh signaling may play a role.

Once cancer cells invade into blood or lymphatic vessels, they attach to distant tissues and anchor-independent growth occurs, forming metastatic tumors. Thus, we investigated the effect of Hh signaling on anchor-independent colony formation in GBC cells. We found that the anchor-independent growth was enhanced by exogenous rhShh and suppressed by Smo inhibition. Furthermore, the effect of *Smo* silencing in a xenograft model led to a decrease in tumor volume and number. These results suggest that Hh signaling may be a potential therapeutic target in GBC. However, the reasons why tumor tissue explants from siSmo do not express Smo or Gli1, even after 28 days, remain to be clarified (Fig. 7f). We think that other signaling relevant to cancer proliferation, such as Akt or Erk, may contribute to tumor growth without Gli1 and Smo expression. It is our aim to investigate a molecule to explain these results.

In conclusion, our data suggest that Hh signaling is active in GBCs and plays a pivotal role in proliferation and invasiveness of GBC. Eligibility criteria is important for establishing Hh inhibitor treatment as standard therapy for GBC. We think that Smo and Gli1 protein-positive patients, as determined by immunohistochemically stained resected tumor specimens, may be eligible for Hh signaling inhibitor treatment. Our results will provide support for the development of new therapeutic strategies to treat refractory GBC.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Misra S, Chaturvedi A, Misra NC, Sharma ID. Carcinoma of the gallbladder. *Lancet Oncol* 2003; **4**: 167–76.
2 Glauser PM, Strub D, Käser SA, Mattiello D, Rieben F, Maurer CA. Incidence, management, and outcome of incidental gallbladder carcinoma: analysis of the database of the Swiss association of laparoscopic and thoracoscopic surgery. *Surg Endosc* 2010; **24**: 2281–6.
3 Gourgiotis S, Kocher HM, Solaini L, Yarollahi A, Tsiambas E, Salemis NS. Gallbladder cancer. *Am J Surg* 2008; **196**: 252–64.
4 Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 2001; **15**: 3059–87.
5 Onishi H, Kai M, Odate S *et al.* Hypoxia activates the hedgehog signaling pathway in a ligand-independent manner by upregulation of Smo transcription in pancreatic cancer. *Cancer Sci* 2011; **102**: 1144–50.
6 Iwasaki H, Nakano K, Shinkai K *et al.* Hedgehog Gli3 activator signal augments tumorigenicity of colorectal cancer via upregulation of adherence-related genes. *Cancer Sci* 2013; **104**: 328–36.
7 Yanai K, Nagai S, Wada J *et al.* Hedgehog signaling pathway is a possible therapeutic target for gastric cancer. *J Surg Oncol* 2007; **95**(1): 55–62.
8 Kubo M, Nakamura M, Tasaki A *et al.* Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. *Cancer Res* 2004; **64**: 6071–4.
9 Dierks C, Beigi R, Guo GR *et al.* Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell* 2008; **14**: 238–49.
10 Peacock CD, Wang Q, Gesell GS *et al.* Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. *Proc Natl Acad Sci U S A* 2007; **104**: 4048–53.
11 Zhao C, Chen A, Jamieson CH *et al.* Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature* 2009; **458**: 776–9.
12 Clement V, Sanchez P, de Tribolet N, Radovanovic I. Ruiz i Altaba A. HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Curr Biol* 2007; **17**: 165–72.
13 A phase III study of oral LDE225 versus temozolomide in patients with hedgehog pathway activated relapsed medulloblastoma. Available from: http://clinicaltrials.gov.show/NCT01708174.
14 Jimeno A, Weiss GJ, Miller WH *et al.* Phase I study of the Hedgehog pathway inhibitor IPI-926 in adult patients with solid tumors. *Clin Cancer Res* 2013; **19**: 2766–74.

15 Poggi L, Kolesar JM. Vismodegib for the treatment of basal cell skin cancer. *Am J Health Syst Pharm* 2013; **70**: 1033–8.
16 Sasaki S, Kuniyasu H, Luo Y *et al.* Significance of epithelial growth factor in the epithelial-mesenchymal transition of human gallbladder cancer cells. *Cancer Sci* 2012; **103**: 1165–71.
17 Sato J, Kimura T, Saito T *et al.* Gene expression analysis for predicting gemcitabine resistance in human cholangiocarcinoma. *J Hepatobiliary Pancreat Sci* 2011; **18**: 700–11.
18 Nagai S, Nakamura M, Yanai K *et al.* Gli1 contributes to the invasiveness of pancreatic cancer through matrix metalloproteinase-9 activation. *Cancer Sci* 2008; **99**: 1377–84.
19 Kameda C, Tanaka H, Yamasaki A *et al.* The Hedgehog pathway is a possible therapeutic target for patients with estrogen receptor-negative breast cancer. *Anticancer Res* 2009; **29**: 871–9.
20 Akiyoshi T, Nakamura M, Koga K *et al.* Gli1, downregulated in colorectal cancers, inhibits proliferation of colon cancer cells involving Wnt signalling activation. *Gut* 2006; **55**: 991–9.
21 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**: 871–90.
22 Von Hoff DD, LoRusso PM, Rudin CM *et al.* Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *N Engl J Med* 2009; **361**: 1164–72.
23 Rudin CM, Hann CL, Laterra J *et al.* Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449. *N Engl J Med* 2009; **361**: 1173–8.
24 Li J, Wu T, Lu J *et al.* Immunohistochemical evidence of the prognostic value of hedgehog pathway components in primary gallbladder carcinoma. *Surg Today* 2012; **42**: 770–5.
25 Kamat AA, Fletcher M, Gruman LM *et al.* The clinical relevance of stromal matrix metalloproteinase expression in ovarian cancer. *Clin Cancer Res* 2006; **12**: 1707–14.
26 Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* 2000; **18**: 1135–49.
27 Lei J, Ma J, Ma Q *et al.* Hedgehog signaling regulates hypoxia induced epithelial to mesenchymal transition and invasion in pancreatic cancer cells via a ligand-independent manner. *Mol Cancer* 2013; **12**: 66.
28 El Khatib M, Kalnytska A, Palagani V *et al.* Inhibition of hedgehog signaling attenuates carcinogenesis in vitro and increases necrosis of cholangiocellular carcinoma. *Hepatology* 2013; **57**: 1035–45.
29 Ota H, Aoyagi K, Fukaya M *et al.* Cross talk between hedgehog and epithelial-mesenchymal transition pathways in gastric pit cells and in diffuse-type gastric cancers. *Br J Cancer* 2009; **100**: 389–98.
30 Nieto MA. The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* 2002; **3**: 155–66.
31 Kwon MJ, Kwon JH, Nam ES *et al.* TWIST1 promoter methylation is associated with prognosis in tonsillar squamous cell carcinoma. *Hum Pathol* 2013; **44**: 1722–9.
32 Ma L, Young J, Prabhala H *et al.* miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol* 2010; **12**: 247–56.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Knockdown efficacy of *Smoothened* (*Smo*) siRNA in GBd15 and TGBC2TKB gallbladder cancer cells.

Fig. S2. Knockdown efficacy of *MMP-2* and *MMP-9*-siRNA in GBd15 and TGBC2TKB gallbladder cancer cells.

Fig. S3. Transfection with *MMP-2* and *MMP-9* siRNA does not affect proliferation in GBd15 and TGBC2TKB gallbladder cancer cells.

Table S1. Polymerase chain reaction primer sequences.

Table S2. Clinical background of the samples used for immunohistochemistry.