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ORIGINAL ARTICLE – CANCER RESEARCH

Possible correlation of sonic hedgehog signaling with epithelial–mesenchymal transition in muscle-invasive bladder cancer progression

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Abstract

Purpose To investigate the role of sonic hedgehog (Shh) signaling and epithelial–mesenchymal transition (EMT) in bladder cancer progression and invasion.

Methods We cultured three bladder cancer cell lines, muscle-invasive T24 and 5637, and non-muscle-invasive KK47, in the presence of a recombinant-Shh (r-Shh) protein or cyclopamine, a Shh signaling inhibitor, to investigate proliferation and expression of EMT markers. Wound-healing assays and transwell assay were performed to evaluate cell invasion and migration. Mice were then inoculated with bladder cancer cells and treated with cyclopamine. Mouse tumor samples were stained for Shh signaling and EMT markers.

Results R-Shh protein enhanced cell proliferation, whereas cyclopamine significantly suppressed cell proliferation, especially in invasive cancer (5637 and T24) ($p < 0.05$). R-Shh protein promoted EMT, suppressed E-cadherin and enhanced N-cadherin and vimentin and Gli1, an Shh downstream molecule, while cyclopamine blocked EMT, especially in 5637 and T24. Cyclopamine also inhibited cell invasion and migration in vitro. In the animal study, intraperitoneal injection of cyclopamine significantly suppressed tumor growth in 5637 and T24 in mice ($p = 0.01$ and $p = 0.004$, respectively) and slightly suppressing KK47 tumor growth ($p = 0.298$). Significant cyclopamine-induced suppression of Gli1 in 5637 and T24 mouse tumors (both $p = 0.03$) was seen, suggesting that muscle-invasive bladder cancer may be more dependent on Shh signaling than non-muscle-invasive bladder cancer.

Conclusions Shh signaling and EMT were especially enhanced in muscle-invasive bladder cancer progression and invasion, and suppressed by the inhibition of Shh signaling.

Keywords Sonic hedgehog · Epithelial–mesenchymal transition · Bladder cancer · Basic study · Oncology

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Introduction

Therapeutic strategies are especially limited in progressive muscle-invasive bladder cancer, and there are no clinically available definitive therapeutic strategies in cases refractory to chemotherapy. Hedgehog (Hh) signaling has been reported in several clinical cancers as well as basic research (Behnsawy et al. 2013; Yamamichi et al. 2014). Hh binds to transmembrane receptor Patched (Ptch) and relieves constitutive repression of Smoothened (Smo). Smo activation promotes activation/translocation of glioma-associated oncogenes (Glis) to the nucleus, causing transcriptional activation of Hh target genes (Shigemura and Fujisawa 2015).

Hh signaling may contribute to development of solid tumors through gene alterations of Ptch/Smo resulting in constitutively activated Hh signaling (Gupta et al. 2010). Although early reports indicated that sonic Hh (Shh) signaling is activated in bladder cancer (McGarvey et al. 1998), these signaling pathways could be activated via various mechanisms including ligand-independent Hh signaling activation. Recently, Shh signaling was also shown to be markedly elevated in human bladder cancer cells during progression from non-muscle invasive to invasive cancer (He et al. 2012). Transforming growth factor (TGF)-β1-induced-Shh signaling also activated epithelial–mesenchymal transition (EMT), tumorigenicity, and stemness in bladder cancer (Islam et al. 2016). EMT is well known as an important mechanism for progression, invasion and migration in cancer cells. EMT involves reprogramming the epithelial cells during embryonic development and tissue repair in adults (Peinado et al. 2007). One of the most characteristic changes during EMT is suppression of E-cadherin, resulting in loss of cell adhesion. EMT also induces loss of basal–apical polarity and tight cell junctions, and increases the expression of mesenchymal markers such as N-cadherin and vimentin in cancer progression and metastatic phenotypes (McConkey et al. 2009). In this study, we investigated whether Hh signaling-activated EMT in bladder cancer cells and compared activation in non-muscle invasive and muscle-invasive cancer to evaluate dependence on Hh signaling. We also investigated the feasibility of Shh signaling inhibition in vitro and in vivo.

Materials and methods

Cells and reagents

Three human bladder cell carcinoma cell lines, KK47 which were derived from non-muscle invasive one, 5637 and T24 which was derived from muscle-invasive ones, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin at 37 °C and 5% CO_2. The Hh signaling inhibitor cyclopamine (LKT Laboratories, St. Paul, MN) was dissolved and diluted with dimethyl sulfoxide (DMSO). Recombinant-human Shh protein (r-Shh: R&D Systems, Minneapolis, MN) was dissolved and diluted by PBS.

Cell proliferation assay

Five hundred KK47, 5637 and T24 cells were seeded for 24 h, then divided into 4 groups and switched to media containing 1 μg/ml r-Shh, 50 μM cyclopamine, both 1 μg/ml r-Shh and 50 μM cyclopamine, or DMSO, respectively. The concentration of r-Shh was based on previous studies (Behnsawy et al. 2013; Yamamichi et al. 2014; Bermudez et al. 2013). After incubation for 0, 48, 72 and 96 h, cell proliferation was investigated by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. All experiments were carried out in triplicate.

Western blotting

Twenty thousand cancer cells were incubated for 24 h, then cells were treated as described above and incubated for an additional 48 h. Cells were washed and lysed in 8 M urea buffer containing 0.1% dithiothreitol. Each sample was added into sample buffer (Nacalai Tesque, Kyoto, Japan) and heated at 95 °C for 5 min. The samples were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with Blocking One (Nacalai Tesque) followed by washing, the membranes were incubated for 1 h at room temperature (RT) with anti-E-cadherin (Abcam, Cambridge, UK), anti-N-cadherin (Santa Cruz Biotechnology, Dallas, TX), anti-vimentin (Santa Cruz Biotechnology), anti-Gli1 (Santa Cruz Biotechnology) or anti-β-actin (Santa Cruz Biotechnology). After another washing, membranes were incubated for 1 h with HRP-conjugated secondary antibodies. Antibody binding to proteins was detected by enhanced chemiluminescence.

Cell immunofluorescence staining

One thousand cancer cells were incubated for 24 h and then treated as described above and incubated an additional 72 h. These cells were fixed by 4% paraformaldehyde for 20 min. After washing, cells were treated with 0.5% Triton-X for 5 min. Cells were blocked with Blocking One Histo (Nacalai Tesque) for 30 min, then incubated with anti-E-cadherin, anti-N-cadherin or anti-vimentin for 1 h. Cells were washed and incubated with Alexa Fluor 488-conjugated secondary antibodies (Life Technologies, Carlsbad, CA) and DAPI at RT for 1 h. After washing, the cells were mounted and viewed under a fluorescent microscope.

Wound healing assay

Wound healing assays were done to investigate the correlation of Shh signaling with invasion and migration in bladder cancer cells as previously described (Islam et al. 2016). 1×10^5 cells were seeded and incubated overnight and then cells with r-Shh for 72 h. To inhibit Hh signaling, cells were treated with cyclopamine or DMSO for 48 h. Cell monolayers were scratched in each well. Then cells were washed and fresh medium was added and incubated for 10 h. Microscopic images (40×) were taken at time points 0 and 10 h.

Transwell invasion assay

Fifty thousand cells were seeded into the upper chambers of Transwell chamber inserts (24-well plate, 8 µm pores, Corning, Corning, NY) and incubated overnight. Then cells were treated with 50 µM cyclopamine with or without r-Shh in serum-free medium for 24 h for the invasion assay. The lower chamber contained standard complete medium with 10% FBS. After incubation, cells inside the upper inserts were wiped off using cotton swabs. Then the invading cells were stained with a Differential Quick Stain Kit (Polysciences, Inc., Warrington, PA) and observed by microscope at 400×.

Animal experiments

Balb/c nu/nu mice 6–8 weeks of age were purchased from CLEA Japan (Tokyo, Japan). 1×10^6 KK47, 5637, or T24 cells were inoculated at day 0 ($n = 10$, respectively). Tumor volume was expressed by the following formula: (longest diameter) × (shortest diameter)^2 × 0.5. After tumor formation, every 10 mice with tumors (KK47, 5637, or T24) were randomly assigned to treatment groups and control groups. 100 mg/kg cyclopamine was injected intraperitoneally with 0.1 ml vehicle (triolein:ethanol, 4:1) for 5 days to 3 weeks in the treatment group (Thayer et al. 2003). After treatment, mice were sacrificed and tumors were collected. Tumors were fixed and embedded with paraffin. All aspects of the experimental design and procedure were reviewed and approved by the institutional ethics and animal welfare committees of Kobe University.

Immunohistochemical staining

Paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in citrate buffer (pH 6.0) at 120 °C for 5 min. Immunohistochemical staining was performed in an automatic tissue processor (Bond-Max, Leica Microsystems, Wetzlar, Germany) following the standard protocol. Briefly, tissue sections were incubated for 60 min with the following primary antibodies: anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-Shh, anti-Ptch1, anti-Smo and anti-Gli1. After washing, sections were exposed to dextran polymer backbone-conjugated secondary antibodies with HRP for 12 min, according to the instrument's standard protocols. Tissue sections were incubated with diaminobenzidine for 10 min and counterstained with hematoxylin. The resulting tissue slides were observed under a microscope.

Immunohistochemical analysis

Immunohistochemical staining was scored by the percentage of positive cells. The staining intensity was scored as 0 (negative), 1+, (weak), 2+ (medium) or 3+ (strong). The percentage of stained cells was categorized as: 1, 0–10%; 2, 11–50%; and 3, more than 50% stained cells. The total IHC score was determined by multiplying the frequency and intensity scores (Yamamichi et al. 2014). Immunohistochemical figures are presented as normal-power (200×) images.

GSE31684 database analysis of Shh signaling pathway gene expression and EMT markers in clinical bladder cancer

To examine the correlation between the Shh signaling pathway and EMT in clinical bladder cancer samples, we analyzed the gene expression of Shh, Ptch1, Smo, Gli1, E-cadherin, N-cadherin and vimentin using the GSE31684 (Riester et al. 2012) RNA expression GEO data sets. We used the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0) with 93 bladder cancer samples ($n = 5$ in pTa, $n = 10$ in pT1, $n = 17$ in pT2, $n = 42$ in pT3 and $n = 19$ in pT4). The raw data were processed using the GEO2R website (https://www.ncbi.nlm.nih.gov/geo/geo2r/), and the correlation between gene expression values and pT stages was analyzed.

Statistical analysis

Comparisons between multiple groups were performed using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer method. The correlations of protein expression in immunohistochemical analyses data and GSE31684 data sets were analyzed by the Spearman rank-order correlation. Statistical differences among mean values were considered significant when $p < 0.05$.

Results

Cell proliferation assay

We determined the cell proliferation in an Hh-dependent manner. After 48 h of culture, cyclopamine significantly inhibited KK47, 5637 and T24 cell growth compared with other treatments ($p < 0.05$) (Fig. 1). In addition, we found that r-Shh signaling significantly compensated for the cyclopamine-induced cell growth inhibition of non-invasive KK47 bladder cancer cells ($p < 0.05$) (Fig. 1). These results indicated that cyclopamine inhibited cell proliferation by blocking Shh signaling at various grades of invasiveness in bladder cancer cell lines.

Detection of EMT markers and Shh downstream Gli1 in bladder cancer cell lines

R-Shh decreased the expression of E-cadherin in all three cell lines, while cyclopamine recovered those inhibitory effects (Fig. 2). In contrast, r-Shh enhanced the expression of N-cadherin and vimentin in all cell lines while cyclopamine suppressed those expressions. Gli1 was also enhanced by r-Shh, while inhibited by cyclopamine. In particular, more intensive Gli1 inhibition by cyclopamine was seen in 5637 and T24 cells compared to KK47.

Immunofluorescence cell staining for EMT detection

R-Shh inhibited the expression of E-cadherin in all three cell lines, while cyclopamine enhanced it (Fig. 3a). Regarding mesenchymal markers, the expressions of N-cadherin (Fig. 3b) and vimentin (Fig. 3c) were enhanced by r-Shh but suppressed by cyclopamine in all cell lines tested. These results indicated that Shh signaling excitation promoted EMT in bladder cancer cells, and blocking Shh signaling could inhibit EMT.

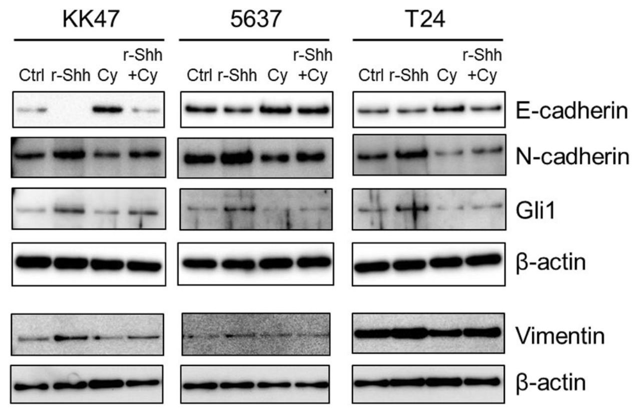

Fig. 2 Western blotting. E-cadherin, N-cadherin, vimentin and Gli1 expression were investigated in the presence of recombinant-sonic hedgehog (r-Shh) protein with or without cyclopamine (Cy) in the KK47, 5637 and T24 cell lines. Cy enhanced activation of E-cadherin. R-Shh activated the expression of N-cadherin, vimentin and Gli1, and the enhanced expressions were decreased by Cy. Vimentin was detected on a separate membrane from other markers, so β-actin is shown

Wound healing assay and transwell assay for migration and invasion

We investigated the correlation of Shh signaling with invasion and migration in bladder cancer cells (Islam et al. 2016). Wound healing assays showed that r-Shh enhanced wound closure in 5637 and T24 cells, but cyclopamine inhibited the wound closure after 10 h cultures compared with all r-Shh-stimulated changes (Fig. 4a). Transwell assays showed

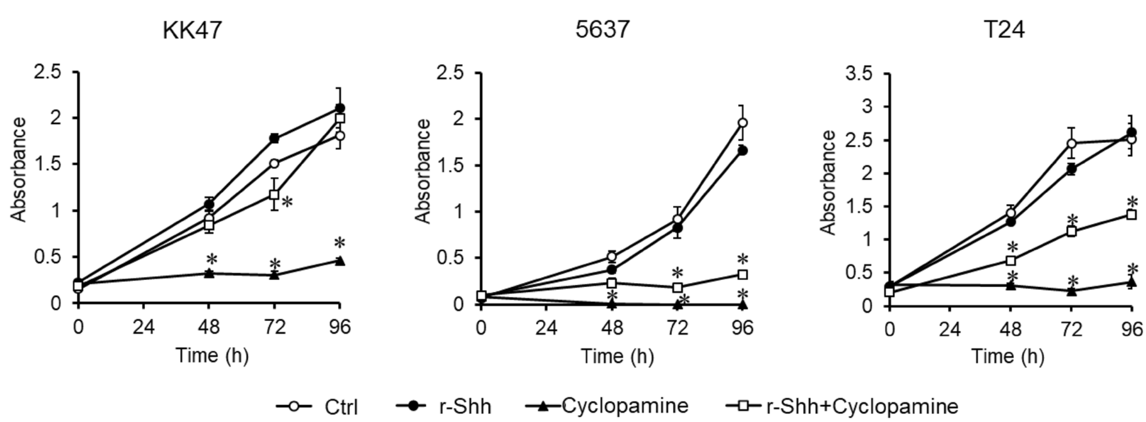

Fig. 1 XTT assay. In vitro cell proliferation assays in KK47, 5637 and T24 cell lines treated with DMSO (vehicle control), 1 µg/ml recombinant-sonic hedgehog protein (r-Shh), 50 µM cyclopamine (Cy) and a combination of 50 µM Cy and 1 µg/ml r-Shh are shown. Cy significantly inhibited cell proliferation in KK47, 5637 and T24 after 48 h treatment ($p < 0.05$). r-Shh treatment significantly restored the inhibitory effect in those three cell lines after 72 h ($p < 0.05$)

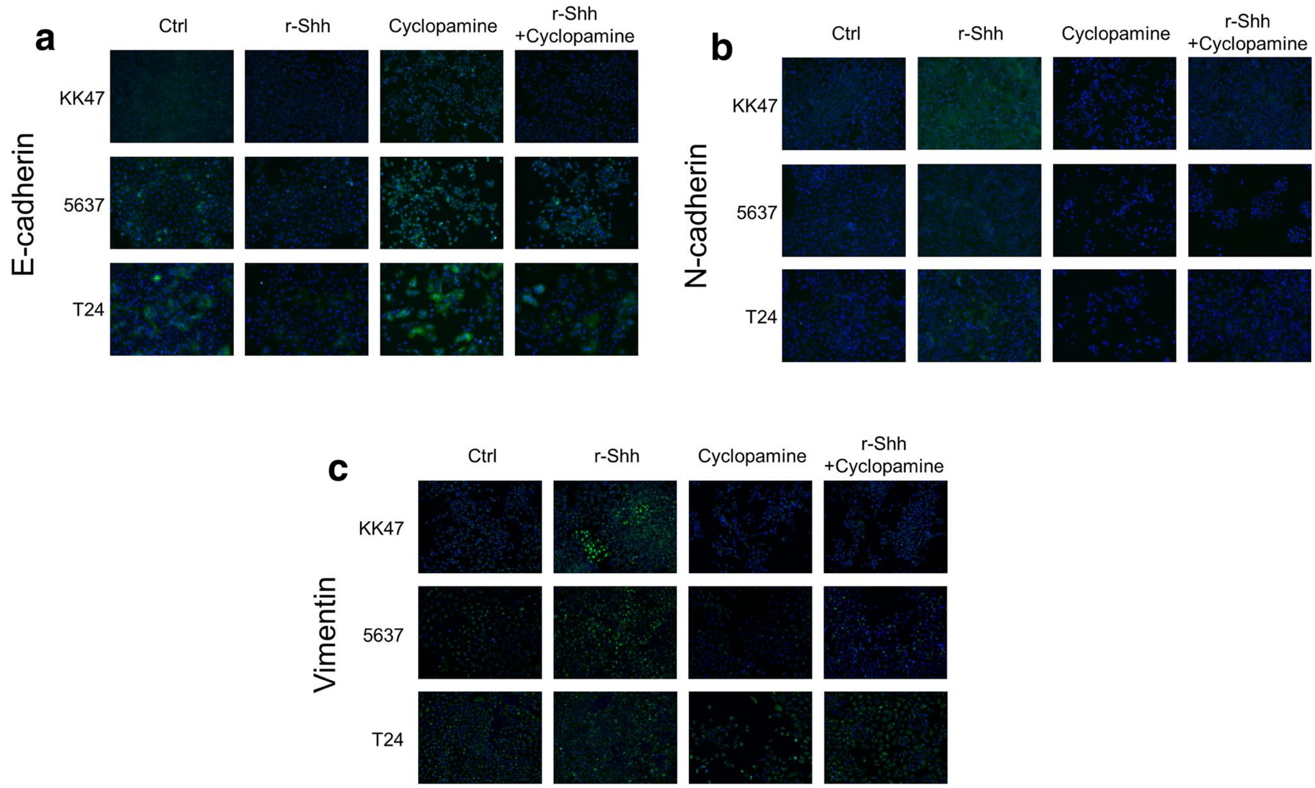

Fig. 3 Immunofluorescence cell staining. The expressions of E-cadherin (**a**), N-cadherin (**b**) and vimentin (**c**) were investigated in the presence of recombinant-sonic hedgehog (r-Shh) protein with or without cyclopamine (Cy) in KK47, 5637 and T24 cell lines (200x). Nuclei were stained with DAPI. E-cadherin, N-cadherin, and vimentin were stained with Alexa Fluor 488-conjugated antibodies. R-Shh induced the expression of N-cadherin and vimentin and the enhanced expression was inhibited by Cy. The expression of E-cadherin was enhanced by Cy in all cell lines

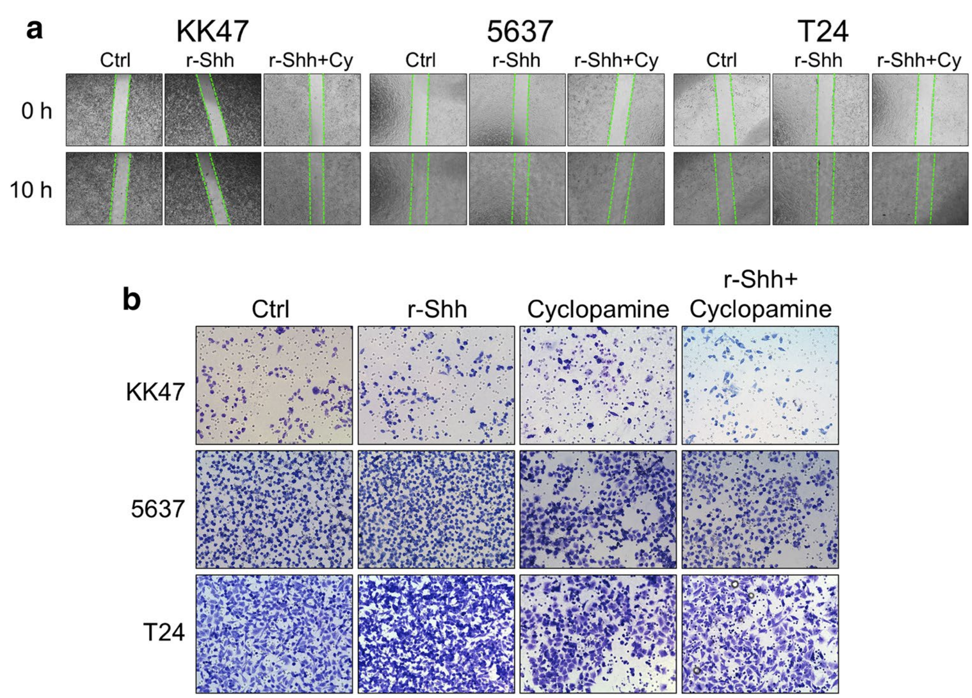

Fig. 4 Wound healing assay and transwell assay. Migration ability was investigated in the presence of recombinant-sonic hedgehog (r-Shh) protein with or without cyclopamine (Cy) in KK47, 5637 and T24 cell lines (×40) up to 10 h culture (**a**). R-Shh promoted migration in 5637 and T24, while Cy inhibited wound closure in 5637 and T24. Green dotted lines indicate the scratch prior to incubation. In the transwell invasion assay, r-Shh treatment promoted invasion in 5637 and T24, and cyclopamine treatment inhibited 5637 and T24 invasion but had a lesser effect in KK47 (**b**) (×400)

that 5637 and T24 were inherently invasive and cyclopamine inhibited their invasiveness (Fig. 4b). KK47 showed only slight invasiveness and cyclopamine did not affect KK47 invasion in the assay.

Mice tumor growth inhibition by cyclopamine

We examined the in vivo effect of cyclopamine in mice. After 3 weeks of treatment, cyclopamine significantly inhibited 5637 ($p = 0.01$) and T24 ($p = 0.004$) mouse tumor growth compared with control, but not KK47 ($p = 0.298$) (Fig. 5). There were no obvious signs of treatment-induced side effects such as severe body weight loss or lethargy.

Immunohistochemical analysis of mouse tumor samples

To examine the in vivo inhibition of EMT by cyclopamine, mouse tumors were extracted and investigated. The expression of E-cadherin was significantly increased by cyclopamine compared with control mice for all tumors ($p < 0.05$) (Fig. 6a, c). As seen in Fig. 6a, d, N-cadherin expression decreased in all tumors, but statistical significance was seen only in T24, the highest-grade tumor, compared with control ($p < 0.05$). In addition, cyclopamine significantly suppressed the expression of vimentin in all tumors compared with control (Fig. 6a, e). Regarding Shh signaling components, major downstream components such as Shh, Ptch1 and Gli1 were inherently overexpressed in mice tumors (Fig. 6b, f–i). Cyclopamine significantly decreased the expression of Shh in T24 ($p = 0.049$), but not in other cell lines (Fig. 6b, f). Cyclopamine did not affect Ptch1 expression (Fig. 6b, g). Cyclopamine significantly decreased the expression of Smo in KK47 ($p = 0.047$) and 5637 ($p = 0.002$) compared to controls, but only slightly decreased Smo in T24 ($p = 0.08$) (Fig. 6b, h). The expression of Gli1 was suppressed by cyclopamine in 5637 and T24 significantly ($p = 0.03$, respectively) compared with controls (Fig. 6b, i).

Analysis of gene expression using the GSE31684 database

We examined the correlation between the Shh signaling pathway and EMT marker gene expression values using the GSE31684 database (Fig. 7). We found significantly increased expression of Smo in higher pT stages ($p = 0.011$). The gene expression of Gli1 and Ptch1 slightly increased in pT3 and pT4 samples, but the correlations with pT stages were not significant ($p = 0.988$ and $p = 0.092$, respectively). The gene expression of Shh showed no change by pT stage ($p = 0.736$). Regarding EMT markers, the gene expressions of N-cadherin and vimentin significantly increased in higher pT stages ($p = 0.034$ and $p = 0.004$, respectively). The gene expression of E-cadherin slightly decreased in higher pT stages, but the correlation was not significant ($p = 0.050$).

Discussion

The Hh signaling pathway activates the genes involved in cell proliferation, differentiation, and cell motility in urological cancers (Chen et al. 2009). Our study showed a difference in Hh signaling dependency between non-muscle invasive and muscle-invasive bladder cancer cell lines in vitro and in vivo. Muscle-invasive cancer depended on Hh signaling more than non-muscle invasive cancer. O'Brien et al. (1995) showed that tumor angiogenesis was elevated in muscle-invasive cancers and facilitated more

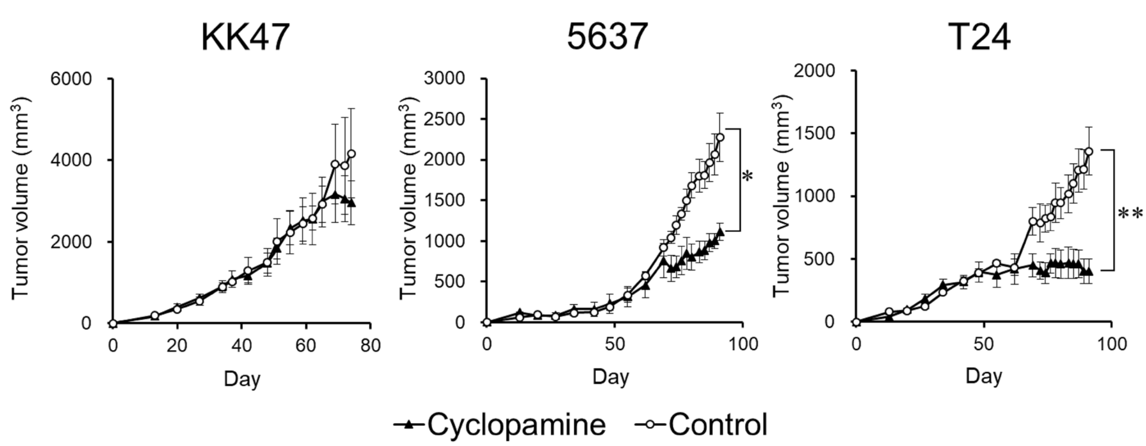

Fig. 5 In vivo tumor inhibitory effects of cyclopamine. Three bladder cancer cell lines, KK47, 5637 and T24, were subcutaneously inoculated into right flanks of Balb/c nu/nu mice. After tumor growth was confirmed, mice were treated with 100 mg/kg cyclopamine or vehicle control 5 days a week for 3 weeks. Cyclopamine significantly inhibited the tumor growth of 5637 and T24 compared with vehicle control treatments (*$p < 0.05$, **$p < 0.01$)

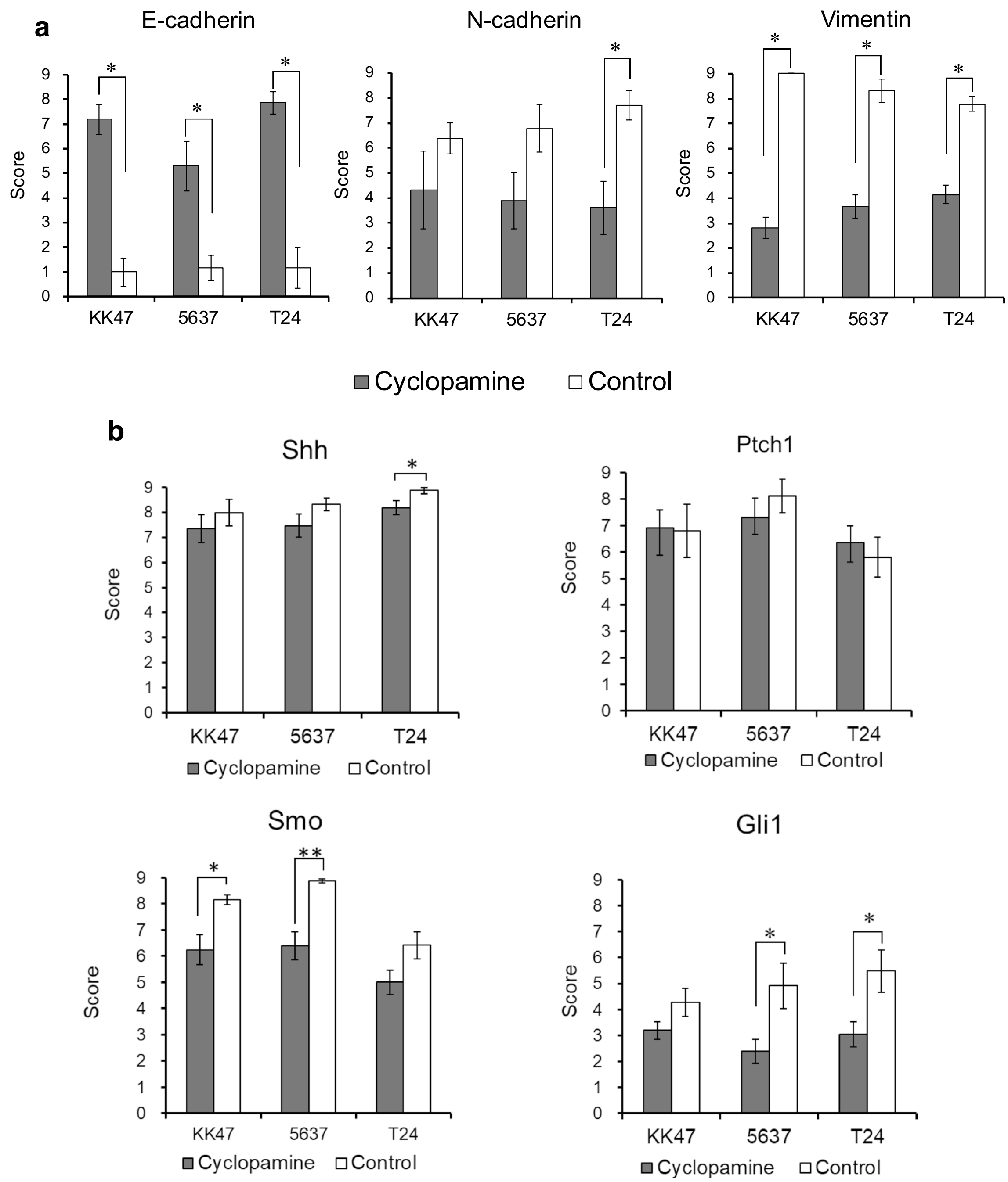

Fig. 6 Immunohistochemical analysis of KK47, 5637 and T24 mouse tumors for EMT markers and Shh signaling. Marker expression was evaluated by staining score (0–9) (**a**). E-cadherin was significantly expressed by cyclopamine compared with vehicle control, while N-cadherin and vimentin were inhibited by cyclopamine ($p < 0.05$). Shh signaling pathway components Gli1 and Smo were significantly suppressed by cyclopamine compared with vehicle control, and Ptch1 was not ($*p < 0.05$, $**p < 0.01$) (**b**). The expression of Shh was significantly decreased by cyclopamine in T24 ($*p < 0.05$) and slightly decreased in KK47 and 5637. Representative staining for each marker is shown in **c** (E-cadherin), **d** (N-cadherin), **e** (vimentin), **f** (Shh), **g** (Ptch1), **h** (Smo) and **i** (Gli1)

tumor growth and progression compared to non-invasive cancers. Our findings supported those of previous studies and suggested that elevated tumor growth was correlated with Hh signaling activation.

Regarding Hh signaling and bladder cancer, Fei et al. (2012) demonstrated that activated Shh signaling occurs in bladder cancer cells in an autocrine/paracrine manner. Our in vitro data suggested that Shh signaling activated EMT

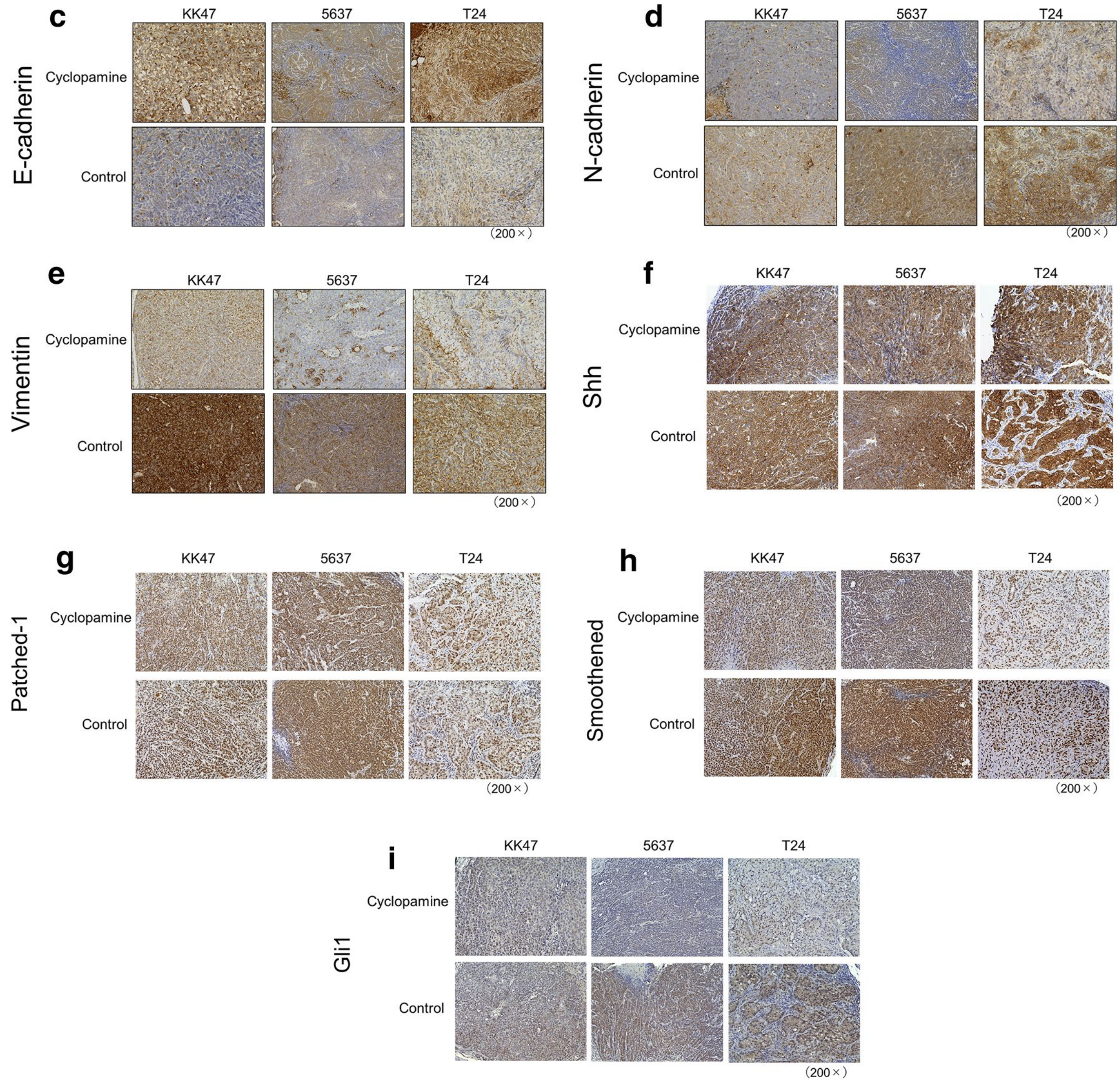

Fig. 6 (continued)

as well as cell growth in those cells especially in muscle-invasive ones. We found some inversion of E-cadherin expression level in non-muscle invasive KK47 and other muscle-invasive cell lines, consistent with the reports of Imao et al. (1999) and Adhim et al. (2011). We showed that the expression of Gli1, a downstream transcriptional factor of Shh signaling (Southgate et al. 2016), was enhanced by r-Shh, while cyclopamine, an Shh signaling inhibitor, especially suppressed muscle-invasive cell lines. In addition, we showed that cyclopamine significantly inhibited the expression of Shh more in T24 (an invasive bladder cancer cell line) than in KK47 (a non-invasive bladder cancer cell line) and 5637 (invasive bladder cancer cell line) and decreased the expression of Smo, a target protein of cyclopamine, and Gli1 in 5637 than in KK47 and T24, suggesting that invasive cancer cells are considered to be dependent on Shh signaling as mentioned previously, and correlated with EMT. Islam et al. (2016) previously found that cyclopamine inhibited the expression of Ptch1 and Gli1 in TGF-β-treated cancer cells in vitro, supporting our results in vitro and in vivo. These data suggest that cyclopamine inhibits the Shh signaling pathway via decreased expression of Smo and Gli1, while not affecting the expression of upstream Ptch1. As to the relationship between cyclopamine and Shh expression, basically Shh locates on the cell surface as an initiator of Shh signaling. It remains controversial if cyclopamine suppresses autocrine Shh, especially in invasive cancer.

EMT is one process of cancer progression, by which epithelial cells acquire mesenchymal properties such as increased motility and invasiveness (Larue and Bellacosa 2005; Kalluri and Weinberg 2009; Rhim et al. 2012). Rhim et al. showed that Shh-Gli1 signals promoted EMT by

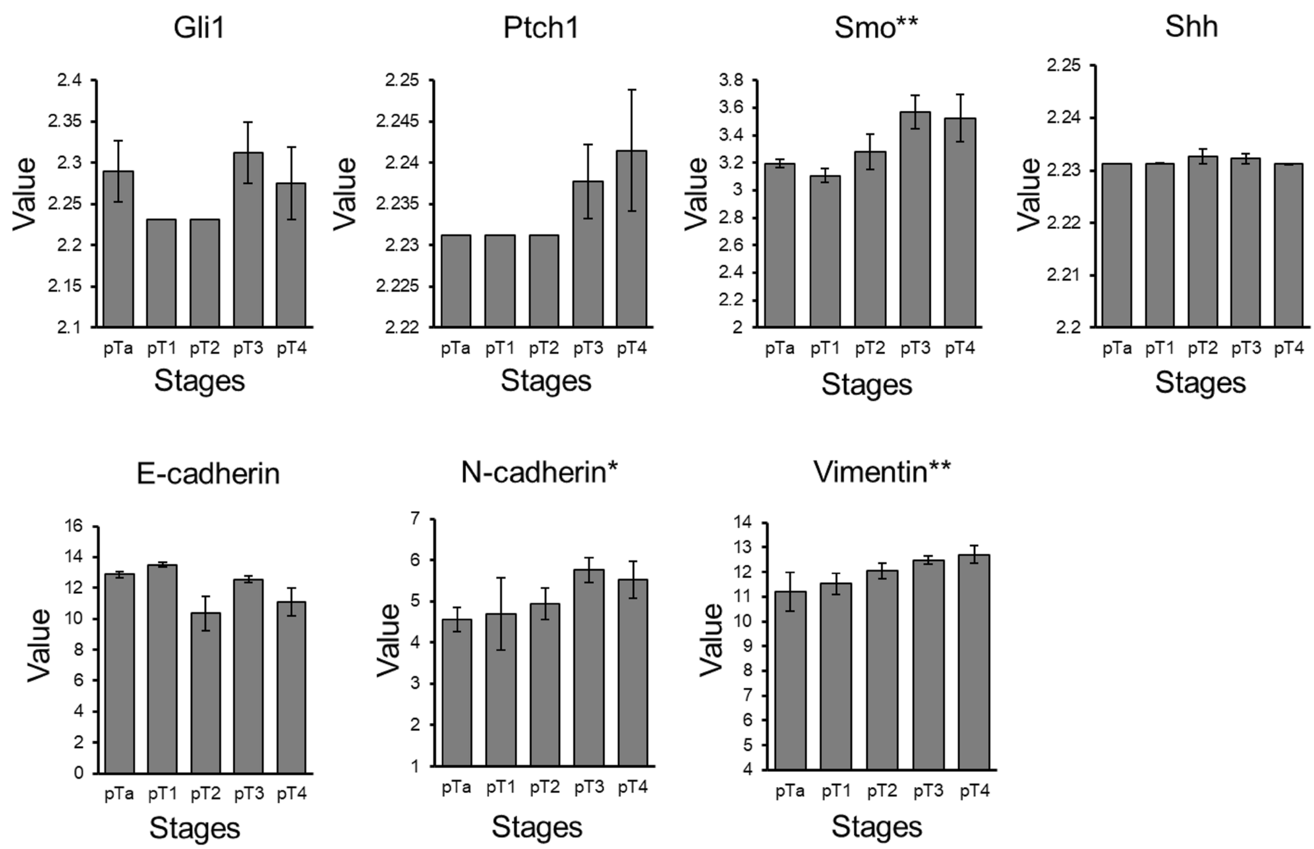

Fig. 7 Gene expression analysis of Shh signaling pathway and EMT markers in clinical samples using the GSE31684 database. Gene expression values of Shh, Ptch1, Smo, Gli1, E-cadherin, N-cadherin and vimentin in GSE31684 (Riester et al. 2012) RNA expression GEO data sets (*$p<0.05$, **$p<0.01$). Gene expression value was calculated by the GEO2R website

activating a complex signaling network in pancreatic tumors (Xu et al. 2012). In this study, we showed elevated Shh signaling by r-Shh-activated EMT in both non-invasive and invasive bladder cancer. Cyclopamine substantially blocked such activation both in vitro and in vivo. We also showed that an activated Shh pathway led to invasiveness in muscle-invasive cell lines, while cyclopamine inhibited wound closure and invasion, a phenomenon was seen more in invasive cancer cells than non-invasive ones, suggesting that invasive cancer depends on Shh signaling for dissemination, while blockade of Shh signaling restrains their invasiveness. Further study will be necessary to fully understand the role of Shh signaling in tumor invasiveness.

Recently, Zhao et al. showed that EMT was significantly correlated with T stage and pathological grade in clinical bladder cancer specimens, and concluded that grade and vimentin expressions were predictors of progression and survival (Zhao et al. 2014). We showed that cyclopamine decreased the expression of vimentin even in high-grade cancer cell lines, implying that Shh could be a target to inhibit cancer progression by suppressing EMT. Cyclopamine may have an even more profound effect in bladder cancer than prostate or renal cancer because the characteristic change to invasive cancer is the main effect on patient prognosis, similar to the findings of Syed et al. (2016).

Our animal experiments showed that cyclopamine had significant anti-tumor effects in muscle-invasive tumors, suggesting that inhibition of Shh signaling is a possible strategy to treat high-grade/invasive bladder cancer. Cyclopamine treatment significantly inhibited EMT in both non-invasive and invasive bladder tumors without any obvious side effects in mice. These results indicated that cyclopamine may promote mesenchymal to epithelial transition and inhibit cancer progression and possible invasiveness in vivo. Our analysis using the GSE31684 data set also showed that gene expression in the Shh signaling pathway and EMT may correlate with higher pT stages in clinical bladder cancer samples. These results also suggest that Shh signaling may be a target for treatment of invasive bladder cancers.

There are both contradictory and supporting studies on the correlation of Shh signaling and bladder cancer progression. Shh expression is lost or attenuated during progression to invasive urothelial carcinoma despite its initial presence in Shh-positive basal urothelial stem cells (Shin et al. 2014a, b). Hh signaling restrains bladder cancer progression and the effect was associated with stromal expression of bone morphogenetic protein signals, which stimulate urothelial differentiation. Our data were consistent with this; 5637 and T24 (invasive) were more susceptible to cyclopamine than KK47 (non-invasive), and demonstrated that non-muscle invasive cancer cells could acquire invasiveness with activated Shh signaling as mentioned above. However, Islam et al. demonstrated that TGF-β1 activates Shh signals, increasing tumorigenicity and stemness via EMT in bladder cancer cells as mentioned before (Islam et al. 2016). Islam et al. also showed that Shh expression correlated with advanced clinical stages and poor prognostic bladder cancer. Pignot et al. reported that constitutive activation of the Hh pathway was observed in most non-invasive bladder cancer and about 50% of muscle-invasive cancers (Pignot et al. 2012). The Hh signaling pathway of Gli1 especially correlated with EMT, tumor invasion and metastasis in bladder cancer (Pignot et al. 2012; Zhang et al. 2016; Singh et al. 2017). In our study, cyclopamine inhibited Gli1 expression and EMT especially in muscle-invasive bladder tumors in mice. Our results supported the relationship of Shh signaling to EMT during cancer progression. Taken together, our results fall into a middle position between these two contradictory hypotheses about Shh signaling and bladder cancer progression. Further studies should be performed to reveal definitive conclusions.

We would like to emphasize the study limitations. First, we only used three cancer cell lines, and therefore have only shown a possibility that the Shh signaling pathway is more significant in muscle-invasive cancer cells. Second, the difference in the effect of cyclopamine on EMT marker expression and Gli1 in muscle-invasive and non-muscle invasive cancer cells is subtle. Third, though we demonstrated a possible correlation between the Shh signaling pathway and bladder cancer progression, we did not show evidence of the canonical Shh signaling pathway in bladder cancer. Fourth, we have not fully analyzed Shh signaling in bladder cancer cells from the aspect of molecular mechanisms, especially the genetic level, though we analyzed the protein level. Fifth, an animal experiment using metastatic bladder tumor is needed to determine the correlation of Shh-signaling and cancer metastasis. Sixth, we showed that cyclopamine might be a possible therapy for muscle-invasive bladder cancer, but it is possible that tumor cells that do not utilize Shh pathway for progression would be selected in the presence of cyclopamine, eventually leading to resistance.

In conclusion, our in vitro and in vivo data showed that Shh signaling and EMT play a role in bladder cancer progression and invasion, and that Shh signaling inhibition may be a possible therapeutic strategy, especially for targeting EMT in muscle-invasive bladder cancer progression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

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