# Pharmacological inhibition of the Hedgehog pathway prevents human rhabdomyosarcoma cell growth

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**Abstract.** The Hedgehog pathway functions as an organizer in embryonic development. Recent studies have shown that mutation of the *PTCH1* gene involved in the Hedgehog pathway affects rhabdomyosarcoma development. However, the expression of Hedgehog pathway molecules in human rhabdomyosarcoma cells has not been well clarified. In addition, the effect of pharmacological inhibition of the Hedgehog pathway is not known. We investigated the expression of the genes involved in the Hedgehog pathway using human rhabdomyosarcoma cell lines and biopsy specimens. Further, we evaluated the effect of pharmacological inhibition of the Hedgehog pathway using cyclopamine or GANT61 by WST assay, cell proliferation assay and cell death detection assay. Real-time PCR revealed that human rhabdomyosarcoma cell lines and biopsy specimens overexpressed the following genes: *Sonic hedgehog, Indian hedgehog, Desert hedgehog, PTCH1, SMO, GLI1, GLI2* and *ULK3*. Immunohistochemistry revealed that rhabdomyosarcoma cell lines and biopsy specimens expressed SMO and GLI2. Inhibition of SMO by cyclopamine slowed the growth of human rhabdomyosarcoma cell lines. Similarly, inhibition of GLI by GANT61 slowed the growth of human rhabdomyosarcoma cell lines. Inhibition of cell proliferation and apoptotic cell death together prevented the growth of rhabdomyosarcoma cells by cyclopamine and GANT61 treatment. Our findings suggest that pharmacological inhibition of the Hedgehog pathway may be a useful approach for treating rhabdomyosarcoma patients.

## Introduction

Rhabdomyosarcomas are the most common soft tissue sarcomas in children. In approximately 20% of patients, rhabdomyosarcoma presents as a disseminated disease at the time of diagnosis. Patients with metastatic disease continue to have a very poor prognosis. Further, local recurrences are common in patients with advanced local disease.

Hedgehog signaling acts through several components, including the transmembrane proteins patched 1 (PTCH1) and Smoothened homolog (*Drosophila*) (SMO), which activate the GLI zinc-finger transcription factors (1,2). Transcriptional activation of the Hedgehog target genes in mammals occurs through the actions of 3 final regulators, namely, GLI1, GLI2, and GLI3 (3,4). The nevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin syndrome or the basal cell nevus syndrome, is an autosomal dominant disorder that predisposes patients to both cancer and developmental defects (5). The estimated prevalence of NBCCS is 1 per 56,000 individuals. The incidence rate of medulloblastomas is 1-2% and basal cell carcinomas is 0.5% in NBCCS patients (6,7). Further, NBCCS patients are at a high risk for ovarian fibromas, meningiomas, fibrosarcomas, ovarian dermoids, cardiac fibromas, and rhabdomyosarcomas, in addition to basal cell carcinomas and medulloblastomas (5,7,8). *PTCH1* mutation promotes NBCCS (9). In addition, mutant mouse models showed that mice heterozygous for *ptch1* develop many of the features characteristic of Gorlin syndrome and rhabdomyosarcoma (10). Although *PTCH1* mutation affects rhabdomyosarcoma development, the expression of Hedgehog pathway molecules in human rhabdomyosarcoma has not been well clarified. In addition, the effect of pharmacological inhibitors on Hedgehog pathway is not clarified. To investigate the involvement of Hedgehog pathway in the pathogenesis of human rhabdomyosarcoma, we examined the expression of the Hedgehog pathway genes in rhabdomyosarcoma and the effect of SMO or GLI inhibitors on the growth of rhabdomyosarcoma cells (11,12).

## Materials and methods

*Cell culture*. The human rhabdomyosarcoma cell lines KYM-1 and RD cells were purchased from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). The human rhabdomyosarcoma cell line RMS-YM cell line was purchased from Riken Bioresource Center (Tsukuba, Japan). The human rhabdomyosarcoma cell line A204 was purchased by American Type

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*Key words:* rhabdomyosarcoma, Hedgehog, cyclopamine, GANT61

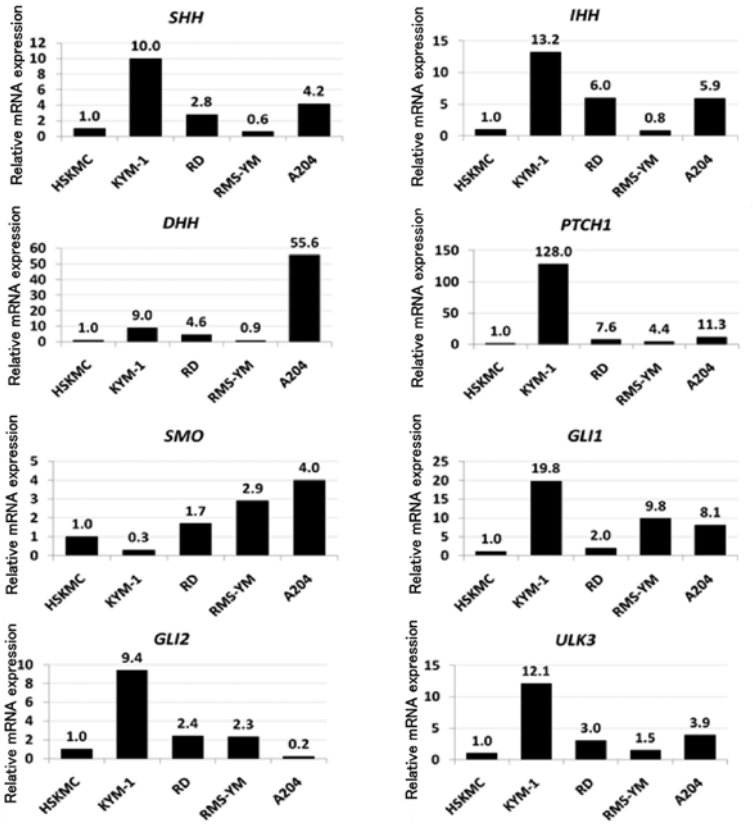


Figure 1. Expression of Hedgehog pathway molecules in human rhabdomyosarcoma cell lines. The total RNA extracted from 4 rhabdomyosarcoma cell lines was used for real-time PCR. The real-time PCR results suggested that 3 human rhabdomyosarcoma cell lines showed increased *SHH* expression from 2.8- to 10.0-fold; 3 rhabdomyosarcoma cell lines showed increased *IHH* expression from 5.9- to 13.2-fold; and 3 rhabdomyosarcoma cell lines showed increased *DHH* expression from 4.6- to 55.6-fold. *PTCH1* was up-regulated from 4.4- to 128.0-fold; *GLI1* was up-regulated from 2.0- to 19.8-fold; and *ULK3* was up-regulated from 1.5- to 12.1-fold in 4 human rhabdomyosarcoma cell lines. *SMO* was up-regulated from 1.7- to 4.0-fold, and *GLI2* was up-regulated from 2.3- to 9.4-fold in 3 human rhabdomyosarcoma cell lines.

Culture Collection (Manassas, VA, USA). HSKMC normal myoblast cell was purchased from Toyobo (Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml).

*Patient specimens*. Human rhabdomyosarcoma biopsy specimens were collected from primary lesions before any diagnostic or therapeutic treatment. Control muscle tissues were collected undergoing operation of scoliosis. The study protocol was approved by the institutional Review Board of Kagoshima University. All patients and controls gave written informed consent.

*WST assay*. Cells were incubated with substrate for WST-1 (Roche, Basel, Switzerland) for 4 h, and washed with PBS and lysed to release formazan from cells. Then cells were analyzed in a Safire microplate reader (Bio-Rad, Hercules, CA, USA). Cyclopamine and GANT61 were purchased from Enzo Life Sciences (Philadelphia, PA, USA). Tomatidine was purchased from EMD4 Biosciences (Darmstadt, Germany).

*Real-time PCR*. Each used primer set amplified a 150-to 200-bp amplicon. Reactions were run using SYBR Green (Bio-Rad) on a MiniOpticon™ machine (Bio-Rad). The comparative Ct ($\Delta\Delta$Ct) method was used to evaluate the fold change of mRNA expression using ACTB as reference. All PCR reactions were performed in triplicate, with 3 different concentrations of cDNA. All primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). The following primers were used; *Sonic hedgehog*: 5'-ACCGAGGG CTGGGACGAAGA-3', 5'-ATTTGGCCGCCACCGAGTT-3'; *Desert hedgehog*: 5'-TGATGACCGAGCGTTGTAAG-3', 5'-GCCAGCAACCCATACTTGTT-3'; *Indian hedgehog*: 5'-AC TTCTGCCTGGTCCTGTTG-3', 5'-AGCGATCTTGCCTT CATAGC-3'; *PTCH1*: 5'-TAACGCTGCAACAACTCAGG-3', 5'-GAAGGCTGTGACATTGCTGA-3'; *SMO*: 5'-GGGAGGC TACTTCCTCATCC-3', 5'-GGCAGCTGAAGGTAATGA GC-3'; *GLI1*: 5'-GTGCAAGTCAAGCCAGAACA-3', 5'-ATAG GGGCCTGACTGGAGAT-3', *GLI2*: 5'-CGAC ACCAGGAAG GAAGGTA-3', 5'-AGAACGGAGGTAGTGCTCCA-3'; *ULK3*: 5'-CCACAGAACCCACCAGTCTT-3', 5'-GTGGGAGAGATG AGGACCAA-3'; *ACTB*:5'-AGAAAATCTGGCACCACACC-3', 5'-AGAGGCGTACAGGGATAGCA-3'.

*Immunohistochemistry*. The following primary antibodies were used; anti-SMO (diluted 1:100, Abcam, Cambridge, UK), anti-GLI2 (diluted 1:50, Abcam). The following secondary antibodies were used: rhodamine-conjugated donkey anti-rabbit IgG antibody (diluted 1:200; Chemicon, Temecula, CA). The cells were counterstained with Hoechst 33258 (Molecular Probes, Carlsbad, CA, USA). Immunohistochemistry with

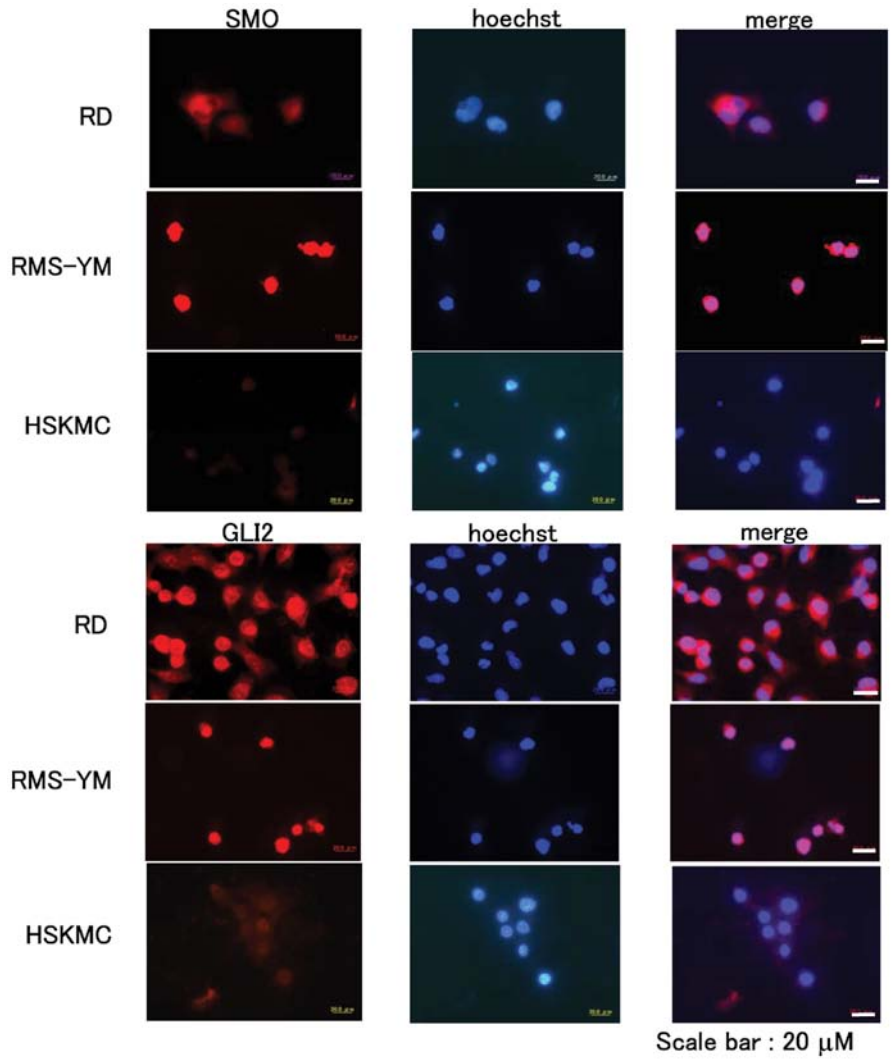


Figure 2. Expression of *SMO* and *GLI2* in human rhabdomyosarcoma cell lines. Immunohistochemistry revealed that *SMO* was expressed in the cytoplasm of RD and RMS-YM human rhabdomyosarcoma cells. GLI2 was localized in the nucleus of RD and RMS-YM human rhabdomyosarcoma cells. The experiment was performed in triplicate with similar results.

each second antibody alone without primary antibody was performed as a control. Sections were examined by confocal fluorescence microscopy: LSM 700 (Carl Zeiss, Göttingen, Germany).

*BrdU cell proliferation assay*. 5-bromo-2'-deoxyuridine (BrdU)-based cell proliferation ELISA kit (Roche Diagnostics) was used for the measurement of cell proliferation according to the manufacturer's protocol. Briefly, 48 h from the start of cultivation, BrdU labeling reagent was performed. BrdU-labeled DNA was stained with peroxidase-conjugated anti-BrdU antibody. Absorbance was measured using a microplate reader (450 nM).

*Cell death detection assay*. Cell Death Detection ELISA$^{PLUS}$ (Roche Diagnostics) was used for the measurement of apoptotic cell death according to the manufacturer's protocol. Briefly, pharmacological inhibitors were added to each well. The plates were then incubated for 48 h at 37˚C. Histone-complexed DNA fragments was stained with anti-histone and anti-DNA antibodies. Absorbance was measured using a microplate reader (450 nM).

*Statistical analysis*. All experiments were performed 3 times, except where otherwise stated, and samples were analyzed in triplicate. For real-time PCR experiments, each sample was tested at 3 different cDNA concentrations. Results are presented as mean (SD). The statistical difference between groups was assessed by applying Student's t-test for unpaired data, using Microsoft Office Excel (Microsoft, Albuquerque, New Mexico, USA) and Statistica (StatSoft, Tulsa, OK, USA).

## Results

*Up-regulation of Hedgehog pathway molecules in human rhabdomyosarcoma cell lines*. To examine the role of Hedgehog pathway in human rhabdomyosarcoma, we analyzed the expression of Hedgehog pathway genes in 4 human rhabdomyosarcoma cell lines. Real-time PCR revealed that *Sonic hedgehog (SHH)* was increased from 2.8- to 10.0-fold in 3 human rhabdomyosarcoma cell lines; *Indian hedgehog (IHH)* expression was increased from 5.9- to 13.2-fold in 3 human rhabdomyosarcoma cell lines; and *Desert hedgehog (DHH)* expression was increased from 4.6- to 55.6-fold in human rhabdomyosarcoma cell lines (Fig. 1).

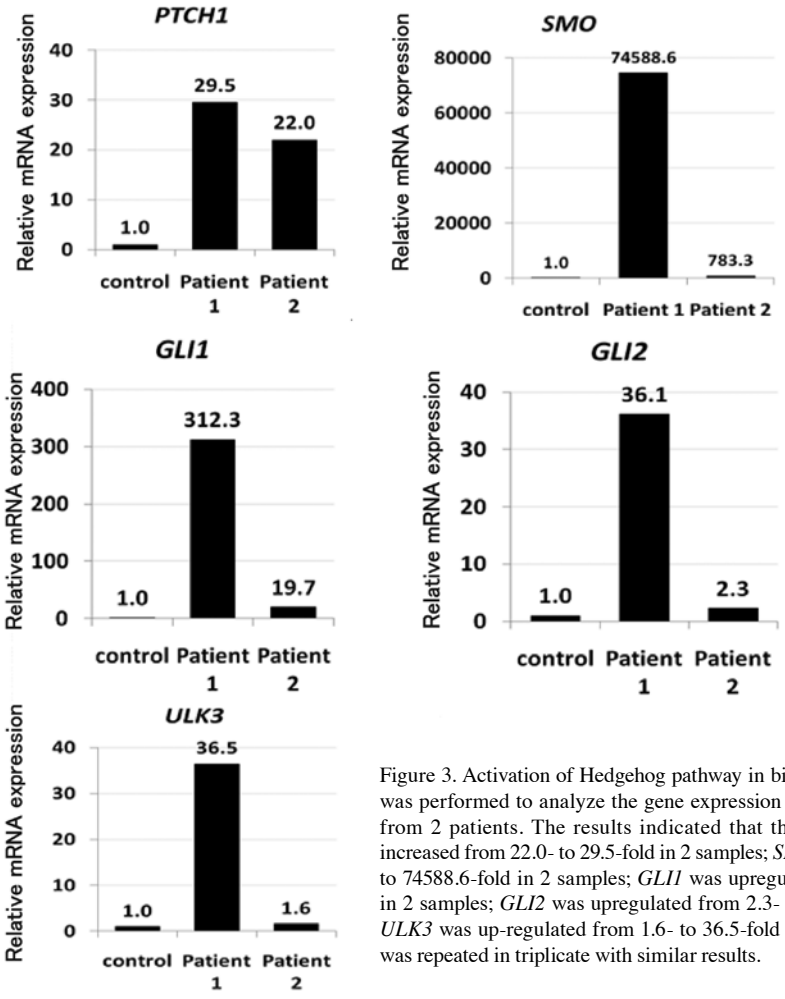


Figure 3. Activation of Hedgehog pathway in biopsy samples. Real-time PCR was performed to analyze the gene expression in 2 biopsy samples obtained from 2 patients. The results indicated that the expression of *PTCH1* was increased from 22.0- to 29.5-fold in 2 samples; *SMO* was increased from 783.3- to 74588.6-fold in 2 samples; *GLI1* was upregulated from 19.7- to 312.3-fold in 2 samples; *GLI2* was upregulated from 2.3- to 36.1-fold in 2 samples; and *ULK3* was up-regulated from 1.6- to 36.5-fold in 2 samples. The experiment was repeated in triplicate with similar results.

Further, we performed real-time PCR for analyzing Hedgehog receptors and target genes expression in 4 human rhabdomyosarcoma cell lines. *PTCH1* expression was up-regulated from 4.4- to 128.0-fold; *SMO* expression was increased from 1.7- to 4.0-fold in 3 human rhabdomyosarcoma cell lines; and *GLI1* expression was up-regulated from 2.0- to 19.8-fold in 4 human rhabdomyosarcoma cell lines. *GLI2* expression was up-regulated from 2.3 -to 9.4-fold in 3 human rhabdomyosarcoma cell lines. *ULK3* expression was up-regulated 1.5- to 12.1-fold in 4 human rhabdomyosarcoma cell lines (Fig. 1). To confirm these findings, we performed immunocytochemistry for SMO and GLI2, and found that rhabdomyosarcoma cells expressed detectable levels of SMO and GLI2 (Fig. 2). GLI2 was located in the nuclei of human rhabdomyosarcoma cells.

*Over-expression of Hedgehog pathway molecules in rhabdomyosarcoma biopsy specimens*. Next, we examined the expression of *PTCH1, SMO, GLI1, GLI2*, and *ULK3* in 2 rhabdomyosarcoma biopsy specimens. Real-time PCR revealed that both the biopsy specimens exhibited increased *PTCH1* expression from 22.0- to 29.5-fold (Fig. 3). In addition, both biopsy samples showed increased *SMO* expression from 783.3- to 74588.6-fold; increased *GLI1* expression from 19.7- to 312.3-fold; up-regulated *GLI2* expression from 2.3- to 36.1-fold (Fig. 3); and increased *ULK3* expression from 1.6- to 36.5-fold (Fig. 3). These findings confirm that Hedgehog pathway is active in human rhabdomyosarcoma cells. To confirm these findings, we performed immunocytochemistry for SMO and GLI2, and found that rhabdomyosarcoma biopsy specimens expressed detectable levels of SMO and GLI2 (Fig. 4). GLI2 was located in the nuclei of human rhabdomyosarcoma cells.

*Pharmacological inhibition of the Hedgehog pathway prevents rhabdomyosarcoma cell growth*. We determined whether Hedgehog pathway activation is required for rhabdomyosarcoma cell growth, by using cyclopamine, which is a pharmacological agent that effectively blocks SMO activation. We had previously reported that 20 $\mu$M cyclopamine effectively inhibits Hedgehog pathway and osteosarcoma growth (13). The WST assay showed that 20 $\mu$M cyclopamine slowed the growth of KYM-1 ($p<0.01$), RMS-YM ($p<0.01$), and RD ($p<0.05$) cells in a dose-dependent manner (Fig. 5A-C). On the other hand, tomatidine, a steriodal alkaloid structurally similar to cyclopamine but which does not inhibit the Hedgehog pathway, did not inhibit rhabdomyosarcoma cell growth (Fig. 5A-C). To confirm whether Hedgehog pathway activation is required for rhabdomyosarcoma cell growth, we examined the effect of GANT61, a pharmacological agent that effectively blocks GLI transcription (12). The WST assay revealed that GANT61 slowed the growth of KYM-1, RMS-YM, and RD cells in a dose-dependent manner ($p<0.01$) (Fig. 5D-F). These findings suggest that the inhibition of Hedgehog pathway prevents human rhabdomyosarcoma cell growth.

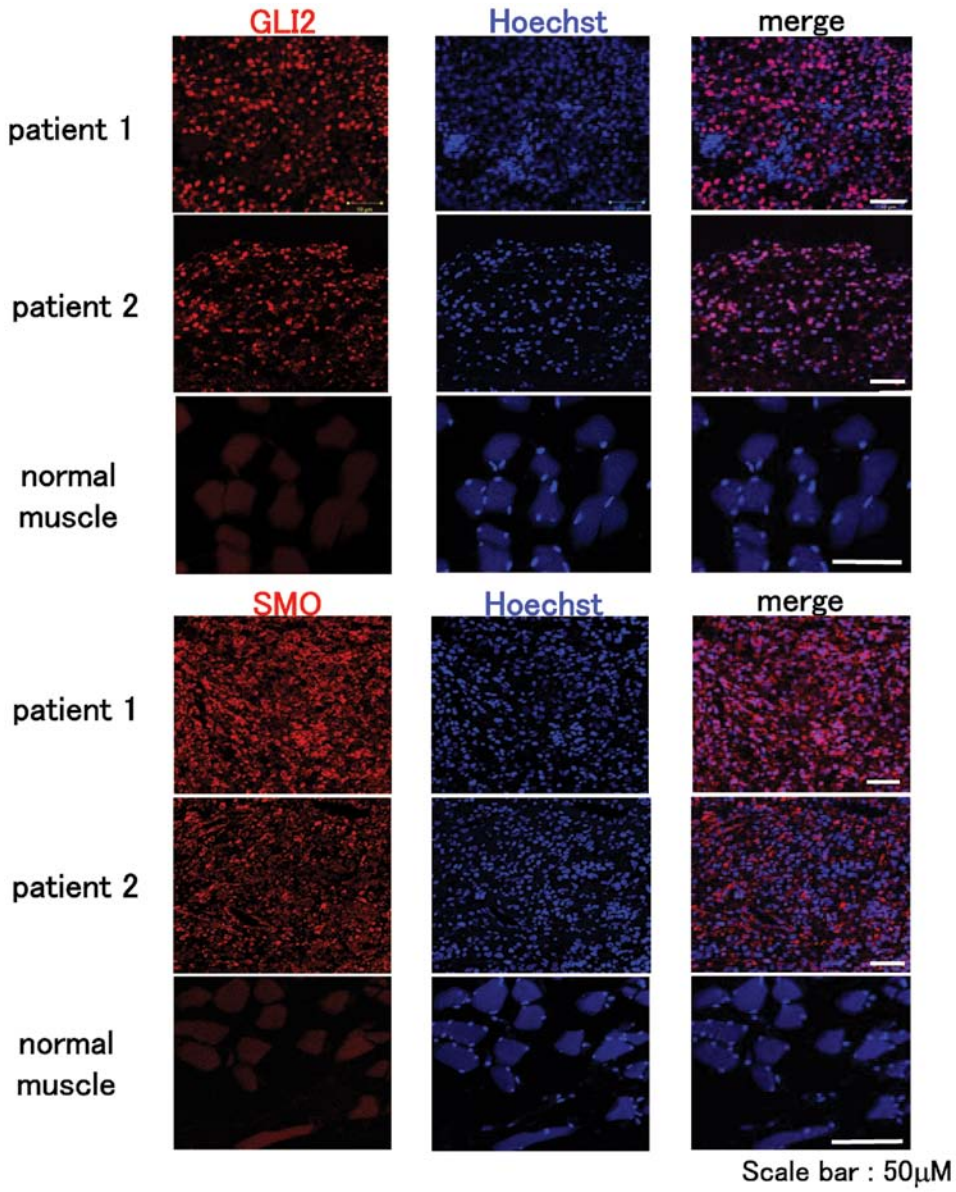


Figure 4. The expression of SMO and GLI2 in human rhabdomyosarcoma biopsy specimens. Immunohistochemical examination revealed that SMO was expressed on the cytoplasm of human rhabdomyosarcoma biopsy specimens. GLI2 was localized in the nucleus of human rhabdomyosarcoma biopsy specimens. The experiment was performed in triplicate with similar results.

*Pharmacological inhibition of the Hedgehog pathway promotes the inhibition of rhabdomyosarcoma cell proliferation and apoptosis*. Both the rate of cell proliferation and cell death reflect the results of WST assay. To determine whether pharmacological inhibition of Hedgehog pathway promotes the inhibition of rhabdomyosarcoma cell proliferation or apoptosis, we examined cell proliferation and apoptotic cell death after treatment with the pharmacological agents. BrdU cell proliferation assay revealed that the proliferation of KYM-1 and RMS-YM cells was inhibited by both cyclopamine and GANT61 ($p<0.05$) (Fig. 6A). Cell death detection assay showed that GANT61 promoted apoptosis of KYM-1 cells, while cyclopamine promoted apoptosis of RMS-YM cells ($p<0.05$) (Fig. 6B). These findings suggest that the inhibition of rhabdomyosarcoma cell growth was achieved by cooperatively inhibition of cell proliferation and apoptotic cell death.

## Discussion

The Hedgehog pathway is activated in various cancers (13-17). Further, *PTCH1* mutations play a role in the pathogenesis of rhabdomyosarcoma (10,18). However, the precise expression and function of the Hedgehog pathway genes in human rhabdomyosarcoma has not been reported. In the present study, real-time PCR revealed that *SHH, DHH, IHH, PTCH1, SMO, GLI1, GLI2*, and *ULK3* transcripts were over-expressed in rhabdomyosarcoma cell lines and biopsy specimens. Recently, it has been reported that human rhabdomyosarcoma specimens express SHH, PTCH1, GLI1, and GLI3 (19,20). Their findings are compatible with our real-time PCR results. In general, it is accepted that enhanced Hedgehog pathway activation induces the expression of downstream target genes, including *PTCH1, GLI1*, and *GLI2*, and the levels of their transcripts often serve as surrogate markers of the

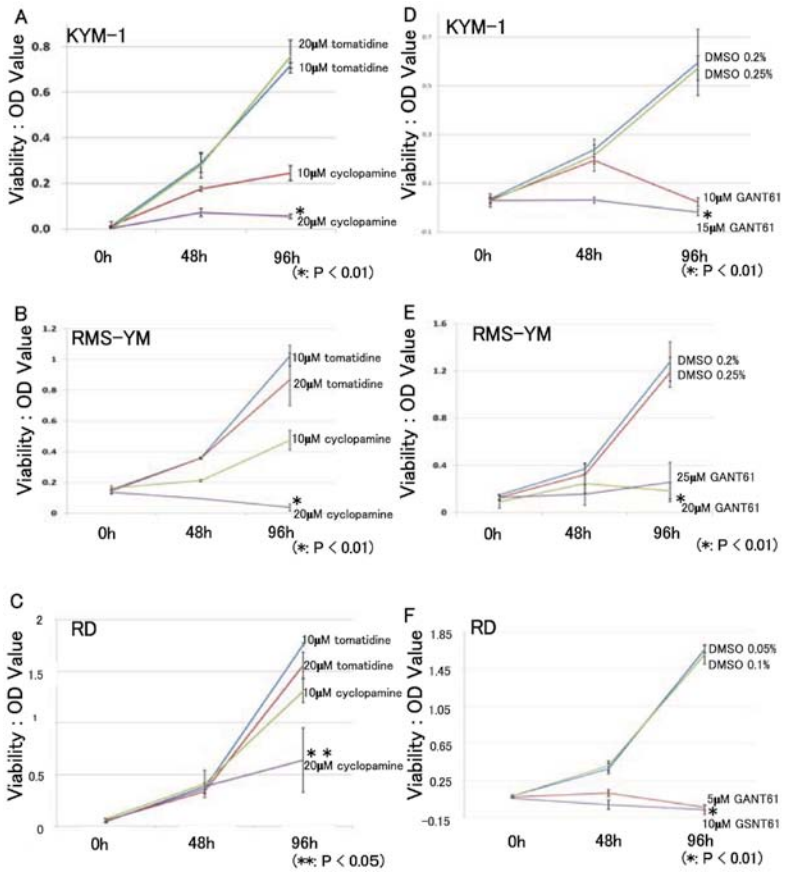


Figure 5. Inhibition of Hedgehog pathway prevents rhabdomyosarcoma cell growth. WST assay revealed a dose-dependent decrease in the growth of viable KYM-1 cells over 4 days after cyclopamine treatment (*p<0.01) (A). A dose-dependent decrease in the growth of viable RMS-YM and RD cells over 4 days was observed after cyclopamine treatment (*p<00.01) (**p<00.05) (B and C). WST assay results indicated a dose-dependent decrease in the growth of viable KYM-1 cells over 4 days after GANT61 treatment (*p<0.01) (D). A dose-dependent decrease was observed in the growth of viable RMS-YM and RD cells over 4 days after GANT61 treatment (*p<0.01) (E and F). The experiment was performed in triplicate with similar results (error bar means ± SD).

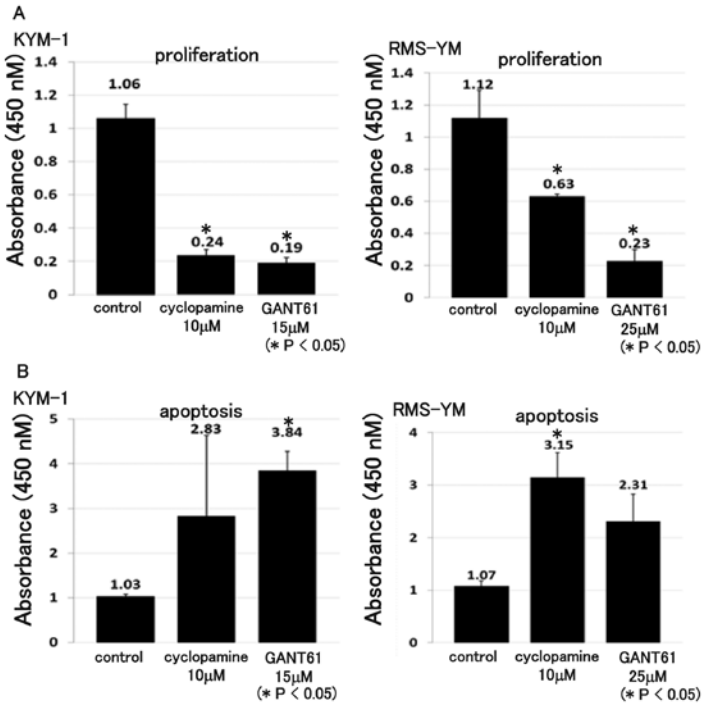


Figure 6. Pharmacological inhibitors of the Hedgehog pathway promote the inhibition of rhabdomyosarcoma cell proliferation and apoptosis. (A) BrdU cell proliferation assay revealed that the proliferation of KYM-1 and RMS-YM cells was inhibited by both cyclopamine and GANT61 (*p<0.05) (error bar means ± SD). (B) Cell death detection assay showed that GANT61 promoted apoptotic cell death of KYM-1 cells, while cyclopamine promoted apoptotic cell death of RMS-YM cells (*p<0.05) (error bar means ± SD).

Hedgehog pathway activity (21). Our findings suggest that the Hedgehog pathway is activated in human rhabdomyosarcomas. Further, we showed that cyclopamine or GANT61 prevented human rhabdomyosarcoma cell growth. This is the first study to suggest that the pharmacological agents inhibit the growth of human rhabdomyosarcoma cell lines. Cyclopamine treatment induces apoptosis in tumor cells (16,22,23). Cell death detection assay showed that cyclopamine induced apoptotic cell death only in RMS-YM cells and not in KYM-1 cells. This discrepancy may be attributed to the existence of other different survival factors or drug export systems, which retain the viability of KYM-1 cells. We showed that pharmacological inhibition of Hedgehog pathway prevented rhabdomyosarcoma growth cooperatively by inhibition of cell proliferation and apoptotic death. Therefore, if rhabdomyosarcoma cells acquired anti-apoptosis resistance, pharmacological inhibition of Hedgehog pathway can prevent rhabdomyosarcoma growth by inhibiting cell proliferation.

We showed that in addition to SMO inhibition, GLI inhibition prevents human rhabdomyosarcoma growth. With respect to the numerous potential mutational targets already discovered within the Hedgehog pathway downstream of SMO, the group of tumors for which direct GLI inhibition is beneficial is substantial and likely to increase. The inhibition of GLI, but not SMO, induced apoptosis in chronic lymphocytic leukemia cells (24).

Several signaling pathways, such as Notch, Wnt, TGF-β, BMP, and Hedgehog are involved in processes essential to the proper development. It is also reported that these pathways play crucial roles in tumorigenesis (reviewed in ref. 15). We have recently found that the activation of Notch pathway promotes the progression of human rhabdomyosarcoma (unpublished data). Additionally, some recent reports have provided evidence for direct interaction or cross-talk between these pathways (reviewed in ref. 15). Further examination is required to elucidate interaction of these pathways in rhabdomyosarcoma pathogenesis.

Several reports have reported that the anti-tumor effect by Hedgehog pathway inhibitors is due to their effect on stromal cells (26,27). On the other hand some reports have reported that the Hedgehog signaling pathway is activated in cancer cells (13,17,28-31). The Hedgehog pathway inhibitors may affect stromal cells *in vivo*, our findings suggest that SMO or GLI inhibitors directly inhibit rhabdomyosarcoma cell growth *in vitro*.

The hypothesis that malignant tumors generated by rare populations of tumor-initiating cells (TICs), also called cancer stem cells, are more tumorigenic than other cancer cells has gained increasing credence (12,32). We and others have shown the existence of TICs in bone and soft tissue sarcomas, including rhabdomyosarcoma (33-37). Further, loss of *Smo* depletes TICs, whereas constitutively active *Smo* augments TICs number and accelerates disease progression (16,38). These findings suggest that the inhibition of Hedgehog pathway might affect the proliferation of TICs of rhabdomyosarcoma.

Our findings suggest that the Hedgehog pathway is functionally activated in human rhabdomyosarcoma. This novel finding has improved the understanding of rhabdomyosarcoma and may prove useful to understand the growth of rhabdomyosarcoma cells. In addition, our findings suggest that if rhabdomyosarcoma cells acquire anti-apoptosis resistance, pharmacological inhibition of Hedgehog can prevent rhabdomyosarcoma growth by inhibition of cell proliferation. Hence, pharmacological inactivation of Hedgehog pathway may be an attractive method to treat rhabdomyosarcoma patients.

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