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 andULK3. 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 GLI1 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 (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
KAWABATA et al.: INHIBITION OF HEDGEHOG PATHWAY PREVENTS Rhabdomyosarcoma CELL GROWTH

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 µg/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).

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 (ΔΔCt) 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'-ATTTGGCCGCCACCCAGGT-3'; Desert hedgehog: 5'-TGATGACCGAGCTGTGTAAG-3', 5'-GCCAGCAACCCA TACTTGTT -3'; Indian hedgehog: 5'-AC TTCTGGCTTGGCTGTTGTTG-3', 5'-AGCGATCTTGCCTT CATAGC-3'; PTCH1: 5'-TAACGCTGCAACAACTCAGG-3', 5'-GAAGGCTGTGACATTGCTGA-3'; SMO: 5'-GGGAGTC TACTTCCTCATCC-3', 5'-GGCAGCTGAAGGTAATGAC-3'; GLI1: 5'-GTGCAAGTCAAGCCAGAACA-3', 5'-ATAGGGGCCTGACTGGAGAT-3', GLI2: 5'-CGAC ACCAGGAAG AAAGGTGA-3', 5'-AGAAGGGGTAGTGATGGCTCCA-3'; ULK3: 5'-CCACAGACACCAACCAGCT-3', 5'-GTGGGAGAGATG AGGAACCA-3'; ACTB: 5'-AGA AAATCTGGCACCACACC-3', 5'-AGAGGCGTACAGGATAGCA-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 werecounterstained with Hoechst 33258 ( Molecular Probes, Carlsbad, CA, USA). Immunohistochemistry with

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. PTCCH1 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.
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 ELISAPLUS (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 human rhabdomyosarcoma cells; and Desert hedgehog (DHH) expression was increased from 4.6- to 55.6-fold in human rhabdomyosarcoma cell lines (Fig. 1).
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.

### 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 µM cyclopamine effectively inhibits Hedgehog pathway and osteosarcoma growth (13). The WST assay showed that 20 µM 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 steroidal 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.
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<0.001) (*p<0.005) (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 may 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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References


