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Hedgehog signaling is synergistically enhanced by nutritional deprivation and ligand stimulation in human fibroblasts of Gorlin syndrome

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A B S T R A C T

Hedgehog signaling is a pivotal developmental pathway that comprises hedgehog, PTCH1, SMO, and GLI proteins. Mutations in *PTCH1* are responsible for Gorlin syndrome, which is characterized by developmental defects and tumorigenicity. Although the hedgehog pathway has been investigated extensively in *Drosophila* and mice, its functional roles have not yet been determined in human cells. In order to elucidate the mechanism by which transduction of the hedgehog signal is regulated in human tissues, we employed human fibroblasts derived from three Gorlin syndrome patients and normal controls. We investigated GLI1 transcription, downstream of hedgehog signaling, to assess native signal transduction, and then treated fibroblasts with a recombinant human hedgehog protein with or without serum deprivation. We also examined the transcriptional levels of hedgehog-related genes under these conditions. The expression of GLI1 mRNA was significantly higher in Gorlin syndrome-derived fibroblasts than in control cells. Hedgehog stimulation and nutritional deprivation synergistically enhanced GLI1 transcription levels, and this was blocked more efficiently by vismodegib, a SMO inhibitor, than by the natural compound, cyclopamine. Messenger RNA profiling revealed the increased expression of Wnt signaling and morphogenetic molecules in these fibroblasts. These results indicated that the hedgehog stimulation and nutritional deprivation synergistically activated the hedgehog signaling pathway in Gorlin syndrome fibroblasts, and this was associated with increments in the transcription levels of hedgehog-related genes such as those involved in Wnt signaling. These fibroblasts may become a significant tool for predicting the efficacies of hedgehog molecular-targeted therapies such as vismodegib.
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1. Introduction

Hedgehog signaling is a pivotal developmental pathway that comprises hedgehog, patched, smoothened, and gli proteins [1]. The hedgehog gene was first identified in *Drosophila* as a segment polarity gene that controls the segmental pattern of the embryo [2]. Hedgehog signaling is known to be crucially involved in morphogenesis, tumorigenesis, osteoblast differentiation, wound repair, and neuroprotection [3,4].

PTCH, the human homolog of *Drosophila* patched, is a twelve-pass transmembrane protein coding a receptor for hedgehog that constitutively suppresses activation of the hedgehog pathway by inhibiting SMO, which transmits hedgehog signaling. When hedgehog ligands bind to PTCH, the inhibition of SMO is released and SMO then activates GLI through several cytoplasmic transduction steps, leading to the translocation of GLI into the nucleus and expression of the targeted gene [5].

Gorlin syndrome (GS) is a rare autosomal dominant disease that is characterized by developmental anomalies including palmer and plantar pits, skeletal abnormalities, falx calcification, and tumorigenesis such as basal cell carcinoma (BCC), ketatocystic odontogenic tumors, and medulloblastoma [6]. This syndrome is caused by mutations in *PTCH1*, and more than 200 mutations have been reported to date [7].

Tumorigenesis is a lifelong problem in patients with GS. These patients develop BCCs in their early 20s, with more than 80% of patients having one or more BCCs throughout their lifetime. Medulloblastoma occurs in 4% of patients with GS at a mean age of 2.3 years [8]. Molecular targeted inhibitors of the hedgehog pathway have recently been developed to treat these tumors. Several small compounds have been applied to clinical trials for these tumors [9–11]. Of these, vismodegib (GDC-0449) was found to be the most effective inhibitor of SMO, reducing the number of BCCs [9].

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Since the hedgehog pathway has not yet been elucidated in detail in human cells, we herein investigated this signaling pathway in GS-derived dermal fibroblasts under diverse cellular stress conditions.

2. Material and methods

2.1. Materials

Recombinant human sonic hedgehog (shh) (C24II), N-Terminus (R&D Systems, Minneapolis, USA), smoothened agonist (SAG) (Calbiochem, Darmstadt, Germany), cyclopamine (BIOMOL, PA, USA), and vismodegib (GDC-0449) (Selleckchem, Huston, TX, USA) were purchased. Mouse monoclonal antibodies against human acetylated tubulin (Sigma–Aldrich, Saint Louis, MO, USA), anti-mouse IgG Alexa 594 (Abcam, Cambridge, UK), and '4',6-diamidini-2-phenylindole, dihydrochloride (DAPI) (Roche Diagnostics, Mannheim, Germany) were obtained.

2.2. Human dermal fibroblasts

All studies described below were approved by the local Ethics Committee of Chiba University. Human dermal fibroblasts were collected from three Japanese GS patients, who were from two families with *PTCH1* mutations as an insertion in one family (G11 and G12; c.3130_3131dupGC) and a deletion in one patient (G72 c.272delG). The ages of G11, G12, and G72 at dermal fibroblast collection were 14, 42, and 36 years old, respectively. After obtaining informed consent from GS patients, dermal specimens were collected from unaffected skin areas at the time of surgical operations. These surgically resected dermal tissues were then cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin, and 1% streptomycin. Fibroblasts were obtained from these dermal tissues, maintained at 37 °C in a humidified atmosphere (5% CO_2 and 95% air), and seeded on 10-mm dishes. The culture medium was renewed every 2 days until cellular confluence was achieved. Three origin-different normal human adult dermal fibroblasts were purchased (PCS-201-012; ATCC, Manassas, VA, USA). These fibroblasts were used during passages 4 to 8 in subsequent experiments.

2.3. Stimulation with agonists and antagonists of the hedgehog pathway

Fibroblasts were seeded on 12-well plates in DMEM with 10% FBS for 24 h, the culture medium was renewed before stimulation experiments, and DMEM with 0.5% FBS was used for serum starvation. These fibroblasts were treated with human recombinant shh (1000 ng/ml), cyclopamine (100 nM), SAG (1 μM), and vismodegib (10 nM) for 48 h after the medium change.

2.4. Immunofluorescence and confocal microscopy

Human fibroblasts grown on slide chambers were fixed with 4% paraformaldehyde. Cells were washed with phosphate buffer saline (PBS) and permeabilized with 0.2% Triton X-100 in PBS, followed by blocking with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with primary antibodies, diluted in 2% BSA in PBS, as mouse monoclonal anti-acetylated α-tubulin (1:1000). After washing with PBS, cells were incubated with secondary antibodies, anti-mouse IgG Alexa 594 (1:500), and DAPI (1:1000) for 30 min. Cells were viewed under a FLUOIVIEW FV1000-D/FV10i confocal microscope (OLYMPAS, Tokyo, Japan) and counted in at least 10 views.

2.5. mRNA quantification by real-time PCR

RNA was extracted from human dermal fibroblasts using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized with SuperScript III First Strand Synthesis SuperMix (Invitrogen). PCR primers were designed against GLI1, GLI2, GLI3, PTCH1b, and SMO which are described in Supplementary Table 1, and the reagent mix was incubated on the cycler (Bio-Rad, CA, USA) with normalization by the internal control gene human GAPDH. PCR reactions were run for 40 cycles twice in triplicate. The log-linear phase of amplification was monitored to obtain threshold cycle values. The comparative threshold cycle method was applied to determine expression levels. The absence of primer dimers was verified by running the PCR product on a 1.5% agarose gel. To explore the relationships among hedgehog pathway components, we employed the RT2 Profiler PCR Array (Qiagen) and analyzed them by RT2 Profiler PCR Data Array analysis v3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

2.6. Statistical analysis

Data were compared by a two-way ANOVA and Dunnett's test using PRISM6 (Graph Pad software). A *P* value less than 0.05 were considered significant. Data were shown as the mean ± the standard error of the mean (SEM).

3. Results

3.1. Hedgehog signaling was enhanced in GS fibroblasts by shh with serum deprivation

To establish the untreated status of hedgehog signaling in GS fibroblasts, we measured the mRNA levels of components of the hedgehog pathway: GLI1, GLI2, GLI3, PTCH1b, and SMO, using real-time PCR. Before the treatment with shh, GLI1 mRNA levels were more than 3-fold higher in GS fibroblasts than in the controls (Fig. 1A). The expression levels of SMO mRNA were also 2-fold higher in GS fibroblasts than in the controls. No significant differences were observed in the mRNA levels of the other hedgehog pathway components between GS fibroblasts and the controls. Although GLI mRNA levels were elevated in all GS fibroblasts, individually different activated levels were observed in each GS cell. For example, GLI1 levels were elevated less in G12 fibroblasts than in other GS fibroblasts.

We then investigated the influence of shh on GS fibroblasts in order to determine whether this condition represented tumorigenesis in GS patients. The sole stimulation of the hedgehog protein did not enhance GLI1 mRNA levels above those in the controls (Fig. 1B).

Since the number of ciliated cells has been shown to increase with nutritional deprivation [12] and hedgehog signaling passes through these primary cilia, we starved fibroblasts and treated them with or without shh. The stimulation of shh and serum deprivation markedly elevated GLI1 levels by at least 3-fold those in the controls (Fig. 1B). GLI1 mRNA levels were lower in G12 fibroblasts than in other GS fibroblasts under the untreated condition; however, these levels were significantly increased by the shh stimulation. These results suggested that GLI1 mRNA levels were constitutively higher in all GS fibroblasts than in the controls, and that hedgehog signaling was synergistically activated by shh stimulation and serum deprivation.

3.2. Formation of primary cilia was increased after serum starvation

We examined the formation of primary cilia by GS fibroblasts and controls using immunostaining for primary cilia and counted

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