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ARTICLE

MicroRNAs profiling in fibroblasts derived from patients with Gorlin syndrome

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Abstract

Gorlin syndrome (GS) is a hereditary disorder with tumorigenicity, caused by constitutive hyperactivity of hedgehog signaling. Smoothened (SMO) antagonists have been effectively used in the clinical treatment of hedgehog signaling-related cancer. However, these treatments have led to problematic side effects, including severe adverse reactions and drug resistance from additional somatic mutations. We profiled microRNAs in GS fibroblasts to explore a novel therapeutic target for controlling hyper-activated hedgehog signaling. To identify GS-related microRNAs, we analyzed dermal fibroblasts from five patients with GS and three normal controls. We used microarray comparative genomic hybridization to screen 632 human microRNAs in GS fibroblasts. We identified 16 down- and 19 upregulated microRNAs with over twofold change in expression. We validated the increased expression of four microRNAs, confirming hsa-miR-196a-5p downregulation and hsa-miR-4485 upregulation using real-time PCR. Moreover, hsa-miR-196a-5p is complementary to sites in the 3′ UTR of *MAP3K1*, which exhibits upregulated expression at mRNA and protein levels in GS fibroblasts. In addition, hedgehog signal induction with exogenous components decreased miR-196a-5p expression and increased *map3k1* expression in a mouse mesenchymal cell line. Given that *MAP3K1* has been reported to activate hedgehog signaling, hsa-miR-196a-5p may contribute to the positive feedback loop in this pathway.

Introduction

Gorlin syndrome (GS, or nevoid basal cell carcinoma syndrome, OMIM #109400) is a hereditary disease caused by constitutive hyperactivity of hedgehog signaling, due to heterozygous mutations in signaling proteins including PTCH1, PTCH2, and SUFU. Core clinical manifestations of GS include congenital anomalies (pit on palms, rib anomalies, calcification of cerebral falx, macrocephaly) and tumorigenesis (basal cell carcinoma, medulloblastoma, keratocystic odontogenic tumors) [1]. Hedgehog signaling is activated locally as a physiological recovery response to wounds, myocardial infarction, and ischemic stroke. Therefore, patients with GS provide a human model of hyperactivity in hedgehog signaling as well as established hereditary diseases.

Over the past decade, hedgehog-signaling inhibitors have been produced as anticancer agents for hedgehog signaling-related cancer, including for patients with GS. Such inhibitors are mainly smoothened (SMO) antagonists, such as vismodegib, which have proven effective clinically [2, 3]. However, these therapies have two major problems: adverse drug reactions [2, 3] and drug resistance from additional somatic mutations [4]. The adverse effects of SMO antagonists are life-threatening, occur in 19–33% of patients, and cause death in 2–11% of patients [2, 3]. Clinical manifestations include weight loss, muscle spasms, alopecia, fatigue, and dysgeusia [2, 3]. Although the pathological mechanism remains controversial, SMO antagonists are thought to

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activate Ca^{2+}-AMPK signaling, known as Type II non-canonical hedgehog signaling, and affect energy metabolism in muscle, adipose tissue, liver, and pancreas [5].

These problems highlight the limitations of small-molecule therapy and the need to investigate novel therapeutic targets for treating and preventing hedgehog signaling-related cancers. We focused on microRNAs (miRNAs) as novel therapeutic targets to control hyper-activated hedgehog signaling. miRNAs are non-coding RNAs that suppress posttranscriptional mRNA expression and modulate cell signaling. Recently, miRNAs have drawn attention in cancer medicine as anticancer agents and biomarkers for determining cancer species, drug efficacy, and patient prognosis [6]. Previous cell-line studies have shown that miRNAs are involved in the control of hedgehog signaling [7, 8], yet no reports are available on miRNA profiling in human samples. Here, we profiled miRNAs in dermal fibroblasts derived from patients with GS to explore novel potential therapeutic agents for hedgehog signaling-related cancer.

Materials and methods

Human fibroblasts and cell lines

The Institutional Review Board (IRB) at Chiba University Hospital approved this retrospective study (IRB number 792). Human fibroblasts were derived from healthy skin of five patients with clinically and genetically diagnosed GS (Table 1). Normal human control fibroblasts (NC) (N1 #58732338, N2 #61447289, and N3 #61683453) and mouse mesenchymal embryonic fibroblast C3H10T1/2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM, supplemented with 10% fetal bovine serum and a 1% penicillin/streptomycin solution at 37 °C with 5% CO_2. Cells were harvested, suspended in QIAzol Lysis Reagent (Qiagene), and immediately stored at −80 °C for future experiments. TE-671 cells were cultured Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum. Experiments were performed using 80–90% confluent cells. TE-671 cells were infected with a lentivirus having shRNA for non-silencing control or MAP3K1. After infection, stable colonies were selected in puromycin-containing medium for 2 weeks. Lentiviral vector plasmids for human MAP3K1 and control non-silencing control shRNA were purchased from Sigma-Aldrich.

Reagents

Recombinant mouse sonic hedgehog (N-shh: R&D Systems, Minneapolis, MN, USA), SMO agonist (SAG; Enzo

Table 1 Clinical and genetic characteristics of patients with Gorlin syndrome

Case no.	Age/gender	Major criteria by Kimonis	*PTCH1* mutations	Other abnormal findings
G4	31 years/female	1,2,3,4,5	c.1261_1262insT	Ulcer colitis, intelligence deficiency, agenesis of corpus callosum, empty sella
G11	14 years/male	2,3,4,5,6	c.3130_3131dupGC	Nasal dermoid, spina bifida, hydrocephalus, dysthyroidism
G12	42 years/female	2,3,4,5,6	c.3130_3131dupGC	Lumbosacral lipoma
G36	7 years/female	3,5,6	Whole gene deletion (1.1 Mb)	
G72	36 years/female	2,3,4	c.272delG	Ovarian fibroma, gastric cancer

1 = more than two basal cell carcinomas (or over one if below 20 years old); 2 = odontogenic keratocysts of the jaw, demonstrable through histology; 3 = three or more palmar or plantar pits; 4 = bilamellar calcification of the falx cerebri; 5 = bifid, fused, or markedly splayed ribs; 6 = first-degree relative with Gorlin syndrome

Life Sciences, Farmingdale, NY, USA), and purmorphamine (Santa Cruz Biotechnology, Dallas, TX, USA) were purchased from commercial sources.

Micro array comparative genomic hybridization (CGH)

Total RNA was extracted from pooled fibroblasts using the miRNeasy Mini kit (Qiagen, Valencia, CA, USA) and quantified with a Nanodrop (Thermo Fisher Scientific, MA, USA) and Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). RNA (1 µg) was labeled with the Flash Tag Biotin HSR RNA Labeling kit, hybridized with a GeneChip miRNA 4.0 Array (Affymetrix, CA, USA), and incubated at 48 °C for 18 h. The array was washed and then scanned. Microarray CGH followed manufacturer protocols.

Real-time RT-PCR for miRNAs and genes

Total RNA was miRNAeasy-extracted and reverse-transcribed using the Universal cDNA Synthesis Kit II (Exicon, Vedbaek, Denmark) for miRNA, as well as the Super Script III First-strand Synthesis Super mix for RT-PCR (Invitrogen, Grand Island, NY, USA) for mRNA. Real-time PCR was performed using the CFX Connect Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA).

microRNA expression was measured using the ExiLENT SYBR Green master mix (Exicon) and individual miRCURY Locked Nucleic Acids™ (hsa-miR-10a-5p, hsa-miR-196a-5p [mm-miR-196a-5p], hsa-miR-615-3p, and hsa-miR-4485) or U6 snRNA PCR Primer Sets (Exicon). Cycling conditions were 95 °C for 10 min; 40 cycles of 95 °C for 10 s; and 60 °C for 1 min.

mRNA expression was measured using the SYBR Green PCR Master Mix (Roche Diagnostics, Mannheim, Germany) and specific primers (Supplemental Table 1). Cycling conditions were 95 °C for 10 min; 40 cycles of 95 °C for 15 s; and primer-specific annealing temperature for 30 s. Melting curves were examined after both PCRs to confirm primer specificity. Threshold cycle values were determined using the comparative CT (ΔΔCT) method. Amplicon amount (2-ΔΔCT) was obtained through normalization to endogenous references (*GAPDH* for human mRNA, *actb* for mouse mRNA, and U6 for miRNA) and compared with controls to determine fold changes in expression.

Immunoblotting and densitometric measurements

Cells were solubilized in RIPA buffer (Nacalai Tesque) according to the manufacture's instruction. Protein concentration was measured with Protein Assay Bicinchoninate Kit (Nacalai Tesque). Samples were resolved on SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with Blocking-One (Nacalai Tesque) and incubated with primary antibodies overnight at 4 °C. MAP3K1 antibody was from proteintech. α-tubulin antibody was from cell signaling. Relative protein amounts were determined by densitometric analysis using ImageJ software (NIH).

Cell proliferation assay

Analysis of BrdU incorporation was performed with BrdU Cell Proliferation ELISA Kit (Abcam) according to the manufacturer's protocol. Five thousand cells per well were seeded into a 96-well plate in triplicate samples for each control and KD clone. The assay was performed following overnight incubation with BrdU.

Statistical analysis

Data are represented as means ± standard error of the mean (SEM), and were compared using a Student's *t* test. Analyses were performed in R version 3.3.1 (R Development Core Team, http://www.R-project.org/) and diagrams were created in GraphPad Prism6 (GraphPad Software Inc.). Significance was set at $p < 0.05$.

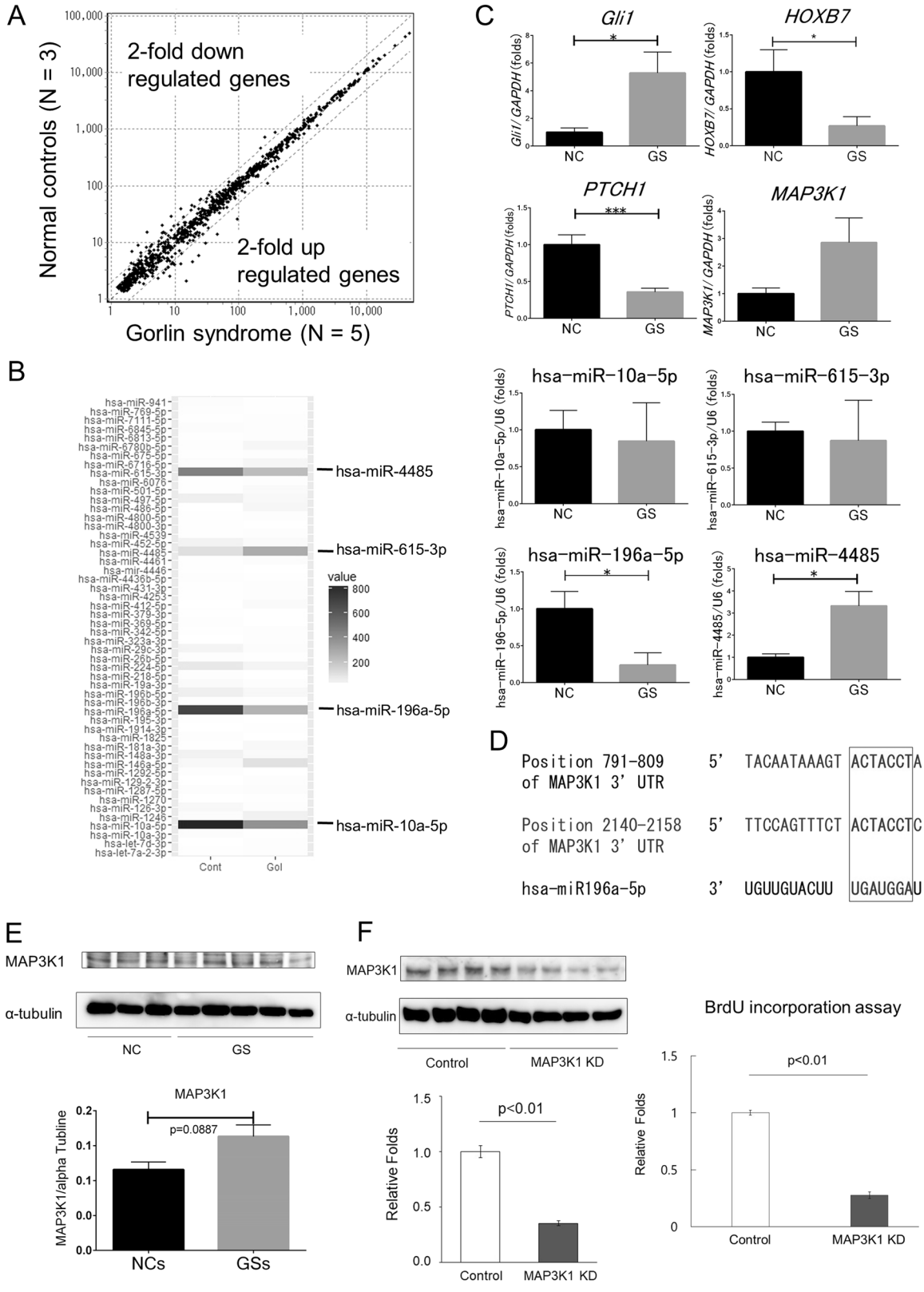
Results

microRNA profiling in GS and NC fibroblasts

To identify miRNAs involved in GS pathogenesis, we profiled miRNA expression in pooled samples from five GS fibroblasts (GSs) and three NC fibroblasts (NCs) using miRNA array. Normalized signal intensities were plotted on a spreadsheet (Fig. 1a; for raw data, see Supplemental Table 2). Among the 632 human miRNAs examined, 35 miRNAs with over twofold difference in expression levels between GS and NC samples were identified (19 upregulated and 16 downregulated) (Table 2 and Fig. 1b). Based on the heat map, four miRNAs (hsa-miR-10a-5p, hsa-miR-196a-5p, hsa-miR-615-3p, and has-miR-4485) were selected as candidates with assessable expression.

Downregulated hsa-miR-196a-5p and upregulated hsa-miR-4485 in GS fibroblasts

We analyzed fibroblasts individually using real-time RT-PCR. As predicted, the hedgehog target gene *Gli1* had significantly higher expression (5-fold; $p < 0.05$) in GS fibroblasts. Conversely, expression of *PTCH1*, which encodes the sonic hedgehog receptor, was significantly lower (0.36-fold; $p < 0.05$) in GS cells than in NC cells.



To validate microarray findings, we performed real time RT-PCR for the four selected candidate miRNAs. Relative miRNA expression levels are represented as the ratio of miRNA to U6 snRNA. hsa-miR-196a-5p expression was significantly lower in GS cells than in NC cells (0.24-fold; $p < 0.05$), and hsa-miR-4485 expression was significantly

◀ **Fig. 1** **a** Scatterplot showing the results of comparative genome hybridization using pooled fibroblasts of patients with Gorlin syndrome and normal controls. **b** Heat map showing upregulated or downregulated microRNAs (miRNAs) with over twofold change in expression. NC, normal control; GS, Gorlin syndrome. **c** Real time RT-PCR analysis of mRNAs and candidate miRNAs in GS. Averages were calculated from a duplicate analysis in each fibroblast and shown as mean ± standard error (Student's *t* test, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). **d** Sequence alignment of miR-196a-5p and complementary sites in the 3′ UTR of *MAP3K1*. **e** Immunoblotting and densitometric measurements of MAP3K1 and α-tubulin in NC ($N = 3$) and GS ($N = 5$). **f** Immunoblotting of MAP3K1 protein and cell proliferation assay in MAP3K1 knockdown ($N = 4$) and non-silencing control TE-671 cells ($N = 4$)

higher in GS cells than in NC cells (3.3-fold; $p < 0.05$) (Fig. 1c). No differences in hsa-miR-10a-5p and hsa-miR-615-3p expression were identified (Fig. 1c). Expression of *HOXB7*, a host gene of hsa-miR-196a, was significantly downregulated (0.75-fold; $p < 0.05$) in GS compared with NC cells (Fig. 1c).

Predictive target genes of miR-196a-5p and miR-4485

We used the miRDB (http://mirdb.org) in silico database to select 22 predicted hsa-miR-196a-5p target genes with a score of over 90 (listed in Table 3). Reference to online databases revealed that among the 22 genes, four are expressed in the skin tissue (*NR2C2*, *MAP3K1*, *CCDC47*, and *RCC2*; The Human Protein Atlas, https://www.proteinatlas.org/). We focused on *MAP3K1* as a miR-196a target gene because the miR-196a-5p sequence matches two regions in the 3′ UTR of *MAP3K1* (Fig. 1d). In addition, *MAP3K1* expression was higher in GS cells than in NC cells (Fig. 1c), and the densitometric measurements of immunereactive bands showed the expression rate of MAP3K1 protein against α-tubulin protein was higher in GS cells than in NC cells (Fig. 1e), but these difference of MAP3K1 at mRNA and protein levels were not statistically significant.

In contrast, the four predicted hsa-miR-4485 target genes (*CTDSPL2*, *LACTB2*, *PLPP7*, and *GALNT14*) with a score of over 80 was identified with miRDB (Table 4). Although *CTDSPL2* is highly expressed in the skin tissue, an interaction of CTDSPL2 with hedgehog signaling has not been reported so far.

The inhibited cell proliferation by MAP3K1 knockdown in TE-671

To confirm the effect of modulating MAP3K1 on cell proliferation, we knockdown MAP3K1 with in TE-671 cells, and evaluated cell proliferation using BrdU. TE-671 cells has been used as a human medduloblastoma cell model [9, 10]. The *MAP3K1* knockdown TE-671 cells have decreased cell proliferation compared with non-silencing control cells (Fig. 1f).

Activation of hedgehog signaling downregulates miR-196a-5p and upregulates *map3k1* in a mouse mesenchymal cell line

To confirm that activation of hedgehog signaling modulates *map3k1* via miR-196a-5p downregulation, we analyzed *map3k1* and miR-196a-5p expression following exogenous activation of hedgehog signaling in C3H10T1/2 cells. C3H10T1/2 cells are a mouse mesenchymal cell line often used to evaluate hedgehog signaling activity [11].

Compared with control, cells treated with recombinant mouse sonic hedgehog (N-shh, 200 nM) expressed significantly more *gli1* ($p < 0.0001$) and less miR-196a-5p (by 0.5-fold; $p < 0.05$) (Fig. 2a). Furthermore, *map3k1* expression was significantly higher in C3H10T1/2 cells treated with N-shh than in control cells (2.5-fold; $p < 0.001$) (Fig. 2a).

Next, because hedgehog signaling occurs via SMO, we treated C3H10T1/2 cells for 24 h with SMO agonists (4 μM SAG or 5 μM purmorphamine) or with vehicle. Stimulation of hedgehog signaling with both SMO agonists increased *gli1* expression, decreased miR-196a-5p expression, and increased *map3k1* expression (Fig. 2b).

Discussion

We identified two GS-related miRNAs, hsa-miR-196a-5p and hsa-miR-4485, through comprehensive analysis of patient-derived fibroblasts. miR-4485 was previously identified in acute lymphoblastic leukemia and in breast cancer, where it regulates mitochondrial function and inhibits tumorigenicity [12]. However, the importance of miR-4485 in a clinical setting has not been established, and only a few of its gene targets have been identified. While, miR-196a is transcribed from HOX B or C clusters [13] and conserved across many vertebrate species. miR-196a regulates proliferation, cell invasion, and migration [14]. The downregulated hsa-miR-196a is reported in melanoma, acute lymphoblastic leukemia, breast cancer, and systemic sclerosis [14]. Furthermore, miR-196a-5p can be quantified in human serum and is a potential biomarker of cervical cancer [15].

Profiling analyses of tumors by other researchers have identified several hedgehog signaling-related miRNAs: miR-124 in glioma [16], and miR-324-5p [17] and miR-1271 [18] in myeloma. Moreover, miR-326 and miR-378 have been reported as a hedgehog signaling modulators: miR-326 provides negative feedback through suppressing Gli2 and SMO, while miR-378 provides positive feedback

Table 2 MicroRNAs with over twofold change in expression

microRNA	Chr	Length	Sequence	NC	GS	Fold change
Downregulated microRNA ($N = 16$)						
hsa-miR-10a-3p	17	22	CAAAUUCGUAUCUAGGGGAAUA	3.775849	14.54688	0.2595642
hsa-miR-369-5p	14	22	AGAUCGACCGUGUUAUAUUCGC	2.686404	8.713036	0.3083201
hsa-miR-218-5p	4	21	UUGUGCUUGAUCUAACCAUGU	2.141825	6.726388	0.3184213
hsa-miR-126-3p	9	22	UCGUACCGUGAGUAAUAAUGCG	8.856215	27.64112	0.3204
hsa-miR-19a-3p	13	23	UGUGCAAAUCUAUGCAAAACUGA	6.383119	19.35522	0.329788
hsa-miR-29c-3p	1	22	UAGCACCAUUUGAAAUCGGUUA	12.50842	37.89977	0.3300395
hsa-miR-196a-5p	12	22	UAGGUAGUUUCAUGUUGUUGGG	262.3991	698.9808	0.3754024
hsa-miR-148a-3p	7	22	UCAGUGCACUACAGAACUUUGU	18.78027	44.09498	0.4259049
hsa-miR-10a-5p	17	23	UACCCUGUAGAUCCGAAUUUGUG	380.9399	841.942	0.4524539
hsa-miR-497-5p	17	21	CAGCAGCACACUGUGGUUUGU	25.7434	56.15898	0.4584022
hsa-miR-26b-5p	2	21	UUCAAGUAAUUCAGGAUAGGU	3.678082	8.021221	0.4585439
hsa-miR-6845-5p	8	19	CGGGGCCAGAGCAGAGAGC	6.726388	14.54688	0.4623939
hsa-miR-196b-5p	7	22	UAGGUAGUUUCCUGUUGUUGGG	25.51946	54.19533	0.4708793
hsa-miR-224-5p	X	21	CAAGUCACUAGUGGUUCCGUU	36.95467	77.8418	0.4747407
hsa-miR-196b-3p	7	22	UCGACAGCACGACACUGCCUUC	3.018979	6.327122	0.4771489
hsa-miR-615-3p	12	22	UCCGAGCCUGGGUCUCCCUCUU	226.0509	452.5642	0.4994891
Upregulated microRNA ($N = 19$)						
hsa-miR-412-5p	14	23	UGGUCGACCAGUUGGAAAGUAAU	15.3889	2.030393	7.5792716
hsa-miR-501-5p	X	22	AAUCCUUUGUCCCUGGGUGAGA	19.45791	4.355919	4.4670046
hsa-miR-181a-3p	1	22	ACCAUCGACCGUUGAUUGUACC	24.35769	5.647525	4.3129849
hsa-miR-146a-5p	5	22	UGAGAACUGAAUUCCAUGGGUU	96.92647	24.73987	3.9178245
hsa-miR-1246	2	19	AAUGGAUUUUUGGAGCAGG	47.25275	13.52334	3.4941627
hsa-miR-6780b-5p	6	23	UGGGGAAGGCUUGGCAGGGAAGA	30.51196	9.525046	3.2033399
hsa-miR-4461	5	23	GAUUGAGACUAGUAGGGCUAGGC	12.56566	4.026038	3.1210982
hsa-miR-4485	11	20	UAACGGCCGCGGUACCCUAA	287.6873	107.2939	2.6813015
hsa-miR-4800-5p	4	21	AGUGGACCGAGGAAGGAAGGA	10.00128	3.947945	2.5332876
hsa-miR-1270	19	23	CUGGAGAUAUGGAAGAGCUGUGU	5.525093	2.275565	2.4280093
hsa-miR-323a-3p	14	21	CACAUUACACGGUCGACCUCU	6.726388	2.814253	2.3901149
hsa-miR-7111-5p	6	22	UGGGGGAGGAAGGACAGGCCAU	9.683009	4.07771	2.3746193
hsa-miR-4253	1	18	AGGGCAUGUCCAGGGGGU	7.297226	3.207621	2.2749652
hsa-let-7a-2-3p	11	22	CUGUACAGCCUCCUAGCUUUCC	7.976401	3.543188	2.2511933
hsa-miR-431-3p	14	22	CAGGUCGUCUUGCAGGGCUUCU	7.779786	3.567373	2.1808165
hsa-miR-6076	14	21	AGCAUGACAGAGGAGAGGUGG	8.849211	4.0814	2.1681803
hsa-miR-342-5p	14	21	AGGGGUGCUAUCUGUGAUUGA	9.357762	4.326972	2.1626583
hsa-miR-1287-5p	10	22	UGCUGGAUCAGUGGUUCGAGUC	12.84063	6.146958	2.0889406
hsa-miR-6716-5p	11	20	UGGGAAUGGGGGUAAGGGCC	28.04681	13.54706	2.0703245

Chr chromosome, *NC* normal controls, *GS* patients with Gorlin syndrome

through suppressing Gli3 [7, 8]. However, our miRNA microarray study did not identify any difference in the expression of these miRNAs in GS and NC cells (Supplemental Table 2).

Among hsa-miR-196a-5p target genes, five genes (*PBX1*, *GATA6*, *RDX*, *CEP350*, and *MAP3K1*) are reported to have interaction with hedgehog signaling (Table 3). *PBX1* hierarchically control Shh expression in the limb development [19]. *GATA6* negatively regulate ectopic expressions of both Shh and hedgehog transcriptional targets in the limb bud [20]. *RDX* is expressed in the response to Shh and attenuates hedgehog signal through reducing levels of Ci [21]. *CEP350* modulate formation of primary cilia [22], which are essential for transduction of the hedgehog signal in mammals [23].

We especially focus on *MAP3K1*, because *MAP3K1* is expressed in the skin tissue and hsa-miR-196a-5p has intriguing complementarity to sites in the 3′ UTR of

Table 3 Hsa-miR-196a-5p target genes

Target rank	Target score	Gene symbol	Linked to hedgehog signaling	High expression in skin
1	100	*ZMYND11*		
2	100	*SLC9A6*		
3	98	*AQP4*		
4	97	*NR2C2*		✓
5	97	*HOXB7*		
6	96	*PBX1*	✓	
7	96	*GATA6*	✓	
8	95	*ERI2*		
9	94	*DENND6A*		
10	94	*MAP3K1*	✓	✓
11	94	*RDX*	✓	
12	94	*CCDC47*		✓
13	94	*HOXC8*		
14	93	*ELAVL4*		
15	93	*CEP350*	✓	
16	93	*ABCB9*		
17	92	*HOXA5*		
18	92	*NTN4*		
19	92	*RCC2*		✓
20	92	*HOXA7*		
21	91	*CCNJ*		
22	91	*NR6A1*		

Table 4 Hsa-miR-4485 target genes

Target rank	Target score	Gene symbol	Linked to hedgehog signaling	High expression in skin
1	93	*CTDSPL2*		✓
2	88	*LACTB2*		
3	88	*PLPP7*		
4	81	*GALNT14*		

MAP3K1 (Fig. 1d). Our study revealed decreased miR-196a-5p expression and increased *MAP3K1* expression in GS fibroblasts (Fig. 1c). Moreover, exogenous stimulation of hedgehog signaling, by Shh ligand and SMO agonist, downregulated miR-196a-5p expression in mouse mesenchymal cell lines (Fig. 2). MAP3K1 is a serine/threonine kinase that functions as a molecular switch of apoptosis regulation [24]. Recent investigations have shown that MAP3K1-targeting miRNAs affect growth and invasive behavior in breast cancer and colorectal carcinoma [25]. Interestingly, in mouse experiments, homozygous deletion of *map3k1′*s kinase domain downregulated *gli2* [26], suggesting the interaction between *MAP3K1* and hedgehog signaling.

This study has two limitations. First, due to small sample size, we could not identify a correlation between miR-196a-5p expression and phenotype or genotype in GS. Patients with GS carrying a *SUFU* mutation have an increased risk for medulloblastoma, but no phenotype–genotype correlation was noted between GS and *PTCH1* mutation or deletion [27]. Therefore, patients enrolled in our study represent a homogeneous population. Second, this analysis was performed using fibroblasts. Tumors that frequently occur in patients with GS include basal cell carcinoma, medulloblastoma, and breast cancer. Basal cell carcinoma is the most common form of skin cancer, but preferentially arises from stem cells within hair follicles and mechanosensory niches [28], not from skin fibroblast cells. Therefore, the findings of this GS fibroblast study may not be directly applicable to GS-associated cancers.

To know the biological characterization of Gorlin syndrome fibroblasts, we have examined the phenotype such as cell survival, proliferation, cell-cycle progression, and morphology including primary cilia. Cell-cycle progression in Gorlin fibroblasts showed aberrant DNA synthesis activity after X-ray irradiation [29]. Although cell survival, proliferation, and morphology, including primary cilia, were not different from controls, the expression of GLI1 mRNA was significantly higher in Gorlin syndrome fibroblasts than in controls. Messenger RNA profiling revealed the increased expression of Wnt signaling and morphogenetic molecules in Gorlin syndrome fibroblasts [30]. Thus, we consider that Gorlin syndrome fibroblasts may become a significant tool for predicting the efficacies of hedgehog molecular-targeted therapies such as vismodegib.

In conclusion, our study revealed that hyper-activated hedgehog signaling contributes to low miR-196a-5p expression and high *MAP3K1* expression in human fibroblasts and mouse cells. These findings suggest that miR-196a is associated with hedgehog signaling in GS patients, raising the possibility of a novel miRNA-based therapeutic measure beyond existing small-molecule therapies. Further studies are needed to clarify whether or not exogenous miR-196a inhibits hedgehog signaling in human cells.

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Author contributions TS: study conceptualization, design, experimental operation, and manuscript preparation. TM and HU: analysis and interpretation of genetic analysis data. TT: experimental operation.

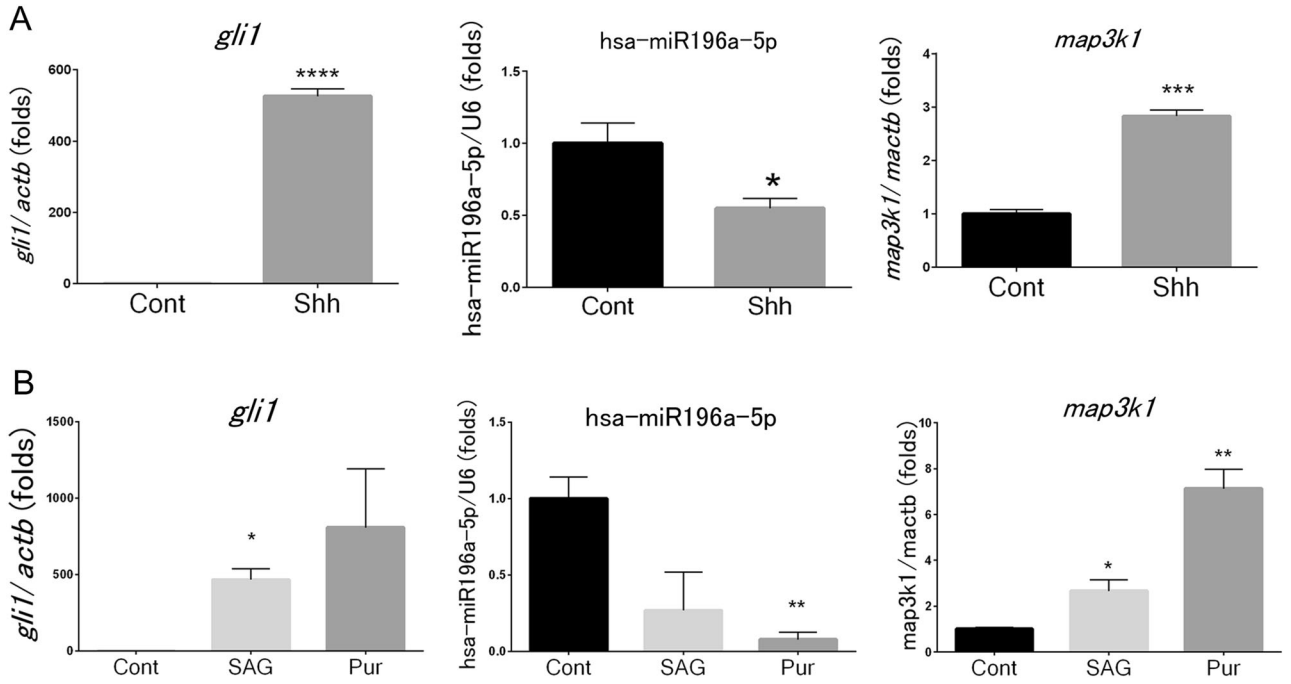

Fig. 2 Exogenous activation of hedgehog signaling decreased miR-196a-5p and increased *map3k1* expression in C3H10T1/2 cells at hedgehog ligand (**a**) and SMO (**b**) levels. Data are mean ± standard error of a triplicated analysis (Student's *t* test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$)

HI, TM and TU: analysis of clinical manifestations. KF and NS: manuscript revision and study supervision.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethics Committee of Chiba University Graduate School of Medicine (IRB number 792) and conformed to the provisions of the Declaration of Helsinki in 1995 (as revised in Seoul 2008).

Informed consent All patients provided informed written consent for molecular and genetic diagnosis.

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