Journal of Pediatric Surgery (2010) **45**, 387–392

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Journal of Pediatric Surgery

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Increased expression of the hedgehog signaling pathway in pediatric solid malignancies

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Received 23 October 2009; accepted 27 October 2009

Key words:
Hedgehog;
Pediatric tumor;
Patched;
Gli1;
Immunohistochemistry

Abstract

Purpose: The activation of the hedgehog (Hh) signaling is involved in the progression of various cancers. However, the correlation between the Hh signaling and tumorigenesis of pediatric malignancies has not been well documented. The present study was undertaken to examine the expression of the Hh signaling pathway in various pediatric tumors to elucidate the role of Hh signaling in pediatric malignancies.

Methods: Surgical specimens were obtained from 68 patients with pediatric malignancies (neuroblastoma, 25; rhabdomyosarcoma, 18; hepatic tumor, 12; and renal tumor, 13). The expression of sonic hedgehog (Shh), its receptor Patched (Ptch), and downstream transcription factor Gli1 was evaluated using immunohistochemical staining.

Results: In neuroblastoma, 96%, 100%, and 68%; in rhabdomyosarcoma, 78%, 100%, and 78%; in Wilms' tumor, 71%, 100%, and 43%; and in hepatoblastoma, 100%, 100%, and 73% of the specimens stained positive for Shh, Ptch, and Gli1, respectively. Differentiated neuroblastoma cells showed more intense Gli1 expression than in immature neuroblastoma cells. In rhabdomyosarcoma, the expression of Gli1 was higher in alveolar type than in embryonal type.

Conclusions: These findings suggest that the Shh-Ptch1-Gli1 signaling pathways are frequently activated in most pediatric malignant tumors. The Hh signaling pathway may therefore play an important role in the differentiation and malignant potential of pediatric malignancies.

The hedgehog (Hh) signaling pathway governs the patterns of cell growth and differentiation in a wide variety of tissues during embryonic development [1,2]. The core components of this signaling pathway are the morphogen sonic hedgehog (Shh) and its receptor patched 1 (Ptch) that inhibits the transmembrane protein smoothened (Smo) [1,3]. When Shh binds with Ptch and the pathway is activated, Smo is unrestrained and also allowed to initiate a signaling cascade that results in the nuclear translocation of the transcription factor Gli [4]. Three vertebrate Gli genes (Gli1, Gli2, and Gli3) have been identified [1]. Among them, Gli1 is a strong positive activator of downstream target genes, and it is itself a transcriptional target of Hh signaling [5].

Presented at the 56th Annual Meeting of the British Association of Paediatric Surgeons, Graz, Austria, June 18-20, 2009.

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0022-3468/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jpedsurg.2009.10.081

Therefore, Gli1 is considered to be a marker of the Hh pathway activation [1,6,7].

Recent data have shown an association between the constitutive activation of the Hh pathway and the initiation of human tumors [8]. The most direct example is Gorlin's syndrome, an autosomal dominant disorder associated with the mutation in *Ptch* [9,10]. Patients with Gorlin's syndrome have an increased incidence of basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma. The somatic mutations of several components of the Hh pathway, including Ptch and Smo, have also been detected in many sporadic basal cell carcinomas and medulloblastomas [8,11]. Furthermore, recent studies have also implicated Hh pathway involvement in a wide range of different tumors including carcinomas of the stomach [12], pancreas [13], breast [14], prostate [15], small cell lung cancer [16], glioblastoma [17], and melanoma [18]. However, the correlation between the Hh signaling pathway and the tumorigenesis of pediatric malignancies has not yet been well documented. The present study was undertaken to examine the expression of the Hh signaling pathway in various pediatric tumors to elucidate the role of Hh signaling in pediatric malignancies.

1. Methods

1.1. Clinical samples and tumor cell lines

A total of 68 pediatric malignant tumor specimens were obtained from patients undergoing surgical resections at Osaka University Medical Hospital (Japan). They included 25 neuroblastomas, 18 rhabdomyosarcomas, 13 renal tumors (7 Wilms' tumors, 2 clear cell sarcoma of the kidney [CCSK], 3 rhabdoid tumor of the kidney [RTK], and 1 renal cell carcinoma), and 12 hepatic tumors (11 hepatoblastomas and 1 embryonal sarcoma). The patients included 30 males and 38 females. Their ages ranged from 0 month to 14 years, with a median age of 1 year 3 months. Written informed consent was obtained for the laboratory analysis according to the institutional requirements before the surgery. In addition, 6 human cell lines derived from childhood pediatric tumors were analyzed. The cell lines were Huh6 (hepatoblastoma), Huh7 (hepatocellular carcinoma), NB19 (*MYCN*-amplified neuroblastoma), RMS-YS, RD (embryonal rhabdomyosarcoma), and RH30 (alveolar rhabdomyosarcoma), and they were obtained from either Riken BRC (Tokyo, Japan) or ATCC (Manassas, Va). All cells were cultured in Dulbecco's modified essential medium with 10% fetal bovine serum and antibiotics.

1.2. Immunohistochemistry

The specimens obtained via either a tumor biopsy or surgical resection were immediately fixed in 10% formalin and embedded in paraffin blocks. The expression of Shh, Ptch, and Gli1 was evaluated using immunohistochemical staining. The paraffin sections were serially cut and stained using the streptavidin-biotin immunoperoxidase technique with the LSAB plus kit (Dako Corp, Carpentaria, Calif). The following monoclonal antibodies were applied as primary antibodies; goat polyclonal anti-Gli1 antibody (1:100; N-16, sc-6153; Santa Cruz Biotechnology, Santa Cruz, Calif), anti-Shh antibody (1:100; N-19, sc-1194), and rabbit polyclonal anti-Ptch antibody (1:200; H-267, sc-9016). The sections were incubated with these primary antibodies overnight at 4°C followed by the secondary antibody. The results were visualized with diaminobenzidine. Specimens of gastric carcinoma in which the Hedgehog pathway is known to be activated were used as positive control specimens [12]. Matched negative controls were stained without primary antibody. In the primary tumors, immunostaining was evaluated by the percentage of positive cells. If more than 10% of the tumor cells were stained, gene expression was considered to be "positive" (<10%, −; 5%-50%, 1+; 50%-90%, 2+; >90%, 3+). To evaluate the reliability of the scoring method, all sections were reviewed 2 times.

1.3. Immunocytochemistry

Six human pediatric tumor cell lines were incubated on Biocoat Culture Slides (Becton Dickinson, San Jose, Calif). The slides were air dried and immersed in ethanol for 15 minutes at room temperature. The remainder of the protocol was identical to that of the immunohistochemistry procedures.

1.4. Statistical analyses

The relationship between the gene expression and tumor stage was estimated using the χ^2 test. A level of $P < .05$ was considered to be significant.

2. Results

2.1. Neuroblastoma

In the 25 cases of neuroblastoma, 24 (96%), 25 (100.0%), and 17 (68%) of the specimens stained positive for Shh, Ptch, and Gli1, respectively (Table 1). The neuroblastoma cell line NB19 showed positive staining of Shh, Ptch, and Gli1. Fig. 1 shows the typical staining of neuroblastoma. The expression was more intense in differentiated cells than in immature cells. All of the Gli1-negative tumors were poorly differentiated. There was a significant relationship between the clinical stage and Gli1 expression (Table 2). Most of the early stage tumors showed a high expression, whereas most of the advanced stage showed negative expression of Gli1. There was no relationship between Gli1 expression and *MYCN* amplification or prognosis.

Table 1 Number (percentage) of tumors with positive gene expression

	Shh, n (%)	Ptch, n (%)	Gli1, n (%)
Neuroblastoma (n = 25)	24 (96)	25 (100)	17 (68)
Rhabdomyosarcoma (n = 18)	14 (78)	18 (100)	14 (78)
Renal tumor (n = 13)			
Wilms' tumor (n = 7)	5 (71)	7 (100)	3 (43)
CCSK (n = 2)	2 (100)	2 (100)	2 (100)
RTK (n = 3)	3 (100)	3 (100)	3 (100)
RCC (n = 1)	1 (100)	1 (100)	1 (100)
Hepatic tumor (n = 12)			
Hepatoblastoma (n = 11)	11 (100)	11 (100)	8 (73)
Embryonal sarcoma (n = 1)	1 (100)	1 (100)	1 (100)

RCC indicates renal cell carcinoma.

2.2. Rhabdomyosarcoma

In 18 cases of rhabdomyosarcoma, 14 (78%), 18 (100%), and 14 (78%) of the specimens stained positive for Shh, Ptch, and Gli1, respectively (Table 1). All of the rhabdomyosarcoma cell lines showed positive staining of Shh, Ptch, and Gli1. Fig. 2 shows the typical staining of rhabdomyosarcoma. The expression was higher in the alveolar type than in the embryonal type; 5 (50%) of 10 alveolar tumors showed high (2+ to 3+) Gli1 expression, whereas 2 (16.7%) of 8 embryonal tumors showed high Gli1 expression.

Table 2 Expression of Gli1 and clinical stage in neuroblastoma

Stage (INSS)	Expression of Gli1 [a]			
	–	1+	2+	3+
1	–	1	3	2
2	–	3	2	1
3	–	-	1	–
4	8	3	1	–

INSS indicates International Neuroblastoma Staging System.
[a] Distribution is significantly different from expected ($P < .05$).

2.3. Renal tumors

Among the 7 Wilms' tumors, 5 (71%), 7 (100%), and 3 (43%) of the Wilms' tumor specimens stained positive for Shh, Ptch, and Gli1, respectively (Table 1). Two CCSKs, 3 RTKs, and 1 renal cell carcinoma showed marked expression of Hh signals.

2.4. Hepatic tumors

In 11 cases of hepatoblastoma, 11 (100%), 11 (100%), and 8 (73%) of the specimens stained positive for Shh, Ptch, and Gli1, respectively (Table 1). One case of embryonal carcinoma and both of the hepatic tumor cell lines (Heh6 and Heh7) showed strong expression of Shh, Ptch, and Gli1,

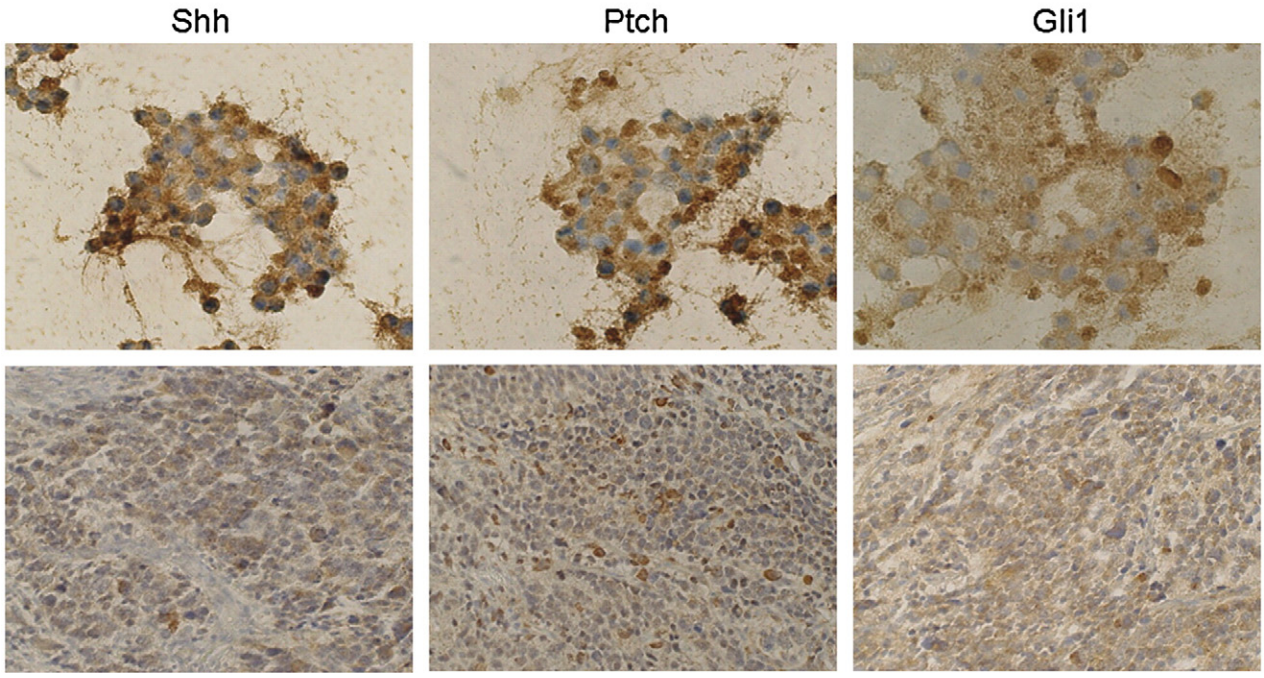

Fig. 1 Immunohistochemical detection of Hh-related genes *Shh*, *Ptch,* and *Gli1* in the human neuroblastoma cell line NB19 (top panel, 400×) and tumor specimens of a 7-month-old girl (bottom panel, 200×). A positive expression was observed in each specimen (brown). The nuclei were counterstained with hematoxylin (purple).

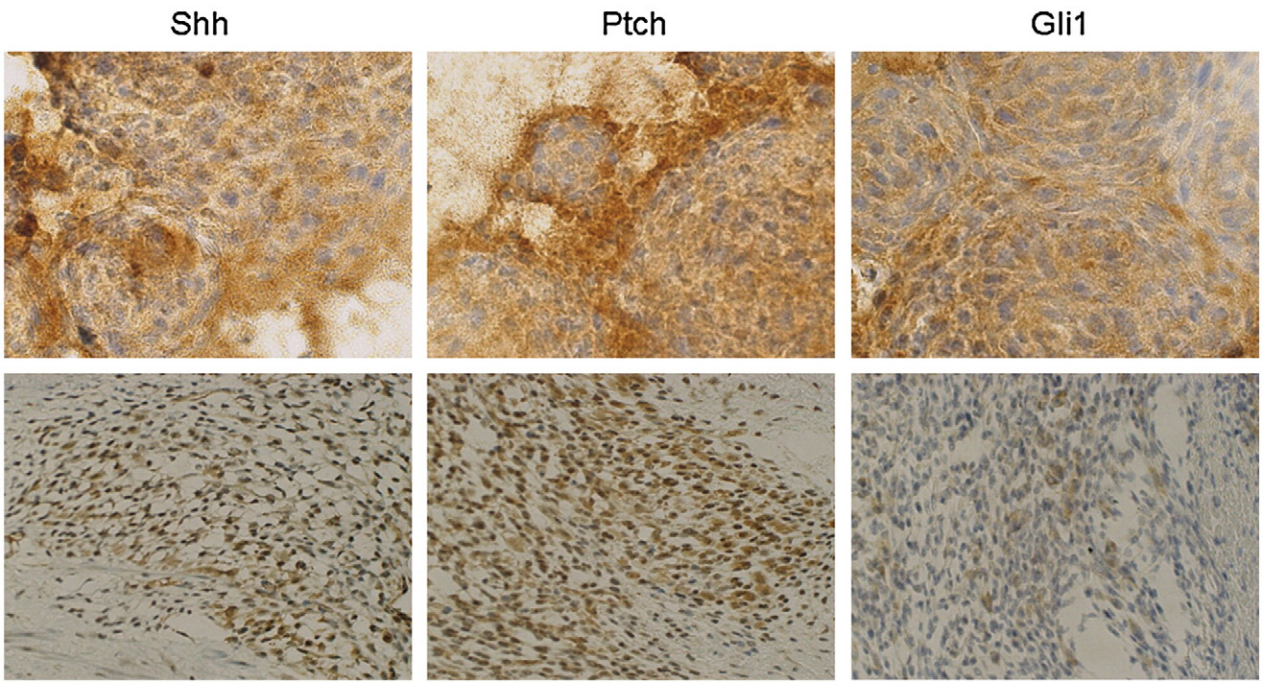

Fig. 2 The immunohistochemical detection of Hh-related genes *Shh*, *Ptch*, and *Gli1* in the human rhabdomyosarcoma cell line RMS-YM (top panel, 400×) and tumor specimens of a 4-year-old girl (bottom panel, 200×).

respectively. Fig. 3 shows the typical staining of hepatoblastoma. No relationship was observed between the Gli1 expression and histologic subtype and either the clinical stage or prognosis.

3. Discussion

The Hh signaling pathway was thought to be active in early-onset pediatric tumors because it plays an important

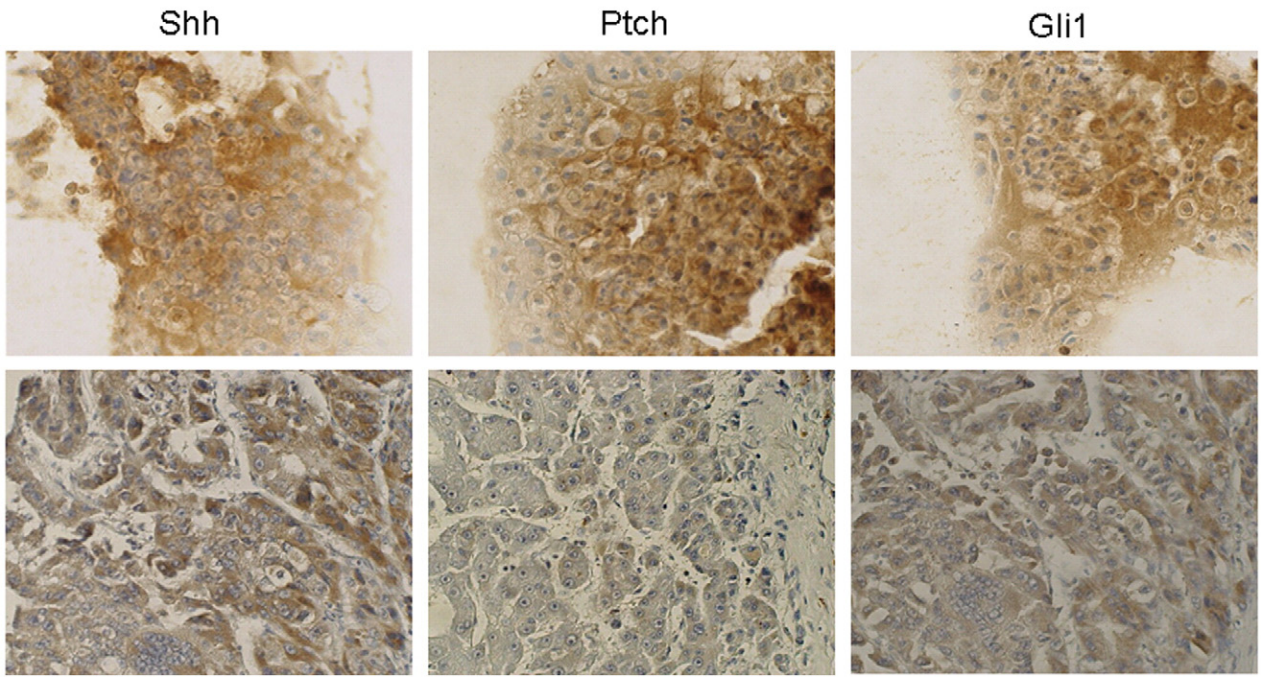

Fig. 3 The immunohistochemical detection of Hh-related genes *Shh, Ptch* and *Gli1* in the human hepatoblastoma cell line Huh6 (top panel, 400×) and tumor specimens of a 2-year-old boy (bottom panel, 200×).

role in embryonic development. The current study showed that the indicators of Hh signaling activity, such as Shh, Ptch, and Gli1 were broadly expressed in various pediatric malignancies and cell lines. Shh and its receptor Ptch were expressed in almost all of the examined pediatric tumors (Table 1). This may indicate that the Hh signaling pathway is broadly present in various pediatric malignancies, and it is also associated with either tumor development or maintenance. On the other hand, the intracellular signaling mediator Gli1 is an activator of downstream target genes, and it is also considered to be a marker of Hh pathway activation [1,6,7]. In this study, Gli1 was expressed in almost 70% of the tumors, in which the activated Hh pathway was considered to play a role in tumor growth and extension.

The germline mutations in the Hh signaling receptor Ptch gene have been found in patients with Gorlin's syndrome, who are predisposed to rhabdomyosarcoma and other tumors [9,10]. In addition, Ptch-deficient mouse strains frequently develop rhabdomyosarcoma [8]. Tostar et al [19] reported that sporadic rhabdomyosarcomas showed an overexpression of Ptch and Gli1 messenger RNA as determined by in situ hybridization. The current study demonstrated the frequent overexpression of these genes at the protein level by immunohistochemistry. These findings lead to the hypothesis that activation of the Hh signaling may contribute to the pathogenesis of sporadic rhabdomyosarcomas. Moreover, the current data demonstrated that a high expression of Gli1 was more frequent in the alveolar type than in embryonal type. Therefore, the activation of Hh signaling may contribute to the biologic aggressiveness of alveolar rhabdomyosarcoma.

The expression of Gli1 in neuroblastoma was more intensive in differentiated cells than in immature cells and also more frequent at an earlier stage than at an advanced stage. These findings indicate that the activation of the Hh signaling pathway may contribute to cell differentiation and maintenance rather than tumor extension in neuroblastoma. Oliver et al [20] used DNA microarrays to identify targets of Shh in Patched knockout mice and found that *cyclin D1* and *MYCN* are important mediators of Shh-induced proliferation and tumorigenesis. If this is true, then the Hh pathways should be activated in MYCN-amplified neuroblastoma. However, in this study, no relationship was observed between the Gli1 expression and *MYCN* amplification. Therefore, the activation of the Hh signaling pathway may not contribute to cell proliferation in sporadic neuroblastoma.

Eichenmuller et al [21] reported that Hh signaling is active in pediatric hepatoblastoma. They also showed that blocking Hh signaling in hepatoblastoma cell lines by the Hh pathway inhibitor cyclopamine leads to a significant decrease in cell viability and apoptosis. Their findings are consistent with the current findings that demonstrate an increased Hh pathway activation in most of the clinical cases of hepatoblastoma.

The expression of Gli1 was observed in only 43% of Wilms' tumors; therefore, the *Hh* pathway is not frequently activated in Wilms' tumors. On the other hand, all of the CCSK and RTK specimens showed intensive expression of Gli1. Cutcliffe et al also reported the activation of Shh genes in CCSK [22]. These findings indicate that the activation of the *Hh* pathway may contribute to the unfavorable biologic behaviors of CCSK and RTK.

Although the treatment of pediatric tumors has dramatically improved for the past 20 years by combining chemotherapy regimens with surgery, the fatal outcome of high-risk patients with either advanced or recurrent cases makes the development of new treatment strategies essential. This study showed the constitutive activation of the Hh pathway in pediatric malignancies using 68 resected specimens. These findings suggest that the Hh pathway is a potentially novel therapeutic target against pediatric malignancies showing an Hh pathway overexpression. Recently, a plant-derived steroidal alkaloid cyclopamine, which inhibits the Hh pathway by antagonizing *Smo* was shown to suppress the growth of various cancer cells [12,14,21]. In this study, the Gli1 expression was observed to increase, especially in alveolar rhabdomyosarcoma and unfavorable renal tumors. It is hoped that the Hh pathway can therefore be a potential therapeutic target for these unfavorable tumors. In this study, the 6 pediatric tumor cell lines showed a marked expression of Shh, Ptch, and Gli1, thus indicating that the Hh pathway is activated. The next step is to investigate the effects of the inhibition of Hh signaling in these cell lines to elucidate whether the Hh pathway can indeed be the novel therapeutic target against pediatric malignancies.

Acknowledgments

This work was supported by grants from the Ministry of Education, Culture, Sports, and Technology of Japan (grant no. 20390452 and 20791299).

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