Involvement of p16 and PTCH in pathogenesis of melanoma and basal cell carcinoma

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Abstract. The involvement of two tumor suppressors p16 and Ptch in pathogenesis of cutaneous melanomas and basal cell carcinomas (BCCs) was studied through expression of Ptch and p16 and genetic alterations in 9p21 region (p16) and in 9q22.3 region (PTCH) of chromosome 9. Immunohistochemical analyses of paraffin-embedded tissues with Ptch and p16 antibodies, typing for 9q22-q31 and 9p21 region with polymorphic markers and p16 and Ptch mutation detection was done. Higher expression of p16 and Ptch in melanoma and BCC of the skin was frequently detected in studied cases. However, allelic loss of PTCH region occurs more frequently in BCCs than loss of heterozygosity of p16 region. Both types of tumors, BCCs and melanomas, suggest involvement of Hh-Gli signaling pathway, but using different mechanisms.

Introduction

The Hh-Gli signaling pathway is receiving increasing attention as a crucial regulator of not only embryonic organogenesis but also as an oncogenic pathway implicated in diverse human tumors. The pathway begins with binding of the ligand protein Hedgehog to the transmembrane receptor Patched (Ptch), which then releases its inhibition of Smoothened (Smo) and a cytoplasmatic cascade of phosphorylation and dephosphorylation events leads to activation of transcription factors Gli1-3 and transcription of target genes, which include Cyclin D, Cyclin E, members of Wnt and TGFß signaling pathways and Ptch itself (1-3).

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The pathway is aberrantly activated in many human cancers and in basal cell carcinomas (BCC) mutations in Patched, Hedgehog and even Smoothened are frequent (4).

In melanoma pathology, indications of involvement of the Hh-Gli pathway started to show promise in therapeutic strategies very recently. Although involvement of p16 in melanoma development has been shown either through loss of heterozygosity or mutation screenings of p16, connections of melanoma and Hh-Gli pathway are very recent (5). The CDKN2A locus encodes a cyclin-dependent kinase inhibitor p16 that acts to inhibit cell cycle progression in the G1 phase by binding and inhibiting CDK4/6 kinases. Loss of p16 leads to deregulated CDK4/6 action and promotion of cell divisions.

We show a combined role of p16 and Hh-Gli signaling pathway in the pathogenesis of skin neoplasia, basal cell carcinoma and melanoma as the two most frequent types of skin neoplasia.

Materials and methods

BCC and melanoma cases. Twenty sporadic malignant melanomas (Table I) (Breslow thickness 3-5 mm, Clark's level 5 or less, classified as superficial spreading melanoma (SSM), nodular melanoma (NM), acral lentigous melanoma (ALM) or lentigo maligna melanoma (LMM) and twenty BCC samples (Table II) classified as carcinoma baseocellulare solidum (CBS), adenocysticum (AC), morpheiphorme (M) or superfitiale multicentricum (SM) were collected from Sestre milosrdnice University Hospital in Zagreb. The main clinical and histopathological features are summarized in Tables I and II. The samples were stripped of identifiers and were collected with consideration to all necessary ethical and legal requirements. Ethics Committee of the hospital approved these studies. The experiments were conducted in accordance with the Declaration of Helsinki.

All cases have been routinely processed and paraffin-embedded. Two 5-μm sections of tumor tissue were cut from paraffin block for each immunohistochemical analysis and 25-μm sections were microdissected for loss of heterozygosity (LOH) analysis (Table III). The tissues were histologically evaluated by a trained pathologist.

Table I. Melanoma samples and IH analyses.

No.	Age	Gender	Type[a]	Clark	Breslow	Size	No. of mitoses	Localization	Immunohistochemistry	
									Ptch	p16
1	55	M	LMM	4	4	13	6	Face	++	++
2	33	F	SSM	3	3	19	3	Abdomen	-	-
3	60	M	SSM	3	3	15	2	Neck	-	-
4	35	M	SSM	4	4	7	8	Back	++	+/-
6	70	M	SSM	4	5	15	5	Face	-	++
8	46	M	SSM	3	5	10	20	Back	++	++
9	47	M	SSM	3	5	11	10	Neck	+	+
18	62	F	SSM	3	4	16	10	Upper arm	+	++
10	74	M	NM	5	5	39	11	Back	++	++
11	62	M	NM	5	5	45	11	Abdomen	++	++
12	73	F	NM	4	5	25	20	Back	+	+/-
13	77	F	NM	4	5	12	3	Leg	++	-
15	48	M	NM	4	5	11	8	Back	++	+/-
16	20	M	NM	3	4	10	13	Neck	++	++
7	56	F	ALM	4	4	15	7	Face	++	++

[a]SSM, superficial spreading melanoma; NM, nodular melanoma; ALM, acral lentigous melanoma and LMM, lentigo maligna melanoma.

Immunohistochemical method. Ptch and p16 proteins were analyzed as follows: slides were deparaffinized, prewarmed in Epitope Retrieval Solution (Dako, Glostrup, Denmark) and kept at 95-99˚C for 40 min and then washed in PBS 3x5 min. Endogenous peroxidase was blocked with 3% H_2O_2/methanol for 10 min, washed 3x5 min in PBS. The sample was surrounded by PAP-PEN (Kiyota, Baltimore, MD). Protein block serum-free (Dako) was added for 10 min.

For Ptch protein: primary anti-PTCH-CTP rabbit antibody in 2X BSA/PBS (a kind gift from Dr Allen Bale) was incubated overnight at 4˚C. For p16 protein: primary anti-p16 mouse antibody was used according to manufacturer's instructions (Dako). After washing in PBS 3x5 min secondary anti-rabbit or anti-mouse antibody (Dako) was added for 60 min at room temperature. Slides were again washed in PBS 3x5 min and PAP (Dako) was added for 60 min. After washing in PBS 3x5 min DAB was added for 7 min. Slides were then counterstained with hematoxylin and embedded in Canada balsam. Negative controls were stained by the same protocol, omitting the primary antibody step. Instead, slides were incubated with 2% BSA/PBS for the same period of time as the samples.

LOH, single-strand conformation polymorphism (SSCP) and sequencing. DNA samples from blood leukocytes and tumor fresh or paraffin tissues were extracted by standard methods. DNA samples were typed for eight short tandem repeat polymorphisms: D9S196, PTCH intra, D9S287, D9S180, and D9S127 spanning chromosome region 9q22.3-q31, and D9S104, D9S126 and IFNA spanning 9p21 region. PCR reactions were performed in 25 μl reaction mixture containing 100 ng of template DNA or 1-5 μl of crude extract from paraffin-embedded tissue prepared after microdissection, 200 μmol/l deoxynucleoside triphosphate (dNTP), 1.5 mmol/l $MgCl_2$, 10 pmol/l each primer, 1.25 U Taq polymerase (AmpliTag Gold® DNA polymerase, Applied Biosystems, Foster City, CA) in 10 mmol/l Tris-HCl buffer. PCR system (Gene Amp PCR system 2400, Applied Biosystems) was set for 25 to 35 cycles, with the parameters published previously (6). For LOH analysis 5-10 μl of PCR product was loaded on 0.1x40x32 cm native 8-12% polyacrylamide gel, at 10-15 V/cm in 1X Tris/borate/EDTA (TBE) buffer and for SSCP 3-5 μl of PCR product was loaded with denaturing buffer, denatured 10 min at 55˚C and then loaded on 0.1x16x18 cm native 6-9% polyacrylamide gel at 250 V in 1X TBE buffer.

DNA was visualized by the silver staining method, briefly: gels were fixed by submersion in ethanol, oxygenated with HNO_3, followed by treatment with $AgNO_3$ and visualized with Na_2CO_3/formalin. The reaction was stopped with acetic acid.

Only the PCR products that showed aberrant SSCP pattern were sequenced. Before sequencing, amplified PCR products were purified with QIAquick purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR products were then sequenced in both directions by using the same primers and the big dye terminator cycle sequencing kit (Applied Biosystems). Sequencing analysis was performed on an automatic sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems).

Statistical analysis. Non-parametric Spearman rank correlation coefficient was used to assess the degree of correlation between pairs of variables. Mann-Whitney test was used to check if there were differences in protein expression between different types and subtypes of skin tumors. Correlations and differences were considered significant if two-tailed p-value was <0.05. All statistical calculations were done using Statistica 7.0 software (Statsoft, Inc., Tulsa, OK, USA).

Table II. BCC samples and IH analyses.

No.	Age	Gender	Type[a]	Localization	Immunohistochemistry	
					Ptch	p16
21	69	F	CBS	Nose	+/-	++
22	81	F	CBS	Face	++	-
23	63	M	CBS	Face	++	++
24	46	F	CBS	Face	+	++
25	76	F	CBS	Nose	+	++
26	76	M	CBS	Face	+	+/-
27	59	M	CBS	Face	+	++
28	54	M	CBS	Nose	++	++
29	60	M	CBS	Abdomen	++	+
30	60	F	CBS	Face	++	++
31	67	M	AC	Nose	++	+
32	72	M	AC	Face	+	++
33	70	F	AC	Face	+	++
34	66	M	AC	Face	++	++
35	75	M	AC	Abdomen	++	+/-
36	75	M	AC	Face	++	-
37	68	F	AC	Face	++	-
38	81	F	AC	face	++	+
39	73	M	M	Nose	+	++
40	81	F	SM	Upper arm	++	-

[a]CBS, carcinoma baseocellulare solidum; AC, carcinoma baseocellulare adenocysticum; M, carcinoma baseocellulare morpheiphorme and SM, carcinoma baseocellulare superfitiale multicentricum.

Table III. Typing with polymorphic markers for 9p21 (D9S104, D9S126, IFNA) and 9q22-q31 (D9S196, PTCH intra, D9S287, D9S180, D9S127) region.

	Polymorphic marker[b]							
BCC sample-type[a]	D9S104	D9S126	IFNA	D9S196	PTCH intra	D9S287	D9S180	D9S127
41 -CBS	het	ho	ho	het	ho	LOH	het	het
42 -CBS	ho	ho	ho	het	het	het	het	het
43 -CBS	-	-	-	ho	-	LOH	-	het
44 -CBS	ho	-	het	ho	ho	het	het	ho
45 -CBS	ho	het	het	ho	ho	het	het	het
46 -CBS	het	ho	ho	ho	LOH	het	ho	ho
47 -CBS	ho	ho	het	ho	ho	LOH	LOH	het
48 -CBS	ho	ho	ho	het	het	het	het	ho
49 -CBS	ho	ho	ho	het	het	het	het	ho
50 -CBS	-	-	-	LOH	ho	LOH	het	LOH
51 -CBS	-	-	-	LOH	LOH	LOH	ho	ho
52 -CBS	-	-	-	LOH	ho	ho	LOH	-
53 -AC	ho	ho	het	LOH	ho	ho	LOH	ho
54 -AC	ho	LOH	het	ho	ho	LOH	ho	het
55 -AC	het	het	het	het	ho	het	het	het
56 -SM, AC, CBS	het	ho	ho	het	ho	het	ho	het
57 -SM, CBS	het	ho	het	het	het	het	ho	ho
58 -SM	ho	het	het	het	ho	LOH	LOH	ho

[a]CBS, carcinoma baseocellulare solidum; AC, carcinoma baseocellulare adenocysticum; M, carcinoma baseocellulare morpheiphorme and SM, carcinoma baseocellulare superfitiale multicentricum. [b]het, heterozygous; ho, homozygous and LOH, loss of heterozygosity.

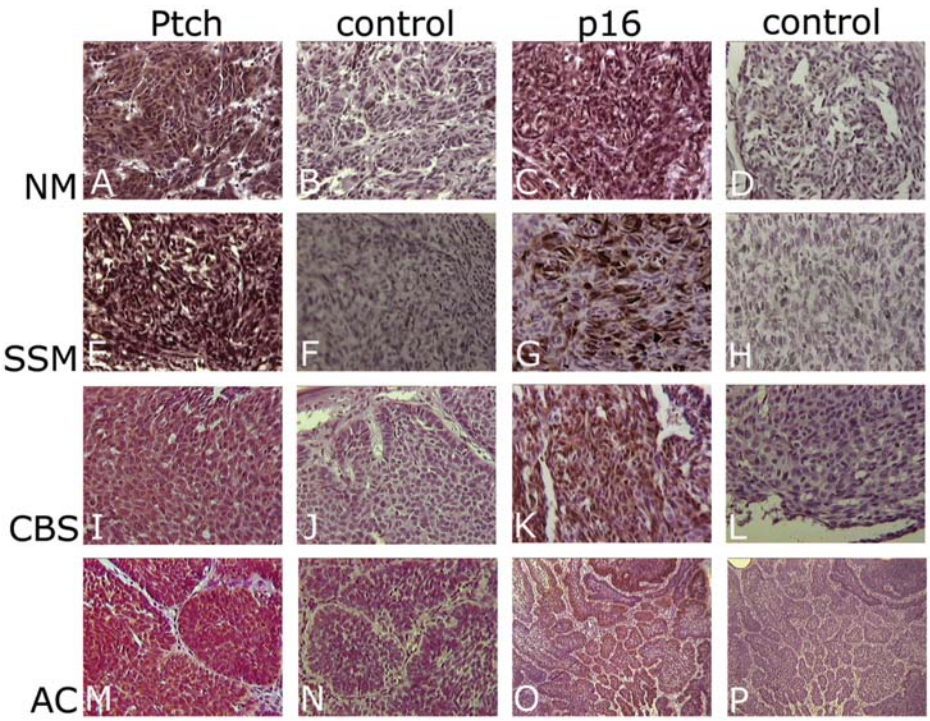

Figure 1. Immunohistochemical staining of NM, SSM, CBS and AC samples with Ptch and p16 antibody with accompanying control.

Results

Fifteen melanoma and 20 BCC samples were stained for Ptch and p16 protein expression. Expression was determined by a trained pathologist and classified as either very strong (++), strong (+), weak (+/-) or no expression (-) (Fig. 1).

Melanoma. Out of 15 melanoma samples, 9 (60%) showed very strong expression of Ptch, 3 (20%) weak and 3 (20%) no expression (Table I). Results for p16 staining were similar in proportion, with 8 (53%) with very strong staining, 1 (7%) strong, 3 (20%) weak and 3 (20%) no expression. However, when we compared individual expression of these proteins per sample the correlation was determined as not significant. Therefore, Ptch and p16 expression do not appear to be correlated in melanoma samples. Also, expression of Ptch and p16 proteins does not significantly differ between melanoma subtypes. Expression of neither of these two proteins does not correlate significantly with Clark index, Breslow index, tumor size or number of mitoses.

BCC. Out of 20 BCC samples, 12 (60%) showed very strong staining for Ptch protein, 7 (35%) strong, and 1 (5%) weak (Table II). There were no BCC samples without Ptch expression. For p16, 11 (55%) samples showed very strong staining, 3 (15%) strong, 2 (10%) weak and 4 (20%) no expression. In these samples, we found a negative correlation between Ptch and p16 expression, as determined by Spearman's rank correlation, with r=-0.5187 (95% confidence interval -0.7872 to -0.0848) and p=0.0191. These results show that expression of these two genes in BCC is inversely correlated, e.g. when Ptch expression is high then p16 is lower and *vice versa*. Also, there is no difference in expression of Ptch and p16 proteins between BCC subtypes.

LOH typing with polymorphic markers for 9p21 (D9S104, S9S126 and IFNA) region where p16 maps, and 9q22-q31 (D9S196, PTCH intra, D9S287, D9S180 and D9S127) region where PTCH mapped was done for 18 BCC samples. LOH was shown for 9p21 region in one case of AC and for 9q22q31 region in 7 of 12 CBS samples, two of three AC samples and one SM sample (Table III).

No p16 mutations were found in any of the melanoma samples analyzed. Of the 12 BCC samples analyzed for PTCH mutations, mutations were found in two samples. Mutation 1450G>A (G484R) in exon 10 was found in sample 58 (SM) and in the sample 44 (CBS) mutation 2066C>T (P689L) in exon 14.

Discussion

Two types of most frequent skin tumors gave indications on alterations of signaling through Hh-Gli pathway and influences on cell cycle machinery.

Basal cell carcinoma is the most common skin tumor and melanoma is the most aggressive, both are increasing in frequency. Although widespread, both are relatively poorly understood at the level of molecular pathogenesis. Progress over the past decade has identified an array of molecular alterations in these tumors that may provide suitable targets for new molecular therapeutics.

A number of genetic alterations have been characterized; some of them involve gain-of-function changes in oncogenes as well as loss-of-function of genes that act as suppressors of tumor formation. The spectrum of changes differs among these tumor types.

Basal cell carcinoma involves Ptch loss-of-function, including gain-of-function in Shh, Smo and Gli proteins, dominantly based on mutations in tumor suppressor PTCH (7).

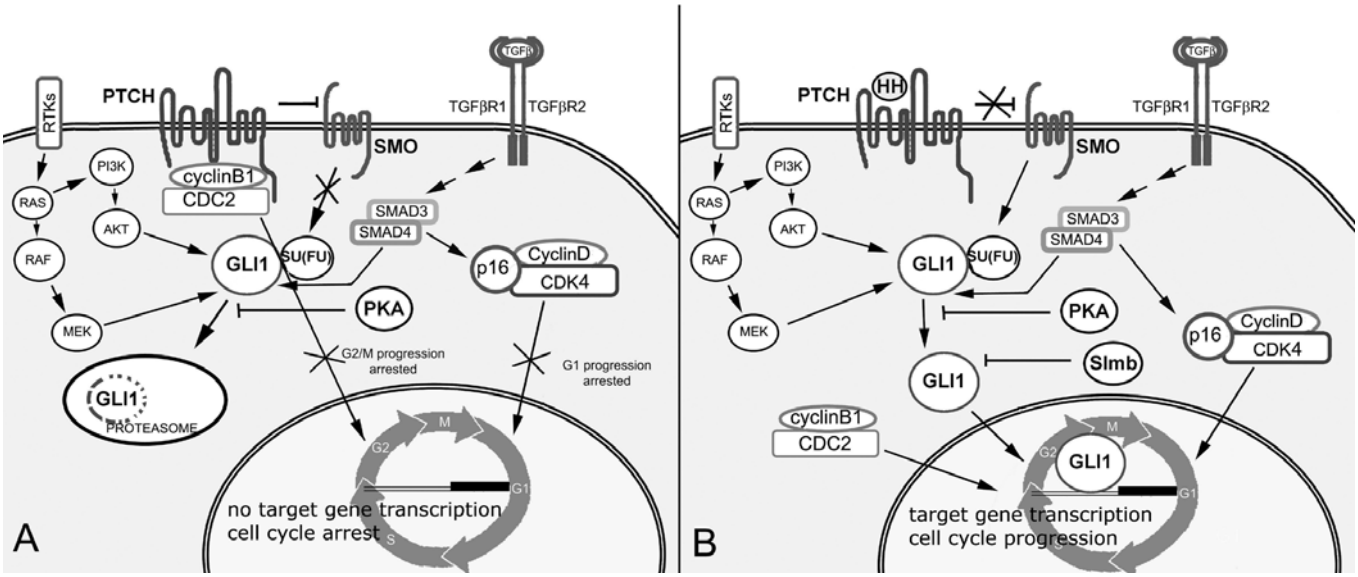

Figure 2. A hypothetical scheme of the interactions of Hh-Gli signaling, receptor tyrosine kinase Ras-MEK-PI3K-Akt signaling, cell cycle regulation, TGFß and proteasomal degradation: (A) In the absence of Hh, Ptch represses Smo and the pathway is inactive, Ptch binds the cyclin B1/CDC2 complex, inhibiting G2/M phase of the cell cycle. Gli1 is bound in the cytoplasm and processed in proteasome, no transcription of target genes; (B) In the presence of Hh, Hh binds to Ptch, Smo is relieved and activates a cascade that leads to transcription of the pathway target genes. Cyclin B1/CDC2 complex is released and contributes to cell cycle progression.

But, involvement of Ptch is no longer restricted only to those tumors, great number of other tumor types require the intact Hh-Gli pathway. Recent results suggest PI3K-dependent Akt activation as essential for Hh-Gli signaling pathway (5,8).

There are also discoveries by others on integration of Hh-Gli and other proliferating pathways, such as Ras/Akt and PI3K (5,8), TGFß (9), FGF (10), K-Ras independently of PI3K pathway (Fig. 2). PI3K pathway is up-regulated in invasive melanomas and activity of Akt is increased in >60% of melanomas. However, K-Ras activation can be mediated toward B-Raf or toward PI3K-Akt direction resulting in increase of Gli activity (11).

Involvement of p16 (cyclin-dependent kinase inhibitor) in melanoma development has been demonstrated either through allelic loss within chromosome 9p21 or mutations of p16 (12). p16 inhibits cell cycle progression in the G1 phase, but loss of p16 leads to deregulation of CDK4/6 and promotion of cell divisions. Our observation on importance of p16 in analyzed melanoma subtypes was confirmed with immunohistochemical staining. Also, role of Ptch in melanoma development is evident, our results (Table I) contribute to findings that great number of tumor types require intact Hh-Gli pathway. In melanoma we have seen that activity of Ptch is not correlated to p16 suggesting that the cell cycle progression and Hh-Gli signal transduction are not related.

Inhibition of Hh-Gli pathway by cyclopamine, acting on transmembrane protein Smo, shows great therapeutic potential; therefore expression of target genes (PTCH is one of target genes) can be blocked affecting transcriptional factor Gli1. There are two other ways to modulate Gli1 function, through down-regulation of Ras/Akt cascades, and another through SUFU, PKA or other Hh-Gli modifiers. Both may normally regulate Gli1 activity in normal and pathological conditions.

Since it was cloned in 1996, importance of Ptch protein was first shown in etiology of BCC, for both sporadic and associated with Nevoid Basal Cell Syndrome (NBCS) (13). No methylation of PTCH promoter in BCC of the skin was shown. Activation of the Hh-Gli pathway in these tumors occurs mainly because of mutations in PTCH (14). In some BCC samples large deleted regions of PTCH were shown (Table III). This is even more pronounced in Gorlin syndrome patients (15).

In this study, mutations in PTCH were found in two of the 12 BCC samples (16.67%). Higher rate of mutations would be expected, as very often previously described. Also, mutations in SHH and SMO are not uncommon. In genetic screenings carried out by us, mutations on those two genes were found (data not shown) but not with as high incidence as was described by others. Rather low rate of mutation detection in those samples can be explained with limitations of the screening method used (SSCP), because only variable patterns were sequenced. Both mutations (1450G>A and 2066C>T) exhibit UV fingerprint (C>T) characteristic for mutations found in BCCs (16). One of the mutations (1450G>A, positioned in exon 10) was previously described in a Gorlin syndrome case (17), while the other (2066C>T, positioned in exon 14) has not been described before. Both mutations are missense (G484R and P689L) leading to changed amino acid sequence of PTCH protein. According to the alignment of the amino acid sequences of human, mouse, chicken, zebrafish and frog PTCH protein (Swiss-Prot accession numbers Q13635, Q61115, Q90693, Q98864 and Q98SW6, respectively), glycine at position 484 and proline at position 689 are highly conserved among species so various tools that predict possible impact of an amino acid substitution on the structure and function of a human protein/PolyPhen (18), PMut (19), Align-GVGD (20)/have predicted that those amino acid changes are most likely to be damaging. Mutation G484R occurs in third transmembrane domain and P689L mutation occurs in third cytoplasmic loop.

Negative correlation of Ptch and p16 expression in BCCs is in agreement with results by others, as with cell cycle regulators Cyclin D (21) and B (22). Ovarian carcinomas (23) and benign ovarian tumors (a model system we have developed on ovarian dermoid cell lines) (24) show an up-regulated Hh-Gli pathway and differential activity during cell cycle in a similar way (data not shown).

The role of Ptch in G2/M phase of cell cycle was demonstrated by activation of M phase promoting factor, giving antiproliferative role to Ptch by interaction with cyclin B1 (25). Cell cycle progression through G1 phase and Ptch interaction with cyclin D and progression through G2 phase by interactions with cubitus interruptus and Cyclin E promoter were published earlier (25-27).

Besides, there are other known tumor suppressors involved in BCC pathology, for example tumor suppressor p53 (21,28). Tumor suppressor p53 is found to be mutated in 50% of all tumors. Protein p53 is crucial for cell cycle regulation and apoptosis. There is a link between Ptch and p53 in BCC development (29).

The above observations suggest that in melanoma pathology the Hh-Gli pathway seems to be a promising target in therapeutic strategies. Although involvement of p16 in melanoma development has been shown either through LOH or mutation screenings of p16, connections to the Hh-Gli pathway have not been shown until recently.

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