Hedgehog-Producing Cancer Cells Respond to and Require Autocrine Hedgehog Activity

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Abstract

A number of Smoothened (SMO) pathway antagonists are currently undergoing clinical trials as anticancer agents. These drugs are proposed to attenuate tumor growth solely through inhibition of Hedgehog (HH), which is produced in tumor cells but acts on tumor stromal cells. The pivotal argument underlying this model is that the growth-inhibitory properties of SMO antagonists on HH-producing cancer cells are due to their off-target effects. Here, we show that the tumorigenic properties of such lung cancer cells depend on their intrinsic level of HH activity. Notably, reducing HH signaling in these tumor cells decreases HH target gene expression. Taken together, these results question the dogma that autocrine HH signaling plays no role in HH-dependent cancers, and does so without using SMO antagonists. Cancer Res; 71(13): 4454–63. ©2011 AACR

Introduction

Hedgehog (HH) signaling plays a critical role in the homeostasis of diverse adult tissues. Consistent with its important role in tissue maintenance, deregulated HH signaling has been implicated in common human cancers of different origins, such as melanoma, leukemia, glioblastoma, and those derived from tumors of the breast, digestive tract, pancreas, lung, and kidney (1, 2). Mammals express 3 HH family members, Sonic hedgehog (SHH), Desert hedgehog (DHH), and Indian hedgehog (IHH), whose binding to their receptor Patched1 (PTCH1; refs. 3, 4) relieves PTCH1-mediated repression of Smoothened (SMO) activity (5–7). This active SMO modulates HH activity through regulation of the GLI family of transcription factors GLI1, GLI2, and GLI3 (8–10) in a manner that is antagonized by the GLI interacting protein suppressor of fused (SUFU; refs. 11–13). Aberrant activation of the HH pathway in cancer results from the overexpression of HH proteins, activating mutations in downstream pathway components such as SMO, PTCH1, or SUFU, or gene amplification of GLI1—a known transcriptional effector and HH target gene (1, 2).

HH has a survival role for diverse cancers in which no mutations in HH signaling components were identified (14–20). Investigators used various SMO antagonists (21–24) to show the HH signaling requirement of cancer cells. Recently, it was suggested that these SMO antagonists exhibit off-target effects when used at the concentrations necessary to inhibit the proliferation of HH-producing cancer cells (25). These arguments were based on the observation that a higher concentration of SMO antagonist is required to inhibit tumor cell proliferation than that required to inhibit HH activity in fibroblasts. Moreover, a lack of association between expression of the HH target gene GLI1 and sensitivity of a large panel of cancer cell lines to SMO antagonists was noted (25). This argument was developed in vivo, using human primary cancers or established cancer cell lines carried as xenografts in athymic mice, or mouse models of pancreatic cancer, to suggest that SMO antagonists act exclusively on the tumor stroma (25–27). One of these studies also showed that, whereas SMO antagonists had marked effects in reducing the expression of stromal derived mouse GLI1, it was unable to reduce human GLI1 expression in the cancer cells themselves (25).

We previously reported that primary non–small cell lung carcinomas (NSCLC) frequently elaborate a constitutively active HH signaling pathway, which appeared to originate in the cancer cells themselves (28, 29). Using 2 structurally distinct SMO antagonists, we showed that a subset of NSCLC cell lines require HH activity for their viability. Given the debate surrounding the specificity of SMO antagonists, this study sought to elucidate the role HH signaling plays in regulating the tumorigenicity of NSCLC cells. This study reports the dependence of NSCLC cells on HH activity for their proliferation and tumorigenesis, primarily using a loss-of-function...
approach that is independent of the use of SMO antagonists. Conversely, overexpression of HH proteins increased the tumorigenic properties of NSCLC cells. Furthermore, reducing GLI1 expression in NSCLC cells significantly reduced the tumorigenicity of NSCLC xenografts in vivo. These findings suggest that NSCLC cells elaborate a highly active HH signaling pathway that is required for their in vitro growth and in vivo tumorigenicity.

Materials and Methods

NSCLC cell lines HOP62, A549, H23, and H522, were obtained from the Developmental Therapeutics Program (DTP), and U1752 and H157 were a gift from Dr. Neil Watkins and Dr. Stephen Baylin (Johns Hopkins University, Baltimore, MD). These cell lines were maintained in RPMI-1640 medium with 10% FBS (Invitrogen) supplemented with penicillin and streptomycin. SHH-Light2 cells (30) and SHH-I cells (23, 31) were cultured as described. The various chemical inhibitors were obtained or purchased (DLF and derivatives: DTP; DMSO and Tomatidine; Sigma Aldrich; SANT1: Calbiochem), and their inhibition of HH (conditioned medium; SHH-I cells) signaling assayed in SHH-Light2 cells (30). All experiments were repeated independently at least 3 times. Unless noted otherwise, data shown are representative, error bars represent SD from the mean, and statistical significance was calculated by Student 2-tailed t test. The values of P < 0.05 were considered statistically significant and denoted by an asterisk (*).

Assays

Expression of the indicated genes was assessed by quantitative real-time PCR (qRT-PCR) as described (ref. 32; and Supplementary Table S1). NSCLC cell lines were plated at 500 cells were transfected with SHH or pcDNA3.1 and then drug selected. 103 cells) in 96-well plates. The concentration of each compound that results in 50% growth inhibition of each cell line (GI50) within the NCI-60 panel is determined. Taken together, individual GI50 across all 60 cell lines, for any specific compound, gives each compound a unique “signature” profile. When queried against the NCI-60 panel such a signature can be used to identify different molecules that inhibit similar biological processes. Previously, we had the growth-inhibitory signature of the SMO antagonist SANT1 (30) determined at the DTP. These results were subsequently confirmed in our laboratory, using the second SMO antagonist cyclopamine and a panel of NSCLC cell lines (28). This work provided a validated SMO inhibitor signature (Fig. 1A).

Contrary to what has been recently proposed (25), our previous results indicated that SMO antagonists inhibit the proliferation of NSCLC cells in a specific manner. If indeed these SMO antagonists specifically attenuated NSCLC cell growth, we reasoned that compounds identified at the DTP based on their similar GI50 signature to that of SANT1 would function to inhibit HH signaling. The DTP database was then queried for compounds that exhibit a similar growth-inhibitory signature to that of SANT1, and 4 compounds were identified that we designated DLF1–4. The ability of these compounds to inhibit HH-dependent activity was tested in SHH-Light2 cells (30), which stably express a HH-dependent luciferase reporter construct (Fig. 1B). Among the compounds tested, DLF3 reduced HH signaling with a similar potency to cyclopamine. Several structural derivatives of DLF3 were available at the DTP, which we subsequently obtained and

Immunohistochemistry and in situ hybridization

Tissue samples resected from NSCLC patients were fixed in formalin, paraffin embedded, and cut into 5-μm thick sections to prepare slides according to an Institutional Review Board–approved protocol or purchased (US Biomax) and then probed for the expression of SHH, PTCH, and GLI1 proteins by using anti-SHH (H-260, Santa Cruz Biotechnology (SCBT)), anti-PTCH (C-20, SCBT), and anti-GLI1 antibody (32), respectively. RNA in situ hybridization on adjacent sections was done to examine the expression of SHH, PTCH, and GLI1 by using digoxigenin-labeled antisense RNA probes as previously described (20, 34). The plasmids used to make RNA probes were a gift from Dr. Ariel Ruiz i Altaba (University of Geneva Medical School, Switzerland). All images were acquired by using a Leica DMIRE2 microscope.
tested. Typically, these derivatives had similar potency to DLF3 (Fig. 1C); however, DLF3b was slightly more potent than DLF3. As SANT1 acts on SMO, and DLF3 and SANT1 displayed similar signatures against the NCI-60 panel, we hypothesized that DLF3 also acts by inhibiting SMO. To explore this possibility, the SMO agonist SAG (30) was used to determine the ability of DLF3b to inhibit SAG-mediated activation of a HH reporter gene in SHH-Light2 cells. Consistent with our hypothesis, DLF3b attenuated SAG-mediated HH activity in a dose-dependent manner, with an IC50 of 500 nmol/L (Fig. 1D). The identification of a novel HH pathway inhibitor, DLF3, based on its similarity to the growth-inhibitory signature of a known SMO antagonist, is consistent with SMO antagonists acting in an on-target manner on HH-producing NSCLC cells. Although these results are consistent with DLF3b acting directly on SMO, they do not rule out that DLF3b may alternatively act downstream of SMO. Interestingly, DLF1, 2, and 4 have a similar growth-inhibitory signature to SANT1 but do not seem able to directly inhibit the HH signaling. We speculated that these compounds might inhibit the proliferation of HH-dependent cancer cells at a step downstream of GLI1 activity.

If NSCLC cells harbor an active HH signaling pathway, we reasoned they should express the key signaling components. We therefore examined the status of the HH signaling pathway in a set of 6 NSCLC cell lines, HOP62, A549, U1752, H23, H522, and H157, using qRT-PCR assays. All these cell lines expressed the 2 HH target genes GLI1 and PTCH1, which are faithful markers of HH activity, and key signaling effectors: SMO and GLI2 (Supplementary Fig. S1A; refs. 10, 35). They also expressed the HH ligands SHH, DHH, and IHH, although the relative expression of the different ligands varied among these cell lines (Supplementary Fig. S1B). The expression of the HH target genes GLI1 and PTCH1 correlated with the expression of SMO in these cells, but the association between target gene expression and expression of HH proteins and GLI2 was less apparent. Interestingly, we and others have also shown...
Increased SMO expression in various primary tumors relative to adjacent normal tissue (28, 36, 37). The widespread expression of HH proteins and of the key effector SMO, when combined with that of the HH target genes GLI1 and PTCH1, indicate that NSCLC cells likely harbor a constitutively active HH signaling pathway.

To uncover a requirement for HH activity in NSCLCs, without using pharmacologic SMO antagonists, a shRNA-mediated knockdown strategy was used. Specifically, 6 positive regulators of the HH signaling pathway (GLI1, GLI2, SMO, SHH, DHH, and IHH) were targeted by using 2 independent, validated shRNAs (Supplementary Figs. S2 and S3) to knock down each component (Supplementary Table S2), and the growth or tumorigenic properties of a panel of NSCLC cell lines examined. Both GLI1-specific shRNAs attenuated proliferation of the majority of these NSCLC cell lines (Fig. 2A and Supplementary Fig. S4A), indicating a widespread requirement of the HH target gene GLI1 for proliferation of NSCLC cells. Consistent with proliferation being dictated by the level of GLI activity, the cell line with the highest level of GLI1 expression, H522 cells, was most resistant to GLI1 knockdown (see Fig. 2A in conjunction with Supplementary Fig. S1A).

Similar results were obtained by using shRNAs against SMO and GLI2 (Fig. 2B and C and Supplementary Fig. S4A). These results showed that there is often a requirement of HH signaling in NSCLC cell proliferation.

HH signaling has been proposed to act as a survival factor in tumor cells (10, 38, 39), preventing programmed cell death (17, 40, 41). To investigate the requirement of HH signaling as a survival factor in NSCLC cells, GLI1, or SMO expression was repressed by using distinct shRNAs in A549 and in HOP62 cells then these cells were assayed for apoptosis induction by using a Caspase cleavage assay (Fig. 2D) or 2 other mechanistically distinct assays of cell death (Supplementary Fig. S4B and C). Reduction of GLI1 or SMO expression by 2 independent shRNAs induced apoptosis in both A549 and HOP62 cells relative to control cells, indicating a requirement for HH signaling to regulate NSCLC cell survival (Fig. 2D, Supplementary Fig. S4B and C).

A requirement for HH signaling in regulating proliferation of NSCLC cell lines (see Fig. 2), when combined with the expression of three distinct HH ligands (see Supplementary Fig. S1B), indicate that the viability of NSCLC cells may prove ligand dependent. To evaluate the requirement of HH ligands

Figure 2. The proliferation of NSCLC cells requires HH signaling components. The knockdown of GLI1 (A), SMO (B), or GLI2 (C) inhibited the proliferation of the indicated NSCLC cell lines. The extent of proliferation was determined 5 days after transduction of the indicated shRNAs and normalized to cells infected with pLKO.1 control virus (Ctrl). D, the attenuation of GLI1 expression induced apoptosis in A549 and HOP62 cells. Apoptosis was measured 5 days after the indicated transduction by using the Caspase-Glo 3/7-assay kit, and the results normalized to the cells transduced with control (Ctrl) virus. Error bars represent ± SEM of 3 independent experiments. The asterisk (*) denotes a statistically significant change (P < 0.05) in apoptosis as compared with the control.
in regulating the proliferation of NSCLC cells, 2 shRNAs independently specific for targeting SHH, DHH, or IHH were each transduced into the studied panel of NSCLC cell lines and effects on cell proliferation were determined (Fig. 3A–C). The sensitivity of NSCLC proliferation to expression of these HH family members varied between the cell lines tested, but in general correlated with the combined expression of HH ligands. The proliferation of NSCLC cells expressing HH ligands at relatively lower levels (HOP62, A549, and U1752) was found more sensitive to reduced HH ligand expression as compared with cells expressing HH ligands at relatively higher levels (H23, H522, H157; see Fig. 3A–C in conjunction with Supplementary Fig. S1B). These findings indicate a requirement for the HH ligand in NSCLC cell proliferation.

A recent report claimed that there was no correlation between basal GLI1 levels and sensitivity to SMO antagonists in a large panel of cell lines, including NSCLC cells, and that SMO antagonists did not affect HH target gene expression in a selected set of cancer cells (25). This work was viewed as evidence that HH-producing cancer cells do not harbor a functional HH pathway. The expression of HH target genes and their subsequent reduction upon knockdown of positive acting components of the HH signaling pathway being our criterion for HH pathway activation, SHH, IHH, or SMO expression was repressed by shRNAs in NSCLC cells and the levels of the HH target genes PTCH1 and GLI1 were determined. Our findings revealed that reduction of HH signaling regulators attenuated the expression of GLI1 and PTCH1 (Fig. 3D left), and reduced GLI1 protein levels, in NSCLC cells (Fig. 3D, right, and data not shown). These results are consistent with HH-producing cancer cells harboring a constitutively active HH signaling pathway. We do not know the reason for our inability to significantly reduce the expression of PTCH1 upon knockdown of SMO but note that other groups have suggested that GLI1 expression is a better biomarker for HH activity than PTCH1 expression (42, 43).

HH expressing tumor cells are themselves thought to be resistant to HH signaling (25). However, exogenous expression of SHH in NSCLC cells HOP62 and A549 induced expression of the HH target gene GLI1 and PTCH1 in a dose-dependent manner (Fig. 4A and Supplementary Fig. S5), indicating the presence of an intrinsic functional HH signaling pathway.

Figure 3. The proliferation of NSCLC cells requires the expression of HH proteins. The knockdown of SHH (A), DHH (B), or IHH (C) inhibited the proliferation of the indicated NSCLC cell lines. The extent of proliferation was determined 5 days after infection with the indicated shRNA virus and normalized to cells infected with pLKO.1 control virus (Ctrl). D, the knockdown of HH signaling components attenuates the expression of HH target genes (left) or the steady-state levels of GLI1 protein (right). A549 cells were transduced with the indicated shRNA virus for 3 to 5 days, RNA or protein was then extracted and the expression of the indicated HH target genes determined by qRT-PCR or immunoblotting. These results were then normalized to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or tublin (Tub). These data are shown relative to the cells transduced with pLKO.1 control virus (Ctrl). All error bars indicate ± SEM of 3 independent experiments. The asterisk (*) denotes a statistically significant change (P < 0.05) in HH target gene expression as compared with the control.

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Furthermore, when HOP62 cells were engineered to stably express the human SHH gene their ability to grow in soft agar was significantly increased (Fig. 4B and C). The effect of downregulating HH signaling in NSCLC cells was also determined, using A549 cells because of their robust ability to grow in soft agar. Two independently targeted shRNAs to each of 6 HH-signaling components significantly reduced the ability of A549 cells to form colonies in a soft agar assay (Fig. 4D). The almost equal efficiency to inhibit residual clonogenicity by knockdown of all three ligands was surprising, as one might anticipate a degree of redundancy within the three HH family members. We did note, however, that the expression of the individual family members was not completely independent, as knocking down one member often affected the expression of the other family members but not the expression of a control gene. Such an observation, while unexpected, might explain some of the results shown here. These results establish an important role of HH signaling in the anchorage-independent growth of NSCLC cells, a recognized in vitro determinant of tumorigenicity (44).

To determine whether NSCLC cells depended on HH activity for their growth and tumorigenicity in vivo, GLI1 levels were knocked down with either of 2 validated shRNAs, or a control shRNA, in A549 cells before implantation of these cells into the flanks of nude mice. The growth of the A549 tumors was measured for a period of 6 weeks (Fig. 5A). The mean tumor volume of A549 cells transduced with GLI1 shRNAs was substantially reduced (>80%) compared with controls (Fig. 5A). The mean tumor volume of A549 cells transduced with GLI1 shRNAs was substantially reduced (>80%) compared with controls (Fig. 5A). A representative mouse, along with the resected tumor, from each experimental group is shown for comparison (Fig. 5B). The tumors isolated from each mice group were sectioned and hematoxylin and eosin stained. A board-certified pathologist (P.A. Bejarano) diagnosed these tumors as poorly differentiated adenocarcinomas (data not shown).
Thus, knockdown of GLI1 also reduced the tumorigenic potential of A549 cells, showing a requirement for HH signaling in the growth of NSCLC tumors. We also determined HH target gene expression in cancer cells (human; A549) and stroma cells (mouse) of the residual tumors formed in the GLI1 targeted group (GLI1 shRNA #1 or 5) and noted that the overall expression of such genes was not significantly different from that in the control group (Ctrl shRNA), consistent with the residual tumors arising from cells uninfected with shRNA, or cells in which the knockdown of GLI1 was inefficient (Supplementary Fig. S6). Together, these results confirm, in a manner independent of use of SMO antagonists, that NSCLC cells harbor a constitutively active HH signaling pathway and that the tumorigenicity of NSCLCs is dependent on this activity.

To compare the levels of HH signaling between stromal and tumor epithelial cells within primary human lung tumors, we examined the expression and localization of SHH and its target genes GLI1 and PTCH1 by RNA in situ hybridization and immunohistochemistry (Fig. 6). SHH and GLI1 were weakly expressed in normal lung tissue but highly expressed in the majority of the lung tumors examined (Fig. 6, Table 1 and data not shown). Furthermore, scoring of tumorigenic epithelial cells and tumor-associated stromal cells in individual lung cancer cases for the protein expression of the HH target GLI1 indicated a prevalence of activated HH signaling in both tumor and stromal cells (Table 1).

Discussion

It was recently proposed that SMO antagonists affect proliferation of HH expressing tumor cells nonspecifically (25). Furthermore, in vitro studies have shown that HH from pancreatic tumor cells acts on the tumor stroma, and this was thought to regulate tumorigenesis (25–27). Together, these results supported a model whereby HH, produced by tumor cells themselves, acts on the tumor stroma in a paracrine fashion. Moreover, the latter study also suggested that such HH-producing cancer cells are themselves incapable of responding to HH, as they likely lack a functional HH signaling pathway (25). It is difficult to reconcile the results presented here, which are consistent with an autocrine model, with the recently proposed paracrine model. Here, using a shRNA-based approach to target 6 distinct positively acting components of the HH signaling pathway, evidence is provided that NSCLC cells indeed elaborate a functional HH signaling pathway, which is functional from HH ligand reception to transcriptional activation of HH target genes. Notably, the exogenous expression of HH ligand induced the expression of HH target genes while inhibition of HH activity via shRNA-mediated knockdown reduced their expression. Evidence is also provided here for the tumorigenicity of NSCLC cells depending on HH signaling (Fig. 5), most likely through an autocrine manner as knockdown of 6 independent HH pathway components reduced NSCLC proliferation whereas exogenous expression of SHH or GLI1 increased the anchorage-independent growth of these NSCLC cells (Fig. 4; ref. 28). Given the controversy about possible off-target effects of pharmacologic inhibitors, the conclusions made here were reached without use of such SMO antagonists. Although our results support the role for ligand-dependent canonical HH signaling in lung cancer, we cannot exclude the possibility that a subset of the activation we observe is a result of noncanonical HH signaling (1, 2).

Our results indicate that a paracrine signaling model (25, 45, 46) alone is insufficient to explain how HH regulates tumor development, as such HH-producing cancer cells themselves harbor a constitutively active HH signaling pathway. Others have argued that autocrine signaling is a major mechanism by which HH regulates HH-dependent tumors (14–20, 47, 48). How then does one resolve these seemingly disparate models?

Figure 5. HH signaling is required for tumor growth. A, the knockdown of GLI1 expression attenuates the ability of A549 xenografts to grow in nude mice. Tumor growth is shown as mean tumor volume (mm\(^3\)) ± SEM (n = 12) measured biweekly for 6 weeks. Scrambled control shRNA (Ctrl) and GLI1 shRNA #5 or #1 transduced cells were injected into the flanks of nude mice. Representative mice harboring A549 tumors from each treatment group along with the resected tumors are shown in (B). A white arrow indicates the site of tumor formation. Adenocarcinoma formation was independently verified by H&E staining of tumor sections, followed by visual inspection by a pathologist (P.A. Bejarano).
One possibility is that both models are correct but dependent on different cell contexts (35, 49). These differences may arise from tumor types studied, differences between human tissue xenografts and cancer cell lines, as well as differences between mouse and human tumors. However, both models seem to be supported by multiple lines of evidence, making such contextual differences difficult to discern. Another possibility is that HH signaling in both cancer cells and stroma is necessary.

Table 1. Summary of the GLI1 immunohistochemistry of tumor samples

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>GLI1 expression (positive cases/total cases)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor cells</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>12/12</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>1/1</td>
</tr>
<tr>
<td>Papillary adenocarcinoma</td>
<td>1/1</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>2/2</td>
</tr>
<tr>
<td>Branchioalveolar carcinoma</td>
<td>1/1</td>
</tr>
<tr>
<td>Large-cell carcinoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Small-cell undifferentiated carcinoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Atypical carcinoid</td>
<td>0/1</td>
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<tr>
<td>Carcinoid</td>
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$^a$As scored by a pathologist (P.A. Bejarano).
for tumorigenesis but in mice the dominant effect is paracrine–attenuation of which debunks the tumor. Alternatively, autocrine HH-dependent cancer cells are a product of growing tumor cells in vitro and are therefore not relevant for understanding tumor growth in vivo. We do not favor this possibility because several groups, including ours, used immunohistochemistry or in situ hybridization analyses to find increased HH target gene expression within the tumor cells themselves (Fig. 6; refs. 14–20, 28, 29, 34, 50). Regardless of such models, the findings presented here shows that the tumorigenicity of human NSCLC cells often depend on HH signaling, and does so in a manner independent of the use of any pharmacologic SMO antagonist.

Disclosure of Potential Conflicts of Interest

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References


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