Hedgehog: an Attribute to Tumor Regrowth after Chemoradiotherapy and a Target to Improve Radiation Response
Jennifer Sims-Mourtada, Julie G. Izzo, Smith Apisarnthanarax, Tsung-Teh Wu, Usha Malhotra, Rajyalashmi Luthra, Zhongxing Liao, Ritsuko Komaki, Albert van der Kogel, Jaffer Ajani, and K.S. Clifford Chao

Abstract

Purpose: Despite aggressive chemotherapy, radiotherapy, surgery, or combination approaches, the survival rate of patients with esophageal cancer remains poor. Recent studies have suggested that constitutive activation of the Hedgehog (Hh) pathway in cancers of the digestive tract may contribute to the growth and maintenance of cancer. However, the relationship between Hh signaling and therapeutic response is unknown.

Experimental Design: The expression and temporal kinetics of Hh signaling and proliferation biomarkers after chemoradiotherapy were examined in esophageal tumor xenografts. Additionally, immunohistochemical analysis of Sonic Hh (Shh) and Gli-1 expression were done on residual tumors from patients who received neoadjuvant chemoradiotherapy followed by surgery. The ability of Shh signaling to induce proliferation in esophageal cell lines was determined. Expression of cell cycle checkpoint proteins was analyzed in cells in which Hh signaling was activated or inhibited. We further determined the effect of inhibiting Hh signaling in sensitizing esophageal tumors to radiation.

Results: We showed that the Shh signaling pathway was extensively activated in esophageal cancer xenografts and residual tumors after chemoradiotherapy and the temporal kinetics of Hh signaling preceded increases in proliferation biomarker expression and tumor size during tumor regrowth. We further showed that Hh pathway activity influences proliferation rates of esophageal cancer cell lines through up-regulation of the G1-cyclin-Rb axis. Additionally, we found that blocking Hh signaling enhanced radiation cytotoxicity of esophageal cancer cells.

Conclusions: These results suggest that activation of the Hh pathway may promote tumor repopulation after chemoradiotherapy and contribute to chemoradiation resistance in esophageal cancers.

Cancer of the esophagus is one of the most virulent malignancies, with a 5-year survival rate of <20% (1). The most commonly used therapy for localized esophageal cancer (stages II and III) is chemoradiotherapy followed by surgery (2–5). However, numerous clinical trials have shown that this preoperative strategy benefits only the 25% of patients who have no cancer cells in the resected specimen (pathologic complete response), whereas the remaining 75% present chemoradiotherapy-resistant and highly aggressive cancers (2–5). Nearly all patients experience severe treatment-related morbidities but only a few patients benefit.

An increase in tumor cell proliferation rates after exposure to chemotherapy (6) and radiation (6–8) contributes to tumor resistance and regrowth (6, 7). As the rate of regeneration of tumor cells increases, the effectiveness of each treatment is reduced, implying repopulation by a chemoradiotherapy-resistant clonogenic population in between treatment fractions (6). Previous studies have suggested that unregulated progenitor cell proliferation induced by abnormal activation of the Hh signaling pathway contributes to carcinogenesis of the digestive tract tissues (9, 10). Activation of Hh signaling by binding of secreted Hh ligands (Sonic, Indian, and Desert) to the membrane receptor Patched results in the nuclear translocation and activation of transcription factors of the Gli family (11, 12), whose targets include genes controlling the cell cycle, cell adhesion, signal transduction, angiogenesis, and apoptosis.
(13). Additionally, Gli-1 is a transcriptional target of the Hh pathway (14), providing positive feedback for Hh signaling. Treatment of cancer cell lines with the Hh-inhibitory compound cyclopamine results in down-regulation of the proliferation marker Ki67 and reduced proliferation rates (9, 15, 16), indicating that Hh pathway activation may be essential for tumor growth and maintenance. However, the significance of Hh signaling in chemoradiotherapy-resistant tumors is not clear. Here, we provide evidence that release of Shh ligand and subsequent activation of the Hh signaling pathway may support tumor repopulation after chemoradiotherapy. Therefore, the Hh pathway is a potential target for improving responses to chemoradiotherapy.

Materials and Methods

Cell lines and antibodies. SEG-1, SKGT4, Bic-1, and BE-3 esophageal adenocarcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum. Polyclonal antibodies to Gli-1 (H-300), cyclin-dependent kinase 4 (C22), Shh (H-160), and cyclin D1 (HD-11) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rb (4H1) and phospho-Rb Ser780 were obtained from Cell Signaling Technologies (Danvers, MA). The antibody to Ki67 (B56) was obtained from BD PharMingen (San Diego, CA). 9F1, a rat monoclonal antibody to mouse endothelium, was obtained from the Department of Pathology, Radboud University Nijmegen Medical Center, The Netherlands. The antibody to bromodeoxyuridine was obtained from Abcam Ltd. (Cambridge, MA).

Patient selection and tissue specimens. Cancer specimens were obtained from 43 patients that participated in clinical trials approved by the University of Texas M.D. Anderson Institutional Review Board and after informed consent from patients. All cancer tissues assigned two categories: no residual carcinoma in the esophagus (negative pathologic response) or residual esophageal cancer–resected specimens. Each tissue sample was considered for scoring purposes. Immunohistochemical reactions were then classified, based on the percentage of nuclear Gli-1, expression using the MetaMorph image analysis system (Universal Imaging Corp., Downingtown, PA) to define and compare tumor regions of interest. Only Shh cytoplasmic and Gli-1 nuclear immunoreactivity in tumor cells were considered for scoring purposes. Staining intensity for Shh was defined as undetectable (0) or detectable (1). Staining intensity for Gli-1 was evaluated on a three-point semiquantitative scale as follows: 0, undetectable; 1, weak to moderate; 2, strong staining. The strength of positive staining was assessed subjectively, taking into account the number of labeled tumor cells, the proportion of positively stained nuclei, and the intensity of the staining. The median (range) staining intensity was 0.4 (0.03-0.9). Immunohistochemical reactions were independently analyzed by three investigators (J.G.I., T.T.W., and J.H.).

Mouse tumor model. Five-month-old Swiss nu/nu mice were obtained from the specific-pathogen-free mouse colony of the Department of Experimental Radiation Oncology, M.D. Anderson Cancer Center, Houston, TX. Single SEG-1 tumor xenografts were generated in the right hind limbs of the mice by i.m. injection of 10^5 tumor cells. Once the tumors reached 5 to 6 mm in diameter, they were centered in a circular field 3 cm in diameter. The tumor was centered in a circular field 3 cm in diameter. The tumor was monitored for growth, and the tumor volume was calculated using the formula: V = 0.52 × L × w^2, where V is the volume, L is the longest diameter, and w is the shortest diameter. The tumor volume was measured every other day to monitor growth.

Proliferation assays. Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, 5,000 cells were seeded in 96-well plates and incubated in culture
NA cleavage product was quantified in a microplate reader (BioTek) at a wavelength of 405 nm. Each assay was done in triplicate and repeated thrice.

**Statistical analysis.** For in vitro studies, significance was determined by unpaired Student’s t test. \( P < 0.05 \) using a two-tailed test was taken as significant for all statistical tests. Computations were carried out using a SAS software package (version 6.12, SAS Institute, Inc., Cary, NC).

**Results**

To determine if activation of the Hh signaling pathway is associated with therapeutic resistance to chemoradiotherapy, we examined expression levels of Shh and nuclear Gli-1 proteins in esophageal carcinoma specimens obtained after esophagectomy of 43 patients who received preoperative chemoradiotherapy. All patients that were resistant to chemoradiotherapy (i.e., achieved pathCR) had residual cancer in the resected specimen. Of the 43 chemoradiotherapy-resistant cancers, 36 (83.7%) had activated Hh signaling (i.e., defined by concomitant cytoplasmic Shh and nuclear Gli-1 expression); 2 (4.6%) had Shh expression without Gli-1 nuclear localization; and 5 (11.7%) were negative for both Shh and Gli-1 expression (Table 1). The median labeling indices for Shh and Gli-1 were 0.08 (range = 0.01-0.8) and 0.4 (range = 0.03-0.9), respectively. Among cancers with activated Hh, 9 had low, 15 had moderate, and 12 had strong Gli-1 nuclear expression (Table 1). Gli-1 nuclear expression was never observed without concomitant cytoplasmic Shh and nuclear Gli-1 expression; 2 patients with high Shh expression showed strong Gli-1 nuclear expression; 1 patient with low Shh expression showed moderate Gli-1 nuclear expression, and 2 patients with moderate Shh expression showed weak Gli-1 nuclear expression.

**Discussion**

The activation of the Hh signaling pathway is associated with resistance to chemoradiotherapy in esophageal adenocarcinoma. The expression of Shh and nuclear Gli-1 proteins is observed in most chemoradiotherapy-resistant cancers, indicating a potential therapeutic target for improving treatment outcomes.
immunochemical detection. Of interest, when analyzing the spatial localization of Shh and Gli-1 immunostaining within a tumor, Shh staining was usually clustered in small patches surrounded by larger areas of Gli-1 nuclear expression, suggesting a regional activation of the Hh signaling pathway. The substantial expression of Shh in patients with residual cancer after chemoradiotherapy suggests that the secretion of Shh and subsequent activation of the Hh signaling pathway support the proliferation and survival of cancer cells after treatment.

To address the role of the Shh/Gli-1 signaling pathway in persistent disease or subsequent regrowth of tumor after chemoradiotherapy, we used an esophageal adenocarcinoma tumor xenograft model. A xenograft model was chosen due to the lack of a suitable murine orthotopic model of esophageal cancer. SEG-1 esophageal adenocarcinoma xenografts were generated by i.m. injection of tumor cells into the hind limbs of nude mice. The resulting tumors were treated with docetaxel (33 mg/kg i.v.) and 15 Gy of ionizing radiation. This in vivo chemoradiotherapy model temporarily delays the growth of tumors but is not sufficient to eradicate the tumor, a scenario that is observed in the clinical setting for treatment-resistant tumors. As shown in Fig. 2A, baseline untreated tumors exhibited chronic hypoxia and a high rate of proliferation occurring close to vessels. However, 2 days after treatment, proliferation rates were strongly reduced. Some proliferating cells were observed, but these were located farther away from vessels. By 6 to 8 days after chemoradiotherapy, tumor repopulation had begun, and small islands of proliferating cells were observed in the stroma. On day 10, high rates of proliferation were observed. In contrast to the diffuse proliferation observed close to vessels at earlier time points, foci of proliferating colonies were evident.

To determine the relationship between Hh expression and proliferation after chemoradiotherapy, we examined temporal changes in expression of Shh ligand or Gli-1 nuclear translocation along with changes in the proliferation biomarker Ki67 and tumor size before and after chemoradiotherapy. As shown in Fig. 2B, a partial treatment response was observed, characterized by an initial decrease in tumor size 4 to 6 days after treatment and subsequent tumor regrowth after day 10. A decrease in Ki67 staining was seen as early as 4 hours after chemoradiotherapy. Proliferation rates continued to decline until 4 days after treatment and increased substantially 8 days after chemoradiotherapy preceding an increase in tumor size.

Low-level expression of cytoplasmic Shh and nuclear Gli-1 (i.e., weak to moderate staining intensity and lower labeling index) was observed before chemoradiotherapy. Similar to the expression patterns observed in the resected cancer specimens, Shh expressing cells were spatially localized in small clusters throughout the tumor, whereas Gli-1 expressing cells seemed more diffusely distributed in the vicinity of Shh-positive clusters. However, 6 to 8 days after chemoradiotherapy, and just before the increase in tumor proliferation rates, we observed a substantial increase in Hh activity, characterized by (a) increased number of clustered cells expressing strong cytoplasmic Shh, (b) distinct Shh staining in the intercellular spaces surrounding the clusters, and (c) significant increase in nuclear Gli-1 labeling indices patching the tumor fields. These observations depict the temporal kinetics of SHH ligand release and Gli-1 activation before repopulation of surviving tumor cells after chemoradiotherapy.

### Table 1. Shh and Gli1 protein expressions in 43 human esophageal cancers resistant to chemoradiation

<table>
<thead>
<tr>
<th>Total (43)</th>
<th>Gli1 expression*</th>
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<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Shh (+)</td>
<td>38</td>
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<tr>
<td>Shh (−)</td>
<td>5</td>
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*Gli1 nuclear staining in tumor cells is defined as negative (0% stained cells), low (1% to <30% stained cells), moderate (≥30 to <60% stained cells), and strong (≥60% stained cells).

† Shh cytoplasmic immunolocalization in tumor cells.

Cancer Therapy: Preclinical

Fig. 2. Temporal kinetics of Hh pathway activation precedes tumor cell proliferation and regrowth after chemoradiotherapy in esophageal adenocarcinoma xenografts. A, multiparameter analysis of SEG-1 tumor sections at 0, 2, 6, and 10 days after chemoradiotherapy. Green, hypoxia; red, proliferation; blue, perfusion; white, vessels. Proliferation of clonogenic foci is apparent 10 days after treatment. B, results of immunohistochemical analysis showing increase of Shh and nuclear Gli-1 expression precedes increases in Ki67 staining (left axis) and tumor size (right axis) during tumor repopulation after chemoradiotherapy. Points, mean of three independent experiments; bars, SD.
To determine the causative relationship between Hh signaling and proliferation of esophageal tumor cells, three esophageal adenocarcinoma cell lines (SKGT4, SEG-1, and Bic1) were treated with exogenous Shh ligand, the Hh pathway antagonist cyclopamine, which blocks SMO receptor activation, or a Gli-1-specific siRNA. Activation or inhibition of Hh signaling was confirmed by detecting Gli-1 nuclear translocation using immunofluorescence-based confocal microscopy (Fig. 3A). Additionally, because Gli-1 is a transcriptional target of the Hh pathway (14), inhibition of Hh signaling was confirmed by a decrease in Gli-1 expression in cells treated with Hh inhibitors or Gli-1-specific siRNA compared with controls (Fig. 3B). After 48 hours of treatment with cyclopamine or Gli-1 siRNA, a significant decrease in proliferation was observed (Fig. 3C), whereas a significant increase in proliferation rates was observed in cells treated with exogenous Shh ligand. The degree of response correlated with level of endogenous Gli-1 expression (Fig. 3D), indicating that this effect is specific to Hh signaling. To further explore the relationship between Hh activation and proliferation of esophageal cancer, DNA synthesis levels of cells stimulated with Shh ligand were measured by 

\[ ^3H \text{-thymidine incorporation} \]

and compared with baseline levels obtained with vehicle-treated control cells. A significant increase in 

\[ ^3H \text{-thymidine incorporation} \]

was observed in Shh treated cells compared with untreated controls.

Similar results were obtained with cells transiently overexpressing nuclear Gli-1 (Fig. 3E). These results show that Hh signaling can promote a significant proliferative response in esophageal tumor cells.

Dysregulation of cell cycle regulatory proteins contributes to the proliferation of tumor cells and subsequent repopulation of tumors after chemoradiotherapy (23). To determine the mechanism of Shh-induced proliferation, we analyzed the expression levels of cyclin D1 in SEG-1 cells upon exogenous stimulation of Hh signaling. As shown in Fig. 4A, there is an increase in cyclin D1 protein expression in Shh stimulated cells compared with controls. We also examined the levels of Rb expression and activation in Shh-treated cells. Although there was no significant change in Rb protein levels, a significant increase in Rb phosphorylation at the cyclin D phosphorylation site (Ser 780) was observed. These results indicate that Shh stimulation in esophageal tumor cells can promote G1-S cell cycle phase transition by increasing activity of the cyclin-Rb axis.

To examine the effect of Hh signaling on the cell cycle distribution of esophageal adenocarcinoma, SEG-1 cells were treated with the Hh pathway antagonists cyclopamine (which

![Fig. 3.](image-url)
blocks receptor activation), forskolin (which inhibits Gli-1 transcriptional activity; ref. 24), or Gli1 siRNA. Inhibition of Hh signaling was confirmed by analysis of Gli-1 protein expression (data not shown). Consistent with previous reports (15, 25), blocking Hh signaling inhibited cell cycle progression, resulting in the accumulation of cells in the G1 phase of the cell cycle (Fig. 4B), as a result of decreased expression of the G1-S checkpoint proteins cyclin D1 and cyclin-dependent kinase 4 (Fig. 4C).

It is well established that the sensitivity of cells to the cytotoxic effects of radiation is cell cycle dependent, with the S phase being more resistant and the G1-S boundary and G2-M phase being more sensitive (26). Treatment of SEG-1 cells with ionizing radiation alone led to a slight but not significant reduction in the radiation-resistant S-phase fraction (Fig. 4D). Treatment with Hh inhibitors alone led to a significant (P < 0.01) reduction in the S-phase fraction, and the combination of radiation and Hh inhibitors caused a greater reduction (P < 0.005) in the S-phase fraction compared with untreated cells. To determine whether inhibition of Hh signaling can sensitize cells to the cytotoxic effects of radiation, SEG-1 or BE-3 cells were treated with Hh pathway inhibitors in combination with increasing doses of ionizing radiation, and clonogenic survival curves were generated. Inhibition of Hh signaling synergistically decreased the clonogenic survival of tumor cells when combined with radiation (Fig. 5A). Blocking Hh signaling also significantly reduced the shoulder region of the ionizing radiation survival curve, indicating that repair of the sublethal damage induced by ionizing radiation was inhibited. We further evaluated whether the synergistic effect of combining Hh inhibition and radiation involves apoptosis. Although treatment with ionizing radiation alone had little effect on cell viability 48 hours after treatment of esophageal adenocarcinoma cell lines (SEG-1, Bic-1, BE-3, and SK5), a significant decrease in cell viability was observed in cells in which Hh signaling was inhibited (Fig. 5B). Cell viability was further reduced by combination of ionizing radiation and Hh inhibition. Minimal apoptosis, as determined by caspase-3 activity, was observed in esophageal adenocarcinoma cell lines treated with ionizing radiation alone (Fig. 5C and D). Treatment with Hh inhibitors alone induced significant apoptosis, and combined treatment with ionizing radiation and Hh inhibitors resulted in a significantly greater amount of apoptosis.

Discussion

The Hh pathway is critical for tissue growth and differentiation during embryonic development of the gastrointestinal tract tissues (27). In adult cells, Hh signaling has been implicated in the maintenance of homeostasis of stem or progenitor cells in a number of epithelial tissues, including intestinal epithelia (28–30). Additionally, Hh signaling contributes to physiologic processes of epithelial repair and regeneration after injury (29). In normal tissues, Hh-induced progenitor cell proliferation is transient and tightly regulated, preventing continuous regeneration. However, aberrant activation of Hh signaling in tumors may allow escape from regulatory mechanisms that cause the return to quiescence that normally follows regeneration. The activation of the Hh signaling pathway observed in both chemoradiotherapy-resistant

Fig. 4. Inhibition of Hh signaling results in G1 arrest and decreases percentage of radioresistant S-phase cells in combination with ionizing radiation (IR). A, Western blot analysis showing up-regulation of cyclin D1 and Rb phosphorylation at Ser780 in cells treated with exogenous Shh ligand. B, increase in percentage of cells in G1 phase of cell cycle in cells treated with inhibitors to the Hh pathway compared with untreated controls. Percentages of SEG-1 cells, with or without treatment with Hh inhibitors, in each cell cycle phase. Representative of the means of three separate experiments. C, Western blot showing down-regulation of the G1 checkpoint proteins cyclin D1 and cyclin-dependent kinase 4 (CDK4) after treatment with Hh inhibitors compared with levels in untreated cells. Actin is shown as a loading control. D, percentage of S-phase cells decreases in cells treated with Hh inhibitors combined with ionizing radiation compared with untreated controls. Percentage of S-phase cells with or without exposure to 4 Gy of ionizing radiation. *, P < 0.01 for combination of Hh inhibitors and ionizing radiation compared with Hh inhibitors alone. #, P < 0.005 for combined treatment compared with untreated controls.
residual esophageal carcinoma specimens (Fig. 1) and animal tumor xenografts (Fig. 2B) suggests that release of Shh ligands may support proliferation and survival of tumor clonogens after chemoradiotherapy in an autocrine-paracrine manner. The observation of the differential labeling indices between Shh and Gli-1, and the organization of Gli-1 staining usually surrounding Shh-expressing cell clusters, underscore our hypothesis. Secretion of Shh ligand by tumor cells could have a double effect, by directly stimulating proliferation of surrounding cancer cells and also by promoting release of growth factors necessary for tumor maintenance, such as vascular endothelial growth factor and epidermal growth factor, from stromal cells.

Our data show that stimulation of esophageal cancer cells with Shh ligand or Gli-1 overexpression results in up-regulation of G1-cyclin activity and increased proliferation. These data suggest that unregulated Hh signaling within tumors may promote a continuous state of tissue repair and clonogenic proliferation once the tumor volume is reduced after chemoradiotherapy, contributing to tumor repopulation. Although a role for Hh signaling has been established in the formation and maintenance of a number of tumors, including basal cell, prostate, breast, and pancreatic carcinomas among others (9, 10, 29, 31–33), our study is the first to examine Hh activation in the context of tumor repopulation after chemoradiotherapy and suggests that in addition to its reported roles in carcinogenesis and tumor maintenance, aberrant activation of the Hh pathway may contribute to tumor repopulation and treatment failure.

Esophageal adenocarcinoma is presently the cancer with the greatest increase in incidence in the United States (34, 35). Currently available treatments of localized cancer of the esophagus are ineffective in >75% of esophageal cancers (chemoradiotherapy resistant), resulting in a 5-year survival rate under 25%. Patients with esophageal cancer and treating oncologists face serious challenge of not being able to optimize therapy. Therapies are currently chosen empirically without concern for tumor and patient heterogeneity. Highly morbid surgery could be avoided if one could identify a molecular signature that would suggest a highly chemoradiation-sensitive cancer. Similarly, understanding the mechanisms resulting in chemotherapy or radiotherapy resistance would allow identification of exploitable targets. The discovery that inhibition of Hh activity can sensitize tumor cells to the effects of radiation suggests that incorporating targeted inhibition of the Hh signaling pathway into current chemoradiotherapy regimens might improve treatment outcome.

There are presently no clinically approved systemic inhibitors of Hh signaling. Topical application of cycloamine has been
shown to inhibit the growth of human basal cell carcinoma (36); however, concerns of neurologic disturbances may limit the systemic application of this drug. Several small molecule compounds that prevent Hh signaling by binding to and inhibiting Smo are currently under development, including Cur61414 (37), which has shown promising results in inhibition of basal cell carcinoma and pancreatic cancer in preclinical models. The therapeutic effects of Hh pathway blockade in combination with current chemoradiotherapy regimens are yet to be investigated. Particularly, the differential regulation and timing of Hh activity in normal and tumor tissue after chemoradiotherapy should be further investigated to determine the most beneficial therapeutic index (i.e., effects on normal tissue repair versus tumor).

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References

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