Role of Hedgehog Signaling in Malignant Pleural Mesothelioma

Yandong Shi, Ubiratan Moura, Isabelle Opitz, Alex Soltermann, Hubert Rehrauer, Svenja Thies, Walter Weder, Rolf A. Stahel, and Emanuela Felley-Bosco

Abstract

Purpose: The aim of this study was to assess the activity of hedgehog signaling pathway in malignant pleural mesothelioma (MPM).

Experimental Design: The expression of hedgehog signaling components was assessed by quantitative PCR and in situ hybridization in 45 clinical samples. Primary MPM cultures were developed in serum-free condition in 3% oxygen and were used to investigate the effects of smoothened (SMO) inhibitors or GLI1 silencing on cell growth and hedgehog signaling. In vivo effects of SMO antagonists were determined in an MPM xenograft growing in nude mice.

Results: A significant increase in GLI1, sonic hedgehog, and human hedgehog interacting protein gene expression was observed in MPM tumors compared with nontumoral pleural tissue. SMO antagonists inhibited GLI1 expression and cell growth in sensitive primary cultures. This effect was mimicked by GLI1 silencing. Reduced survivin and YAP protein levels were also observed. Survivin protein levels were rescued by overexpression of GLI1 or constitutively active YAP1. Treatment of tumor-bearing mice with the SMO inhibitor HhAntag led to a significant inhibition of tumor growth in vivo accompanied by decreased Ki-67 and nuclear YAP immunostaining and a significant difference in selected gene expression profile in tumors.

Conclusions: An aberrant hedgehog signaling is present in MPM, and inhibition of hedgehog signaling decreases tumor growth indicating potential new therapeutic approach.

Cancer Therapy: Preclinical

Introduction

Malignant pleural mesothelioma (MPM) is associated with asbestos exposure. Chronic tissue inflammation and tissue repair have been postulated to be the central mechanism leading to tumorigenesis (1, 2). Tissue repair involves the activation of stem cells and the expression of stem cell renewal genes. Activated stem cell signaling has already been suggested in MPM with the presence of an 11-gene signature, correlating with a stem cell–like expression profile, which is associated with a poor prognosis in patients with MPM (3). Cells staining positive for nuclear β-catenin, a marker for Wnt signaling activation, have been reported in a few studies (4–6). A significant transcriptional downregulation of the secreted frizzled-related proteins (sFRP), which are negative modulators of the Wnt signal transduction pathway, has been observed in MPM primary tissues and cell lines (7). More recently, the downregulation of several miRNAs antagonizing Wnt signaling has been described in MPM (8). Another stem cell signaling pathway that has been investigated in vitro is Notch, whereby Notch1 has been found to control PTEN expression in MPM lines (9). Concerning bone morphogenetic proteins (BMP), which are members of the TGF-β superfamily and are critical mediators of early embryonic patterning, methylation of BMP3b and BMP6 promoters has been observed in MPM (10). In addition, in a case of biphasic malignant mesothelioma with osseous and cartilaginous differentiation, expression of BMP2 has been observed (11).

Our study now identifies aberration in hedgehog signaling in MPM. Hedgehog signaling has a key role for normal organ development and is dysregulated in several types of cancer (12). We recently observed increased expression of PTCH1 (patched, the receptor binding hedgehog ligands) in mesothelioma side population–derived tumors which exhibited a tendency to have increased tumor-initiating properties and developed tumors with precursor phenotype similar to tumors in patients who relapse after chemotherapy (13). This prompted us to investigate whether hedgehog pathway is activated in MPM and the effect of its inhibition in primary mesothelioma cell cultures and in a xenograft.

Authors’ Affiliations: 1Laboratory of Molecular Oncology, Clinic and Polyclinic of Oncology, 2Division of Thoracic Surgery, 3Institute of Surgical Pathology, University Hospital Zurich; and 4Functional Genomic Center Zurich, Zurich, Switzerland

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Corresponding Author: Emanuela Felley-Bosco, Laboratory of Molecular Oncology, Haedelweg 4, Zurich 8044, Switzerland. Phone: 41-44-6342872; Fax: 41-44-6342872; E-mail: emanuela.felley-bosco@usz.ch

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Translational Relevance

Malignant pleural mesothelioma (MPM) are particularly resistant to current chemotherapy. In this study, we observed hedgehog signaling pathway activation in MPM, and we show that inhibition of this pathway decreases cell proliferation in cell culture and inhibits the growth of MPM in vivo in a xenograft model. These findings suggest that hedgehog signaling inhibitors may have therapeutic potential for MPM.

Materials and Methods

Tissue samples

Forty-five tumor specimens were collected at the time of surgery and were immediately processed for primary culture or total RNA extraction using Qiagen RNAeasy. In addition, parts of tumor specimens were embedded in Tissue-Tek O.C.T Compound (Sakura) and immediately frozen. Non-tumoral pleural tissue was received from 10 patients undergoing mesothelioma unrelated thoracic surgery. The study was approved by the Institutional Review Board of Zurich University Hospital (Zurich, Switzerland), and a written informed consent was obtained for each patient.

Gene expression analysis

Selected gene expression analysis was conducted as previously described (14). Additional primers are listed in the Supplementary Table SI. To compare the profile of hedgehog pathway gene expression in clinical samples versus primary cultures, the ΔΔCt method was used where all ΔCt were normalized to 12.2, the lowest ΔCt determined. The heatmap of genes expressed in the xenografts was produced as previously described on raw data (13, 14), and only tumors with the same levels of human housekeeping normalizer gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were considered.

Primary MPM cultures

Primary MPM cultures were established from surgical specimens as previously described (13), except that at the end of enzymatic digestion, cells were resuspended in culture medium [Dulbecco’s Modified Eagle’s Media (DMEM):F12, 0.4 μg/mL hydrocortisone, 10 ng/mL EGF, 20 ng/mL basic fibroblast growth factor (bFGF), 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 μg/mL selenium, 1 mmol/L sodium pyruvate, 100 μmol/L β-mercaptoethanol] supplemented with nonessential amino acids and 30% conditioned medium (15) and incubated in 3% oxygen. The paired cultures of SDM61, SDM62, SDM74, and SDM76 grown in the presence of serum have been previously described (16). All cultures used in this study were authenticated by DNA fingerprinting (Microsynth). Primary cultures were used between passage 3 and 20.

Smoothed inhibition and measurement of cell growth

Cells were treated with cyclopamine (Toronto Research Chemicals), HhAntag (Genentech), both being specific antagonists of SMO, or with tomatidine (Sigma-Aldrich), a structurally similar compound with nonspecific inhibition of hedgehog signaling. Cell growth was determined as previously described (17).

Western blot analysis

Primary cultures were characterized for mesothelioma marker expression as described elsewhere (16), and expression of apoptosis or survival markers was achieved using rabbit polyclonal antibody anti PARP (polyclonal, 1:1,000 dilution, Cell Signaling), anti-survivin (polyclonal, 1:1,000 dilution, R&D), anti-caspase-3 (polyclonal, 1:1,000, Cell Signaling), anti-phospho-histone (polyclonal, 1:1,000, Millipore), anti Gli-1 (polyclonal, 1:1,000 dilution, Cell Signaling), anti-YAP (polyclonal 1:1,000 Cell Signaling), and anti-P-YAP (polyclonal 1:1,000, Cell Signaling).

In situ hybridization and immunohistochemistry

Tissue-Tek O.C.T-embedded tumors were used to prepare 12-μm thick sections which were processed for in situ hybridization with digitonin-labeled sense and antisense riboprobes as described (18). Human PITCH1, GLI1, and Sonic hedgehog (SHH) encoding plasmids (kindly provided by Dr. Ruiz-I-Altaba, Department of Genetic Medicine and Development, University of Geneva Medical School, Switzerland) were linearized with Xhol (sense) and Xhoi (antisense PATCH1) and HindIII (antisense GLI1, SHH), respectively. Human desert hedgehog (DHH) encoding plasmid (kindly provided by Dr. McMahon, Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts) was linearized with Ndel (antisense) and Xhoi (sense).

Immunohistochemistry was conducted on paraffin-embedded sections using polyclonal anti-YAP (1:25), Ki-67 (Mib-1, clone B126.1, 1:50, Abcam), and HHIP (clone M01, 1:100, Abnova) as detailed in Supplementary Methods.

Transfection of GLI1 or constitutively active YAP

ZL55SPT cells plated at a density of 3,500 cells/cm² were transfected with either pcDNA3.1-encoding human GLI1 (ref. 19; kindly provided by Drs. Bert Vogelstein and Sasaki) or pcDNA3.1 using DMRIE-C (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after transfection, 400 μg/mL G418 (Roche Applied Biosciences) was applied for selection. For constitutively active YAP expression, transient transfection of pcDNA3.1-encoding human YAP with the 5 LATS phosphorylation sites mutated to alanine (Ser61, Ser109, Ser127, Ser164, and Ser397; ref. 20; kindly provided by Dr. X. Yang) was used applying the same protocol.

RNA interference

For downregulation of GLI1 with siRNA, ZL55SPT cells were transfected with ON-TARGET plus SMARTpool siRNAs targeted to GLI1 or control nontargeting (NT) siRNA (Thermo Scientific Dharmacon), according to the manufacturer’s reverse transfection protocol. Cells were then plated at 7,000 or 700 cells/cm² to extract RNA or determine...
effect on cell growth, respectively. RNA was extracted after 48 and 72 hours. Cell growth was investigated by crystal violet staining after 12 days.

Assay for hedgehog pathway activation
NIH3T3 cells were plated into 12-well at the density of 80,000 cells per well. The next day, cells were transfected with 8xGli Bswt-luc reporter, GLI-binding site–mutated 8xGlimut-luc reporter (21) provided by Dr. Sasaki and Renilla luciferase (pRL-TK) at 50:1 ratio using DMRIE-c. After 9 hours, ZL55SPT conditioned medium with or without 300 nmol/L HhAntag was added, and cells were incubated for another 40 hours before luminometric detection (dual luciferase assay, Promega). Results are expressed as firefly luciferase activity normalized to Renilla luciferase activity. The presence of DHH in the conditioned medium was determined by ELISA as detailed in Supplementary Methods.

Animal studies
Human mesothelioma ZL55 cells (10⁶ per animal) were subcutaneously injected under general anesthesia into the left flank side of 8-week-old CD1 nude mice (from Charles River and Harlan). Treatments started when the tumor volumes reached 31 ± 8mm³. Mice were treated with HhAntag 38 mg/kg body weight or vehicle alone, by oral gavage twice per day 5 d/wk for 2 weeks. Tumor volume was measured by caliper and calculated with the formula width² × length/2. All animal experiments were carried out in accordance with the ethical principles and guidelines for
experiments on animals of Swiss Academy of Medical Sciences.

Results

**Hedgehog pathway expression in mesothelioma**

To address the activation of hedgehog signaling in MPM, we examined fresh-frozen tumor tissues from 39 patients. There were 34 males and 5 females, with a median age of 62 years (range, 42–77 years). In 6 patients, tumor samples were obtained before and after neoadjuvant chemotherapy or at progression giving a total of 45 tumor samples. In addition, 4 samples of chronic inflammation of the pleura were analyzed as well as 6 samples of normal pleura as control (16). The histopathology showed 25 tumors (64%) being of epithelioid type, 13 (33%) of the biphasic type, and one sarcomatoid type.

**SHH** gene expression could be detected in tumor tissue but not in nontumoral pleural samples (Fig. 1A). Downstream hedgehog targets **GLI1** and **human hedgehog interacting protein (HHIP)** mRNA levels were 2- and 6-fold higher, respectively, in MPM tissue than in nontumoral pleural tissue. No significant expression difference was observed for **PTCH1** (Fig. 1A). **Indian hedgehog, DHH, SMO, and GLI2** expression (Supplementary Fig. S1A) was mostly associated with tumor cells and correlated with quantitative PCR data. Data on overall survival were available for 23 patients who had received chemotherapy. In an exploratory analysis, we looked for a possible association of high **GLI1** expression with overall survival. There was a significant association of high **GLI1** expression with poor survival (P = 0.042, Supplementary Fig. S1B).

**Hedgehog pathway expression and activity in mesothelioma cultures**

Although only one fifth of MPM primary culture grows in the absence of serum compared with the ones that grow in medium containing serum, growth in the presence of their own conditioned medium, and 3% instead of 20% oxygen culture conditions allowed increased expression of "stemness" genes compared with serum-free medium at 20% oxygen (Supplementary Fig. S2). In primary cultures established in these conditions, we observed a profile of relative expression of the components of hedgehog pathway (Supplementary Fig. S3A) similar to the one determined in tumors, thereby allowing functional studies.

Treatment of mesothelioma primary cultures with cyclopamine resulted in a significant downregulation of **GLI1** expression in 4 of 6 primary cultures tested (Fig. 2A), whereas tomatidine, which was used as control for specificity, had no or little effect. Response to cyclopamine correlated with higher basal levels of **HHIP** protein basal levels (data not shown). In addition, we observed that conditioned medium from a primary mesothelioma culture specifically increased a hedgehog pathway–specific reporter (21) in NIH3T3 mouse cell line.
Hedgehog Inhibition in Malignant Pleural Mesothelioma

Altogether these data suggest that hedgehog signaling has an autocrine activity in MPM.

Inhibition of hedgehog signaling decreases MPM growth

The growth of primary MPM cultures without serum was very slow, and long-term cultures were obtained only with ZL55SPT and SDM103T2 which were originated from MPM xenografts in mice. Therefore, most of the following studies were conducted with these 2 lines.

Treatment with HhAntag resulted in a dose-dependent decrease of cell survival, indicating a role for hedgehog signaling in mesothelioma growth (Fig. 3A). The involvement of hedgehog signaling was confirmed by HhAntag-induced downregulation of GLI1 target *HHIP* (Supplementary Fig. S4). To gather a better understanding of the mechanisms leading to decreased cell survival, we investigated apoptosis markers PARP and caspase-3, mitotic marker phospho-histone and survivin, the latter being essential for mesothelioma survival (24), in cells treated during 48 hours with either 5 µmol/L cyclopamine or 5 µmol/L HhAntag (Fig. 3B). Although no PARP cleavage or caspase-3 decrease could be detected, we observed a significant decrease of phospho-histone mitotic marker and survivin levels in cells treated with HhAntag. Furthermore, a significant decrease in survivin mRNA expression was observed (Fig. 3C).

To investigate adequate targeting of HhAntag, we tested the effect of the treatment in ZL55SPT cells transfected with GLI1 or control vector. Western blot analysis of GLI1 expression (Fig. 4A) using a commercial antibody against the region surrounding amino acids 420 recognized the diverse forms of GLI1: full-length, the partially active 130 kDa, the weak repressor 100 kDa (25), and an additional 70 kDa band not yet identified, which were most visible in the transfected cells. The 100-kDa inactive form was the most abundant consistent with the notion that it is the more stable GLI1 form (25), nevertheless increased GLI1 activity was confirmed by increased *HHIP* expression (Supplementary Fig. S5). GLI1 transfection rescued survivin decreasing effects of HhAntag (Fig. 4A). In addition, it rescued the expression of hedgehog target SOX2 (Fig. 4B) which is expressed in ZL55SPT (13) and was downregulated by HhAntag.

The role of hedgehog signaling in MPM growth was further confirmed in ZL55SPT cells by downregulation of GLI1 expression using siRNA (Fig. 4C). Silencing GLI1 was already observed after 48 hours (data not shown) but was more efficient after 72 hours (Fig. 4C) and resulted in decreased clonal cell growth comparable with the effect of cyclopamine. In addition, decreased levels of *HHIP*, survivin, and SOX2 expression compared with nontargeting siRNA were observed.

**HhAntag suppression of survivin expression is associated with decreased YAP**

Survivin is not described as a direct target downstream hedgehog pathway. Hence, we sought for other transcription activators known to be expressed in MPM and to

embryonic fibroblasts cells (Fig. 2B). Conditioned medium activity was abolished in the presence of HhAntag, a recently developed potent synthetic SMO inhibitor (22), indicating that biologically active hedgehog ligands are present in the conditioned medium from mesothelioma culture as it had been shown in another model system (23). The presence of DHH in the conditioned medium (10.5 ± 4.5 pg/mL, n = 5) was confirmed by ELISA. Finally, we determined that in MPM xenografts (13), the expression of human DHH correlated with human GLI1 and *PTCH1* expression ($r^2 = 0.77, P = 0.004$; $r^2 = 0.54, P = 0.037$, respectively), whereas no correlation was observed with murine GLI1 or *PTCH1*; similarly, no correlation was observed between the expression of mouse DHH and human GLI1 or *PTCH1*.

![Figure 3. Hedgehog pathway controls MPM growth in vitro. A, HhAntag dose dependently inhibited MPM cell proliferation. *P* < 0.05; **P** < 0.001, compared with vehicle control. B, blocking hedgehog pathway with HhAntag does not induce apoptosis, assessed by determination of PARP and caspase-3 cleavage but decreases phospho-histone and survivin protein expression. MW, molecular weight. C, HhAntag significantly (P < 0.05) decreases survivin mRNA expression.](image-url)
regulate survivin expression and the most obvious was YAP. YAP is a transcriptional coactivator which localizes in the nucleus unless it is inactivated by phosphorylation by LATS kinase (26) downstream of NF2 signaling. YAP is constitutively active in more than 70% of primary MPM (27), it has been originally described to be involved in size control (14); hedgehog and YAP signaling. The latter was accompanied by increased HHIP immunohistochemical analysis. Indeed, to get further insight into the mechanism of decreased tumor growth observed in HhAntag-treated mice, gene expression analysis was conducted investigating the relative expression of mesothelioma markers calretinin, podoplanin, and mesothelin (14); hedgehog pathway components GLI1, PTCH1, DHH, and HHIP; ABC transporters ABCC1 and ABCC2; stem cell markers Nestin, OCT4A, CD90, and HES1, osteoblastic differentiation markers BMP2 and runx2; hypoxia controlled CAIX and Wisp2; and matrix remodeling Slug, Twist, and PAI-1. Some mouse genes (mGLI1, mPTCH1, mSca-1, mABCG2, and mABCC1) were also included to take into account mouse stromal components. We observed (Fig. 6B) a treatment-induced significant 2-fold increase in nestin, human ABCC1 (P < 0.001 for both), and HHIP (P < 0.005) expression levels. The latter was accompanied by increased HHIP immunoreactivity in samples from HhAntag-treated mice (Supplementary Fig. S7). An almost 2-fold increase expression was also observed for runx2, human PTCH1 (P < 0.01 for both), and Bmp-2 (P < 0.05), whereas the increase of CAIX, twist, and podoplanin was less extended (P < 0.05). The highest increase was observed for mouse GLI1 (P < 0.01) where a basis of therapeutic range reported in the literature (22). HhAntag led to a significant (P < 0.05, t test) 35% decrease of the tumor volume after the 2 weeks of treatment (Fig. 6A).

**Effect of HhAntag as a single agent on MPM xenografts in SCID mice**

Finally, we tested the effect of HhAntag in vivo in ZL55 xenografts. Tumor-bearing mice were randomized in 2 groups receiving either solvent or HhAntag. The in vivo HhAntag treatment dosage (38 mg/kg body weight, administered twice daily by oral gavage, 5 d/wk) was chosen on the...
3-fold increase was determined. The effect of HhAntag on tumor volumes was also accompanied by a significant \((P < 0.05, \text{Mann–Whitney} \ U \text{ test})\) 43% decrease in Ki-67 labeling index (Fig. 6C). Furthermore, consistent with in vitro experiments, we observed a significant \((P < 0.05, \text{Mann–Whitney} \ U \text{ test})\) 32% decrease in nuclear YAP immunostaining in HhAntag-treated tumors (Fig. 6C).

All in all, these data support a role for hedgehog signaling in MPM growth.

Discussion

Hedgehog signaling has been implicated in several cancers (reviewed in ref. 30), however, it is the first time that it is documented to have a role in mesothelioma cell growth. We observed a significant increased expression of GLI1 in tumor tissue indicating the presence of an active pathway. Levels of both SHH ligand and HHIP, which is a negative regulator by binding all ligands with nanomolar affinity (31), were also significantly upregulated in mesothelioma tumors. Because
expression levels of HHIP were in some samples 100-fold higher than in the other pathway components, this may indicate a differential negative feedback mechanism. A high variation of HHIP expression compared with nontumoral tissue has already been observed in lung tumors (32), but whether it corresponds to modulation of hedgehog signaling in the tumor has not been investigated yet, although it is known that HHIP is essential for normal lung development (33).

Hedgehog signaling in tumors can be ligand-independent and driven by mutations in signal transducers as observed in basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma, whereas in several cancers, ligand-dependent hedgehog autocrine activity has been shown (reviewed in ref. 34). On the other hand, a tumor-promoting activity via a paracrine effect of hedgehog ligands secreted from the tumor on stroma (35) or vice versa has been observed (36). We have 3 lines of evidence suggesting that an autocrine activity is present in some mesothelioma: SMO inhibitors could decrease cell growth and GLI1 expression, conditioned medium could stimulate a GLI1-reporter activity, and human sonic hedgehog ligand expression was correlated with human GLI1 and PTCH1 but not with murine GLI1 or PTCH1 expression in tumor xenografts.

Sensitivity to SMO inhibitors was not the same in all primary cultures tested, and this did not seem to be related to HHIP expression which was similar in the 3 cultures tested which had differential sensitivity. One other possibility could be the differential expression of glypican-3 (GPC-3), a proteoglycan expressed at the surface of the cell, which is frequently inactivated by promoter methylation in mesothelioma (37). GPC-3 is known to inhibit hedgehog signaling (38), and its overexpression has been shown to inhibit cell growth in mesothelioma (39). Alternatively, another member of glypican family, glypican-5, which has been recently shown to activate hedgehog signaling (40), is expressed in MPM and at different levels, thereby controlling hedgehog signaling. Finally, it is possible that the negative regulator of hedgehog signaling, suppressor of...
fused (41, 42), is differentially operational in each primary culture. Further studies will address this question.

Potential ligands present in the conditioned medium are either DHH which was expressed in primary cultures or oxysterols, which can be derived from endogenous cellular biosynthesis and are efficient stimulators of hedgehog signaling (43). DHH is known to be expressed in gonads, including Sertoli cells of testis and granulosa cells of ovaries (reviewed in ref. 44), and there are no obvious reasons why it is expressed in mesothelioma. However, homogeneous increase of DHH, but not of SHH, has been recently described in osteosarcoma cell lines (45), suggesting that DHH expression is aberrantly activated in cancer.

Decreased survivin expression upon inhibition of hedgehog signaling has been described in at least one study in colon cancer cells in culture (46). The fact that we could antagonize the SMO inhibitor effect by GLI1 overexpression and that this phenomenon was also observed after GLI1 silencing indicates that this effect is specific. Many signals control survivin expression (47), the one relevant to mesothelioma and linked to hedgehog signaling is active YAP. Indeed, YAP is constitutively active in more than 70% of primary MPM (27), and we confirmed YAP activation in this study. The observation that HhAntag decreases YAP protein expression is consistent with the role of hedgehog in maintaining YAP protein stability (48).

In addition to a decrease in survivin by HhAntag, we observed a decrease in the expression of the stem cell marker SOX2. The latter is controlled by hedgehog signaling in neural stem cells (49), but YAP has also been described to directly positively regulate SOX2 expression (50). Functional studies are necessary to identify whether GLI1 transcription factors or YAP-dependent transcription are involved in SOX2 expression in mesothelioma.

In the xenograft model, the inhibition of hedgehog was accompanied not only by a decrease of nuclear YAP but also by a significant change in gene expression. The increase in HHIP and PTCH1 might be relevant for a negative signaling regulator, whereas the increase in ABCG1 transporter expression might be linked to drug-induced adaptation, assuming that HhAntag is effluxed by ABCG1. The selected gene expression analysis included genes (runx2 and Bmp-2) along mesenchymal stem cell differentiation toward osteoblast (51, 52). This is due to the fact that we recently observed that mesothelioma primary cultures express mesenchymal stem cell markers CD90, CD105, and CD73 (13), therefore if hedgehog signaling is involved in maintaining stemness, its inhibition should result in promoting differentiation, as it has been recently observed in chronic myeloid leukemia (53). The increase in runx2 and Bmp-2 expression upon HhAntag treatment is consistent with knowledge about hedgehog control of osteoblastic differentiation (54) and with defects in bone structure observed in young mice treated with this agent (55). This result is also consistent with the recent observation that YAP reduces the expression of Bmp-2 (20).

The increase in nestin and mouse GLI1 expression is more intriguing. An unexpected increased vasculature and decreased stroma have been observed in a mouse model of pancreatic ductal carcinoma treated with a hedgehog inhibitor for the same length of time (56), but no such changes were identified by histopathologic analysis in our study.

In conclusion, upregulation of hedgehog signaling was observed in MPM tumors, and SMO inhibitors decreased cell growth both in vitro and in vivo in sensitive mesothelioma. Growth control was associated with downregulation of YAP and its target survivin. Further studies identifying factors associated with response will allow defining patient who may potentially benefit from hedgehog antagonist therapy.

References


Disclosure of Potential Conflicts of Interest

A. Soltermann has received fees as consultant for NSCLC Advisory Board Switzerland for Pfizer. Funding agencies had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: W. Weder, E. Felley-Bosco
Development of methodology: Y. Shi, A. Soltermann, W. Weder, E. Felley-Bosco
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Shi, U. Moura, A. Soltermann, E. Felley-Bosco
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Opitz, A. Soltermann, H. Rehrauer, S. Thies, E. Felley-Bosco
Writing, review, and/or revision of the manuscript: I. Opitz, A. Soltermann, S. Thies, R.A. Stahel, E. Felley-Bosco
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Shi, S. Thies
Study supervision: W. Weder, E. Felley-Bosco

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