



Review

Inhibition of the Hedgehog pathway in lung cancer

A. Dimou^{a,*}, A. Bamias^b, H. Gogas^c, K. Syrigos^d^a University of Colorado, Division of Medical Oncology, 12801 E. 17th Avenue, Mail Stop 8117, Research 1 South, Aurora, CO, USA^b Alexandra Hospital, National and Kapodistrian University of Athens School of Medicine, Department of Clinical Therapeutics, Alexandra Hospital, 80 Vasilisis Sofias Avenue, Athens, Greece^c Laiko General Hospital, National and Kapodistrian University of Athens School of Medicine, 1st Department of Medicine, 17 Agiou Thoma St. Athens, Greece^d Sotiria Hospital, National and Kapodistrian University of Athens School of Medicine, 3rd Department of Medicine, 152 Masogeion Avenue, Athens, Greece

ARTICLE INFO

Keywords:

Smoothered

GLI

Vismodegib

Arsenic trioxide

GANT61

Primary cilium

ABSTRACT

Inhibitors of the hedgehog pathway are effective in patients with basal cell carcinoma and a subgroup of patients with medulloblastoma with active hedgehog signaling. Despite preclinical work suggesting otherwise, clinical trials in solid tumors of epithelial origin have not shown added benefit with these drugs. Here, we review the preclinical and clinical data of hedgehog pathway inhibition in the most common histologic types of lung cancer. We focus on highlighting areas of uncertainty, where further research might define a niche for hedgehog pathway inhibition in patients with lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer related mortality globally and is classified as either Small Cell Lung Cancer (SCLC) or Non-Small Cell Lung Cancer (NSCLC) upon pathology review [1,2]. The main histologic types of NSCLC include squamous cell carcinoma and adenocarcinoma [2]. The majority of patients have non-curable disease stage at the time of diagnosis. Advances in systemic treatments including chemotherapy, targeted therapies and immune check point inhibitors have improved prognosis in recent years. Nevertheless, most patients with metastatic lung cancer die within a few years from diagnosis, stressing the need for more and novel therapeutic approaches. Targeted therapies benefit patients classified in well-defined molecular groups identified by the presence of activating mutations in key genes like *EGFR*, *ALK*, *ROS1* and *BRAF* that follow the oncogene addiction paradigm [3]. The list of driver oncogenes with actionable mutations is expanding over time. On the other hand, targeting molecular pathways in the absence of an addicting oncogene mutation is a more challenging goal.

The Hedgehog signaling pathway (HH pathway hereafter) is vital for development and tissue homeostasis [4]. In recent years, studying

the role of the HH pathway in carcinogenesis and cancer stemness has highlighted the HH inhibitors as putative cancer therapeutics [5]. However, clinical application of HH inhibition in cancer improves outcomes only for patients with basal cell carcinoma [6] and a subgroup of patients with medulloblastoma with active HH signaling [7]. Both these tumor types harbor oncogenic mutations in key molecules of the HH pathway. In contrast, clinical trials have largely yielded negative results for unselected populations of patients with solid tumors of epithelial origin despite preclinical rationale [8,9]. Herein, we review the preclinical and clinical data on the use of HH inhibitors with respect to the major histologic types of lung cancer, including small-cell lung cancer (SCLC), lung adenocarcinoma and squamous cell lung carcinoma. The goal of this review is to highlight areas of uncertainty and guide future research for HH pathway inhibition in lung cancer.

2. Hedgehog pathway overview

Stem cells are capable for both self-renewal and evolution to more differentiated states, a feature known as asymmetric cell division [10]. The identification of stem cells originated from the observation that a limited number of bone marrow cells are able to reconstitute the entire

Abbreviations: HH, Hedgehog; SCLC, small-cell lung cancer; NSCs, neural stem cells; CICs, cancer initiating cells; SHH, sonic Hedgehog; IHH, Indian Hedgehog; DHH, desert Hedgehog; PTCH, patched; GPCR, G-protein coupled receptor; SMO, smoothered; SUFU, suppressor of fused; KIF7, kinesin family member 7; CK1α, casein kinase 1α; NRP1, neuropilin 1; NRP2, neuropilin 2; GSK3β, glycogen kinase 3β; HHIP, hedgehog inhibitory protein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ATO, arsenic trioxide; PFS, progression free survival; OS, overall survival; PKCι, protein kinase iota; EMT, epithelial to mesenchymal transition

* Corresponding author at: University of Colorado School of Medicine, Division of Medical Oncology, 12801 E. 17th Avenue Mail Stop 8117, Research 1 south, Anschutz Medical Campus, Aurora, CO, 80045, USA.

E-mail addresses: anastasios.dimou@ucdenver.edu (A. Dimou), abamias@med.uoa.gr (A. Bamias), hgogas@med.uoa.gr (H. Gogas), ksyrigos@med.uoa.gr (K. Syrigos).

<https://doi.org/10.1016/j.lungcan.2019.05.004>

Received 6 February 2019; Received in revised form 2 May 2019; Accepted 5 May 2019

0169-5002/ © 2019 Elsevier B.V. All rights reserved.

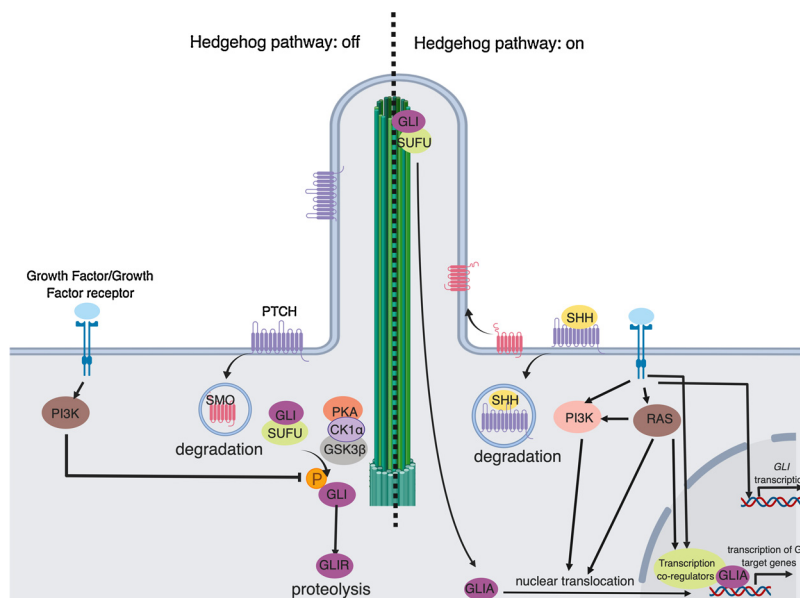


Fig. 1. Canonical and non-canonical activation of Hedgehog pathway. The canonical Hedgehog pathway in vertebrates requires the primary cilium, a solitary plasma membrane structure with controlled entrance for plasma bound molecules. In the absence of Hedgehog ligands, the pathway is inactive (left). The patched (PTCH) receptors inhibit smoothened (SMO) which subsequently undergoes endocytosis and degradation. In this state, GLI transcription factors are sequestered by Suppressor of Fused (SUFU), phosphorylated successively by protein kinase A (PKA), glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α) and either degraded (GLI2), or truncated to a HH pathway repressor form, GLIR (GLI3). In the active state of the pathway (right), sonic hedgehog (SHH) and other hedgehog ligands bind to their receptor PTCH which then undergoes endocytosis and degradation. SMO then re-localizes into the primary cilium. Also, the SUFU/GLI complex localizes at the tip of the cilium, GLI dissociates from SUFU, exits the primary cilium and enters the nucleus in its active form, GLIA (mainly GLI2), where it activates the transcription of *GLI* responsive genes. GLI1 functions as an amplifier of the pathway. In the non-canonical activation of the pathway, PI3K, RAS and growth factors (like EGF and TGFβ) might either inhibit the phosphorylation of GLI by PKA, induce the nuclear localization of GLI, activate transcription co-regulation factors *in cis* with GLI or induce the transcription of the *GLI* genes. Created with BioRender.

hematopoietic system when transplanted in syngeneic mice [11]. Since then, a number of stem cell populations have been characterized both in normal adult and embryonic tissues including the hematopoietic stem cells [12], the neural stem cells (NSCs) [13], the embryonic stem cells [14] and others [15,16]. Likewise to stem cells in normal tissues, the stem cell theory of cancer introduces the concept of the cancer initiating cell (CIC) sitting at the top of cancer cell hierarchy [17]. According to this theory, a limited number of CICs can form a tumor xenograft when implanted in mice [18]; CICs are responsible for resistance to drug therapy, disease relapse and metastasis [17]. Importantly, both normal stem cells and CICs activate common molecular pathways including morphogens WNT, NOTCH and HH [19]. Especially the HH pathway activation follows a concentration HH ligand gradient during the development of the normal lung and is also important for the maintenance of adult stem cells in many tissues [20,21].

The HH pathway (illustrated in Fig. 1) was initially described in *Drosophila melanogaster* where it was found to be required for segment polarity and ventral-dorsal differentiation [22]. In vertebrates, the SHH, IHH and DHH (Sonic, Indian and Desert Hedgehog respectively) ligands bind to the 12-pass transmembrane receptors Patched1 and Patched2 (PTCH1 and PTCH2 respectively) [23]. In the absence of ligands, the PTCH receptors suppress the 7-pass transmembrane G-protein coupled receptor (GPCR)-like protein Smoothened (SMO) [24]. The binding of lipid modified SHH, IHH and DHH ligands to the PTCH receptors [25,26], activates SMO in the primary cilium, a solitary organelle with sensory function in most mammalian cells [27–29]. The exact interaction of PTCH with SMO is not fully elucidated but involves control of cholesterol availability [30,31]. This process inhibits the processing of the zinc finger transcription factors GLI. Following SMO activation, full length GLI enters the cilia in the form of a complex with the HH negative regulator Suppressor of Fused (SUFU) [32]. The complex accumulates at the cilia tip where GLI disassociates [32] and exits the cilia in a Kinesin Family Member 7 (KIF7) dependent process to function as a transcriptional activator for HH pathway target genes [4]. There are currently three identified GLI transcription factors in vertebrates, GLI1, GLI2 and GLI3. GLI3 functions mainly as a pathway repressor in its truncated form. GLI1 lacks the N-terminal repressor sequence and has activating effect only, whereas GLI2 is the main pathway activator [33–35].

Several layers of regulation for this canonical activation of the HH pathway exist. First, the protein kinase A (PKA), the casein kinase 1α (CK1α) and GSK3β can phosphorylate and mark for proteosomal

degradation the GLI transcription factors [36]. Second, Suppressor of Fused (SUFU) binds to GLI and sequesters GLI in the cytoplasm in the absence of hedgehog ligands [37]. Third, genes encoding for negative regulators of the HH pathway, such as *PTCH* 1 and 2 and hedgehog inhibitory protein (*HHIP*) are GLI target genes, therefore they engage in a negative feedback loop with GLI [38,39]. Also, neuropilin 1 and 2 (NRP1 and NRP2), activate a positive feedback loop with GLI as described recently [40,41]. Neuropilins enhance HH signaling in both a PKA dependent [41] and independent [42] way. In the mouse, non-canonical Hedgehog pathway also exists, where astrocyte derived Shh activates nestin in a medulloblastoma model. Importantly, this paracrine loop is *Ptc1* and *Gli* independent but *Smo* dependent [43]. Last but not least, pathways other than the HH pathway, for example RAS, TGFβ and PI3K, can induce *GLI* expression in cancer in a number of ways [44,45]. First, RAS, as well as PI3K induce the nuclear localization and activate GLI1 in melanoma models [46]. Further, PI3K/AKT signaling inhibits GLI phosphorylation by PKA and prevents its degradation [47]. Third, signaling through EGFR activates c-JUN via MAPK which functions as a transcription co-regulator factor with GLI for certain GLI targets [48]. Fourth, SMAD transcription factors are regulated by TGFβ and synergize with GLI1 to induce TGFβ and HH dependent *CCND1* expression [49]. Finally, TGFβ signaling increases transcription of the *GLI* genes [50]. Fig. 1 illustrates the canonical and non-canonical HH pathway and its regulation.

3. HH pathway inhibitors

There are currently several strategies to inhibit the hedgehog pathway in the clinic. Interfering directly with SMO activity is a well-studied means to inhibit the HH pathway. Cyclopamine belongs to *Veratrum* alkaloids, plant derived compounds known to cause teratogenesis including cyclopia [51,52]. Cyclopamine binds to the extracellular loops of the transmembrane domain of SMO and is a SMO inhibitor [51,53]. Vismodegib, saridegib and sonidegib are more potent inhibitors of SMO which also bind to the transmembrane domain [54]. On the contrary, oxysterol and other oxidized derivatives of cholesterol activate SMO by binding to the cysteine rich domain located in the extracellular domain of SMO [55–57]. Indeed, statins which are known to block cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) inhibit medulloblastoma growth *in vivo* [58]. Additionally, cholesterol is necessary and sufficient to activate SMO and might represent the missing link between PTCH1 and SMO [30,31].

In this model, PTCH1 negatively regulates plasma membrane cholesterol in the vicinity of SMO further supporting the rationale for cholesterol synthesis inhibition as a HH targeting strategy.

Itraconazole, a triazol antifungal agent was identified as an inhibitor of HH signaling in a library screen of 2400 drugs with FDA approval or in post phase I drug development process [59]. Despite the well-established target of itraconazole, 14- α -lanosterole demethylase which is necessary for ergosterol synthesis in fungi and cholesterol in mammals with higher potency for the fungal enzyme, the inhibitory effects of itraconazole on the HH pathway result from direct binding and inhibition of SMO at a site different from the binding site for oxysterols or cyclopamine [59]. Itraconazole effectively prevents the accumulation of SMO in the primary cilium and inhibits the growth of Hh dependent medulloblastoma *in vivo* [59]. The itraconazole doses necessary to inhibit SMO are higher compared to doses used to inhibit ergosterol synthesis in fungi but still clinically feasible.

Another approach to inhibit the HH pathway is to target the transcription factors GLI. The active form of GLI2 is the most significant mediator of HH activity in mammals, GLI3 is mostly a suppressor and GLI1 serves as a pathway output amplifier [34,35,60]. Arsenic trioxide (ATO) is used clinically for the treatment of acute promyelocytic leukemia because it degrades PML-RARA [61]. ATO inhibits GLI1 in a primary cilium independent manner, blocks Gli2 accumulation in the primary cilium and exerts anti-tumorigenic effects in a wide array of cancer cell lines and HH dependent *in vivo* mouse models [62,63]. GANT-58 and GANT-61 were identified in a drug screen as inhibitors of GLI1 as they prevent binding of GLI1 transcription factor to DNA [64]. SSTC3 is an agonist for CK1 α , which along with GSK3 β phosphorylates the GLI transcription factors marking them for degradation [65]. SSTC3 inhibits *TRP53* mutated, *MYCN* amplified medulloblastoma mouse models by promoting GLI degradation [65].

3.1. Hedgehog pathway inhibition in small cell lung cancer

The HH pathway is active in the developing airway as well as in regeneration of neuroendocrine cells following airway injury [66]. In the latter case, diffuse intraepithelial expression of Gli1 and Shh occurs right before the appearance of neuroendocrine cells in the lungs of mice during regeneration following injury with naphthalene [66]. Additionally, the same group reported juxtacrine activity of SHH in human SCLC cell lines, expression of multiple members of the HH pathway in SCLC cell lines and tumors and anti-proliferative and anti-tumorigenic activity of cyclopamine in these models [66]. GLI1 is frequently expressed in SCLC tumors but not in cell lines [67]. In transgenic mouse SCLC models with conditional knockout of *Rb1* and *Trp53* cell autonomous Hedgehog signaling is activated and promotes the development and maintenance of the SCLC tumors [67,68]. Activation of HH pathway has been demonstrated to be involved in development of resistance to chemotherapy in human SCLC models, *in vitro* and *in vivo* [67]. Interestingly, the proportion of cells having primary cilia increases in cell culture and mouse xenograft models upon development of resistance [67].

Despite the preclinical data, incorporation of SMO inhibitors in the treatment of patients with SCLC does not yield significant benefit in unselected patients. Vismodegib failed to improve response rates, progression-free survival (PFS) and overall survival (OS) when added to cisplatin and etoposide compared with chemotherapy alone [8]. In a phase I trial, sonidegib in combination with cisplatin and etoposide induced a durable response for at least 27 months in a patient with SCLC and SOX2 amplification [69].

There are several possibilities that might explain the discrepancy between clinical trial data and preclinical models. First, it is possible that cell lines and animal models might not fully recapitulate the complexity of human tumors. Second, although the HH pathway might contribute to pro-tumorigenic phenotype in SCLC, other molecular pathways might salvage cancer cells from HH pathway inhibition in an

adaptive resistance model. Additionally, timing of HH pathway inhibition might be important for optimal activity. In this respect, the findings of Park et al [67] suggest that SMO inhibitors should follow chemotherapy as HH pathway is activated as part of a resistance phenotype. Last, targeting other molecules of the HH pathway, like SHH or the GLI transcription factors could be more effective than targeting SMO, as these might promote SCLC progression in a non-canonical fashion. In a recent report, Szczepny et al. showed a Smo independent role of Shh ligand in activating cyclin B1 and inducing chromosomal instability in SCLC conditional Tp53/Rb1 mutant mouse models, in addition to the effect in the canonical pathway [68].

3.2. HH inhibitors in squamous cell NSCLC

Squamous cell histology accounts for up to 30% of all NSCLC cases [70]. Despite advances in targeted treatment for patients with lung adenocarcinoma, there is little progress for patients with squamous cell lung cancer. An important step for better molecular understanding of this disease is the classification of squamous cell lung cancers into four categories, primitive, classical, basal and secretory, on the basis of gene expression profiles [71]. This classification schema is more informative compared to the existing morphological WHO classification as it correlates with outcomes and is also validated in the TCGA genomic classification for squamous cell lung carcinomas [72]. Importantly, GLI1 and GLI2 expression as well as the GLI target PTCH, are upregulated and the expression of negative regulators of the HH pathway, GLI3 and SUFU is downregulated in the classical but not the other molecular groups [73]. Interestingly, inhibition of the GLI transcription factors with GANT61 increases apoptosis, reduces cell proliferation in a GLI dependent fashion and decreases tumor growth *in vivo*. Conversely, inhibition of SMO has a modest effect on these processes [73].

Amplification of chromosome 3q is a hallmark of classical subtype of squamous cell lung cancer [74]. SOX2 and PRKCi are localized in this area of the genome and they are frequently amplified in these tumors [75]. A recent study showed that SOX2 and PRKCi amplifications are enriched in lung spheres representing lung squamous cancer initiating cells. Consistently with the CICs phenotype, they induce higher anchorage independent growth in soft agar assays and tumorigenic capacity compared to parental cells [76]. Interestingly, these spheres are also dependent on the canonical HH pathway. Mechanistically, protein kinase C iota (PKCi) encoded by PRKCi, phosphorylates SOX2 at threonine in position 118 (T118) and induces SOX2 occupancy of the HHAT (Hedgehog acyltransferase) promoter. Subsequent induction of HHAT, palmitoylation of SHH and activation of an autocrine canonical HH pathway is required for CICs maintenance [76]. GLI1 also enhances cancer initiating properties in lung squamous cell carcinomas with *FGFR1* amplification [77]. This alteration is present in up to 16.6% in this population and often associated with SOX2 amplification [77].

Clinical data are sparse and limited to case reports. A clinical response was documented in a patient with squamous cell lung cancer and a germline SMO mutation treated with vismodegib [78]. Whether there might be a role for HH pathway inhibition in the absence of mutations is unknown.

3.3. HH inhibitors in lung adenocarcinoma

HH target genes are expressed in lung adenocarcinoma cell lines and clinical specimens [79–81]. The majority of lung adenocarcinoma cases (76%) have some expression of GLI1 by immunohistochemistry according to a study, however almost half of the GLI1 positive cases do not express any SMO [82]. The same report showed frequent methylation of the SMO promoter in lung adenocarcinoma cell lines to explain low SMO levels. In contrast, GLI1 expression in SMO-low cell lines is induced by the MAPK pathway in a non-canonical fashion. Inhibition and knock down of GLI1 with GANT61 and small interfering RNA (siGLI) respectively inhibits proliferation and decreases the expression

Table 1
Overview of Hedgehog inhibitors.

Mechanism of Hedgehog inhibition	Example drugs	Clinical trials in lung cancer (ongoing)
Inhibition of oxysterol synthesis: Oxysterols are derivatives of cholesterol, they bind to and activate SMO.	• Statins	• NCT01441349: Irinotecan/Cisplatin plus simvastatin for patients with chemotherapy naïve extensive SCLC.
Inhibition of SMO	• Vismodegib, sonidegib • Triazole antifungals (e.g. itraconazole)	• MATCH trial (NCT02465060): Vismodegib arm for patients with SMO or PTCH mutations • NCT02357836: Neoadjuvant itraconazole in NSCLC • NCT03664115: Itraconazole plus chemotherapy vs. chemotherapy alone for patients with treatment naïve metastatic NSCLC
Inhibition of GLI	• ATO • GANT61	• NCT02066870: Icotinib and ATO in Patients NSCLC and EGFR mutations, with Resistance to EGFR-TKI
Activation of CK1α (Hedgehog pathway regulation)	• SSTC3	none

SMO: Smoothened, CK1α: Casein Kinase 1α, ATO: arsenic trioxide, NSCLC: Non-Small Cell Lung Cancer, SCLC: Small Cell Lung Cancer, TKI: tyrosine kinase inhibitor.

of proteins associated with a cancer initiating phenotype in these cell lines. Other preclinical data also demonstrate the anti-tumor efficacy of SMO inhibitors or shRNA for SMO or GLI *in vitro* [79,81].

In a phase 2 study, addition of the SMO inhibitor itraconazole to pemetrexed in second line treatment of patients with lung non-squamous cell carcinoma prolonged median PFS (5.5 months) compared to pemetrexed alone (2.8 months). However, this difference was not statistically significant as the study was stopped prematurely and included only 23 patients [83]. Noteworthy, a low dose of itraconazole (200 mg) was used in this study.

In the mouse, Cyclin E overexpression induces HH pathway activation in both cancer and dysplasia lesions indicating a role for HH in lung carcinogenesis [84]. Additionally, in a transgenic *KRAS*^{G12D} mouse model, loss of *EphA2* accelerates the tumorigenic process mediated by conditional activation of *KRAS*. Interestingly, these tumors have ERK dependent activation of the canonical HH pathway and cells from these tumors respond to SMO inhibitors sonidegib and vismodegib in cell viability assays [85].

A number of groups have reported activation of the HH pathway in *EGFR* mutation positive lung adenocarcinoma models developing resistance to *EGFR* inhibitors via epithelial to mesenchymal transition (EMT) [86–88]. EMT is a cellular process that allows epithelial cells to detach from a basic membrane and migrate by adopting a mesenchymal morphology [89]. It involves the activation of a network of transcription factors and plays crucial roles during the implantation of the embryo and organ development. In cancer biology, EMT promotes cancer cell migration, invasion, metastasis and drug resistance. Especially for lung cancers with activating *EGFR* mutations, EMT drives secondary resistance independently from other known mechanisms of resistance to *EGFR* tyrosine kinase inhibitors (TKIs) [90]. GLI promotes EMT in lung adenocarcinoma [91]. The role of HH pathway in resistance to *EGFR* inhibition has also been demonstrated in head and neck cancer and glioblastoma [92,93].

Della Corte et al showed that HH pathway activation by SMO amplification is a mechanism of secondary resistance to gefitinib *in vitro* [94]. They also showed that SMO forms a complex with MET, creating a rationale for combined SMO and MET inhibition [94]. In a follow up study, they developed a series of *EGFR* TKIs resistance xenograft models from *EGFR* mutation positive HCC827 parental cells [87]. Interestingly, many of these *in vivo* models demonstrate up regulation of SMO and GLI1 protein levels along with an EMT profile. Additionally, combination of SMO and *EGFR* inhibition effectively decreases proliferation, invasion and anchorage independent growth of resistant cases [95]. In another study, Bora-Shingal et al showed the HH pathway and *EGFR* co-operate to activate the stem cell inducing transcription factor SOX2 in cell lines with *EGFR* activating mutations [88]. Also, in this study, GLI1 was enriched in the side population, thought to represent the CICs of these cells in culture.

4. Mechanism of HH pathway activation in NSCLC

Despite the role of the HH pathway in basal cell carcinoma and medulloblastoma, it has been postulated that epithelial tumors do not demonstrate cell autonomous HH ligand activity. The main evidence comes from the study by Yauch et al showing no correlation between *GLI1* expression levels and sensitivity of a large range of colorectal, pancreatic and lung cancer cell lines to SMO inhibitors [96]. Additionally, the concentrations of HH inhibitors required to inhibit proliferation in pancreatic and lung cancer cell lines are in the micromolar range whereas the concentrations needed to inhibit GLI1 luciferase activity as well as proliferation in known hedgehog responsive mesenchymal cell lines are 10 and 20 times lower respectively. Instead, the HH ligands secreted by the epithelial neoplastic cells may have a paracrine effect in mesenchymal cells in the tumor microenvironment [96]. This paracrine loop in HH ligand expressing tumor models is pro-tumorigenic supporting a therapeutic role for SMO inhibitors. Nevertheless, a growing body of evidence, including experiments with specific knock downs of SMO, supports the autocrine activity of HH ligands in lung cancers in addition to the paracrine model [79–81]. The autocrine model is particularly active in the subpopulation of cancer initiating cells rather than cancer cells from the bulk of the tumor [76].

5. Ongoing clinical trials with HH inhibitors in lung cancer

HH pathway inhibition has been explored in clinical trials in unselected populations over the past decade. Despite approval in patients with non-resectable basal cell carcinoma and activity in patients with SHH type of medulloblastoma, the trials in epithelial solid tumors yielded negative results. Currently, few clinical trials are ongoing as summarized in Table 1. In one of them (NCT02357836), itraconazole is administered at a dose of 600 mg twice daily for 10–14 days as neoadjuvant treatment prior to surgery for patients with resectable NSCLC. This dose of itraconazole was selected to reflect the higher dose required to inhibit the HH pathway compared to the typical itraconazole dose when the drug is used for its antifungal properties. The study, which has a strong translational rationale will evaluate the resection specimen for changes in the HH pathway and tumor angiogenesis.

6. Areas of uncertainty and concluding remarks

Clinical trials did not show any benefit from HH inhibition in SCLC or other solid tumors of epithelial origin. Given the role of the HH pathway in lung carcinogenesis and lung cancer biology, we believe that further study of the mechanisms of lack of response to HH inhibitors might explain the failure to translate preclinical work. To this end, we identify three areas of uncertainty, where further research might propose novel directions for this domain. First, formation of complexes of SMO with other transmembrane receptors like MET [94] or generation of oxysterols that function as SMO activators [57] might limit the activity of SMO inhibitors and could support combination

strategies with MET inhibitors and statins respectively. Second, activity of HH inhibition in selected squamous cell lung cancer population on the basis of *SOX2* and *PRKCI* amplification is currently unknown and could be the focus of future studies. Last but not least, GLI inhibition could be a novel strategy in the treatment of lung cancer and the clinical value of GLI inhibitors should further be explored.

Disclaimer

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author declaration

I wish to confirm the following disclosures:

Anastasios Dimou has received honoraria from Roche/Genentech (April 2019). The rest of the authors do not have any relevant disclosures.

I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm and approve the order of authors listed in the manuscript.

I confirm that I have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing I confirm that we have followed the regulations of our institutions concerning intellectual property.

I understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. I confirm that I have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from anastasios.dimou@ucdenver.edu.

References

- [1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, *CA Cancer J. Clin.* 68 (2018) 7–30 2018.
- [2] W.D. Travis, E. Brambilla, A.G. Nicholson, et al., The 2015 World Health Organization classification of lung tumors: impact of genetic, clinical and radiologic advances since the 2004 classification, *J. Thorac. Oncol.* 10 (2015) 1243–1260.
- [3] J.M. Pacheco, A. Dimou, P.A. Bunn, Advances in lung cancer, *Oncotarget* 8 (2017) 78247–78248.
- [4] J. Briscoe, P.P. Thérond, The mechanisms of Hedgehog signalling and its roles in development and disease, *Nat. Rev. Mol. Cell Biol.* 14 (2013) 416–429.
- [5] L.L. Rubin, F.J. de Sauvage, Targeting the Hedgehog pathway in cancer, *Nat. Rev. Drug Discov.* 5 (2006) 1026–1033.
- [6] D.D. Von Hoff, P.M. LoRusso, C.M. Rudin, et al., Inhibition of the hedgehog pathway in advanced basal-cell carcinoma, *N. Engl. J. Med.* 361 (2009) 1164–1172.
- [7] G.W. Robinson, B.A. Orr, G. Wu, et al., Vismodegib exerts targeted efficacy against recurrent sonic Hedgehog-subgroup medulloblastoma: results from phase II pediatric brain tumor consortium studies PBTC-025B and PBTC-032, *J. Clin. Oncol.* 33 (2015) 2646–2654.
- [8] C.P. Belani, S.E. Dahlberg, C.M. Rudin, et al., Vismodegib or cixutumumab in combination with standard chemotherapy for patients with extensive-stage small cell lung cancer: a trial of the ECOG-ACRIN cancer research group (E1508), *Cancer* 122 (2016) 2371–2378.
- [9] D.V. Catenacci, M.R. Juntila, T. Karrison, et al., Randomized phase IIb study of gemcitabine plus placebo or vismodegib, a Hedgehog pathway inhibitor, in patients with metastatic pancreatic cancer, *J. Clin. Oncol.* 33 (2015) 4284–4292.
- [10] J.A. Knoblich, Mechanisms of asymmetric stem cell division, *Cell* 132 (2008) 583–597.
- [11] G.J. Spangrude, D.M. Brooks, D.B. Tumas, Long-term repopulation of irradiated mice with limiting numbers of purified hematopoietic stem cells: in vivo expansion of stem cell phenotype but not function, *Blood* 85 (1995) 1006–1016.
- [12] L.D. Wang, A.J. Wagers, Dynamic niches in the origination and differentiation of haematopoietic stem cells, *Nat. Rev. Mol. Cell Biol.* 12 (2011) 643–655.
- [13] H. Kimura, T. Ouchi, S. Shibata, et al., Stem cells purified from human induced pluripotent stem cell-derived neural crest-like cells promote peripheral nerve regeneration, *Sci. Rep.* 8 (2018) 10071.
- [14] S. Nishikawa, L.M. Jakt, T. Era, Embryonic stem-cell culture as a tool for developmental cell biology, *Nat. Rev. Mol. Cell Biol.* 8 (2007) 502–507.
- [15] Y.X. Wang, M.A. Rudnicki, Satellite cells, the engines of muscle repair, *Nat. Rev. Mol. Cell Biol.* 13 (2011) 127–133.
- [16] C. Nombela-Arrieta, J. Ritz, L.E. Silberstein, The elusive nature and function of mesenchymal stem cells, *Nat. Rev. Mol. Cell Biol.* 12 (2011) 126–131.
- [17] B.B. Zhou, H. Zhang, M. Damelin, et al., Tumour-initiating cells: challenges and opportunities for anticancer drug discovery, *Nat. Rev. Drug Discov.* 8 (2009) 806–823.
- [18] S.C. Bendall, G.P. Nolan, From single cells to deep phenotypes in cancer, *Nat. Biotechnol.* 30 (2012) 639–647.
- [19] N. Takebe, L. Miele, P.J. Harris, et al., Targeting notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update, *Nat. Rev. Clin. Oncol.* 12 (2015) 445–464.
- [20] Y. Litington, L. Lei, H. Westphal, et al., Sonic hedgehog is essential to foregut development, *Nat. Genet.* 20 (1998) 58–61.
- [21] C. Adolphe, M. Narang, T. Ellis, et al., An in vivo comparative study of sonic, desert and Indian hedgehog reveals that hedgehog pathway activity regulates epidermal stem cell homeostasis, *Development* 131 (2004) 5009–5019.
- [22] T. Tabata, T.B. Kornberg, Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs, *Cell* 76 (1994) 89–102.
- [23] S.M. Shimeld, The evolution of the hedgehog gene family in chordates: insights from amphioxus hedgehog, *Dev. Genes Evol.* 209 (1999) 40–47.
- [24] X. Zhan, J. Wang, Y. Liu, et al., GPCR-like signaling mediated by smoothened contributes to acquired chemoresistance through activating Gli, *Mol. Cancer* 13 (2014) 4.
- [25] N. Fuse, T. Maiti, B. Wang, et al., Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for patched, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999) 10992–10999.
- [26] B. Roberts, C. Casillas, A.C. Alfaro, et al., Patched1 and Patched2 inhibit smoothened non-cell autonomously, *Elife* 5 (2016).
- [27] J. Taipale, M.K. Cooper, T. Maiti, et al., Patched acts catalytically to suppress the activity of smoothened, *Nature* 418 (2002) 892–897.
- [28] J. Kim, E.Y. Hsia, A. Brigui, et al., The role of ciliary trafficking in Hedgehog receptor signaling, *Sci. Signal.* 8 (2015) ra55.
- [29] V. Singla, J.F. Reiter, The primary cilium as the cell's antenna: signaling at a sensory organelle, *Science* 313 (2006) 629–633.
- [30] B.R. Myers, L. Neahring, Y. Zhang, et al., Rapid, direct activity assays for smoothened reveal Hedgehog pathway regulation by membrane cholesterol and extracellular sodium, *Proc. Natl. Acad. Sci. U. S. A.* 114 (2017) E11141–E11150.
- [31] Y. Zhang, D.P. Bulkley, Y. Xin, et al., Structural basis for cholesterol transport-like activity of the Hedgehog receptor patched, *Cell* 175 (2018) 1352–1364 e14.
- [32] H. Tukachinsky, L.V. Lopez, A. Salic, A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes, *J. Cell Biol.* 191 (2010) 415–428.
- [33] J. Kim, M. Kato, P.A. Beachy, Gli2 trafficking links Hedgehog-dependent activation of smoothened in the primary cilium to transcriptional activation in the nucleus, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 21666–21671.
- [34] C.B. Bai, W. Auerbach, J.S. Lee, et al., Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway, *Development* 129 (2002) 4753–4761.
- [35] Y. Abe, N. Tanaka, Roles of the Hedgehog signaling pathway in epidermal and hair follicle development, homeostasis, and cancer, *J. Dev. Biol.* 5 (2017).
- [36] Y. Pan, C.B. Bai, A.L. Joyner, et al., Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation, *Mol. Cell Biol.* 26 (2006) 3365–3377.
- [37] P. Kogerman, T. Grimm, L. Kogerman, et al., Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1, *Nat. Cell Biol.* 1 (1999) 312–319.
- [38] J. Hepker, Q.T. Wang, C.K. Motzny, et al., *Drosophila cubitus interruptus* forms a negative feedback loop with patched and regulates expression of Hedgehog target genes, *Development* 124 (1997) 549–558.
- [39] J. Jeong, A.P. McMahon, Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1, *Development* 132 (2005) 143–154.
- [40] M.G. Hayden Gephart, Y.S. Su, S. Bandara, et al., Neuropilin-2 contributes to tumorigenicity in a mouse model of Hedgehog pathway medulloblastoma, *J. Neurooncol.* 115 (2013) 161–168.
- [41] X. Ge, L. Milenkovic, K. Suyama, et al., Phosphodiesterase 4D acts downstream of neuropilin to control Hedgehog signal transduction and the growth of medulloblastoma, *Elife* 4 (2015).
- [42] J.M. Pinsky, N.E. Franks, A.N. McMellen, et al., Neuropilin-1 promotes Hedgehog signaling through a novel cytoplasmic motif, *J. Biol. Chem.* 292 (2017) 15192–15204.
- [43] Y. Liu, L.W. Yuelling, Y. Wang, et al., Astrocytes promote medulloblastoma progression through Hedgehog secretion, *Cancer Res.* 77 (2017) 6692–6703.
- [44] M. Lauth, R. Toftgard, Non-canonical activation of GLI transcription factors: implications for targeted anti-cancer therapy, *Cell Cycle* 6 (2007) 2458–2463.
- [45] J. Zhou, G. Zhu, J. Huang, et al., Non-canonical GLI1/2 activation by PI3K/AKT signaling in renal cell carcinoma: a novel potential therapeutic target, *Cancer Lett.* 370 (2016) 313–323.
- [46] B. Stecca, C. Mas, V. Clement, et al., Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 5895–5900.
- [47] N.A. Riobo, K. Lu, X. Ai, et al., Phosphoinositide 3-kinase and Akt are essential for sonic hedgehog signaling, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 4505–4510.
- [48] H. Schnidar, M. Eberl, S. Klingler, et al., Epidermal growth factor receptor signaling synergizes with Hedgehog/GLI in oncogenic transformation via activation of the

- MEK/ERK/JUN pathway, *Cancer Res.* 69 (2009) 1284–1292.
- [49] M.D. Nye, L.L. Almada, M.G. Fernandez-Barrena, et al., The transcription factor GLI1 interacts with SMAD proteins to modulate transforming growth factor beta-induced gene expression in a p300/CREB-binding protein-associated factor (PCAF)-dependent manner, *J. Biol. Chem.* 289 (2014) 15495–15506.
- [50] D. Javelaud, V.I. Alexaki, S. Denner, et al., TGF-beta/SMAD/GLI2 signaling axis in cancer progression and metastasis, *Cancer Res.* 71 (2011) 5606–5610.
- [51] J. Taipale, J.K. Chen, M.K. Cooper, et al., Effects of oncogenic mutations in smoothened and patched can be reversed by cyclopamine, *Nature* 406 (2000) 1005–1009.
- [52] J.K. Chen, I only have eye for ewe: the discovery of cyclopamine and development of Hedgehog pathway-targeting drugs, *Nat. Prod. Rep.* 33 (2016) 595–601.
- [53] J.K. Chen, J. Taipale, M.K. Cooper, et al., Inhibition of Hedgehog signaling by direct binding of cyclopamine to smoothened, *Genes Dev.* 16 (2002) 2743–2748.
- [54] E.F.X. Byrne, R. Sircar, P.S. Miller, et al., Structural basis of smoothened regulation by its extracellular domains, *Nature* 535 (2016) 517–522.
- [55] S. Nachtergaele, D.M. Whalen, L.K. Mydock, et al., Structure and function of the smoothened extracellular domain in vertebrate Hedgehog signaling, *Elife* 2 (2013) e01340.
- [56] N. Sever, R.K. Mann, L. Xu, et al., Endogenous B-ring oxysterols inhibit the Hedgehog component smoothened in a manner distinct from cyclopamine or side-chain oxysterols, *Proc. Natl. Acad. Sci. U. S. A.* 113 (2016).
- [57] D.R. Raleigh, N. Sever, P.K. Choksi, et al., Cilia-associated oxysterols activate smoothened, *Mol. Cell* 72 (2018) 316–327 e5.
- [58] R.E. Gordon, L. Zhang, S. Peri, et al., Statins synergize with Hedgehog pathway inhibitors for treatment of medulloblastoma, *Clin. Cancer Res.* 24 (2018) 1375–1388.
- [59] J. Kim, J.Y. Tang, R. Gong, et al., Itraconazole, a commonly used antifungal that inhibits Hedgehog pathway activity and cancer growth, *Cancer Cell* 17 (2010) 388–399.
- [60] Q. Ding, J. Motoyama, S. Gasca, et al., Diminished sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice, *Development* 125 (1998) 2533–2543.
- [61] R. Nasr, M.C. Guillemin, O. Ferhi, et al., Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation, *Nat. Med.* 14 (2008) 1333–1342.
- [62] E.M. Beauchamp, L. Ringer, G. Bulut, et al., Arsenic trioxide inhibits human cancer cell growth and tumor development in mice by blocking Hedgehog/GLI pathway, *J. Clin. Invest.* 121 (2011) 148–160.
- [63] J. Kim, J.J. Lee, J. Kim, et al., Arsenic antagonizes the Hedgehog pathway by preventing ciliary accumulation and reducing stability of the Gli2 transcriptional effector, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 13432–13437.
- [64] M. Lauth, A. Bergstrom, T. Shimokawa, et al., Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 8455–8460.
- [65] D.J. Robbins, J. Rodriguez-Blanco, B. Li, et al., A CK1alpha activator penetrates the brain, and shows efficacy against drug-resistant metastatic medulloblastoma, *Clin. Cancer Res.* (2018).
- [66] D.N. Watkins, D.M. Berman, S.G. Burkholder, et al., Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer, *Nature* 422 (2003) 313–317.
- [67] K.S. Park, L.G. Martelotto, M. Peifer, et al., A crucial requirement for Hedgehog signaling in small cell lung cancer, *Nat. Med.* 17 (2011) 1504–1508.
- [68] A. Szczepny, S. Rogers, W.S.N. Jayasekara, et al., The role of canonical and non-canonical Hedgehog signaling in tumor progression in a mouse model of small cell lung cancer, *Oncogene* 36 (2017) 5544–5550.
- [69] M.C. Pietanza, A.M. Litvak, A.M. Varghese, et al., A phase I trial of the Hedgehog inhibitor, sonidegib (LDE225), in combination with etoposide and cisplatin for the initial treatment of extensive stage small cell lung cancer, *Lung Cancer* 99 (2016) 23–30.
- [70] T.Y. Cheng, S.M. Cramb, P.D. Baade, et al., The International epidemiology of lung cancer: latest trends, disparities, and tumor characteristics, *J. Thorac. Oncol.* 11 (2016) 1653–1671.
- [71] M.D. Wilkerson, X. Yin, K.A. Hoadley, et al., Lung squamous cell carcinoma mRNA expression subtypes are reproducible, clinically important, and correspond to normal cell types, *Clin. Cancer Res.* 16 (2010) 4864–4875.
- [72] Cancer genome atlas research N: comprehensive genomic characterization of squamous cell lung cancers, *Nature* 489 (2012) 519–525.
- [73] L. Huang, V. Walter, D.N. Hayes, et al., Hedgehog-GLI signaling inhibition suppresses tumor growth in squamous lung cancer, *Clin. Cancer Res.* 20 (2014) 1566–1575.
- [74] M. Brunelli, E. Bria, A. Nottogari, et al., True 3q chromosomal amplification in squamous cell lung carcinoma by FISH and aCGH molecular analysis: impact on targeted drugs, *PLoS One* 7 (2012) e49689.
- [75] B.R. Kim, E. Van de Laar, M. Cabanero, et al., SOX2 and PI3K cooperate to induce and stabilize a squamous-committed stem cell injury State during lung squamous cell carcinoma pathogenesis, *PLoS Biol.* 14 (2016) e1002581.
- [76] V. Justilien, M.P. Walsh, S.A. Ali, et al., The PRKCI and SOX2 oncogenes are coamplified and cooperate to activate Hedgehog signaling in lung squamous cell carcinoma, *Cancer Cell* 25 (2014) 139–151.
- [77] L. Toschi, G. Finocchiaro, T.T. Nguyen, et al., Increased SOX2 gene copy number is associated with FGFR1 and PIK3CA gene gain in non-small cell lung cancer and predicts improved survival in early stage disease, *PLoS One* 9 (2014) e95303.
- [78] A.S. Tsao, I. Wistuba, D. Xia, et al., Germline and somatic smoothened mutations in Non-small-cell lung cancer are potentially responsive to Hedgehog inhibitor vismodegib, *JCO Precision Oncol.* 1 (2017) 1–10.
- [79] S. Singh, Z. Wang, D. Liang Fei, et al., Hedgehog-producing cancer cells respond to and require autocrine Hedgehog activity, *Cancer Res.* 71 (2011) 4454–4463.
- [80] J. Rodriguez-Blanco, N.S. Schilling, R. Tokhunts, et al., The hedgehog processing pathway is required for NSCLC growth and survival, *Oncogene* 32 (2013) 2335–2345.
- [81] Z. Yuan, J.A. Goetz, S. Singh, et al., Frequent requirement of hedgehog signaling in non-small cell lung carcinoma, *Oncogene* 26 (2007) 1046–1055.
- [82] A. Po, M. Silvano, E. Miele, et al., Noncanonical GLI1 signaling promotes stemness features and in vivo growth in lung adenocarcinoma, *Oncogene* 36 (2017) 4641–4652.
- [83] C.M. Rudin, J.R. Brahmer, R.A. Juegens, et al., Phase 2 study of pemetrexed and itraconazole as second-line therapy for metastatic nonsquamous non-small-cell lung cancer, *J Thorac Oncol* 8 (2013) 619–623.
- [84] Y. Ma, S. Fiering, C. Black, et al., Transgenic cyclin E triggers dysplasia and multiple pulmonary adenocarcinomas, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 4089–4094.
- [85] N. Yeddula, Y. Xia, E. Ke, et al., Screening for tumor suppressors: loss of ephrin receptor A2 cooperates with oncogenic KRas in promoting lung adenocarcinoma, *Proc. Natl. Acad. Sci. U. S. A.* 112 (2015) E6476–85.
- [86] C.M. Della Corte, U. Malapelle, E. Vigliar, et al., Efficacy of continuous EGFR-inhibition and role of Hedgehog in EGFR acquired resistance in human lung cancer cells with activating mutation of EGFR, *Oncotarget* 8 (2017) 23020–23032.
- [87] C.M. Della Corte, V. Ciaramella, C. Cardone, et al., Antitumor efficacy of dual blockade of EGFR signaling by osimertinib in combination with selumetinib or cetuximab in activated EGFR human NCLC tumor models, *J. Thorac. Oncol.* 13 (2018) 810–820.
- [88] N. Bora-Singhal, D. Perumal, J. Nguyen, et al., Gli1-mediated regulation of Sox2 facilitates self-renewal of stem-like cells and confers resistance to EGFR inhibitors in non-small cell lung cancer, *Neoplasia* 17 (2015) 538–551.
- [89] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, *J. Clin. Invest.* 119 (2009) 1420–1428.
- [90] L.A. Byers, L. Diao, J. Wang, et al., An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance, *Clin. Cancer Res.* 19 (2013) 279–290.
- [91] H. Li, D. Yue, J.Q. Jin, et al., Gli promotes epithelial-mesenchymal transition in human lung adenocarcinomas, *Oncotarget* 7 (2016) 80415–80425.
- [92] S. Eimer, F. Dugay, K. Airiau, et al., Cyclopamine cooperates with EGFR inhibition to deplete stem-like cancer cells in glioblastoma-derived spheroid cultures, *Neuro Oncol.* 14 (2012) 1441–1451.
- [93] S.B. Keysar, P.N. Le, R.T. Anderson, et al., Hedgehog signaling alters reliance on EGF receptor signaling and mediates anti-EGFR therapeutic resistance in head and neck cancer, *Cancer Res.* 73 (2013) 3381–3392.
- [94] C.M. Della Corte, C. Bellevicine, G. Vicidomini, et al., SMO Gene amplification and activation of the hedgehog pathway as novel mechanisms of resistance to anti-epidermal growth factor receptor drugs in human lung cancer, *Clin. Cancer Res.* 21 (2015) 4686–4697.
- [95] F. Morgillo, G. Amendola, C.M. Della Corte, et al., Dual MET and SMO negative modulators overcome resistance to EGFR inhibitors in human nonsmall cell lung cancer, *J. Med. Chem.* 60 (2017) 7447–7458.
- [96] R.L. Yauch, S.E. Gould, S.J. Scales, et al., A paracrine requirement for hedgehog signalling in cancer, *Nature* 455 (2008) 406–410.