INTRODUCTION

The Hedgehog (Hh) signaling pathway, also known as Hedgehog-Patched (Hh-Ptch), Hedgehog-Gli (Hh-Gli) or Hedgehog-Patched-Smoothened (Hh-Ptch-Smo), is an evolutionarily conserved pathway of signal transmission from the cell membrane to the nucleus. The Hh signaling pathway plays a significant role in the normal embryonic development of invertebrates and vertebrates [1]. The Hh signaling pathway plays an important role in the embryonic development. It exerts its biological effects through a signaling cascade that culminates in a change of balance between activator and repressor forms of glioma-associated oncogene (Gli) transcription factors. The components of the Hh signaling pathway involved in the signaling transfer to the Gli transcription factors include Hedgehog ligands (Sonic Hh [SHh], Indian Hh [IHh], and Desert Hh [DHh]), Patched receptor (Ptch1, Ptch2), Smoothened receptor (Smo), Suppressor of fused homolog (Sufu), kinesin protein Kif7, protein kinase A (PKA), and cyclic adenosine monophosphate (cAMP). The activator form of Gli travels to the nucleus and stimulates the transcription of the target genes by binding to their promoters. The main target genes of the Hh signaling pathway are \textit{PTCH1}, \textit{PTCH2}, and \textit{GLI1}. Deregulation of the Hh signaling pathway is associated with developmental anomalies and cancer, including Gorlin syndrome, and sporadic cancers, such as basal cell carcinoma, medulloblastoma, pancreatic, breast, colon, ovarian, and small-cell lung carcinomas. The aberrant activation of the Hh signaling pathway is caused by mutations in the related genes (ligand-independent signaling) or by the excessive expression of the Hh signaling molecules (ligand-dependent signaling – autocrine or paracrine). Several Hh signaling pathway inhibitors, such as vismodegib and sonidegib, have been developed for cancer treatment. These drugs are regarded as promising cancer therapies, especially for patients with refractory/advanced cancers.

KEY WORDS: Hedgehog signaling pathway; Hh; tumorigenesis; signal transduction

INTRODUCTION

The Hedgehog (Hh) signaling pathway, also known as Hedgehog-Patched (Hh-Ptch), Hedgehog-Gli (Hh-Gli) or Hedgehog-Patched-Smoothened (Hh-Ptch-Smo), is an evolutionarily conserved pathway of signal transmission from the cell membrane to the nucleus. The Hh signaling pathway plays a significant role in the normal embryonic development of invertebrates and vertebrates [1]. The Hh gene is also relevant for proper segregation, i.e. the polarity of the organism and the development of many tissues and organs.

The Hh pathway is mostly inactive or poorly active in the adult organism. If necessary, it can be activated, for example, in wound healing [2]. Furthermore, the pathway is involved in the maintenance of somatic stem cells and pluripotent cells important for tissue repair [3], such as mammary [4], skin [5], neural [6], erythropoietic [7], and lung stem cells [8], as well as some epithelial cells of internal organs [9]. Accordingly, Hh signaling is critical for regeneration of the lung epithelium [8], prostate epithelium [10], and exocrine pancreas cells [11].

In other tissues, the Hh signaling pathway is present only in primary cilia (PC), organelles that consist of microtubules and emanate from the cell surface, receiving mechanical, chemical, and thermal signals [12]. All components of the Hh signal transduction pathway are found in the PC [13].

Some studies indicate that Hh signaling can be involved in various stages of carcinogenesis in different tumors. For example, in pancreatic and esophageal cancer, the activation of this signaling pathway is found in the early stages of tumor as well as in metastatic tumors [14,15]. In other tumors, such as gastric cancer and prostate cancer, the activation of the Hh signaling pathway is associated with tissue invasion and increased metastatic potential. In accordance with those findings, the inhibition of the Hh signaling pathway reduces tumor cell proliferation in prostate and gastric cancer [16].
HH SIGNALING PATHWAY

Three proteins are involved in Hh signaling activation: Hedgehog (Hh) ligand, Patched (Ptch) and Smoothened (Smo) [17].

In the absence of Hh ligand, Ptch localizes to the base of the PC and represses the activity of Smo by inhibiting its translocation into the PC [18]. This leads to proteolytic cleavage of full-length glioma-associated oncogene (GliFL) to Gli repressor (GliR) after phosphorylation by protein kinase A (PKA), glycogen synthase kinase-3 (GSK3), and casein kinase 1 (CK1) [19]. GliR binds to Hh target gene promoters and keeps Hh target genes switched off (Figure 1A) [20].

The Hh signaling cascade is activated by Hh binding to Ptch protein. The Hh-Ptch complex is internalized, and both proteins are degraded in lysosomes [21]. The binding relieves the Smo inhibition, and the Hh signal is now able to be transmitted downstream of Smo via cytoplasmic protein complex composed of kinesin protein (Kif7), Suppressor of fused (Sufu), and GliFL. Smo travels to the tip of the PC and signals Sufu to release Gli activator (GliA). GliA then migrates to the nucleus and activates the expression of the target genes (Figure 1B) [22]. This pathway of signal transduction, where Hh regulates the Gli family of transcription factors, is called the canonical Hh signaling pathway. In the non-canonical Hh signaling pathway, Hh proteins signal through Gli-independent mechanisms.

ELEMENTS OF HH SIGNAL TRANSDUCTION

Hh gene/protein

The Hh gene is highly conserved from fruit flies to humans, and is a key regulator in embryonic development [1]. Unlike Drosophila melanogaster (D. melanogaster), where one Hh gene has been identified, in vertebrates, three Hh gene family members have been detected: the Sonic Hedgehog (SHh), Indian Hedgehog (IHh), and Desert Hedgehog (DHh) gene [23-25]. The products of any of these three genes can bind to Ptch1 receptor and activate the Hh signaling pathway [26], but they perform this activity in various organ systems [23,25]. Hh proteins can act as mitogens, morphogens, and differentiation factors at longer or shorter distances, during different stages of development and in different tissues [17].

The most studied Hh ligand is SHh. It has the highest activity and is involved in the development of various organs during embryogenesis. SHh is expressed in the central nervous system, lungs, teeth, intestines, and hair follicles during development [27-30]. SHh signaling can be autocrine (binds to the same cell from which it is excreted) or paracrine (binds to the nearby cells or induces changes in cells at longer distances).

Hh is involved in endochondral bone formation, as a negative regulator of chondrocyte differentiation [31], and participates in the development of gastrointestinal tract [32] and mammary glands [4].

Of all discovered Hh proteins, DIHh is the closest homologue of D. melanogaster Hh ligand [33]. Its expression is largely restricted to gonads, including Sertoli cells in the testis, where it plays a key role in the differentiation of germ cells [34].

All Hh proteins undergo maturation before the active ligand is generated and released from the cell and before the activation of Hh signaling pathway [35]. After the translation, N-terminal signal sequence is removed from a ~45 kDa long polypeptide, which is then autocatalytically cleaved between glycine and cysteine to form an N-terminal fragment. The C-terminal domain (autoprocessing domain) of the Hh polypeptide promotes the binding of cholesterol to glycine.

FIGURE 1. A simplified display of the Hedgehog signaling pathway. (A) In the absence of Hedgehog ligand, full-length Gli is phosphorylated by protein kinase A, glycogen synthase kinase-3, and casein kinase 1. This leads to proteolytic cleavage of the full-length Gli into Gli repressor. Gli repressor represses the expression of target genes, (B) after binding of Hedgehog ligand, Smoothened protein is phosphorylated by protein kinase A and casein kinase 1. The inhibitory effect of Sufu is removed and Gli activator is formed. Gli activator induces the transcription of target genes. Red symbols represent the inhibitory effect, and green arrows show the activating effect. Hh - Hedgehog; Ptch - Patched; Smo - Smoothened; Gli - glioma-associated oncogene; GliFL - full-length Gli; GliA - Gli activator; GliR - Gli repressor; CK1 - casein kinase 1; PKA - protein kinase A; GSK3 - glycogen synthase kinase-3.
Ptch gene/protein

Ptch is the receptor for Hh protein. Two Ptch homologs have been isolated in vertebrates: Ptch1 and Ptch2 [38]. SHh, IHH, and DHH ligands bind with similar affinity to both of them, and both proteins may repress the activity of Smo protein [39]. The human Ptch gene is located on chromosome 9q22.3; it contains 23 exons and encodes a glycoprotein of 1147 amino acids [40,41]. The Ptch gene is located on 1p34.1 and encodes a 1203-amino acid protein. Ptch1 and Ptch2 genes have different functions based on their different expression during epidermal development [42]. The Ptch is primarily expressed in mesenchymal cells that produce SHh proteins, while the Ptch2 is expressed in skin and testicular epithelial cells [43].

Ptch protein is a 12-pass transmembrane protein with two large extracellular loops and two large intracellular loops [25]. Within the extracellular loop structure, there is a sterol-sensing domain (SSD) that is thought to interact with cholesterol bound to Hh protein [44]. In the absence of Hh, Ptch blocks the pathway activity [45]. However, when Hh ligand binds to Ptch, Hh relieves the inhibition of Ptch to activate the signal transduction [46]. Subsequently, there is no Smo blockade anymore, which results in the modulation of Gli transcription factors. Ptch also sequesters Hh ligand and restricts the range of signaling of the free ligand [47]. In cells where Ptch is absent, Hh protein is further dispersed [48] and it induces the targeted gene expression at longer distances [49]. The mechanism by which Ptch regulates Smo is still not understood, but it has been shown that Hh binding causes the internalization of Ptch from the cell surface and promotes the accumulation of Smo at the cell surface [18].

Ptch and Hh proteins regulate the cell cycle in two ways [50]. First, Ptch without Hh ligand binds maturation promoting factor (MPF), which is composed of cyclin B1 and cyclin-dependent kinase 1 (CDK1). Binding or retaining MPF in the cytoplasm, prevents its activity. When Hh binds to Ptch, cyclin B1 is released and the cell cycle continues [57]. Second, the Hh signal transmission pathway leads to the transcription of cyclin D and cyclin E, which also promotes the cell cycle progression [51].

Several observations suggest that, besides Ptch, other receptors bind Hh ligands and participate in the Hh signaling pathway activation [52,53]. Negative receptor of Hh signaling is Hedgehog-interacting protein (Hhip) [54], while Cdo and Boc bind vertebrate Hh proteins and positively regulate Hh signaling. It is not yet fully known if they synergize or compete with Ptch for binding to Hh [55]. Some evidence has shown that the membrane protein growth arrest-specific 1 (GAS1), known as a negative regulator of Hh signaling, also modulates Hh signaling positively [56]. These negative and positive receptors most likely play a key role in monitoring the magnitude and range of Hh signaling pathway [57].

Smo gene/protein

As previously mentioned, Smo protein is a co-receptor in the Hh signaling pathway, i.e. it represents a signaling component of the receptor complex. It is a seven-pass transmembrane protein, structurally similar to G-protein-coupled receptors [58], and has an extracellular cysteine-rich domain (CRD) that is indispensable for its function [59].

Smo is considered to be a positive Hh signaling pathway regulator because it is constitutively active in the absence of inhibitory Ptch1, and it promotes the activation of downstream components of this signaling pathway [60].

The mechanism by which Ptch1 inhibits Smo is still not clear. The suggested mechanism [61] based on the physical interaction between Smo and Ptch1, in which they form a membrane-associated receptor complex, has not been confirmed in vivo [47]. Ptch1 possibly functions through the changes in the distribution or concentration of a small molecule that affects Smo [62]. Ciliary localization of Ptch1, enabled by the ciliary localization sequence (CLS) within its carboxyl-terminal tail, has one of the main roles in the inhibition of Smo. CLS also unlocks Ptch transport into the PC [63]. Oxysterols [64] and Vitamin D3 derivatives [65] may function as endogenous regulators of Smo activity. Despite these insights on the regulators of Smo activity, the mechanisms by which Ptch1 represses Smo and how Hh ligand counters this effect remain unknown.

After the binding of Hh ligand and degradation of Ptch1 protein, Smo is phosphorylated by PKA and CK1, and its endocytosis and degradation are blocked [66]. Smo transmits a signal to the cytoplasm in a phosphorylation cascade, where Gli protein is the final target (Figure 1b).

Gli gene/protein

Gli family consists of zinc finger proteins, and is named after glioblastoma from which they were initially isolated [67].

In vertebrates, there are three members of Gli gene family: Gli1, Gli2, and Gli3 [68]. Gli1 protein acts as a transcriptional activator [69]. Hh ligands induce the expression of Gli1, which also provides a positive feedback for Hh signaling [70].
The activator domain of GLI1 consists of 18 amino acids, and it probably forms a negatively charged helix that is similar to viral protein 16 [71]. GLI2 primarily functions as a transcriptional activator, while GLI3 mainly functions as a repressor in Hh signaling [72].

GLI proteins regulate the expression of target genes by directly binding to their promoters [73]. GLI1 and GLI3 proteins recognize the 5′-GACCACCCA-3′ sequence in target gene promoters [74], and GLI2 recognizes almost the identical 5′-GAACCCACCA-3′ motif [75].

In the absence of Hh ligand, GLI-FL is phosphorylated by PKA, GSK3 and CK1, and recognized by β-transducin-repeat containing protein (β-TrCP). This leads to proteolytic cleavage of GLI-FL into C-terminally truncated repressor form, GLI-R [76]. GLI-R translocates to the nucleus where it binds to Hh target gene promoters and represses their expression (Figure 1A). The binding of Hh ligand leads to the release of Gli from Suppressor of fused protein (Sufu) and the formation of GliA [77]. GliA then translocates to the nucleus, binds to target gene promoters, and activates the transcription of Hh target genes (Figure 1B).

The activation of canonical Hh signaling results in the suppression of proteolytic degradation of Gli proteins, thereby increasing their cytoplasmic and nuclear levels, and hence, the transcription of target genes in the Hh signaling pathway. Studies have shown that the expression of Gli transcription factors and their activation is also regulated by other signaling pathways. For example, in esophageal carcinoma, the mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase beta-1 (S6K1) signaling pathway activates GLI1 by phosphorylation, leading to the release of GLI1 from the Sufu protein complex and its translocation into the nucleus [78]. In addition, it has been reported that the transforming growth factor (TGF-β), epidermal growth factor receptor (EGFR), mitogen-activated protein kinases (MAPK), and fibroblast growth factor (FGF) signaling pathways can also induce the expression of Gli transcription factors [79].

Suppressed fusion protein (Sufu)

Sufu is a crucial negative regulator of the Hh pathway, and it functions between Smo and Gli transcription factors. It binds directly to Gli proteins [80]. In a stimulated Hh signaling pathway, active Smo leads to the recruitment of Sufu-Gli to cilia, followed by a rapid dissociation of the complex and initiation of target gene transcription [81]. Sufu inhibits Gli proteins by preventing their translocation into the nucleus [82]. It plays a key role in the stabilization and processing of Gli and thus maintains accurate Hh signaling [77]. Sufu also localizes to the nucleus where it can bind to Gli-binding sequences in DNA molecule and prevent gene transcription [83].

Kif7

The kinesin protein Kif7 is a component of Hh signaling that can act as both a positive and negative regulator [84]. In response to Hh activation, Kif7 localizes to the tip of the PC, and it can control the cilium structure and organize a specialized compartment necessary for Hh signaling [85]. When Hh signaling is induced, Gli proteins also translocate to the PC tip [86]. The mechanisms of Kif7 positive and negative roles are not completely understood. One possible mechanism is the post-translational control of Kif7 phosphorylation by protein phosphatase 2A (PP2A) [87].

Cyclic AMP (cAMP)-dependent PKA

cAMP-dependent PKA is also an important negative regulator of the Hh pathway (Figure 1) [88]. It is localized at the base of cilia and regulates the formation of GliR/GliA complex [89]. PKA activity is crucial for Hh signaling. In the

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**TABLE 1. Hh signaling pathway target genes**

| Gene | Full name of protein | Effect of the gene product | References |
|------|---------------------|---------------------------|------------|
| Patched 1 | GLI1 | Inducer of the activated signaling pathway, negative feedback | [93] |
| Patched 2 | GLI2 | Inducer of the activated signaling pathway | [93] |
| Glioma-associated oncogene 1 | GLI1 | Inducer of the activated signaling pathway, positive feedback | [93] |
| Hedgehog-interacting protein | Hhip | Hh signaling pathway regulation | [54] |
| G1/S-specific cyclin D2 | CCND2 | Cell cycle regulator | [51] |
| G1/S-specific cyclin E1 | CCNE1 | Cell cycle regulator | [51] |
| N-myc proto-oncogene protein | MYCN | Cell proliferation and tumorigenesis | [95] |
| B-cell lymphoma 2 | BCL2 | Apoptosis regulator | [94] |
| ATP-binding cassette subfamily G member 2 | ABCG2 | Transport of many positive and negatively charged hydrophobic molecules | [96] |
| Fibroblast growth factor 4 | FGF4 | Mitogenic activity, involved in various biological processes (embryonic development, morphogenesis, and tissue repair) | [97] |
| Vascular endothelial growth factor A | VEGFA | Angiogenesis, vasculogenesis, endothelial cell growth | [98] |
| Paired box protein Pax-6, 7, and 9 | PAX6, PAX7, PAX9 | Transcription factors during embryogenesis | [99] |
| Notch ligand and Wnt signaling pathway | JAG1 | Notch ligand and Wnt signaling pathway | [101] |
| Forkhead box protein M1 | FOXM1 | Transcription factor | [100] |
absence of Hh ligand, PKA phosphorylates Gli proteins, which are then cleaved to the repressor form of GliR and repress Hh target gene expression (Figure 1A) [90]. PKA may also inhibit Gli proteins by modulating their interaction with Sufu. One explanation as to how PKA inhibits Hh signaling is by blocking the localization of Sufu-Gli complex to cilia and inhibiting the Sufu-Gli dissociation [81]. The basal level of PKA activity maintains the suppression of Hh signaling pathway in the absence of Hh ligand. If the PKA activity is reduced, it causes ectopic expression of Hh target genes [91].

Target genes

The main target genes of Hh signaling pathway (Table 1) include the *Ptch1, Ptch2, and Gli1* genes; their activation results in elevated levels of the respective mRNAs and proteins [92]. Increased expression of the *Ptch1, Ptch2, and Gli1* genes is a highly reliable indicator of activated signaling pathway and provides negative (Ptch1) and positive (Gli1) regulation of Hh signaling with negative and positive feedback loop mechanisms [93].
Other target genes include Hedgehog-interacting protein (Hhip) [54], cell cycle regulators (CCND2 and CCNE1) [51], apoptosis regulator (BCL2) [94], MYCN [95], ABCG2 [96], FGF4 [97], VEGFA [98], PAX6, PAX7, PAX9 [99], FOXMi [100], JAG1, and members of the Wnt signaling pathway [101]. Recent studies show the existence of an interaction between the Wnt and Hh signaling pathways [32,102].

Activation and deactivation of these Hh genes may contribute not only to the development of normal tissues and organs, but also to tumorigenesis.

THE ROLE OF THE HH SIGNALING PATHWAY IN CANCER

Dysfunction or aberrant activation of the Hh signaling pathway is associated with developmental deformities and cancers [103], such as basal cell nevus syndrome (BCNS), also known as Gorlin syndrome, sporadic basal cell carcinoma (BCC), medulloblastomas (MBs), rhabdomyosarcomas, meningiomas [104], and others. According to the latest estimates, the Hh signaling pathway contributes to the development of one-third of all malignant tumors [60]. Derepression of any component within the Hh pathway leads to its aberrant activation, resulting in malignant transformation. There are three proposed mechanisms of aberrant Hh signaling activation in different cancer types [22]. These are as follows:

- Type I - autonomous and ligand-independent type of Hh signaling (Figure 2A);
- Type II - ligand-dependent oncogenic Hh signaling in autocrine/juxtacrine manner (Figure 2B);
- Type IIIa/b - ligand-dependent Hh signaling in paracrine or reverse paracrine manner (Figure 2C).

Type I - ligand-independent signaling

The ligand-independent activation of Hh signaling in Type I is caused either by the activating mutations in the SMO plus the SMO-negative regulators leading to constitutive activation of Hh signaling in the absence of the ligand (Figure 2A) [105]. This link was first found in patients with a rare autosomal dominant disorder, i.e., BCNS (Gorlin syndrome), where a Ptch1 mutation on chromosome 9 was found [106]. These patients are at high risk for the development of BCCs and have an increased risk of developing other tumors, especially MBs, meningiomas, and rhabdomyosarcomas [104].

Somatic mutations of these genes have also been found in sporadic BCCs and MBs [107]. Sporadic BCC is the most common cancer in humans, and almost all cases are caused by a gene mutation leading to a constitutive activation of Hh signaling pathway.

The confirmation of these findings was found in many preclinical and clinical models. For example, an inactivating mutation in the Ptch1 was found in about 85% of sporadic BCCs [106]. These observations have been supported by the fact that in Ptch1 heterozygous mice the formation of UV-induced BCC was more frequently observed [108]. Activating SMO mutations that reduce its inhibition by Ptch1 have been found in 10% of sporadic BCCs. Rare mutations of the Sufu gene (<10% somatic) have also been found [105]. However, although the Sufu is a tumor suppressor gene, its targeted inactivation in mouse skin does not result in BCC development, suggesting that the Sufu heterozygosity is not sufficient for tumorigenesis [109]. Furthermore, it has been found that the hyperactivation of Hh signaling after Gli overexpression and activation of atypical protein kinase C (aPKC) leads to tumor cell proliferation and survival.

MB is a rare, aggressive tumor, predominantly present in children, and is another malignancy that occurs in 5% of patients with Gorlin syndrome [111]. The Hh signaling pathway plays an important role in the embryonic development of the cerebellum, and consequently, its role in the formation of MB is not surprising. Purkinje cells release SHh, which induces mass proliferation of granule neuron progenitor (GNP) cells and delays neural differentiation. After early postnatal development, Hh signaling is normally downregulated in the brain. The formation of MB occurs when the Hh pathway is constitutively activated in GNP cells and the proliferation continues outside the normal developmental period [112]. More than one-third of sporadic MB cases are associated with aberrant activation of Hh pathway that is either linked to Ptch1 mutation or Ptch1 locus chromosomal loss (45% of cases) [113]. Somatic inactivating SMO or Sufu mutations each occur in about 14% of cases. While SMO mutations are more frequent in adults, mutations of Sufu are mostly found in pediatric patients (0–3 years) [114]. In preclinical animal models, Ptch1+/− heterozygous mice and Sufu−/− heterozygous mice, deficient also in p53 alleles, both develop MBs [112].

In both BCCs and MBs, dysregulation of PKA and guanine nucleotide-binding protein (GNAS), was also found. PKA is the main negative regulator of the Hh pathway, while G-protein alpha subunit, encoded by the GNAS gene, promotes PKA-dependent cAMP activity. Therefore, it is not
surprising that reduced PKA activity triggers oncogenic Hh signaling and formation of MBs and BCCs. Furthermore, GNAS gene mutations have been found in human MBs [110] and in lesions similar to BCC [115], confirming their important role in Hh signaling. Ptchi mutations, although rare, can be found in MBs and in BCCs as well [42].

Ptchi gene mutations were detected in trichoepitheliomas [116], esophageal carcinomas [117], and bladder carcinomas [118], whereas mutations of Ptchi and Sufu have been found in a rare muscle tumor, rhabdomyosarcoma [119].

Type II - ligand-dependent autocrine/juxtacrine signaling

Type II is ligand-dependent and responds to Hh in an autocrine/juxtacrine manner leading to tumor formation and growth (Figure 2B). Since the Hh pathway is activated in a cell-autonomous manner, Hh ligand is produced by and taken up by the same or surrounding tumor cells. The over-expression of the ligand-dependent autocrine/juxtacrine Hh signaling pathway has been found in various tumors including stomach, esophageal, pancreatic [120], colorectal [121], ovarian and endometrial [122], breast [123], prostate [16], lung [8], melanomas [124], gliomas [125], and other extracutaneous tumors. Apart from the Hh ligand overexpression, most of these tumors show ectopic expression of Ptchi and Gli.

Studies of the activation of Hh signaling pathway in colorectal carcinomas (CRCs) are contradictory. A few studies [126,127] found an increased level of the Hh pathway components in CRC. In addition, in CRC cells in vivo, increased SHh expression was detected at both, the mRNA and protein level. These findings suggest that SHh is required for the development of CRC. In contrast, other reports [120,128,129] claim that the Hh signaling pathway is inactivated during the CRC progression.

Type III - ligand-dependent paracrine signaling

Type III is ligand-dependent and uses paracrine signaling. Although paracrine Hh signaling plays an important role during normal embryonic development and is required for the growth and maintenance of many tissues [130], paracrine activation of Hh pathway in stromal cells has also been associated with various cancers, such as those of prostate, pancreas, and colon. Namely, Hh ligands, secreted by tumor cells, bind to Ptchi receptors on tumor stromal cells, which then undergo Hh pathway activation. In a feedback loop, the stromal cells transmit the growth signals (vascular endothelial growth factor [VEGF], insulin-like growth factor [IGF], Wnt, PDGF, and BMP) to tumor cells, supporting and promoting their proliferation and differentiation (Figure 2C-1) [131]. In addition, Fan et al. [132] suggested that some prostate cancer cells signal to stromal cells in ligand-dependent and paracrine manner, and Theunissen and Sauvage [133] recently supported this finding. This suggests that, while some Hh ligand-expressing epithelial cancer cells do not respond to the ligands themselves, there is an activation of Hh signaling pathway in their surrounding stromal cells.

The “reverse” paracrine signal model has also been recognized, but only in hematological malignancies such as B-cell lymphomas, multiple myelomas, and leukemias. In this model, tumors receive Hh ligand secreted directly from bone marrow or lymph node stromal cells. Thus, in the reverse paracrine ligand-dependent cancers, stromal cells provide a microenvironment that is favorable for tumor growth (Figure 2C-2). For that reason, surrounding stromal cells can also be considered as a therapeutic target [133].

Cancer stem cells (CSCs)

Recently, CSCs have emerged as an important factor in both tumor initiation and progression [134,135]. Within each tumor, there is a set of cells that act as stem cells: they divide slowly but, if necessary, they can very quickly proliferate and create a new population of tumor cells [136]. Activated signaling pathways, including Hh, are involved in their growth, survival, migration, and proliferation [137]. Tumor stem cells have already been detected, for example, in multiple myeloma [137], pancreatic adenocarcinoma [138], breast cancer [139], and chronic myelogenous leukemia (CML) [140]. Due to their slow growth, CSCs are potentially resistant to conventional chemotherapy and radiotherapy, and they are thought to be the major cause of tumor relapse after such therapies. Therefore, new generations of antitumor drugs are being designed in an attempt to target signaling pathways, including Hh, specifically in CSCs [141].

Epigenetic changes

Recent studies show that, apart from mutations, the Hh signaling pathway can be disrupted by epigenetic changes, or more accurately, by methylation of gene promoters. So far, the methylation of the promoter of the Ptchi gene has been described in dermoid cysts and ovarian fibromas [142] and in breast cancer [143], whereas in MBs, SHh ligand induces a local switch of epigenetic cofactors that cooperate with Gli in controlling the transcription outcomes [144].

CLINICAL AND THERAPEUTIC IMPLICATIONS

Recent findings in the Hh signaling pathway and its role in the tumorigenesis have opened new views toward the development of molecular targeting and tumor prevention associated with the Hh pathway. Special attention has been
TABLE 2. Targeted tumor therapy associated with Hh signaling pathway

| Group                  | Drug                        |
|------------------------|-----------------------------|
| Hh ligand inhibitors   | Neutralizing antibodies (5E1), robotnikinin, cycloamine, saridegib, vismodegib, sonidegib |
| Smo antagonists        | Glasdegib, taladegib, LEQ506, TAK-441, XL-139, itraconazole, HPI-1, HPI-2 |
| Gli inhibitors         | GANT-56, GANT-61, ATO       |
| BET inhibitors         | JQ1, PSI                    |
| aPKC inhibitors        |                             |
| Phosphodiesterase      | NVP-ABE171, cilomilast, deguelin |
| inhibitors             |                             |
| Natural products       | Siegesbeckia glabrescens extracts, Vitamin D3 |

HPI - Hedgehog, Ptc - Patched, Smo - Smootherned; Gli - glioma-associated oncogene; BET - bromodomain and extra-terminal domain family of proteins; aPKC - atypical protein kinase C

paid to the targeted Hh signaling pathway inhibition (HPI) as a treatment for locally aggressive BCCs and metastatic BCCs, when radiotherapy and surgery are not effective treatment modalities. Therefore, HPI therapy approach is a new hope for patients with difficult-to-treat BCCs. More than 50 HPI molecules have been identified, which act at different levels of the Hh signaling pathway. HPIs are categorized as Hh ligand inhibitors, Smo antagonists, Gli inhibitors, inhibitors of bromodomain and extra-terminal domain (BET) family of proteins, atypical protein kinase C (aPKC) inhibitors, and phosphodiesterase inhibitors (Table 2).

Hh ligand inhibitors act at the highest level of the signaling pathway by inhibiting the binding of Hh protein to Ptc receptor [145]. On the other hand, Smo antagonists bind to the Smo drug-binding pocket, thus preventing the downstream activation of the Hh signaling cascade [146]. Smo inhibitors currently used in clinical trials are IPI-926 (saridegib), BMS833923 (XL-139), PF04449913 (glasdegib), LY2940680 (taladegib), LEQ506, and TAK-441, whereas GDC-0449 (vismodegib) and LDE225 (sonidegib) have been approved by the US Food and Drug Administration, but are not in use yet [22,147,148].

Due to its pharmacokinetic properties, sonidegib is a highly effective drug [149]. In addition to treating BCC, there is a significant interest in the use of sonidegib in the treatment of MB and renal, lung, pancreatic, and ovarian carcinomas, along with hematologic malignancies, such as myeloid leukemia and lymphoma.

Vismodegib has a similar safety profile as sonidegib, but a Phase II clinical trial with vismodegib revealed its lower therapeutic efficacy and more serious side effects, such as significant fatigue, hyponatremia, hypocalcemia, muscle spasms, and atrial fibrillation [150].

Although Smo inhibitors possess a great potential for the treatment of BCC, mutations in drug-binding pocket may result in the resistance of tumor cells to these drugs. Therefore, the main focus is on the antagonists of Smo receptors that do not bind to the same binding site as sonidegib and vismodegib, such as itraconazole [151] and on the common antagonists of Hh signaling pathway, such as Gli-transcription factor inhibitors HPI-1, HPI-2, GANT-56, GANT-61, and arsenic trioxide.

The viability and proliferation of tumor cells due to aberrant Hh signaling activity in Smo-resistant tumors can also be decreased by BET inhibitors. JQ1 is, among others, a BET inhibitor commonly used in research studies. It was shown that BET inhibitors also inhibit the growth of MB and BCC and increase the survival in mouse models [152].

Phosphodiesterase inhibitors were useful in the treatment of Smo-resistant MB in vivo [153] and aPKC inhibitors could be useful in the treatment of resistant BCCs [154]. Several natural molecules have also shown some benefits in cancer treatment. For example, deguelin is a flavonoid with anticarcinogenic and antiproliferative activities. It induces apoptosis and cell cycle arrest and inhibits blood vessel formation [155]. Many studies refer to deguelin as the regulator of the Hh signaling pathway, and several studies have already shown its excellent potential in the treatment of various malignant tumors such as gastric, lung and breast cancers, and more recently, pancreatic cancer [155]. Furthermore, it has been found that Siegesbeckia glabrescens sesquiterpenes suppress Gli-mediated transcriptional activity and proliferation of human pancreatic cells [156]. Also, recent studies indicate that Smo protein can be repressed by the secretion of Ptc-dependent (pro-)Vitamin D3 [65].

CONCLUSIONS AND FUTURE DIRECTIONS

Novel findings reveal multiple roles of the Hh signaling pathway in the development and progression of various cancers. Although the link between the Hh signaling pathway and tumorigenesis is very heterogeneous, it is known that the aberrant activation of Hh signaling leads to the growth, proliferation, and invasion of tumor cells. Therefore, further research and understanding of the specific role of deregulated activation of Hh signaling in different cancer types will hopefully contribute to the development of novel anti-cancer treatment modalities. Smo inhibitors represent a new and promising treatment option with possible benefits for some cancers. Their entry into clinical use provides a new avenue and hope for many patients with advanced and
chemorefractory BCCs. Their use in the treatment of other cancer types, such as pancreatic cancer or MB, has also been proposed. However, due to harmful and potentially toxic side-effects of Smo inhibitors, undetermined safety in children, and the evidence that some patients develop resistance to Smo-inhibitors, efforts are being made to develop new classes of drugs. CSCs seem to be critical for tumorigenesis and HPI resistance in various tumors, so the combination of systemic HPI with other cytotoxic inhibitors is required. Further research studies should elucidate other mechanisms of Hh actions and translate their findings into novel, better, and safer anti-cancer therapies.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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