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Overview

Epigenetic regulation of the Hedgehog-Gli signaling pathway in cancer

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The term epigenetics refers to the modulation of gene expression caused by means other than changes in the DNA sequence.

Alongside with genetic alterations, epigenetic changes can also be considered a hallmark of cancer. Epigenetic silencing of tumor suppressors can serve as one of the hits in the Knudson's two hit model of tumor initiation when in conjunction with genetic mutations or deletions. Also, loss of methylation can be implicated in tumorigenesis by activating normally silent regions of the genome.

The Hedgehog-Gli (Hh-Gli) signaling pathway has been highly conserved through evolution and plays a crucial role during embryonic development from *Drosophila* to humans.

Malfunction of the Hh-Gli signaling pathway in humans has first been observed in Gorlin syndrome, characterized by developmental malformations and cancer susceptibility. In the last decades aberrant activation of the Hh-Gli signaling pathway has been implicated in various sporadic tumors, both benign and malignant ones.

Genetic alterations of the Hh-Gli signaling pathway components and their implication in the development of various types of cancer and developmental malformations are well known. In the last few years it has become obvious that epigenetic changes also play a crucial role in the regulation of this pathway.

EPIGENETIC GENE REGULATION IN CANCER

The term epigenetics refers to the modulation of gene expression caused by means other than changes in the DNA sequence. These modulations play a crucial role in development and differentiation, but they can also occur in adult tissues, by random change or under the influence of environment *(1)*.

Epigenetic mechanisms that modify the chromatin structure can be divided into four categories: DNA methylation, covalent histone modifications, non-covalent modifications and non-coding RNAs including microRNAs. In normal cells these modifications act together to regulate gene expression patterns in different cell types or different developmental stages *(2)*.

Alongside with genetic alterations, changes in DNA methylation can also be considered a hallmark of cancer. DNA methylation is a covalent modification of the postreplicative DNA where DNA methyltransferases add a methyl group to the cytosine ring and form 5-methyl cytosine. In mammals, this modification is predominant in cytosines of

the CpG dinucleotide sequence. CpG dinucleotides are more common in certain regions of the genome and form so called CpG islands. These islands are frequently located at the 5' end of genes and occupy about 60% of human gene promoters. Most of the CpG sites in the genome are methylated, but the majority of the CpG islands remain unmethylated during development and in differentiated tissues *(2)*. However, some CpG islands can become methylated during development, which results in long term gene silencing. It has been reported that such events occur during the development of some tumors, where the promoters of tumor suppressor genes become hypermethylated *(3)*. Epigenetic silencing of tumor suppressors can serve as one of the hits in the Knudson's two hit model of tumor initiation when in conjunction with genetic mutations or deletions (Figure 1) *(2)*. Also, loss of methylation can be implicated in tumorigenesis by activation of normally silent regions of the genome. These regions can contain silenced genes, inserted viral genes or repeat elements which are potentially harmful when expressed abnormally. Loss of methylation in regions other than genes can affect chromosomal stability in cancer *(3)*. Such an event can activate oncogenes and initiate tumor formation.

HEDGEHOG-GLI SIGNALING PATHWAY

The Hh-Gli signaling pathway (Figure 2) has been highly conserved through evolution and played a crucial role during embryonic development from *Drosophila* to humans. It has been first discovered in the fruit fly, when Nüsslein-Volhard and Wieschaus (*5*) identified mutations in the Hedgehog gene that caused a short and spiked phenotype of the *Drosophila* larvae, similar to hedgehog spikes. Despite the fact that the key components of the signaling pathway are conserved, this pathway in mammals has some alterations compared to the fruit fly (*6*).

In humans, there are three different homologues of the pathway ligand Hedgehog: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), which are all tissue specific. Prior to activation and secretion from the producing cell, the Hh protein is subjected to autocatalytic cleavage, as well as dual lipid modifications at the N- and C- termini *(7)*. Any of these forms of the Hh protein can bind to the receptor Patched (Ptch), a 12-pass transmembrane protein that represses another transmembrane protein, Smoothened (Smo), in the inactive state. There are two human homologues of the PTCH gene, PTCH1 and PTCH2, whereas there is only one human homologue of Smoothened (SMOH). After ligand binding, Ptch relieves its repression of Smo, allowing activation of downstream signaling through the Suppressor of Fused (SuFu), which, in turn, leads to the activation and nuclear translocation of the zinc finger transcription factor Gli *(8)*. In the absence of Hh signal, SuFu acts as a negative regulator of the signaling pathway because it inhibits Gli-mediated transcription by recruiting a histone deacetylation complex to Gli target genes. SuFu also sequesters Gli to the cytoplasm (*9*). Vertebrates have three isoforms of the Gli transcription factor, Gli1, Gli2 and Gli3 (*10*). Among direct Gli1 transcriptional targets are PTCH1 and GLI1, representing a negative and positive feedback loop, respectively *(11)*.

Malfunction of the Hh-Gli signaling pathway in humans has first been observed in Gorlin syndrome, a medical condition characterized by developmental malformations as well as susceptibility to various tumors. The syndrome is caused by a germline mutation in the PTCH gene. PTCH haploinsufficiency is the cause of anomalies, whereas a somatic mutation of the second PTCH allele leads to tumor formation *(8)*.

In the last decades aberrant activation of the Hh-Gli signaling pathway has been implicated in various sporadic tumors, both benign and malignant ones. There are many known genetic alterations associated to the components of this signaling pathway that cause its aberrant activation in adult tissues. Once aberrantly activated it promotes tumor formation and progression by upregulating the transcription of direct target genes. These targets include regulators of cell cycle progression (D-type

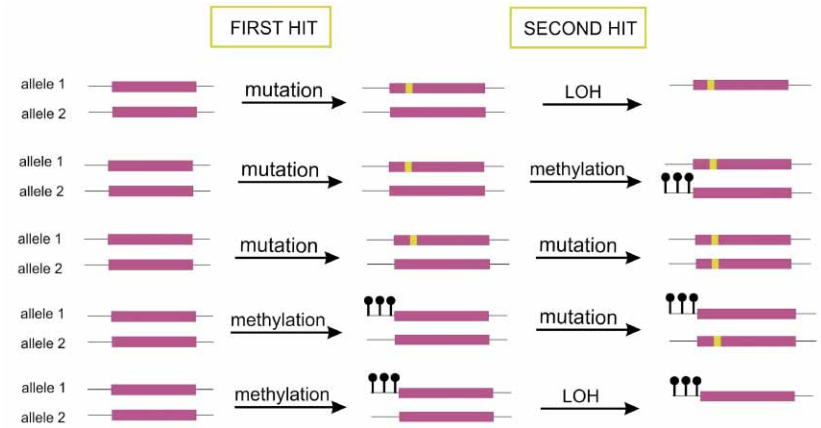

Figure 1. *Knudson's two hit model. Two hits are necessary to inactivate tumor suppressor genes and lead to cancer development. Promoter hypermethylation can represent the first or the second hit and lead to tumorigenesis in conjunction with point mutations or loss of heterozygosity by deletions of the functional allele.*

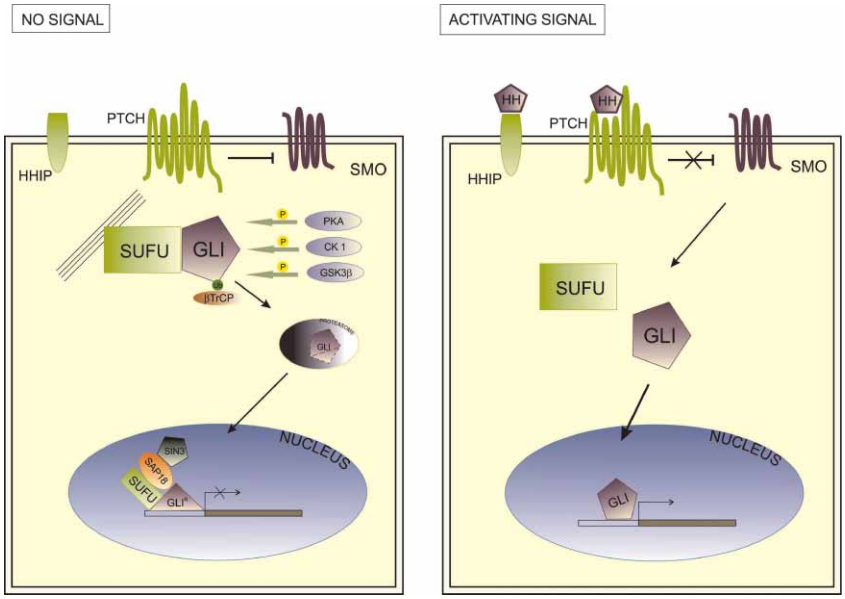

Figure 2. *Illustration of the Hedgehog signaling pathway. (A) In the absence of Hh ligand, Ptch represses Smo and consequently the pathway is inactive. SuFu tethers Gli to the cytoplasm and inhibits Gli-mediated transcription by recruiting a histone deacetylase complex to Gli target genes (4). (B) In the presence of Hh ligand, Hh binds to Ptch, which relieves its repression of Smo and activates the pathway that leads to target gene expression. Hedgehog-interacting protein (Hhip) also binds Hh and regulates ligand availability.*

cyclins), proliferation (myc proto-oncogene) and metastasis (Snail, a transcription factor that is associated with increased invasiveness and metastasis). Also, the Gli transcription factors can promote cell survival by upregulating the anti-apoptotic protein Bcl2 *(4)*.

Tumors usually associated with Gorlin syndrome, such as basocellular carcinoma, medulloblastoma, rhabdomyosarcoma, fibramas of the heart and ovary, meningioma and odontogenic cysts, have been associated with changes in the Hh-Gli signaling pathway as sporadic cases *(12–15)*. Apart from these tumors, there is growing evidence of Hh-Gli pathway involvement in many other lethal tumors: melanoma *(16, 17),* trichoepithelioma *(18),* rhabdomyoma and rhabdomyosarcoma *(19, 20),* digestive tract tumors *(21),* prostate *(22),* small cell lung cancer *(23),* squamous lung cancer *(24),* pancreatic cancer *(25),* bladder cancer *(26, 27),* pituitary adenoma *(28),* breast cancer *(29),* ovarian cancer *(30, 31)* and chronic myeloid leukemia *(32)*.

EPIGENETIC REGULATION OF THE MEMBERS OF THE HEDGEHOG-GLI SIGNALING PATHWAY

Genetic alterations of the Hh-Gli signaling pathway components and their implication in the development of various types of cancer and developmental malformations are well known. In the last few years it has become obvious that epigenetic changes are as crucial in the regulation of this pathway as the genetic ones. Promoter hypermethylation has mostly been reported in the tumor suppressor PTCH and has been implicated in the development of various cancers. In 2007, our laboratory reported higher methylation of the PTCH1 promoter region in ovarian dermoids and fibromas, compared to normal ovarian tissue. In several cases the expression of GLI1 was elevated, but Ptch did not seem to be consequently activated in either of them. The analysis showed that tumors were mostly methylated in the CpG island located in the PTCH1 promoter region near the Gli binding sites, whereas normal tissues showed no methylation at those sites. We suggested that hypermethylation of the Gli-binding site on the PTCH promoter contributes to tumor growth in ovarian fibroma and dermoids because it obstructs the pathway negative feedback loop in which GLI1 stimulates the expression of PTCH. In the cases where there was no elevation in the expression of GLI, tumor proliferation most likely did not rely on Gli activity. On the other hand, there was no change observed in the PTCH promoter methylation status in basal cell carcinomas, compared to healthy skin *(33)*.

The PTCH promoter was also found to be methylated in the ER positive, well differentiated breast cancer cell line MCF-7 as well as breast cancer samples. Methylation was identified in the promoter that regulates the transcription of the exon 1b, which encodes the N-terminal part of the fully active Ptch protein. These samples showed low Ptch expression, which was also validated by immunohistochemistry using an antibody specific for this N-terminal region. This suggests that the downregulation of the active form of Ptch by promoter hypermethylation may be associated with the Hh pathway activation *(34)*.

Du *et al*. analyzed the promoter methylation status of the PTCH1a transcript in a gastric cancer cell line AGS, as well as in gastric cancer tissues. Their results indicated that the transcriptional regulation region of PTCH1a

was hypermethylated in the AGS cell line. After treatment with 5-Aza-dC, almost all sites became unmethylated, which was followed by an upregulation of PTCH1 expression and induction of apoptosis. They also found hypermethylation of the PTCH1 promoter in 32% of gastric cancer tissues, compared to no methylation in adjacent normal tissue. This methylation correlated negatively with the PTCH1 gene expression and was not related to clinical features of gastric cancer. This suggested that hypermethylation of the PTCH1a transcriptional regulating region is an early event in gastric carcinogenesis *(35)*.

Another negative regulator of the Hh-Gli signaling pathway that has been shown to be downregulated in various cancer types by promoter hypermethylation is the Hh-interacting protein (HHIP). Hhip is a membrane-bound protein that can bind all human Hh proteins with an affinity similar to that of Ptch1. Thereby it regulates the availability of ligand and attenuates signaling *(36)*. Tada *et al*. showed that HHIP promoter methylation reduced HHIP mRNA transcription in a subset of hepatoma and hepatoblastoma cell lines. Also, this promoter was hypermethylated in more than 50% of HCC tissues, but no methylation was detected in the corresponding healthy tissues. This led to downregulation of HHIP transcription, and consequently to elevated transcription of GLI1 and PTCH, suggesting that the downregulation of HHIP leads to the Hh signal activation in HCC. Treating the hepatoma cell lines with a demethylating agent restored HHIP expression and attenuated Hh signaling. Thus, signal activation through the inactivation of HHIP may be implicated in the tumorigenesis of HCC *(37)*.

HHIP promoter hypermethylation was also observed in a subset of pancreatic cancer cell lines and pancreatic cancer tissue samples. Epigenetic reactivation of HHIP expression reduced hedgehog pathway activity *(37)*.

In their investigation of the Hh signaling pathway activation in medulloblastoma, glioblastoma and neuroblastoma, Shahi *et al*. found higher expression of GLI1 and lower expression of PTCH1 in a subset of medulloblastoma and glioblastoma cell lines. After the treatment of these cell lines with demethylating agents, PTCH1 expression increased, which would support its promoter methylation as a means of activating the Hh signal.

They also demonstrated the methylation of the promoter of SMO, an activator of the Hh signaling pathway, in cell lines and tumors despite a normal level of SMO expression in these cell lines and samples. They observed an increase in SMO expression after treatment with demethylating agents but, nevertheless, they suggest that methylation does not play a significant role in SMO silencing *(39)*. Wang *et al*. investigated the expression of SHH and altered methylation of its promoter in gastric cancer and its related lesions. The level of SHH expression was significantly higher in advanced gastric cancer than in the early stages. Analysis of the promoter methylation status revealed partial methylation in only one of 63 carcinoma samples, and no methylation in other lesions. However, promoter methylation was frequent in normal gastric pit. They suggested that SHH is the link between chronic tissue injury and cancer and that promoter (de) methylation is the major regulatory mechanism of expression *(40)*.

All these results have shown classic examples of epigenetic deregulation in tumorigenesis, such as tumor suppressor inactivation by promoter hypermethylation (PTCH and HHIP) and oncogene activation caused by promoter demethylation in tumors. This indicates the importance of epigenetic regulation of the Hh-Gli signaling components. Therefore, investigations in this field need to be deepened in order to fully understand the mechanisms of epigenetic regulation of this pathway and its potential as a therapeutic target. It would also be interesting to see whether SUFU, another negative regulator of the pathway, undergoes such regulation, which may also be an important step in tumorigenesis. Furthermore, other mechanisms of epigenetic regulation of this pathway have not been investigated to the same extent as promoter methylation, so this is something for future investigations to focus on.

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