Hedgehog Signaling
Pathway and Cancer

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Cancer is one of the most serious health problems worldwide. It is also the leading cause of death. World Health Organization estimates that more than 7 million people died of cancer in 2008, and more than 13 million people might die of this disease by 2030. In the United States, cancer has led to the death of about 580 thousand people in 2013, and there were more than 1.66 million of new cancer cases in the same year.\[^1\] Unfortunately, there is still no single solution that can completely resolve this problem. Traditional chemotherapy of cancer focuses on impairing the replication of DNA or cell division in cancer cells. However, those cytotoxic agents used in chemotherapy are equally toxic to normal, fast dividing cells in the body, such as hair follicle, epithelial cell on the gastrointestinal tract, and the bone marrow. This lack of selectivity leads to severe toxicity which can sometimes be fatal. The development of drug resistance is another problem that challenges the use of the existing cytotoxic agents. Due to the complexity of cancer, a full understanding of the disease’s nature is necessary before more efficient new therapy can be derived.

Cancer is a disease characterized by unregulated cell growth. The disease is thought to be originated from a single cell which acquires the ability to divide uncontrollably. The loss of growth control is the result of accumulated abnormalities
in multiple regulation systems. The unregulated cells may further undergo multiple steps of alternation and eventually become malignant.\[^2\] One major alteration in cancer cells is the aberrant activation of the embryonic signaling systems. Many embryonic signaling pathways had been found to be involved in cancer development. These pathways include the Hedgehog (Hh) signaling pathway, the Wnt signaling pathway, and the Notch signaling pathway.\[^3\]

The Hedgehog signaling pathway is one of the pathways that draws intensified attention because of its multiple roles in cancer development. The major components of the Hh pathway include the Hh proteins, the Patched (Ptc) receptor, the Smoothened (Smo) protein, and the Gli transcription factors. Patched is a transmembrane receptor of the hedgehog pathway. Hh protein is the ligand of Ptc. Under normal condition, the activity of the Smoothened protein is inhibited by Ptc when the Hh protein is absent, and the canonical transduction pathway of hedgehog is blocked.\[^4\] After the Hh protein has bound to Ptc, the suppression of Smo by Ptc is released. Activated Smo blocks the constitutive processing of Gli which will in turn triggers the transcription of the Hh pathway target genes.\[^4\] Before Ptc is activated by the Hh protein, Smo is localized to the plasma membrane and the intracellular vesicles. The binding of Hh to Ptc triggers the translocation of Smo into
the primary cilia.\(^5\) Although the mechanism is not known yet, it is now clear that
primary cilia, the motor organelles located on the surface of various cell types, are
involved in the activation of the canonical Hh pathway in mammals.\(^5\) The
movement of Smo into the cilia is actively mediated by a family of kinesins (KIFs) and
intraflagella transport (IFTs) proteins.\(^4\) Defects of these proteins or loss of the cilia
results in disruption of Hh signal transduction.\(^5\) However, localization of Smo into
the primary cilia alone is not enough to activate the downstream elements of the
pathway. This G-protein couple receptor like molecule has to be activated before it
can function.\(^4\)

The Gli proteins are zinc-finger transcription factors which regulate the
transcription of the Hh target genes. Human Gli family consists of Gli1, Gli2, and
Gli3. Gli1 is not an effector at the onset of the signaling. In fact, the transcription
of Gli1 depends on Hh signaling pathway. Loss of Gli1 function does not disrupt the
Hh pathway activation and normal development.\(^5\) In contrast, Gli2 and Gli3 are
both very important effectors of the pathway. Gli2 is the primary positive mediator
of the Hh pathway, and Gli3 is the major repressor. Loss of Gli2 results in multiple
defects, whereas mutations in Gli3 causes severe polydactyly in all limbs during
development.\(^5\) Tukachinsky found that cytoplasmic Gli proteins form complexes
with the suppressor of fused homolog (SUFU) proteins which prohibit the translocation of Gli into the nucleus. Upon Hh activation, Smo and the SUFU-Gli complexes are up-taken into the primary cilia, and the SUFU-Gli complex is dissociated with the aid of active Smo. Free Gli is then translocated into the nucleus and triggers the transcription of the target genes.\(^6\)

Hedgehog signaling pathway plays an important role in embryogenesis. The pathway is involved in patterning, proliferation, differentiation, and cell fate determination.\(^8\)\(^{10}\) However, this signaling pathway is hijacked in various types of cancer. The Hh pathway has been reported to be activated aberrantly in breast cancer, bladder cancer, lung cancer, colon cancer, and many other types of cancer. Furthermore, the Hh pathway was found not only responsible tumorigenesis of these cancers, but also involved in metastasis, and drug resistance development in some cancers.\(^8\)\(^{10}\)

Evidence has shown that the Hedgehog pathway has a substantial effect on tumor growth and tumor survival. Using immunostaining, Yoshikawa showed that the Hh pathway expression was increased in adenoma and cancer tissue. In the experiment, cell samples of normal tissue, colorectal adenoma, hyperplastic polyp,
and colorectal cancer were fixed on separated plates. Antibodies of Shh, Ptc, and Smo were added to react with the samples followed by the addition of the secondary antibodies. The secondary antibodies were linked to enzymes which produced the detectable signals. It was shown that the cellular contents of Shh, Ptc, and Smo were increased more significantly in adenoma than in cancer tissue. This result showed that the Hh pathway was more active in early stage of cancer development and implied that the pathway might play an important role in early tumorigenesis.\(^{[12]}\)

In another experiment by Yue, the expression level of Gli was found, by both immunostaining and real time reverse transcription-PCR (RT-PCR), to be increased in nasopharyngeal carcinoma.\(^{[7]}\) In the RT-PCR experiment, mRNA extractions of Gli, Patch1, and Smo from the cell samples were converted into DNA separately using reverse transcriptase. The product DNA segments were then applied to regular PCR processes. The amounts of DNA were measured after each amplifying cycle. It was found that Smo, Gli, and Patch1 were actively transcribed in nasopharyngeal carcinoma whereas the transcription levels of these genes were relatively low in normal tissue.\(^{[7]}\) Cell counting kit-8 (CCK-8) viability essay performed on three cancer cell lines before and after treatment of Hh inhibitors showed that treating the cancer tissues with the SMO inhibitor, cyclopamine, reduced cell viability in these
CCK-8 is a type of microculture tetrazolium (MTT) assay. This assay makes use of water soluble tetrazolium salt. The tetrazolium is converted into formazan by cellular reductase in living cells. The amount of formazan is measured using light spectrometer, and the amount of formazan formed is proportional to the number of living cells. The CCK-8 results showed that the number of living cancer cells decreased significantly after the cells were treated with Hh inhibitors and suggested that the Hh pathway plays an important role in cell viability in some cancer types.\(^7\)

In addition, the Hh pathway might be indispensable in the development of some cancers. Using RT-PCR and MTT assay, Takezaki found that cyclopamine could strongly inhibit the proliferation of two glioma cancer stem cells which maintained active notch, Hh, and Wnt signaling pathways. Treating the same cancer cell lines with the notch inhibitor or Wnt inhibitor did not give the same effect of proliferation suppression.\(^9\) RT-PCR showed that the biomarkers of notch, Hh, and Wnt signaling pathway were actively expressed in the sample cancer cell lines. MTT assay showed that although all three pathways were active in the two cancer cell lines, only the Hh inhibitor was able to suppress the growth of both cell lines significantly. These results reflected that the Hh pathway was critical to the growth and maintenance of
some cancer types.

Furthermore, the Hedgehog pathway is also crucial to cancer cell survival. Eichenmuller found that blockage of the Hh pathway by cyclopamine led to decreased viability in liver cancer cells and triggered apoptosis in the sample cell lines. [8] Apoptosis assay which measures the cellular content of cleaved caspase-3 showed that cyclopamine triggered program cell death in the sample cancer cells. Caspase-3 is a protease which exists as an unreactive proenzyme in normal cells. Activated caspase-3 plays a central role in the execution phase of cell apoptosis. In the experiment, cancer cells were incubated in media with or without cyclopamine and then fixed on separated plates. Afterwards, these cells were treated with anti-human cleaved caspase-3 antibodies followed by enzyme-linked secondary antibodies. The signal produced by the enzyme on the second antibody was then measured to evaluate the level of activated caspase-3. The apoptosis assay showed that the level of cleaved caspase-3 in cyclopamine treated cells was significantly higher than the cells treated with the empty control, suggesting that cyclopamine triggered apoptosis in sensitive cancer cells. Similar conclusions were reached by Samarzia, Yue, and Han on cervical cancer cells, nasopharyngeal carcinoma cell lines, and gastric cancer cells using different biomarkers of apoptosis. [7][11]
Evidence from Han’s experiment showed that the effect of the Hh pathway on cellular survival was contributed to the influence of Hh pathway on Bcl-2 expression.\(^{[11]}\) By using Western blot, Han showed that the cleaved caspase-9 level and Bcl-2 level had increased while Gli-1 level decreased after exploring the gastric cancer cells to cyclopamine. In order to compare the levels of caspase-9, Bcl-2, and Gli-1 in cancer cells treated respectively with tomatidine, phosphate buffer solution (PBS), and cyclopamine, the protein contents were extracted from the lysed cells and then separated with gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane after separation and probed with specific anti-bodies of caspase-9, Bcl-2, and Gli-1. Like caspase-3, caspase-9 is an important protease in apoptosis. The increased level of cleaved caspase-9 indicated that cyclopamine induced apoptosis in the gastric cancer cells. Han noticed that Bcl-2 and Gli-1 protein levels were suppressed while more caspase-9 proteases were turned into its active form. He thus proposed that the effects of cyclopamine on apoptosis were co-related with the suppression of Bcl-2. Bcl-2 is an important anti-apoptotic protein. This protein might exert its effect on apoptosis by regulating the permeability of the mitochondrial membrane. Using immunofluorescence staining, Han found that cytochrome c was released from the mitochondria. Han used
anti-cytochrome C primary antibodies and fluorescein isothiocyanate (FITC) conjugated secondary antibodies to probe the protein after treating the cells with cyclopamine or tomatidine. Diffused fluorescence was found in the cyclopamine treated cells whereas the fluorescence was relatively concentrated in cells treated with the empty control. This indicated that some mitochondrial contents had leaked into the cytoplasm after cyclopamine treatment. Reduced Bcl-2 level might responsible for this leakage which ultimately triggered apoptosis.\textsuperscript{[11]} This finding was supported by Mazumda’s study. Using Western blot, Mazumda found that the Hh inhibitor GANT61 caused up-regulation of the death receptors FAS and CD95, but down-regulated the expression of Bcl-2 and platelet-derived growth factor receptor α (PDGFR α). PDGFR α is a receptor tyrosine kinase on the cell surface which transduces the stimulating signal for cell growth, cell division, and cell survival into the cell upon activation. Suppressed expression of this receptor inhibits cell growth and cell proliferation. The down regulation of Bcl-2 might further weaken the cell survival signal and eventually causes apoptosis.\textsuperscript{[10]}

Not only does the Hh signaling pathway have a great influence on cancer survival and cancer growth, it also plays an important role in the development of cancer drug resistance and metastasis. It is known that hypoxia triggers epithelial
to mesenchymal transition (EMT). EMT is an important step in cancer invasion and metastasis. A major characteristic of EMT is the loss of E-cadherin on the cell surface. E-cadherin is a protein that mediates cell-cell adhesion. Loss of E-cadherin results in increased motility and detachment from the adjacent cells. In cancer cells, loss of E-cadherin promotes cancer invasion and metastasis.\textsuperscript{[13]}

Studies conducted by Lei showed that the Hh pathway played an important role in hypoxia triggered EMT in pancreatic cancer cell. By Western blot analysis, Lei showed that the expressions of Smo and Gli were up-regulated in pancreatic cancer cells under hypoxia, and this activation of the Hh pathway led to the suppression on E-cadherin and stimulated the production of vimentin, an intermediate filament specific for mesenchymal cells. These effects could be reversed by applying cyclopamine or GLI-1α siRNA to the pancreatic cell lines.\textsuperscript{[13]}

Similar results of Western blot analysis were reported in a study by Yoo. In the study, metastasis and invasion were shown to be promoted by aberrant activation of the Hh pathway, and these effects could be inhibited by cyclopamine. Yoo’s study clearly showed that treating the cells with N-Shh reduced the content of cellular E-cadherin, and this effect could be reversed by cyclopamine. Besides showing a
suppressed level of E-cadherin in Hh signaling active cancer cells, the study also showed that the expression of matrix metallopeptidase 9 (MMP-9) was increased along with the increased Hh signaling activity.\[16] MMP-9 is a protease which can digest the extracellular matrix. Because of this ability, MMP-9 is thought to be involved in metastasis by helping the cancer cells to migrate across the extracellular matrix. Yoo showed, with RT-PCR, that the addition of N-Shh promoted the expression of MMP-9 in the gastric cancer cell lines whereas treating the cancer cells with cyclopamine inhibited the up-regulated expression of MMP-9. This result suggested that MMP-9 activity in cancer cells was also regulated by the Hh pathway, and the Hh pathway promoted metastasis via multiple mechanisms.\[16]

Lastly, Hh signaling pathway may play an important role in the development of drug resistance. Using water-soluble tetrazolium salt-1 (WST-1) assay, Kobune found that the Hh signaling inhibitor cyclopamine could sensitize the response of cytarabine(Ara-C) resistant CD34+ leukemic cell line to the nucleotide analog chemotherapy agent cytarabine. The combination of Ara-C and cyclopamine strongly reduced the survival rate of the Ara-C resistant leukemic cells.\[14] WST-1 is a form of MTT assay used to assess the survival rate of the cells. In living cells, water soluble tetrazolium salt is turned into the yellowish formazan. The ratio of survived
cells can be assessed by comparing the absorbance of light at 450nm. It was shown that cyclopamine enhanced the effect of Ara-C significantly. This enhancement was not a simple addition of the effect of these two compounds, hinting that the two compounds might be able to increase the sensitivity of each other. Consider that Ara-C is a nucleotide analog and exerts its effect on cancer through interfering the synthesis of DNA, this synergy effect must be brought about by cyclopamine and its suppression on Hh pathway.

Chen showed that Hh signaling mediated up-regulation of permeability glycoprotein (P-gp) could be an important mechanism in the development of drug resistance. P-gp is an ATP-dependent efflux pump which belongs to the ATP-binding cassette (ABC) transporter family. With Western blot analysis, Chen showed that, the addition of N-Shh to the multi-drug resistant human breast cancer cell lines up-regulated the expression of P-gp. This higher level of P-gp was responsible for the low concentration of doxorubicin (DOX) in drug resistant cells. Making use of the property that DOX emits red fluorescence light after excitation, Chen found that the Hh signaling inhibitor norcantharidin (NCTD) could significantly elevate the concentration of DOX inside the drug resistant breast cancer cells. This effect was achieved by suppressing the expression of P-gp through Hh signaling inhibition.\(^{[15]}\)
Being one of the major embryonic signaling pathways, Hedgehog is involved in many cellular events, such as cell proliferation, differentiation, and cell fate determination. However, the deregulation of this pathway might also lead to the occurrence and development of cancer. It is known that the Hedgehog pathway is critical to cancer growth, cancer cell survival, metastasis, and drug resistance in various types of cancer. Because of its multiple roles in cancer development, the study of the Hh pathway becomes an important part of the study of cancer. Understanding the Hh pathway not only help us to know more about cancer and cancer features, but also provides us some clues on how to develop more efficient drugs and therapies to treat cancer.

Works Cited


