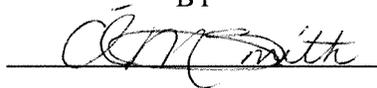EPIGENETIC REGULATION IN LIVER CANCER

AN ABSTRACT

SUBMITTED ON THE SEVENTH DAY OF APRIL 2019

TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF MEDICINE

OF TULANE UNIVERSITY FOR THE DEGREE
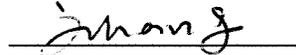
OF

MASTER OF SCIENCE

BY

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ÁNNA M. SMITH

APPROVED BY:

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Dr. Chang Han, M.D., Ph.D., Advisor

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Dr. Jinqiang Zhang, M.D., Ph.D.

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Dr. Tong Wu, M.D., Ph.D.

ABSTRACT

In 2016, liver cancer was declared to be the second most common cancer-related death world-wide. The American Cancer Society has estimated that 42,030 cases have been reported this year and that 31,780 cases have resulted in death. Looking toward the future from a global perspective, as many as 1 million cases are expected to be reported by 2030.

At present, there are extremely limited treatment options, targeted and non-targeted in nature, available for liver cancer patients. There are only three FDA-approved targeted therapies available for treatment; Lenvatinib, Sorafenib and Regorafenib. These have not proven to be extremely effective and produce unwanted side-effects. Therefore, the need for the development and implementation of a more precise therapy is great.

Present data show a significant relationship between liver cancer and EZH2 expression. Overall, an increase in EZH2 expression has been linked to decreased survival. More specifically, EZH2 expression increases as both tumor grades and the cancer stages advance. EZH2 is the catalytic portion of PRC2 that is responsible for tri-methylation of Lysine 27 of Histone H3.

When treated with EZH2 inhibitor GSK 126, Hep3B and PLC cell lines displayed a dose-dependent response to the treatment. Both cell proliferation and tumorigenesis were significantly decreased. The data gathered from this study suggest that inhibition of EZH2 by GSK 126 could be used to develop and implement a new and effective treatment for liver cancer.

Keywords: EZH2, GSK 126, H3K27, Hep3B, Liver Cancer, PLC, PRC2

ACKNOWLEDGMENTS

First, I would like to thank my Creator for the gifts of grace and of life itself. I am truly blessed to have been accepted into this program and to be surrounded by such caring and supportive faculty and staff.

I would like to thank my family, especially my grandparents, for all of their love, support and encouragement prior to, and during this journey.

I would like to thank the members of my thesis committee, Dr. Chang Han, Dr. Jinqiang Zhang, and Dr. Tong Wu, along with all members of the Wu Lab, for their guidance and graciously accepting me as a student into the laboratory. You all have provided me with a nurturing environment where I was able to work, learn, think and thrive.
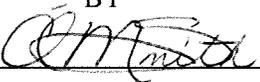
I would also like to thank Dr. Haitao Zhang, Dr. Gilbert Morris and Dr. Byron Crawford for their guidance and assistance throughout my graduate school journey.

Finally, I would like to thank Dr. Diane Blake and the BMS Office for their advising and assistance as well.

You are all greatly appreciated.

EPIGENETIC REGULATION IN LIVER CANCER

A DISSERTATION

SUBMITTED ON THE SEVENTH DAY OF APRIL 2019

TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF MEDICINE

OF TULANE UNIVERSITY FOR THE DEGREE
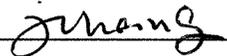
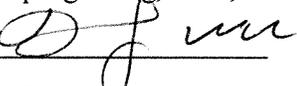OF

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FOREWORD

The purpose of this thesis is not just to document my own findings throughout this laboratory experience, but also to aide in the research project of whoever reads it.

Graduate school has been no easy task but I have enjoyed every moment of it; the ups, the downs, the repeat experiments, all of it. Whether you are a seasoned researcher, or fresh out of undergrad, the repetition that comes with laboratory work may seem like every experiment is being done in vain. I assure you that this could not be farther from the truth. Why? Because every result, be it good or bad, expected or unexpected, is important.

Thus said, I hope this will remind you to keep the faith each day as we all move one step closer to finding the cures for the ailments that plague the people of our world; our friends, our families, everyone. They are important. Therefore, the work that we do, to help them, is important.

- Ánna

Chapter One: Introduction

In general, liver cancer is described as the presence of malignant cells in the liver; a vital organ. Equipped with its own filtration system, the liver is tasked with filtering the blood. Additionally, the liver detoxifies and metabolizes the chemicals that enter our bodies. Malignancy of the cells in the liver, known specifically as hepatocytes, can cause anatomical and physiological changes. These changes are detrimental in that they can prevent the liver from functioning normally, thus causing an accumulation of toxic waste. In more severe cases, liver function may be halted altogether and often results in death. This is due mostly to the limited availability of treatment options, more specifically targeted therapies, to increase survival. (16, 24, 25)

For more targeted therapies to be implemented, we must first understand the target itself. Broadly speaking, the target in recent cancer studies has been epigenetic in nature. (20) The question becomes, do the processes that are involved in the initiation, progression and metastasis of cancer undergo epigenetic regulation? Specifically, do those epigenetic regulatory processes exist in liver cancer? Finally, how can research take advantage of the knowledge gained from a better understanding of epigenetic regulation in liver cancer to develop more precise therapies? (4, 13, 14)

In essence, the goal of this project was to explore these questions by investigating the role of Enhancer of zeste homolog 2 (EZH2), a "master modulator" of chromatin, in liver cancer. (28) The role of EZH2 in liver cancer cells' ability to proliferate, and eventually form tumors, was studied and evaluated using cell culture and Western blot techniques. Epigenetic analyses were also integrated into this project. Collectively, the results indicate

1

that EZH2 can be pharmacologically targeted through inhibition for the purpose of developing a possible liver cancer treatment.

1. Incidence and mortality associated with Liver Cancer.

While this cancer type is more prevalent among individuals in Asia and Sub-Saharan Africa, due to an elevated incidence of Hepatitis B Virus (HBV) infection, the United States has seen a surge in the number of cases reported per year. This can be attributed to complications from Hepatitis C Virus (HCV) infection. (9, 17) Other non-viral factors that put individuals at risk for developing liver cancer include non-alcoholic steatohepatitis (NASH), cirrhosis, alcohol use disorder (AUD), genetic haemochromatosis, ingestion of foods contaminated with Aflatoxin, diabetes, obesity, and hyperlipidemia. (21) It is important to understand that liver cancer development is a complex, multi-faceted process. Therefore, it is believed that the manifestation of liver cancer may very well be the result of two or more of the known risk factors working together over time. (19)

In 2016, liver cancer was declared to be the second most common cancer-related death world-wide. (6) The American Cancer Society has estimated that 42,030 cases have been reported this year and that 31,780 cases have resulted in death. Looking toward the future from a global perspective, as many as 1 million cases are expected to be reported by 2030. (22) At present, there are extremely limited treatment options, targeted and non-targeted in nature, available for liver cancer patients. (16) In addition to this, many treatments have proven to be ineffective and, in many cases, have become a barrier to patients receiving alternative therapies. Another barrier that has prevented liver cancer patients from

receiving adequate treatment is the lack of substantial early detection technique practices. Most patients are not aware of the diagnosis until the cancer has progressed to its later stages. The later the detection and diagnosis, the less likely the patient is to respond well to treatment. (1, 15, 24)

2. What current targeted therapies are available for Liver Cancer Patients?

There are three FDA-approved drugs available for clinical use; two first-line therapies and one second-line therapy. Those drugs are Sorafenib/Lenvatinib and Regorafenib respectively. The latter can only be used after the Sorafenib shows promising results. Therefore, a patient who does not respond well would be at a significant disadvantage. Furthermore, some patients may not be a good candidate for other treatment options such as surgery. Patient eligibility for other treatment options can be due to factors directly related to, or outside of, their liver cancer diagnosis. This creates even more of a health disparity among cancer patients. When compared to other cancer types, liver cancer patients have access to a very limited pool of options that may, or may not, be beneficial to their diagnosis. (6)

 Although these drugs are being implemented, they have proven to be somewhat ineffective and very toxic. Toxicity is often an indication that a drug is not targeting the molecule in a manner which is accurate and highly specific. This further justifies that there is a need to explore a new arena of targeted therapeutic treatment outside of multi-kinase inhibitors.

By approaching liver cancer treatment from an angle that prioritizes precision medicine, toxicity and poor response to treatment may be drastically reduced. (1, 12, 23)

3. The role of EZH2 in Liver Cancer.

The exploration of epigenetic drugs as a means of treatment options for cancer has garnered much attention within the past decade; rightly so as liver cancer patient survival rates have shown little to no improvement. Henceforth, a shift in focus on Enhancer of zeste homolog 2 (EZH2), a gene that encodes for methylation, as a targeted therapy.

EZH2 is the catalytic component of Polycomb repressive complex 2 (PRC2). PRC2 alone is responsible for monitoring methylation of Histone H3 of Lysine 27 (H3K27). The role of EZH2 directly is tri-methylation of the K27. This highly specific catalytic activity makes it a candidate for pharmacological targeting through inhibition. (7)

Across different cancer types, EZH2 is actively promoting cancer in several different ways. Most importantly, EZH2 participates in cell cycle regulation. This means that EZH2 is directly involved in cell proliferation. (20) This makes liver cancer cells a good candidate for experimental cell growth inhibition.

EZH2 is believed to play a major role in the initiation, progression and possibly the metastasis, of many types of cancer. (1, 5, 7) Although many details about the specific role of EZH2 are still in the early phases of discovery, some studies have demonstrated that EZH2 plays a key role in viral carcinogenesis. The viral risk factors associated with liver cancer, HBV and HCV infections, deem EZH2 worthy of exploration as a treatment option.

EZH2 has been shown to be overexpressed in liver cancer. (3) Therefore, successful

inhibition of EZH2 could lead to a promising therapy that may one day deepen the shallow

pool of limited liver cancer treatment options.

Figure 1.1

Expression of EZH2 in LIHC based on Sample types
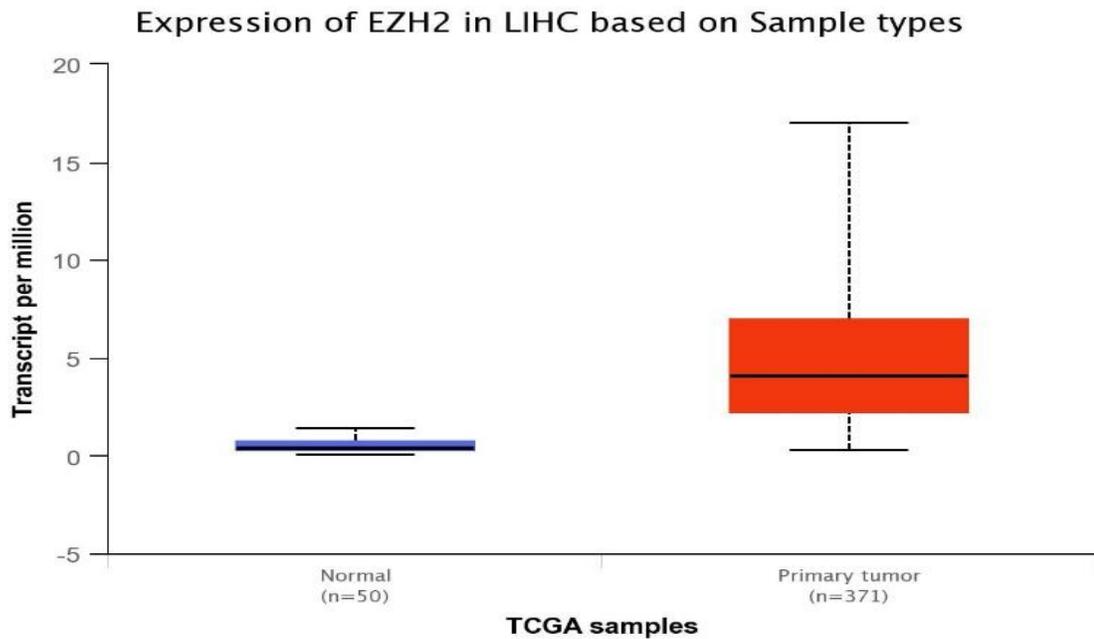
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Figure 1.1 Expression of EZH2 in LIHC based on sample types. Analysis of EZH2 mRNA levels in LIHC (Liver Hepatocellular Carcinoma) samples from TCGA (The Cancer Genome Atlas) database. 371 HCC samples were compared with 50 non-tumor adjacent liver tissue samples, and the results demonstrated that EZH2 mRNA was increased significantly in HCC (t-test p-value $< 1 \times 10^{-12}$).

4. Inhibition of EZH2 by GSK 126

Because of the toxicity associated with inhibitory drugs, it is of great importance that a drug which seeks to inhibit EZH2 does so with accuracy and precision. If implemented as a treatment for liver cancer, indirect targeting of EZH2 could be potentially fatal. Indirect targeting can cause severe damage in other organs of the body; ultimately doing more harm than good. Therefore, more emphasis should be placed on providing not only more treatment options for liver cancer, but options that are safe and have minimal side effects. This was one of the first factors considered during the process of screening and choosing an inhibitor for this project. The utilization of GSK 126 for the treatment of liver cancer is highly favorable because of its known specificity. When compared to other EZH2 inhibitors, GSK 126 demonstrates a 1,000-fold greater selectivity for EZH2. (8,18, 29)

GSK 126 belongs to a division of drugs known as S-Adenosyl Methionine (SAM) Analogues. These drugs block the interaction between EZH2 and other PRC2 subunits. Specifically, it competes with SAM; the donor of methyl groups to EZH2. This competitive interaction in turn inhibits the methyltransferase activity of EZH2. (26)

By incorporating the highly specific inhibitory properties of GSK 126 into this project, its effects on cellular proliferation and tumorigenesis in liver cancer can hopefully be better understood.

Chapter Two:

The Effect of EZH2 Inhibitor, GSK 126, on the Proliferation of Liver Cancer Cells

1. Overview

To evaluate the effect of EZH2 inhibition on the proliferation of liver cancer cells, two distinct cell lines were used; Hep3B and PLC.

The cells were plated in 2-dimensional fashion in 96-well plates and treated with the inhibitor for 24, 48 or 72 hours at doses of 10, 20, 30, 40 or 50 µM.

Cell viability, post-treatment, was determined via WST-1 proliferation assay after a period of 45 minutes.

2. Methods & Materials

Plating

Hep3B and PLC cells (ATCC, Manassas, VA, USA) were sub-cultured. From the sub-culture, each cell line was plated at low-to-medium confluency in three, 96-well plates (Flat bottom with Lid, Tissue Culture Treated, Nonpyrogenic, Sterile, Corning, Corning, NY, USA), and allowed to incubate overnight (37˚C). Cell culture medium consisted of EMEM with L-Glutamine, Sterile-filtered (ATCC, Manassas, VA, USA), 10% FBS (Cell-culture tested, ATCC, Manassas, VA, USA) and 5% Anti-biotic/Anti-mycotic (100X, Gibco, Grand Island, NY, USA).

Treatment

Upon attachment of cells, the cell culture medium was removed from each well via light vacuum suction and replaced with treatment-containing medium. To prepare the treatment-containing medium, fresh cell culture medium was distributed amongst six 50mL conical vials. Varying amounts of EZH2 inhibitor (GSK 126, 10mM, Cayman Chemical Company, Ann Arbor, MI, USA) were added to each vial to produce treatments with concentrations ranging from 0-50 µM. As a control, DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added in lieu of the inhibitor.

After thoroughly mixing the cell culture medium with the inhibitor, the treatment-containing medium was administered such that one concentration was distributed per column. Finally, the plates were incubated with the treatment-containing medium for either 24, 48 or 72 hours (37˚C).

Evaluation

To evaluate the dose-response experiment, the cells underwent WST-1 testing at the end of each treatment time point. The treatment-containing medium was removed via light vacuum suction and replaced with a working solution of WST-1. The working solution was generated from serum-depleted medium (1X, Opti-MEM, Gibco, Grand Island, NY, USA) and WST-1 (Roche Diagnostics, Basel, Switzerland).

Each plate was read via a microplate reader after 45 minutes of exposure to WST-1. The mean value of the optical density obtained for each concentration was recorded and translated to a graph.

3. Results: Hep3B

Figure 2.1

Figure 2.1 Hep3B growth curve. A dose-response curve, with data table, showing the effect of GSK 126 on Hep3B cells after multiple treatment days and 45 minutes of WST-1 exposure.

4. Discussion: Hep3B

After viewing the growth curve, it appears that doses 20-50µM are the most effective at decreasing the viability of the cells. Significant results were not seen until after Day 3 of treatment. It is possible that a longer treatment length should be implemented; perhaps 5 days.

10µM is an ineffective dose; given the current length of treatment. The spike in cell proliferation on the second day of treatment supports this.

The results also indicate that 20µM is just as effective as 50µM. This is important because excessive doses of EZH2 inhibitor could be toxic to healthy surrounding tissues; if administered in a clinical setting. The goal is to inhibit the growth of cancer cells as much as possible without damaging healthy cells. This data supports a low, yet effective dose for the treatment of liver cancer.

5. Results: PLC

Figure 2.2

	Day 1	Day 2	Day 3
0 µM	0.744	1.089	1.375
10 µM	0.734	1.091	1.38
20 µM	0.529	0.426	0.307
30 µ	0.362	0.274	0.253
40 µM	0.285	0.268	0.257
50 µM	0.272	0.263	0.252

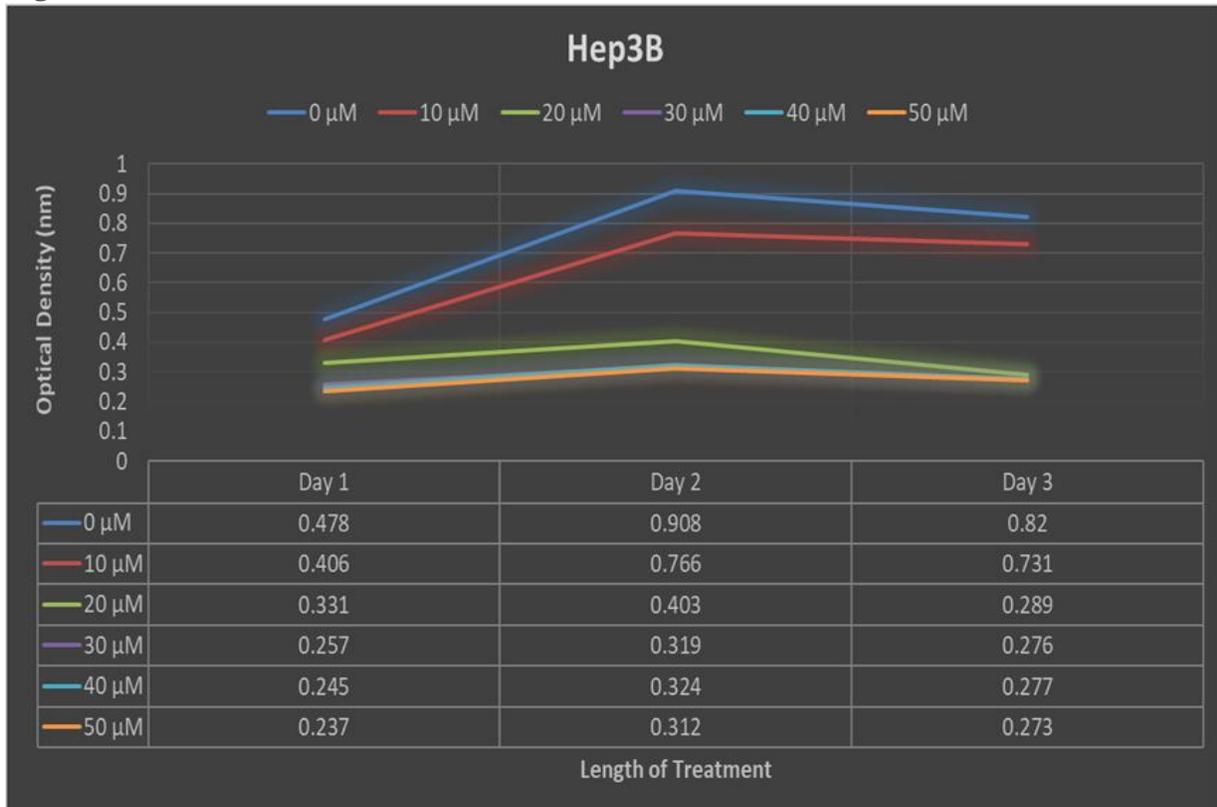**Figure 2.2 PLC growth curve.** A dose-response curve, with data table, showing the effect of GSK 126 on PLC cells after multiple treatment days and 45 minutes of WST-1 exposure.

6. Discussion: PLC

The same pattern of anti-proliferative effects can be seen in this cell line as well. The most effective doses are 20-50µM. Also, 10µM of treatment proved to be extremely ineffective as the proliferation of the cells receiving that dose surged significantly on the final day.

It is important to note that the group that received the 20µM dose was the only one to show a steady and significant decrease in cellular proliferation. Again, this dose appears to be effective despite being on the lower end. Studies showing the results of a longer treatment with this dose should be further investigated.

Chapter Three:
The Effect of EZH2 Inhibitor, GSK 126, on Liver Cancer Tumorigenesis

1. Overview

To evaluate the effect of EZH2 inhibition on the ability of liver cancer cells to form tumors, two distinct cell lines were used; Hep3B and PLC.

The cells were cultured in 3-dimesional fashion in 24-well plates; low-attachment plates were employed to encourage actual spheroid formation. Cells were treated with the inhibitor at doses of 10, 20, 30, 40 or 50 µM for five days.

Cell spheroid-forming ability post-treatment was assessed via photographic images taken while being viewed under light microscopy.

2. Methods & Materials

Plating

Hep3B and PLC cells (ATCC, Manassas, VA, USA) were sub-cultured. From the sub-culture, each cell line was plated at low-to-medium confluency in a 24-well plate (With Lid, Flat Bottom, Ultra-Low Attachment Surface Polystyrene, Corning, Corning, NY, USA) and allowed to incubate for four days until spheroids, or cell colonies, were formed (37˚C). Cell culture medium consisted of EMEM with L-Glutamine, Sterile-filtered (ATCC, Manassas, VA, USA), 10% FBS (Cell-culture tested, ATCC, Manassas, VA, USA) and 5% Anti-biotic/Anti-mycotic (100X, Gibco, Grand Island, NY, USA).

Treatment

Upon successful cell spheroid or colony formation, treatment-containing medium was added to each well. To generate the treatment-containing medium, fresh cell culture medium was distributed among six separate 50mL conical vials. Varying amounts of EZH2 inhibitor (GSK 126, 10mM, Cayman Chemical Company, Ann Arbor, MI, USA) were added to each vial to create treatments with concentrations ranging from 0-50µM. As in the previous experiment, DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added in lieu of the inhibitor to generate a control.

However, the treatment-containing medium was added in addition to the pre-existing cell culture medium in each well. Therefore, a more potent set of concentrations had to be used to consider that the cell culture medium would dilute the treatment and decrease the effectiveness of each dose. Ultimately, the treatments were decreased to the target concentrations previously described after being added to each well.

Lastly, the cells were incubated with treatment for five days (37˚C). The plates were allowed to remain in the incubator undisturbed throughout the treatment period. This was done as a precaution to avoid perturbation of any cell spheroid formations that were unaffected by the treatment.

Evaluation

To evaluate the ability of the inhibitor to prevent tumorigenesis, each well was carefully viewed under light microscopy. Images were taken with an iPhone 6 camera to survey the contents of each well for characteristics indicative of anti-tumorigenesis. This method was chosen due to traditional imaging methods placing limitations on the amount of area captured per image.

3. Results: Hep3B

Figure 3.1
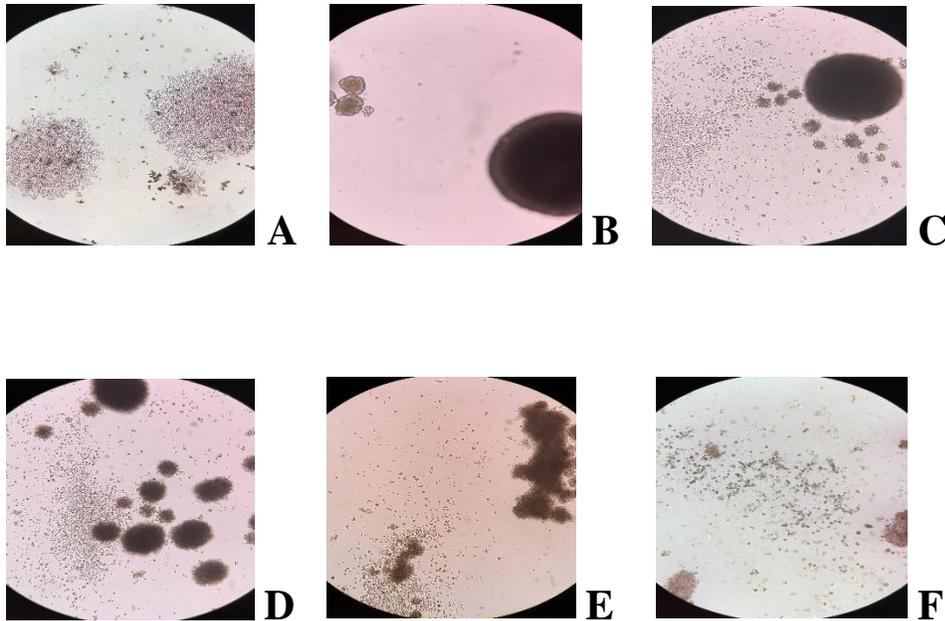
Figure 2.1 Hep3B cells grown in 3-D culture and treated with GSK 126 for five days
A. 0µM **B.** 10µM **C.** 20µM **D.** 30µM **E.** 40µM **F.** 50µM

4. Discussion: Hep3B

The goal of this experiment was to evaluate the effect of GSK 126 inhibitor on the ability of Hep3B cells specifically to form cell spheroids. Therefore, a range of doses was administered to determine which was most effective. The most effective dose in this situation would translate as the least number of well-defined spheroids, the greatest number of poorly-defined spheroids, and the greatest amount of cellular debris present around the spheroids; if any spheroids remain after treatment.

Evidenced by the presence of the very well-defined spheroids, 10µM is not an effective dose. Although some small spheroids can be seen, one of the spheroids is exceptionally large. The spheroids treated with this dose appear to have a glowing encasement which may have been impenetrable by low doses of GSK 126. The inability of the GSK 126 to penetrate the casing of the spheroids could have led to the retention of its spherical shape. The texture and pattern on the surface of this group of spheroids is also much more visible in comparison to the other groups. Most notably, there is no cellular debris present in the well.

It was not until 20µM was administered that cellular debris could be seen around well-defined spheroids. Also, while a large spheroid is present in the well, it is visibly smaller than the large spheroid of the previous group. The spheroids also appear to be less dense than the previous group. When 30µM of treatment was administered to the cells, no exceptionally large spheroids were present in the wells. It is also important to note that the spheroids themselves are greater in number yet smaller in size in comparison to all previous groups. As expected, cellular debris can be seen surrounding the spheroids.

Moving forward, after 40µM of treatment, the shape of the spheroid was completely lost. While a mass is visible, it is not uniform, nor does it possess a clearly defined margin. There appears to be "budding" taking place as evidenced by the smaller masses protruding from the larger mass. Inclosing the budding portions are cellular debris.

Finally, when the 50µM treatment was administered, the well consisted largely of cellular debris. The masses that are visible, despite a tinge of pink because of staining from the media, are translucent. This is in comparison to the masses and spheroids collectively of the previous groups. After taking the various characteristics of the cells after treatment into consideration, the most effective doses appear to be 40 and 50µM. While the 20 and 30µM doses show a decrease in

spheroid size and cellular debris, the 40 and 50μM doses prove to discourage spheroid formation. Spheroids were either not present or dismantled after being exposed to the effective doses.

5. Results: PLC

Figure 3.2
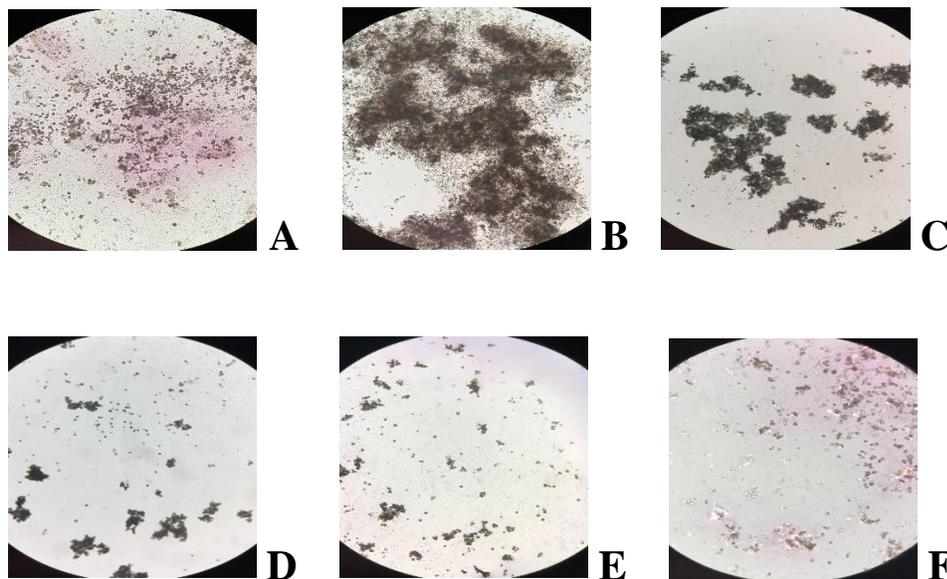
Figure 3.2 PLC cells grown in 3-D culture and treated with GSK 126 for five days
A. 0μM **B.** 10μM **C.** 20μM **D.** 30μM **E.** 40μM **F.** 50μM

6. Discussion: PLC

The goal of this experiment was to evaluate the effect of GSK 126 inhibitor on the ability of PLC cells specifically to form spheroids. As with the previous cell line, a range of doses was administered to determine which was most effective. Ideally, the most effective dose in this situation would translate as the least number of well-defined spheroids, the greatest number of

poorly-defined spheroids, and the greatest amount of cellular debris present around the spheroids; if any spheroids remain after treatment. Since there was no concrete spheroid formation but rather colony formation amongst the cells in this experiment, effectiveness of each dose was assessed mostly via the latter.

After undergoing the 10µM treatment, the cells form the largest colony in comparison to the remaining groups. Also, a lot of cellular debris, more plentiful than that of all the other treatment groups, is present. I theorize that this dose, although low, is somewhat effective at preventing spheroids because there are no concrete spheroids present in the well; large or small.

When given 20µM of treatment, the colony sizes decreased significantly and became more isolated. Also, some cellular debris can be visualized in the clearing of the well. Colony formation may be prevented early on thus being the reason for the absence of more cellular debris as would be expected. Although this dose is on the lower end, it proves more effective than the previous dose in a sense because smaller colonies are present.

It is not until 30µM of treatment are administered that the colonies become excessively disseminated. The clearing of the wells become much more pronounced following this dose. The number of colonies is sparse and there is not much cellular debris.

When 40µM of treatment was administered, both the colony sizes and their overall density decreased. The colonies that remained in this group are slightly smaller than those of the previous group. Some cellular debris is present.

Finally, after administering the highest dose of 50µM, colony formation no longer took place and the cells were largely translucent. Uncaptured by the photo, the cells took on a sharp, glass-like appearance. It appeared that the cells had been emptied of all their contents and only the cytoskeleton remained.

Collectively, the inhibitor is effective at preventing spheroid formation at any dose equal to or greater than 10µM. Due to the combination of a lower density and dissemination of the cell colonies which remain, 40µM proves to be most effective at preventing colony formation. This is important because colony formation could ultimately give rise to cell spheroids.

7. Tumorigenicity and EZH2

There exists an important link between patient prognosis and the grade of a tumor. The more advanced a tumor is on the grading scale, the poorer the patient prognosis becomes. (16, 27) Tumorigenicity alone is highly influenced by EZH2 across many types of cancer. EZH2 inhibition influences cancer by altering the expression of tumor suppressor genes which in turn encourages tumorigenicity. (28) It is quite possible that this altered expression of genes is what is taking place as liver tumors advance on the grading scale. (10,11) When compared to normal tissue, liver tumors express an increased amount of EZH2. That amount increases as the grade of the tumor advances.

Figure 3.3

Expression of EZH2 in LIHC based on tumor grade
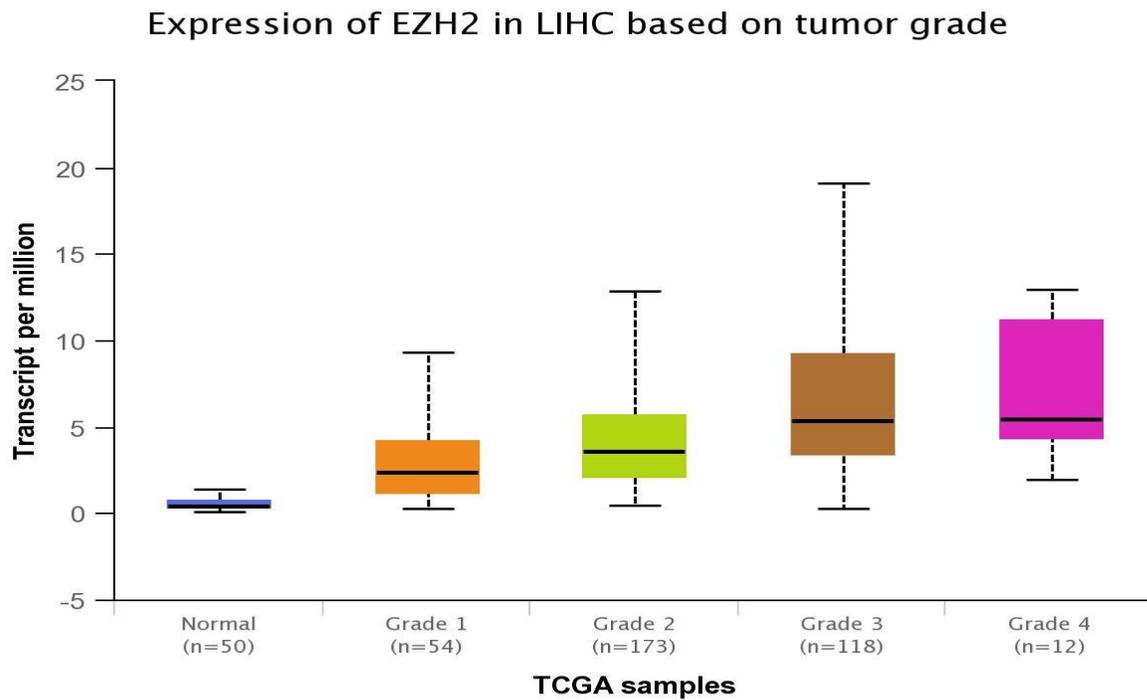
Figure 3.3 Expression of EZH2 in LIHC based on tumor grade. Analysis of EZH2 mRNA level in LIHC (liver hepatocellular carcinoma) samples from TCGA (The Cancer Genome Atlas) database. HCC samples of various tumor grades were compared with 50 non-tumor adjacent liver tissue samples, and the results demonstrated that EZH2 mRNA increases significantly in HCC as tumor grades advance.

Chapter Four:

Lysate Preparation of Liver Cancer Cells After EZH2 Inhibition by GSK 126

1. Overview

To identify the presence and expression of specific apoptotic proteins relative to EZH2 inhibition in liver cancer, Hep3B and PLC cells were plated in Petri dishes and given treatment doses of 0, 10, 20 or 40 µM.

A cell lysis was performed on the cells after being treated with the inhibitor for two days.

The lysates were then subjected to Western Blot analyses using β-actin, Caspase 8 and PARP.

2. Methods & Materials

Plating
Hep3B and PLC cells (ATCC, Manassas, VA, USA) were sub-cultured. From the sub-culture, each cell line was plated at medium-to-high confluency in four Petri dishes (100mm X 20mm, Nonpyrogenic, Polystyrene, Corning, Corning, NY, USA) and allowed to incubate overnight (37˚C). Cell culture medium consisted of EMEM with L-Glutamine, Sterile-filtered (ATCC, Manassas, VA, USA), 10% FBS (Cell-culture tested, ATCC, Manassas, VA, USA) and 5% Anti-biotic/Anti-mycotic (100X, Gibco, Grand Island, NY, USA).

Treatment

Upon attachment of cells, the cell culture medium was removed via motorized pipette at low speed and replaced with treatment-containing medium. To generate the treatment-containing medium, cell culture medium was distributed amongst four separate 50mL conical vials. Varying amounts of inhibitor (GSK 126, 10mM, Cayman Chemical Company, Ann Arbor, MI, USA) were added to each vial to create treatments with concentrations ranging from 0-40µM. Like all prior experiments of this project, DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added in lieu of the inhibitor. Upon successful mixing of the cell culture medium with the inhibitor, the treatment-containing medium was administered and evenly distributed upon the monolayer of each dish. Finally, the dishes were incubated with the treatment-containing medium for 48 hours (37˚C).

Lysate Preparation

Each dish was removed from incubation, washed thoroughly with PBS (Sigma-Aldrich, St. Louis, MO, USA) and placed on ice. The dishes were dried with light vacuum suction.

Cells were lysed in RIPA buffer (NaCl 150mM, EDTA 5mM, Tris 50mM, NP-40 1%, SDS .1%, and Na deoxycholate .5%) supplied with phosphatase and protease inhibitor cocktails (Roche Diagnostics, Basel, Switzerland).

Upon visualization of lytic activity in the dish, the contents were collected with a cell scraper and transferred to chilled microcentrifuge tubes.

Samples were sonicated thrice in 20 second on/off cycles.

The sonicated samples were then placed in a refrigerated centrifuge for 10 minutes at 14,000 RPM.

The supernatants were transferred to new, chilled microcentrifuge tubes.

Western Blot

After being denatured, samples were separated on SDS-PAGE gel and then transferred onto the nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The milk-blocked blots were incubated with different primary antibodies at 4°C overnight.

After washing with PBS-T thrice, the blots were incubated with IRDye 680RD Goat anti-Mouse or IRDye 680RD Goat anti-Rabbit conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) and scanned using the LI-COR Odyssey Imaging system. Primary antibodies for Caspase 8 and PARP were purchased from Cell Signaling (Danvers, MA, USA) while that of β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Results: Hep3B and PLC

Figure 4.1
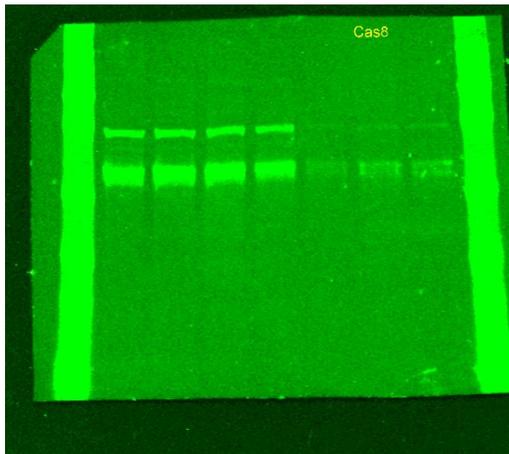
-Proform Caspase 8 (55kD)
-p43/p41 (Caspase 8, cleavage fragments) 43/41kD

Figure 4.1 Western blot utilizing Caspase 8 only. Wells 1-4: PLC (0, 10, 20, and 40µM). Wells 5-7: Hep3B (0, 10, and 20µM).

Figure 4.2

-42 kD

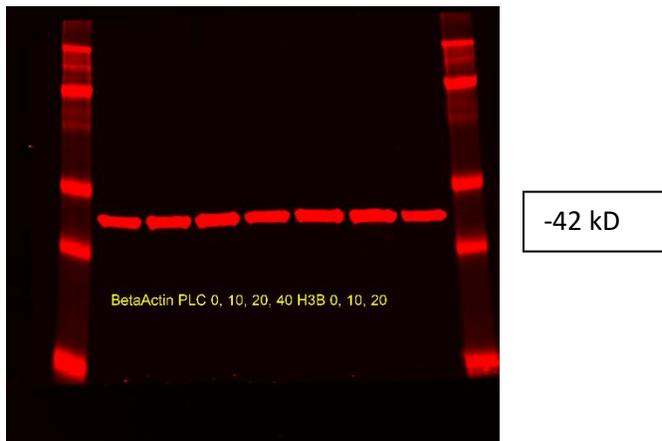**Figure 4.2 Western blot utilizing β-actin only.** Wells 1-4: PLC (0, 10, 20, and 40µM). Wells 5-7: Hep3B (0, 10, and 20µM).

Figure 4.3

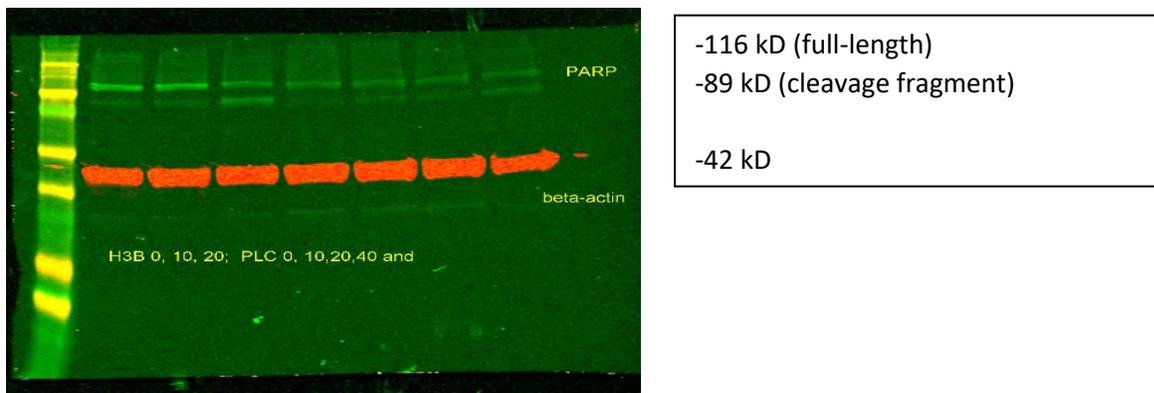-116 kD (full-length)
-89 kD (cleavage fragment)

-42 kD

Figure 4.3 Western blot analysis utilizing β-actin and PARP. Wells 1-3: Hep3B (0, 10, and 20µM). Wells 4-7: PLC (0, 10, 20, and 40µM).

4. Discussion: Hep3B and PLC

While conducting this experiment, a Western Blot analysis could not be performed for Hep3B cells given 40μM of treatment. This was because the cells were destroyed after treatment and obtaining protein from that group would have proven obsolete as no protein remained. The destruction of this group of cells indicates that the Hep3B cells were sensitive to that dose.

In contrast, samples were able to be obtained from all doses of the PLC cells. This indicates that this cell line is more resistant to EZH2 inhibition when compared to Hep3B cells.

Chapter Five:

The Impact of EZH2 on Staging and Overall Survival in Liver Cancer

1. Overview

If EZH2 inhibitors are to be used as a future therapy in clinical settings to treat liver cancer, it is important to identify its expression pattern within two important arenas; patient survival overall and the stages of cancer progression. The analyses were obtained from The National Cancer Genome Atlas (TCGA).

2. The correlation between EZH2 expression in Liver Cancer and patient survival.
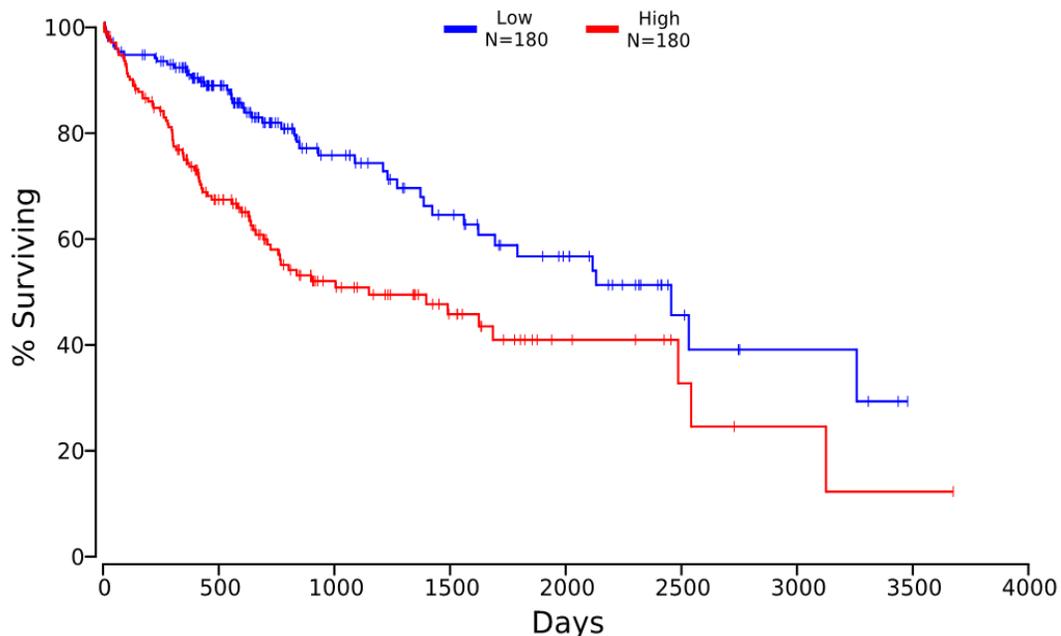
Figure 5.1

Figure 5.1 Expression of EZH2 in LIHC based on survival. HCC patients in TCGA-LIHC cohort were divided into high- and low-EZH2 groups with median EZH2 expression level as the cut-off value. Then, the overall survival of these two groups was assessed. As shown in the Kaplan–Meier survival curves, patients of low-EZH2 group have longer overall survival when compared to patients of high-EZH2 group (Log-rank test p-value=5.87×10^{-5}).

The data obtained from this analysis indicates an apparent correlation between EZH2 expression and survival. The greater the EZH2 expression, the least likely the patient is to survive. In turn, lower EZH2 expression predicts an increase in survival. This relationship justifies further exploration of EZH2 inhibition for liver cancer treatment.

This data also suggests that tests identifying EZH2 expression levels may be beneficial if implemented in clinical practice for prognosis purposes. This may be ideal in that a clinical trial to investigate the effects of EZH2 inhibition by GSK 126 may not happen immediately. More pre-clinical studies proving its effectiveness in liver cancer must take place. The utilization of EZH2 expression in a manner only to predict prognosis is likely to manifest before a clinical trial to develop a treatment.

3. The relationship between EZH2 expression and Liver Cancer stages.

Figure 5.2
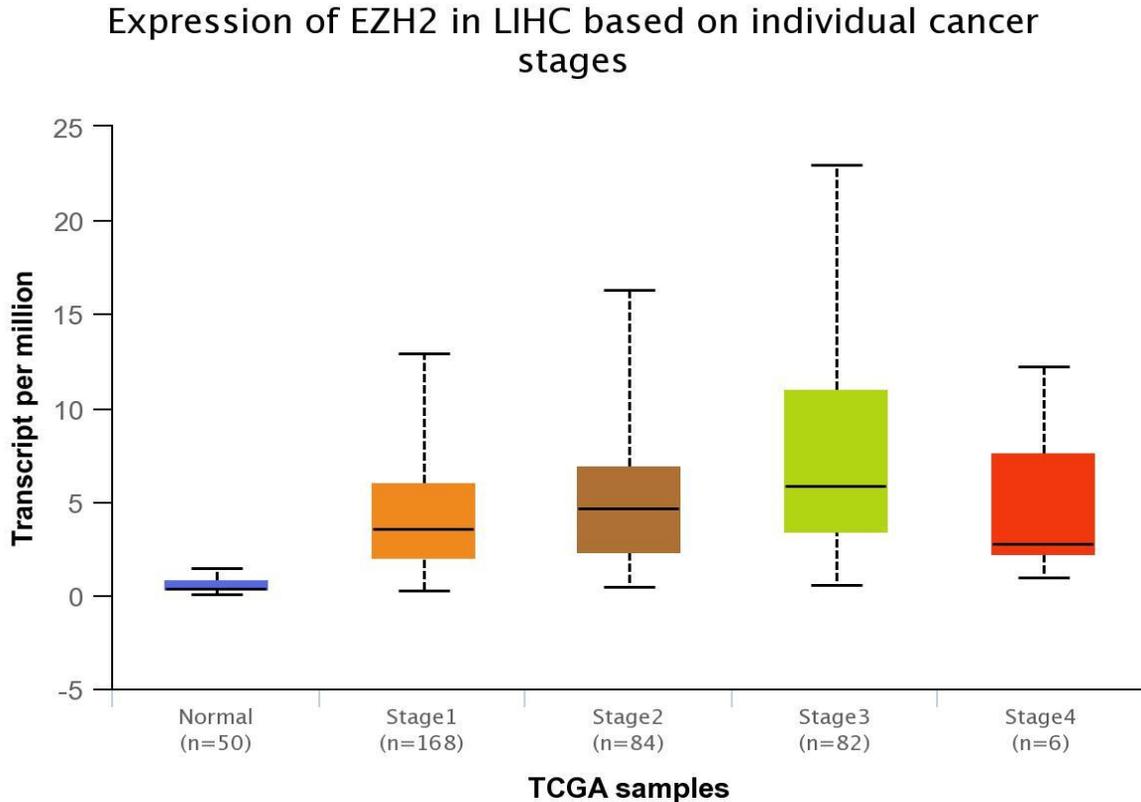
Figure 5.2 Expression of EZH2 in LIHC based on individual cancer stages. HCC patients in TCGA-LIHC cohort were divided into groups based on cancer staging. Then, the expression of EZH2 was assessed. As shown, EZH2 expression increases as the cancer stages progress.

The data obtained from this analysis shows that EZH2 expression increases as the cancer advances. This could explain the reason for poorer treatment outcomes in patients whose cancer is detected at a later stage. It appears that EZH2 plays a significant role in patient outcomes given what is already known about liver cancer in the latter stages.

This data also suggests that EZH2 inhibition can be implemented as a liver cancer therapy for later stage liver cancer. By doing so, patients may have a better chance for survival. Currently, the prognosis for late stage liver cancer does not indicate an increased chance for survival.

Project Summary

The liver is a vital organ tasked with the responsibility of filtering the blood. However, as with any organ, there comes the potential to develop cancer. Therefore, cancer of this type is generally defined as the presence of malignant cells in the liver.

The presence of these cells can alter the structure and function of the organ ultimately leading to a decrease in, or total loss of, the liver's function. Toxic build-up accumulates in the organ giving way to illness, and often death. In regard to survival, liver cancer patients have shown minimal improvement over the years due to a limited availability of treatment options.

At present, only three FDA-approved targeted therapies are available; Sorafenib, Lenvatinib and Regorafenib. Only after a patient exhibits a good response from Sorafenib can Regorafenib be used. This implementation of a second-line therapy creates a barrier to receiving prompt treatment; if any at all.

The factor that continues to complicate the daunting task of developing a new therapy for liver cancer is that there are more cases being reported each year world-wide. Therefore, the demand for an effective therapy is great.

In recent years, researchers are beginning to understand the initiation, progression and metastasis of all cancers in terms of epigenetics. Thus said, what epigenetic factors influence cancer? What factors can be exploited to develop precise therapies for liver cancer?

To explore these questions, the study focused on EZH2 as a potential therapeutic target. EZH2 is an epigenetic regulator that has been found to be overexpressed in liver cancer. It serves as the catalytic component of Polycomb Repressive Complex 2 (PRC2).

To investigate the role of EZH2 in liver cancer, the inhibitor GSK 126 was employed. The inhibitor was administered to Hep3B and PLC cells in various doses to determine which were most effective at inhibiting their proliferation and tumorigenesis.

Epigenetic analyses were also performed to determine the expression of EZH2 in liver cancer itself, in patients with increased or decreased survival, and across the stages of cancer and tumor grades.

All data obtained for this study demonstrated that EZH2 is an important factor in liver cancer and that its inhibition could serve as a novel therapeutic target. The data also suggest that there is a correlation between increased EZH2 expression and decreased survival in liver cancer. EZH2 expression also increased as liver cancer stages and tumor grades advanced.

Looking toward the future, more studies should be implemented to determine the effects of equal, as well as lesser, doses of treatment over longer time periods. Hopefully, this project and its continuation will bring researchers a step forward in the pursuit of an effective targeted therapy for liver cancer.

Abbreviations

ATCC = American Type Culture Collection

AUD = Alcohol Use Disorder

EZH2 = Enhancer of Zeste Homolog 2

FBS = Fetal Bovine Serum

H3K27 = Histone H3 of Lysine 27

HBV = Hepatitis B Virus

HCC = Hepatocellular Carcinoma

HCV = Hepatitis C Virus

LIHC = Liver Hepatocellular Carcinoma

NASH = Non-alcoholic Steatohepatitis

PBS = Phosphate Buffered Saline

PRC2 = Polycomb Repressive Complex 2

RPM = Rotations per minute

SAM = S-Adenosyl Methionine

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