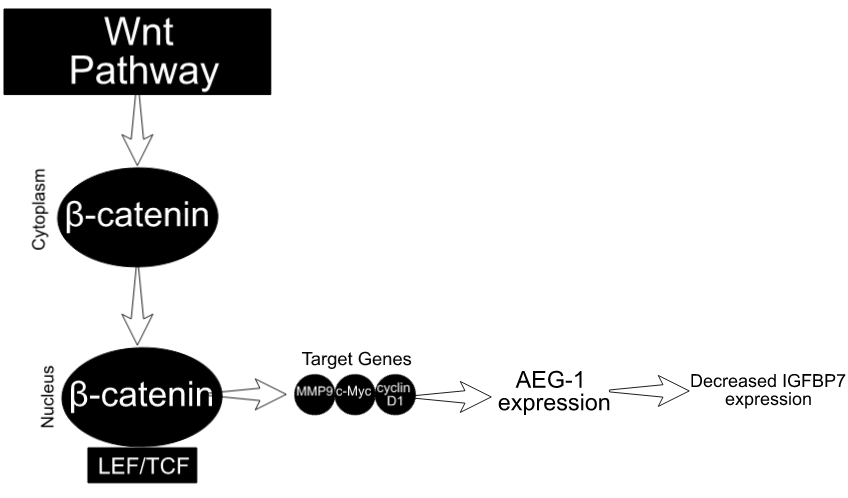
AEG-1 alters methylation status of IGFBP7 promoter via activation of the Wnt/β-catenin signaling pathway in HCC

1. **Introduction**

Hepatocellular carcinoma (HCC) is a common malignancy of the liver, where it develops due to mutations that cause the cells to increase in replication and sidestep apoptosis (Yoo et al 2011). In regard to treatments for HCC, surgery can be a major option; however less than one-third of patients qualify as surgical candidates’ due to various limiting factors (Tomimaru et al 2012). So, in many cases there is not an effective therapy to different HCC cases (Yoo et al 2009). Insulin-like growth factor binding protein-7 (IGFBP7) is a secreted protein consisting of two growth factors known as IGF-I and IGF-II along with their corresponding receptors. IGFBP7 has been known to play major roles in growth, differentiation, and proliferation and it displays potential tumor suppressive activity and downregulation as the tumor progresses (Chen et al 2013). Although this possible tumor suppressor exists, the malignancy of HCC continues due to the overexpression of astrocyte elevated gene-1 (Akiel et al 2014) which acts as an oncogene and helps with progression and development of HCC (Li et al 2017).

AEG1 is known as a mediator of tumor malignancy and has been regarded as a key converging point in the network of oncogenic signaling pathways (Yoo et al 2011). Previous studies demonstrate that AEG1 was significantly overexpressed in various malignant cells and plays a major role in tumorigenesis, proliferation, invasion and metastasis (Xu et al 2014). This oncogene is positioned on chromosome 8q22 and this region is intensified among multiple cancers, including HCC (Zhao et al 2014); also, greater than 90% of HCC patients display the overexpression of this oncogene (Chen et al 2011). Further studies have made the connection between IGFBP7 and AEG1, where IGFBP7 is one of the most downregulated genes via expression of AEG1 (Chen et al 2013). The overexpression of AEG1 can be accounted for due to a number of pathways, including MAPK, P13K/Akt/mTOR pathway, NF –kB pathway, and the Wnt/β-catenin signaling pathway. These pathways can be used in studying different types of HCC. In this study, we will be focusing on the Wnt signaling pathway for its close association with various processes involved in tumor development, proliferation and invasion (Yoo et al 2009).

****

**Figure 1. Proposed Pathway of AEG-1 Overexpression on Status of IGFBP7**

The figure shows the basic process of the Wnt signaling pathway, where β-catenin is the main downstream effector. The activation of the pathway leads to β-catenin binding to Lymphoid Enhancer Factors (LEF) and T-Cell Factors (TCF), thus inducing expression of multiple target genes. Further, β-catenin in the nucleus acts as a signal for an active Wnt pathway, and so does AEG-1. Both interact and activate the pathway, causing for decreased IGFBP7 expression.

I.A. Wnt/β-catenin Signaling Pathway

The Wnt signaling pathway affects the activated site of multiple downstream effector molecules. β-catenin is the main downstream effector of this signaling pathway. When the pathway is activated, β-catenin accumulates in the cytosol at high levels, binds to T-cell factor (TCF)/lymphoid enhancer factors (LEF), and shifts to the nucleus. Once in the nucleus, it induces expression of target genes, including MMP9, cyclin D1, and c-Myc (Li et al 2017). These target genes play roles in regulation of cell proliferation, migration, invasion, cell cycle progress, and metastasis propagation (Weisberg et al 2015). Also, the induction of c-Myc, an oncogenic transcription factor, causes for increased hepatocarcinogenesis, which is the production of cancer in the liver. (Srivastava et al 2015). The high level of β-catenin in the nucleus acts as an indicator of an active Wnt signaling pathway.

In HCC, AEG-1 was found to activate the Wnt signaling pathway (Emdad et al 2013). Zhang et al did a study on AEG-1’s effects on invasion via the β-catenin signaling pathway; they found a correlation between high AEG-1 expression and high β-catenin accumulation (Zhang et al 2012). This would suggest the Wnt signaling pathway is activated by the combination of the two, where AEG-1 and β-catenin interact, causing for an increase of migration and invasion in hepatocellular carcinoma. Figure 1 shows the proposed pathway on the relationship between the Wnt pathway and AEG-1 and how this interaction leads to decreased IGFBP7 expression.

**The purpose of this experiment is to show how AEG1 alters the methylation status of IGFBP7 promoter in human HCC via activation of the Wnt/ β-catenin signaling pathway.**

1. **Experiment**

In order to determine if AEG1’s activation of the Wnt/ β-catenin signaling pathway will cause for altered methylation status of IGFBP7 in human HCC, an *in vitro* experiment focused on effects of AEG-1 silencing will be performed. This experiment will, in many ways, be modeled after Li et all, who performed similar experiments to show how AEG1 promotes breast cancer proliferation via activation of the Wnt/β-catenin signaling pathway. This experiment will differ from Li et al, where we will look into the effect of IGFBP7 from AEG-1 overexpression using this pathway. HCC cell lines will be grown, maintained, and separated into two different experimental groups. The first group will be the Lenti-AEG-1 infected cells, which will have the lentivirus expressing AEG-1. A lentivirus can infect dividing and non-dividing cell types. The second group will be the Lenti-control-infected cells (Li et al 2017), where there will not be a lentivirus expressing AEG-1. The Lenti-AEG-1 will be targeted by a short hairpin RNA (shRNA). The sequence will be known as ‘5’-AACTTACAACCGCATCATT-3’ (Li et al 2017). The following shRNA will be used because it is able to down regulate lentiviruses, meaning it can decrease gene expression of both dividing and non-dividing cells. In this case, the shRNA will be used to downregulate AEG-1. Western blotting will be performed on each of these groups to detect the relative mRNA, protein levels of axin and adenomatous polyposis coli protein (APC), and IGFBP7 levels. Axin is known as an inhibitor of the Wnt signaling pathway (Nakamura et al 1998) and APC is known as a tumor suppressor gene (Aoki et al 2007). With the information gained from these studies, we will be able to determine if AEG1 alters the methylation of IGFBP7 via the Wnt/β-catenin signaling pathway.

II.A. Cell Culture and Colony-Formation

Primary rat hepatocytes will be isolated and cultured. SNU-423 HCC cells will be obtained from ATCC and used for this experiment. MTT assay will be done in order to act as an indicator of cellular metabolic activity. The assay works by viable cells metabolizing a water-soluble salt into a water-insoluble formazan product. DMSO acts as the most suitable solvent for dissolving the water-insoluble product (Twentyman et al 1987). The HCC cell lines will be supplemented with 10% fetal bovine serum (FBS) due to its high content of growth factors, along with antibodies, 100 IU/mL penicillin, and 100mg/mL streptomycin, in 5% CO2 at 37˚C (Tomimaru et al 2012). Penicillin and streptomycin are commonly utilized to prevent bacterial contamination of the cell cultures. And again, the HCC cells will be divided into the two experimental groups mentioned above of the Lenti-AEG1 group and the Lenti-control group.

II.B Methylation-Specific Polymerase Chain Reaction (MS-PCR) and Bisulfite Genomic Sequencing (BGS)

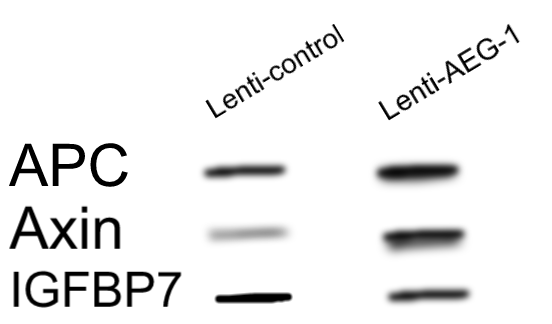
Genomic DNA from the HCC cell lines will be used in bisulfite conversion and purified by EpiTect Fast Bisulfite Conversion Kit (QIAGEN). The kit will allow for the conversion of unmethylated cytosine to uracil. The bisulfite-modified DNA will be used in Methylation-Specific PCR (MS-PCR) and bisulfite genomic sequencing (BGS) (Chen et al 2015). MS-PCR allows for the analysis of DNA methylation patterns in CpG islands. CpG islands play a role in the regulation of gene expression, so their overexpression, in turn, would lead to repressed gene expression. In cancers, gene silencing is occurred through methylation in the promoter of tumor suppressor genes (Ku et al 2011). BGS allows for the determination of the pattern of methylation using bisulfite treatment. Bisulfite is used for its deamination capabilities to unmethylated cytosine, causing for chemical conversion to uracil (Darst et al 2010). The methylated and unmethylated specific-PCR conditions for IGFBP7 will be performed and the PCR for both products will be visualized on 2% agarose gel (Chen et al 2015).

II.C. Western Blotting

HCC cells will be washed using ice-cold Phosphate buffered saline (PBS). PBS is a balanced salt solution and is commonly used in this method in order to maintain a constant pH and osmolarity of the cells. The cells will then be lysed using ice-cold RIPA buffer, phosphatase inhibitor sodium fluoride (NaF), and sodium orthovanadate (Na3VO4). NaF will be used in order to inactivate endogenous phosphatases and to protect protein phosphorylation. Following cell lysis, the proteins in the cell will undergo separation by 5-10% SDS-PAGE. SDS is a detergent that denatures proteins by altering their tertiary structure via unfolding them, after their binding of one SDS per two amino acids (Nielson et al 2007). This process charges the protein, causing for the protein to move down the polyacrylamide gel after an electric current is induced. And so, smaller proteins will move farther down the gel, towards the anode, whereas larger proteins will not move very far down the gel and remain towards the cathode.

The proteins will then be moved onto a PVDF membrane. PVDF membranes are highly hydrophobic, so they will be treated with either methanol or ethanol prior to submersion in the transfer buffer. Then the polyacrylamide gel will be placed against the membrane between two sheets of porous polyethylene facing toward the cell, creating a transfer stack. The porous polyethylene is very rigid and can facilitate in handling, making it a better choice in comparison to the alternative of filter paper (Goldman et al 2015). Again, the use of an electric current will cause for the protein to transfer over to the membrane because of the negative charges of the proteins.

After transferring the proteins to the PVDF membrane, the membranes will then be blocked in 1xTBST (Tween-Tris buffered saline) with 5% skim milk for 1 hour and will be incubated with their corresponding antibodies overnight. In this experiment, the primary antibodies include Rabbit anti-AEG1, rabbit anti-axin, rabbit anti-APC, and rabbit anti-β-catenin. After incubation with the primary antibodies, the membranes will be washed with TBST 3 times and then incubated with the secondary antibody for 2 hours at room temperature. The secondary rabbit antibodies will be used to probe the primary antibodies and will add horseradish peroxidase (HRP). Following the 3 washes of TBST, each protein will be detected using ECL Western Blotting. ECL western blotting substrate will interact with horseradish peroxidase (HRP), thus allowing for visualization. The substrate is able to detect antigen via imaging and visualize the presence of HRP. The experiment will be repeated three times. Figure 2 shows the expected results.

****

**Figure 2. Possible Western Blot Results**

Western blotting in various cell groups will detect the relative mRNA and proteins levels of APC and Axin, and IGFBP7. The following results would also show IGFBP7 levels would be significantly higher in the control group, meaning AEG-1 silencing inhibits activation of the Wnt/β-catenin signaling pathway (Li et al 2017).

1. **Discussion**

From this experiment, the level of AEG1 mRNA expression could be higher or lower dependent on which experimental group it was placed in. In the Lenti-AEG-1 group, the expression would be significantly lower compared to the Lenti-control group. Further, the effect of AEG-1 silencing on the activation of the Wnt/ β-catenin signaling pathway can be assessed. The mRNA levels of axin and APC in the Lenti-AEG-1 group will be significantly higher compared to the Lenti-control group. This would suggest that AEG-1 silencing inhibits the activation of the Wnt/ β-catenin signaling pathway. Looking back to the IGFBP7 as mentioned previously, it is one of the most downregulated genes from the overexpression of AEG1. The purpose of this experiment was to show that AEG1 activates the Wnt/ β-catenin signaling pathway in HCC, thus causing for altered methylation of IGFBP7.

If western blot data of mRNA levels among APC and axin shows the Lenti-AEG-1 cells to be lower in expression than the Lenti-control cells, then this would demonstrate how AEG-1’s overexpression causes tumorigenesis. If the western blot analysis shows the Lenti-AEG-1 cells to be darker than the control cells, then this would show the effect of AEG-1 silencing on the activation of the Wnt/ β-catenin signaling pathway. If these results show AEG-1 decreases expression, the connection to IGFBP7 can be made, where AEG-1 overexpression will decrease its expression. If the described results were achieved, then this would also show this experiment was effective in restructuring the work of Li et al.

However, there are other possible, opposing outcomes to this experiment. If AEG-1 mRNA expression in the experimental group increases or stays the same, this would suggest IGFBP7 is not downregulated via AEG-1, but by another factor. Also, this could suggest that AEG-1 silencing does not inhibit the Wnt/ β-catenin signaling pathway because the mRNA levels of APC and axin in the experimental group would be lower than the control group cells, this would suggest the Wnt/ β-catenin pathway is not regulated via AEG-1 in this experiment setting. More likely, AEG-1 hypermethylation does affect the status of IGFBP7, but a different signaling pathway could be more plausible versus the Wnt/ β-catenin signaling pathway.

1. **References**
2. Chen, D., Yoo, B.K. et al. 2011. Insulin-like Growth Factor-Binding Protein-7 Functions as a Potential Tumor Suppressor in Hepatocellular Carcinoma. Human Cancer Biology. Pgs. 6693-6701. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3207018/>
3. Yoo, B.K., Emdad, L. et al. 2009. Astrocyte elevated gene-1 regulates hepatocellular carcinoma development and progression. The Journal of Clinical Investigaation. Volume 119. Pgs. 465-477. <https://www.ncbi.nlm.nih.gov/pubmed/19221438>
4. Li, M, Dai, Y., Wang, L., and Li, L. 2017. Astrocyte elevated gene-1 promotes the proliferation and invasion of breast cancer cells by activating the Wnt/β-catenin signaling pathway. Oncology Letters. Volume 13. Pgs. 2385-2390. <https://www.ncbi.nlm.nih.gov/pubmed/28454408>
5. Zhao, J., Wang, W., Huang, Y., Wu, J., Chen, M., Cui, P., Zhang, W., Zhang, Y. 2014. HBx Elevates Oncoprotein AEG-1 Expression to Promote Cell Migration by Downregulating miR-375 and miR-136 in Malignant Hepatocytes. DNA and Cell Biology. Volume 33. Pgs. 715-722. <https://www.ncbi.nlm.nih.gov/pubmed/25050974>
6. Srivastava, J., Siddiq, A. et al. 2015. Astrocyte elevated gene-1 (AEG-1) and c-Myc cooperate to promote hepatocarcinogenesis. Hepatology. Volume 61. Pgs. 915-929. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4309751/>
7. Waisberg, J., Saba, G.T. 2015. Wnt-/-β-catenin pathway signaling in human hepatocellular carcinoma. World Journal of Hepatology. Volume 7. Pgs. 2631-2635. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4651907/pdf/WJH-7-2631.pdf>
8. Emdad, L., Das, S.K., Dasgupta, S., Hu, B., Sarkar, D., Fisher, P.B. 2013. AEG-1/MTDH/LYRIC: Signaling Pathways, Downstream Genes, Interacting Proteins, and Regulation of Tumor Angiogenesis. Advance Cancer Research. Pgs. 1-29. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3928810/>
9. Zhang, F., Yang, Q., Meng, F., Shi, H., Li, H., Liang, Y., Han, A. 2012. Astrocyte elevated gene-1 interacts with β-catenin and increases migration and invasion of colorectal carcinoma. Molecular Carcinogenesis. 52: 603–610. <http://onlinelibrary.wiley.com/doi/10.1002/mc.21894/abstract>
10. Darst, R.P., Pardo, C.E., Ai, L., Brown, K.D., Kladde, M.P. 2010. Bisulfite Sequencing of DNA.Curr Protoc Molecular Biology. Pgs. 1-20. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3214597/>
11. Chen, L.H., Liu, D.W., Chang, J.L., et al. 2015. Methylation status of insulin-like growth factor-binding protein 7 concurs with the malignance of oral tongue cancer. Journal of Experimental and Clinical Cancer Research. Volume 34. Pgs. 1-12. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4355468/>
12. Ku, J.L., Jeon, Y.K., Park, J.G. (2011). Walker, J.M (ed). 2011. Methods in Molecular Biology. Humana Press. 791: 23-32.
13. Nielson, M.M., Andersen, K.K., Westh, P., Otzen, D.E. 2007. Unfolding of β-Sheet Proteins in SDS. Biophysical Journal. Volume 92. Pgs. 3674-3685. <https://www.ncbi.nlm.nih.gov/pubmed/17351005>
14. Goldman, A., Spelcher, D.W. 2016. Unit 10.7 Electroblotting and Polyacrylamide Gels. Curr Protein Protein Sci. Volume 82. Pgs.1-24. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4663689/>
15. Xu, C., Kong, X., Wang, H., Zhang, N., Kong, X., Ding, X., Li, X., Yang, Q. 2014. MTDH Mediates Estrogen-Independent Growth and Tamoxifen Resistance by Down-Regulating PTEN in MCF-7 Breast Cancer Cells. Cellular Physiology and Biochemistry. Volume 33. Pgs. 1557-1567. <https://www.ncbi.nlm.nih.gov/pubmed/24854844>
16. Sarkar, D., Lebedeva, I.V., Su, Z., Park, E., Chatman, L., Vozhilla, N., Dent, P., Curiel, D.T., Fisher, P.B. 2007. Eradication of Therapy-Resistant Human Prostate Tumors Using a Cancer Terminator Virus. Cancer Research. Volume 61. Pgs. 5434-5442. <https://www.ncbi.nlm.nih.gov/pubmed/17545625>
17. Kozera, B., Rapacz, M. 2013. Reference genes in real-time PCR. J Appl Genetics. Volume 54. Pgs. 391-406. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3825189/>
18. Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, K., Akiyama, T.1998. Axin, an inhibitor of the Wnt signaling pathway, interacts with β-catenin, GSK-3β and APC and reduces the β-catenin level. Genes to Cells. Volume 3. Pgs. 395-403. <https://www.ncbi.nlm.nih.gov/pubmed/9734785>
19. Aoki, K., Taketo, M.M. 2007. Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. Journal of Cell Science. Volume 120. Pgs. 3327-3335. <https://www.ncbi.nlm.nih.gov/pubmed/17881494>
20. Akiel, M., Rajasekaran, D., Gredler, R., Siddiq, A., Srivastava, J., Robertson, C., Jariwala, N., Fisher, PB., Sarkar, D. 2014. Emerging role of insulin-like growth factor-binding protein 7 in hepatocellular carcinoma. Journal of Hepatocellular Carcinoma. Pgs. 9-19. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4918263/>
21. Yoo, BK., Santhekadur, PK., Chen, D., Emdad, L., Bhutia, S., Pannell, L., Fisher, PB., Sarkar, D. 2011. Increased RNA-Induced Silencing Complex (RISC) Activity Contributes to Hepatocellular Carcinoma. Volume 53. Pgs.1538-1548. <https://www.ncbi.nlm.nih.gov/pubmed/21520169>
22. Tomimaru, Y., Eguchi, H. et al. 2012. IGFBP7 downregulation is associated with tumor progression and clinical outcome in hepatocellular carcinoma. International Journal of Cancer. Volume 130. Pgs.319-327. <https://www.ncbi.nlm.nih.gov/pubmed/21328580>
23. Chen, D., Siddiq, A. et al. 2013. Insulin-like Growth Factor-binding Protein-7 (IGFBP7); A Promising Gene Therapeutic for Hepatocellular Carcinoma (HCC). Molecular Therapy. Volume. Pgs. 758-766. <https://www.ncbi.nlm.nih.gov/pubmed/23319057>
24. Twentyman, P.R., Luscombe, M. 1987. A study of some variable in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br. J. Cancer. Volume 56. Pgs. 279-285. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2002206/pdf/brjcancer00508-0035.pdf>