

Deciphering The Role Of Heparin Sulfate On SLIT2 Mediated Carcinogenesis



SUPERIOR UNIVERSITY

Thesis Submitted to

The Superior University Lahore

In Partial Fulfillment of the

Requirement for the Degree of

MS Zoology

By

SABA IFTIKHAR

Roll No. SU92-MSZGW-S23-012

Session: 2023-2025

Faculty of Sciences

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Designation: Assistant Professor

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No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Faculty of Sciences, The Superior University, Lahore in partial fulfillment of the requirements for the degree of Master of Science/Master of Philosophy in the field of “**MS Zoology**” in Faculty of Sciences at The Superior University, Lahore.

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Dedication

Dedicated to **ALLAH ALMIGHTY**, who blesses me to continue this journey with dedication and positive energy.

I dedicate my research work to my beloved **husband**, whose unwavering support and encouragement have been my guiding light throughout this journey. Your support has enabled me to pursue my dreams, and for that, I shall always be grateful to you.

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List of Abbreviations

cDNA	Complementary Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
E-CAD	E-Cadherins
GAG	Glycosaminoglycan
HAPGs	Heparin sulfate proteoglycans
LRR	Leucine-rich repeat
MTT	Tetrazolium salt
N-CAC	N-Cadherins
ROBO	ROBOundants
RT-PCR	Real Time Polymerase Chain Reaction
TGF- β	Transforming growth factor

ABSTRACT

Cancer is a destructive disease that has been causing a number of mortalities globally. Cancer cells not only cause a tumor at their place, but they can also spread the tumor to other parts of the body as well. SLITs engage with ROBOs and then perform a significant part in organ system and tumor formation by attracting different gateway molecules or enzymes to generate a series of signaling pathways. The emergence of SLIT/ROBO pathway along with heparin sulfate has provided a new insight to deal with the cancerous cells. The present study aims to determine the role of *TGF- β* , SLIT, and Heparin sulfate in showing their relative expression in hepatocytes, HepG2 cancerous cells. The HepG2 cells were taken and cultured under normal conditions. Different treatments were provided to the cultured cells of HepG2, and the expression of genes involved in EMT in cancerous cells was observed. Cell proliferation was observed by using assays like the MTT assay and the Neutral Red assay. The results indicated that these proteins have a significant role in the SLIT2-mediated progression of cancer. In the future, these proteins can be involved in extending their positive role in treating malignancies.

CHAPTER 1

INTRODUCTION

1.1. Cancer

Cancer is the second biggest cause of mortality universally and a significant public health concern [1]. Though they reflect important and growing facts, the most recent classifications have fallen behind the understanding that the cancer cell is changing and transforming itself. Tumors, which are expanding masses of tissue, were the original definition of cancer. They knew how it looked, how quickly it expanded, and how it frequently seemed to "grab" into the body [2].

The cells that make up benign tumors resemble normal cells quite a bit. They only become an issue if they enlarge significantly and become severe or become unsightly and noticeable, pressing against other organs to produce chemicals that alter bodily functions. Cancer cells are the building blocks of malignant tumors. They typically develop more quickly than benign tumors and destroy surrounding tissues [3].

Although cancer has proved to be fatal in several cases, several entities help in the suppression of cancer-causing entities, of which tumor suppressor genes are vitally important. The presence of some tumor suppressor genes in some specific cancers including SLITs and ROBOs, has been linked to multiple studies on tumor growth promotion. Members of the SLIT/ROBO family have since been discovered to play a role in vascular development, organ formation, and tumor growth by controlling cell adhesion, proliferation, and survival in a wide range of tissues [3].

1.2. Role Of SLIT/ROBO Receptors

Recent studies have demonstrated the possible involvement of Roundabout receptors (ROBO) and SLIT glycoproteins (SLIT) in angiogenesis, cancer metastases, and cell adhesion, as well as the modification of the tumor microenvironments of a number of tumor types, including brain tumors [4]. Large proteins compose the ROBO receptors that play a critical neurodevelopmental role in the CNS, controlling growth of neurons, cell mobility, and axon coordination in numerous anatomical locations, notably in cortical and thalamocortical axons, but also in the cervical spine [5]. SLITs are secreted polypeptides that are evolutionarily conserved with variable physiological activities. All species share a significant level of conservation in SLIT proteins [6].

They are often released in order to attach to the extracellular matrix and the cell membranes [7].

1.3. SLIT Genes

There are three SLIT genes SLIT 1, 2, and 3 were found in the majority of vertebrates. It illustrates that SLIT proteins have four leucine-rich regions. As a result, the SLIT/ROBO signaling pathway helps cells of different systems interact to control cell movement, cardiovascular development, immune system orientation, and the division and navigation of both neuronal and non-neuronal cellular types [8].

1.4. SLIT/ROBO Signaling Pathway

In both physiological and pathologic processes, including angio- and organogenesis of the kidney, heart, lung, and mammary glands, as well as inflammatory reactions, tumorigenesis, and tumor blood flow, SLITs directly binds and interacts with ROBOs. By controlling the direction and polarization of both neurological and non-neuronal cellular types as well as cell movement, blood vessel development, and immune system navigation the SLIT/ROBO signaling pathway contributes to the process of cellular interaction between various systems. More specifically, SLITs and ROBOs play a crucial role in the preservation of normal organ shape and function. When this system is dysregulated, it can contribute to congenital cardiac problems [4].

1.5. SLIT/ROBO Receptors

Elements of the axon guidance molecule family, including SLIT and their ROBO receptors, are essential for the development of the nervous systems of both vertebrates and invertebrates. The group includes three ligands (SLIT1, SLIT2, SLIT3) and four receptors (ROBO1, ROBO2, ROBO3, and ROBO4). SLITs were initially identified as a ROBO ligand in *Drosophila melanogaster*. ROBOs are membrane-based receptors that are members of the immunoglobulin-like superfamily [9,10].

In *Drosophila*, three ROBO genes have been identified. There are four ROBO counterparts in zebrafish and mammals, three in chicks and *Xenopus* [5]. Three SLIT ligands have been discovered thus far in vertebrates that exhibit a temporal expression pattern in the peripheral tissue, various organs, and the nervous system throughout development [6].

In vertebrates, SLIT proteins exist in three distinct isoforms: SLIT1, SLIT2, and SLIT3. A laminin G-like domain, nine epidermal growth factor (EGF)-like repeats, four leucine-rich repeat (LRR) domains, an N-terminal signal peptide, and a cysteine-rich C terminus are all present in the mammalian SLIT protein. SLIT1 is primarily expressed in the developing nervous system, while SLIT2 and SLIT3 are also found in the kidney, lungs, heart, and immune cells, among other places. An essential function of SLIT2 was to guide the axonal course of the developing embryonic nervous system by acting as a chemorepellent to prevent incorrect midline crossing by the developing commissural neurons in *Drosophila melanogaster* [7]. The ventral midline of the neural tube and other areas, such as the gut's entry, express at least three of the SLIT protein's members, which have been identified in both mammalian and avian species gut [8].

Four ROBO receptors have been identified in animals because of their similarity. ROBO1 and ROBO2 are highly expressed in the nervous system across the embryonic phase and serve critical roles in directing axons and cell migration. Besides the nervous system, ROBO1 and ROBO2 show cell and organ-specific expression patterns, and their expression is required to govern formation in the kidney, lung, and heart. ROBO3, on the other hand, is exclusively produced by commissural neurons and is essential for the regulation of commissural axons that cross the central nervous system's midline [6]. Since ROBO receptors are single transmembrane proteins with no biochemical or autocatalytic activity, their function is probably regulated by downstream signaling and structural molecules [11]. By acting as a repellent to enable precise axon pathfinding and neuronal migration during development, the SLIT axon directing molecules and their receptors, sometimes referred to as ROBO (Roundabout), constitute one of the most important ligand-receptor combinations among the traditional axon guidance signaling pathways [6]. Building on decades of knowledge about the cellular signaling pathways mediated by glycoconjugates and the role of glycan structural features in defining specific cellular responses, researchers are utilizing the diverse properties of glycosaminoglycans to develop new tools for improving the clinical management of cancer.

1.6. GAG's

One of the main classes of macromolecules present in all mammalian cells is GAGs, which are lengthy linear chains of diverse saccharides. Their interactions with growth

factors, morphogens, chemokines, extracellular matrix (ECM) proteins, and their bioactive fragments, receptors, lipoproteins, and pathogens have all been thoroughly investigated and documented. From crucial stages in embryogenesis and early development to ECM (re) modeling and cell signaling regulation in a variety of physiological and pathological situations, this dynamic network coordinates a number of vital processes [12].

Glycans found in the extracellular matrix (ECM) and on the surfaces of cell membranes in mammals are known as sulfated glycosaminoglycans (GAGs). The amino acids that are produced when they are covalently bound to their particular core proteins can either be secreted and form an essential component of the extracellular matrix (ECM) or they can be transmembrane and connected to the membrane by a glycosylphosphatidylinositol anchor. The most well-known member of the GAG family is probably heparin. The GAG chains of the proteoglycan serglycin are a strongly negatively-charged, linear polymer that is present in the secretory granules of connective tissue mast cells. D-glucosamine (GlcN) and uronic acid (D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)) are two of the 1-4 connected repeating disaccharide units that make up heparin/heparan sulfate (HS) [9]. As a regulatory molecule in the glycocalyx, HS glycosaminoglycans (GAG) evolved over 500 million years under rigorous evolutionary selection constraints. It exhibited DNA recognition and information-storage capabilities that allowed it to regulate cellular behavior and a variety of vital physiological life processes [13].

1.7. Heparin Sulfate

Heparan sulfates are found in the extracellular space and on the outside of cells. They bind with various ligand particles to mediate biological processes. Additionally, heparin interacts with SLIT2 and creates a ternary complex that stabilizes the contacts between SLIT2 and ROBO. Additionally, directional migrations and invasions mediated by SLIT2 are said to occur only when heparin is present. It participates in SLIT-ROBO signaling as a co-receptor as well [10]. It is frequently found in mammalian tissues, Heparan sulphate proteoglycans are a varied set of extremely useful proteins with renowned roles in tissue formation and healing processes. More than 400 heparin sulphate binding protein members are among the many interaction ligands found in Heparin sulphate proteoglycans [11]. Heparin sulphate chain structures are crucial elements that define the actions of Heparin sulphate

proteoglycans, which communicate a variety of metabolic and cellular regulation mechanisms. The extracellular matrix structural proteins, cell-adhesion molecules, neurons, cell-surface morphogens, proteases, and protease inhibitory proteins are among the proteins that interact with Heparin sulphate. [14].

1.8. Heparin Sulfate And SLIT/ROBO Pathway

Furthermore, it has been demonstrated that heparan sulfate is essential for SLIT-ROBO signaling. It was demonstrated that the C-terminal cap region of SLIT's second leucine rich repeats domain has a heparan sulfate binding site. Heparin, the highly sulfated form of heparan sulfate, increased the binding capacity of the SLIT-ROBO connection, according to biochemical and functional investigations. Additionally, heparan sulfate binding sites in ROBO Ig domains were discovered by ROBO structure-based mutagenesis studies employing ROBO mutants, which aided in SLIT-ROBO signaling [7]. Heparan sulfate proteoglycans (HSPGs) are necessary additional receptors for SLIT/ROBO signaling. Heparan sulfate chains surround a large peptide core protein that makes up HSPGs, stabilizing the connection between SLIT2 and ROBO1 [15]. Although SLIT-ROBO expression has been found in a variety of human cancer types, its function in the disease's biology is still up for debate. According to certain theories, SLIT-ROBO may act as a tumor suppressor system in malignancies of the breast, ovary, non-small cell lung, and cervical regions. On the other hand, it has been documented that endometrial, hepatic, colorectal, and prostate cancers have elevated expression of SLIT-ROBO family members. The expression of SLIT-ROBO proteins in hematological malignancies is poorly understood. Nonetheless, it is known that ROBO4 is expressed on the surface of stem cells that produce blood (HSC) and contributes to HSC niche-finding.

1.9. Role Of SLIT/ROBO In Cancer

In a variety of malignancies, including small cell lung cancer, the SLIT/ROBO signaling pathway has pro-apoptotic and anti-migratory characteristics. The cognate partners and their mediators may have mutations that decrease these tumor-suppressive effects. Most of the time, pulmonary dysmorphogenesis in ROBO1 knockout mice led to embryonic mortality, while those who survived displayed excessive bronchial growth [15].

SLIT2 has been shown to increase tumor cell activity and motility, metastatic spread, and therapy resistance, especially in colon cancer, digestive, and osteoblast cancers. It also plays a proangiogenic role in tumor settings. However, other research found that SLIT2/ROBO signaling has a tumor-suppressive effect in breast and lung malignancies. While some research has linked SLIT/ROBO signaling to more aggressive Glioblastoma behavior, other studies have suggested that SLIT2 signaling may limit tumor growth in the context. It was unclear if SLIT/ROBO signaling might be utilized therapeutically to stop the growth of cancer because of its various and context-dependent effects [16].

1.10. Endothelial To Mesenchymal Transition

During the development of embryos and mature tissue, a state of equilibrium epithelial cells can adopt a morphological phenotype through a biological reprogramming process called EMT. Because EMT gives cancer cells hostile phenotype-associated traits, it is essential for embryonic development, wound healing, and the spread of cancer. The growth of the embryo, wound healing, and the advancement of malignancy all depend on EMT. E-cadherin functions as a glue to keep epithelial cells in healthy tissue attached to one another. The expression of epithelial indicators like E-cadherin, ZO-1, and occludin declined following EMT activation, while mesenchymal markers, including N-cadherin, vimentin, fibroblast-specific protein 1, and fibronectin, increased [11].

A while ago, EMT was thought to be an "all or nothing" process in which cells may either be migratory or epidermal. However, new discoveries have revealed that EMT can give cells an incomplete or mixed phenotype that concurrently possesses morphological and epithelial traits, rather than always manifesting as a binary flip between both extremes. Cancer cells have a greater capacity for adaptation in response to microenvironmental stimuli because of this novel idea, which was recently characterized as epithelial-mesenchymal plasticity (EMP). EMT was thought to be an "all or nothing" process in which cells may either be morphological or epithelial. Nevertheless, new discoveries have revealed that EMT can give cells a partial or hybrid phenotype that concurrently possesses mesenchymal and epithelial traits, rather than always manifesting as a binary flip between two extremes. Cancer cells have a greater capacity for adaptation in response to microenvironmental stimuli because of this novel idea, which was recently characterized as epithelial-mesenchymal plasticity

(EMP) [12]. Crucial processes such as organ fibrosis, tissue healing, cancer progression, and embryonic cell migration are all based on epithelial plasticity. In each of these situations, cell plasticity is triggered by the epithelial-to-mesenchymal transition (EMT), demonstrating its pleiotropy and inherent complexity [17].

1.11. E-Cadherin

An epithelial protein, E-cadherin, which plays a significant role in cell adhesion, has been linked to metastasis when lost. It regulates the epithelial-mesenchymal transition associated with the invasion and migration of cancer cells. However, recent research has shown that E-cadherin promotes the survival and growth of metastatic cancer cells [18].

The growth of tumors has been directly associated with the loss of E-cadherin. The lower levels of E-cadherin have been observed in various cases of carcinoma that are related to the destruction caused by tumors, leading to poor survival of patients. The interruption of E-cadherin among cells may result in the easy spread of tumors to other healthy and uninfected cells. It has been observed that the cancer cells lose E-cadherin and attain the mesenchymal markers when the epithelial tumor has invaded enough surrounding cells. The role of E-cadherin in the suppression of tumors has been studied in various cancerous cells. When E-cadherin is present on two different molecules, it binds to the plasma membrane of cancer cells, contributing to the growth regulatory signaling [19].

Cadherins are the transmembrane glycoproteins that promote attachment from a cell to cell while also preserving healthy tissue structure. Moreover, cadherins have been detected in physiological fluids, particularly blood, as well as between tumor cells. Standard cadherins, a family of adhesion molecules with several subclasses, interact with catenins through the cytoplasmic domain. The classical cadherins are classified into three types: epithelial, placental, and neural. Many studies have been conducted to determine the role of cadherins in cancer formation in the last few decades. Cadherins have been studied in many malignancies, including breast, gastric, glioblastoma, hepatocellular, pancreatic, and melanoma.

Many research studies have been conducted to determine the significance of E-cadherin as a tumor inhibitor. Recently, the motility, invasion, and development of tumor cells have been observed due to E-cadherin in the later stages of cancer. The

formation of cancer is related to the genetic defects in the cell surface regulation of E-cadherin. Many studies on the metastatic colonization have disclosed that E-cadherin re-expresses itself during the mesenchymal-to-epithelial transition. Some studies on the invasive ductal carcinoma models have proved the role of E-cadherin in promoting metastasis. The overexpression of N and P-cadherin has also been observed in the bad prognosis of breast cancer patients [13].

1.12. N-Cadherin

N-cadherin is involved in promoting the survival of tumor cells, migration, and their invasion. The overexpression of N-cadherin is associated with poor prognosis and diagnosis. N-cadherin has also been produced by the endothelial cells, which assists in the development and maintenance of normal and tumor-associated angiogenic arteries. The recent experimental findings have revealed that N-cadherin might be used as a therapeutic target against cancer cells [20].

N-cadherin, just like E-cadherin, promotes the movement and penetration of cancer cells. Hazan et al. 1997 illustrated that N-cadherin expression was correlated with incursion and invasion. They proposed that N-cadherin-mediated interactions between breast cancer cells and stromal cells accelerated invasion [21]. Another study exposed that N-cadherin plays an explicit role in promoting the invasion process by generating an isolated phenotype produced by oral squamous carcinoma-derived cells. The enforced expression of N-cadherin led to the downregulation of endogenous E- and P-cadherins. This caused a difficulty in differentiating between the motility-promoting and motility-suppressing characteristics of N-cadherin and E-cadherin, respectively. In contrast to this, N-cadherin has been involved in enhancing the inhibition of contact in normal skeletal muscle cells, thus inhibiting the migration of cells upon touch without affecting their motility.

1.13. Vimentin

Vimentin, an essential helical protein, assists in the organization and functioning. During the early phases of cancer development, the levels of vimentin are usually lower, but the levels rise when the cancer spreads to the nearby cells. Vimentins have been known to play a number of roles in the growth of cancer and expansion to obscure areas of the body. A recent study on vimentins has highlighted the role of the vimentin gene in cancer, although the impact of vimentin on cancer growth and development is

doubtful. For better medical treatment and survival of patients, modern technologies have been utilized to target vimentin and its action [22,23].

1.14. Integrin

The physiology of cancer disrupts the integrin-mediated attachment of cells to the extracellular matrix that is responsible for the normal development of tissue and function. The cancer cells are actually capable of crossing the tissue borders and entering the bloodstream and venous arteries. They survive by floating in body fluids, managing themselves biologically or analytically because of the irregular regulation of integrin receptors and the endothelial cell matrix's ligands. The adhesion activity of integrin receptors can be impacted by the alterations in various mechanisms at cellular and molecular levels due to cancer cells. The defects of tumor cell integrin-mediated extracellular matrix adhesion and the components of tumor microenvironment all contribute to the cancer genesis and progression. Resistance can be overcome by the effective use of integrin inhibitors. The recent study has supported the role of integrin inhibitors against the resistance to anti-cancer medications [24].

AIMS AND OBJECTIVES

The main aims of this study are the following:

- 1.** To estimate expression levels of SLIT2 in cancer cells.
- 2.** To analyze the effect of exogenous administration of SLIT2 on cancer progression.
- 3.** To examine the role of heparin sulfate in SLIT2-mediated cancer progression.

CHAPTER 2

LITERATURE REVIEW

The function of heparin sulfate glycosaminoglycans in the promotion of tumors in glioblastoma was monitored. They cultured Murine GBM tumor-spheres that were extracted from xenografts of patients. The dissociated tumor-spheres were dissociated, from which GAGs were purified and analyzed. Quantification of HS proteins was carried out. The tumor-spheres were subjected to the analysis of RNA and protein. The HPSE was targeted to make the lentivirus. The cells were then exposed to various assays to validate their invasion and adhesion. It was concluded that after the enzymatic degradation, the HS content can be altered directly or indirectly [25].

The role of heparin on hepatocarcinoma cells was studied. Human hepatocarcinoma cells were cultured under special conditions. Different tests were conducted to detect the effects of different heparins. Various assays were performed for the determination of cell motility, cell invasion, luciferase activity, cross-linking, co-immunoprecipitation, gelatin zymography, immunoblotting, sulforhodamine B, real-time cell growth, and flow cytometry. After performing all these assays, qPCR was carried out. They concluded that heparin stimulates c-Met-mediated invasion and migration of Hepatocellular Carcinoma cells [26].

The accumulation of heparin sulfate in tissues affected with tumors predicting low relapse-free survival of glioblastoma patients, was researched. Tissue samples from about thirty-one patients who were diagnosed with high-grade glioma were obtained after getting their consent. The patients were provided with adjuvant therapy to control the dissemination of glioma cells and reversion of disease. The embedded tissues were then subjected to immunostaining. RT-PCR was performed after the extraction of RNA. It was deduced that HS content increased in glioma cells showing distinct dissemination in glioblastoma tissues. This research also indicated that the level of heparin sulfate is not linked with the activity of glioblastoma cells while they are related to low relapse-free survival of such patients as high levels of HS cause the up-regulated amount of syndecan-1 and perlecan [27].

The effect of SLIT2 and ROBO1 in determining the survival rate of patients diagnosed with osteosarcoma was studied. Human osteoblast and osteosarcoma were taken, and

cultivation was done under specific conditions. Different protocols like transfection of siRNA and plasmid were performed. qPCR was carried out. Various assays such as Western blotting, IHC staining, CCK-8, MTT, colony formation, BrdU cell proliferation, cell apoptosis, cell analysis, CHIP, luciferase, and many other assays were brought out. It was assumed that SLIT2 and ROBO1 are up-regulated in osteosarcoma promoting the proliferation of Osteosarcoma cells and inhibiting the apoptosis of cells [28].

The function of heparin hexa-saccharide in the inhibition of stem cells of colon cancer using the process of kinase activation was reviewed. Human colorectal and pancreatic cells were cultured. Xenografts of different animals were generated providing different treatments. The tumor tissues were collected and prepared for further analysis. After performing various assays, the flow cytometry and western blot analysis were done. The results showed the inhibition of colon CSC production after several treatments [29].

The adverse and resistant effects of exogenous SLIT2 on VEGF-induced endothelial cell migration and tube formation were investigated. Human CECs were taken and cultivated under specific conditions. Several assays were brought out to determine the cell migration and formation of the tube. Immunocytochemistry, western blot analysis, and RT-PCR were carried out. The results summarized that CECs mostly express the presence of Factor-VIII antigens, SLIT2, and ROBO4. It was witnessed that the exogenous SLIT2 directly accelerates the ROBO4 protein and inhibits the VEGF-induced cell migration as well as the tube formation [30].

The activation of the SLIT2/ROBO1 signaling pathway that promotes tumor metastasis by activation of the *TGF- β* signaling pathway was studied. The serum samples from 23 volunteered patients who were diagnosed with colorectal carcinoma and some 18 healthy volunteers as a control for this research were collected. They took mice for further research and were given treatments according to the protocols. The tissues were chopped off from the mice and cultured under specific conditions. Various assays were performed to validate the presence of tumor metastasis including an assay for wound healing, the transwell migration assay, the Matrigel invasion assay, and the Western blot assay. The histological and immuno-histological analyses were carried out on the intestinal tissues. The over-expression of SLIT2 was observed in CRC. It was concluded that the tumor growth and metastasis of CRC were inhibited

by blocking the SLIT2/ROBO1 pathway at specific sites, leading to the suppressed growth of cells, migration of cells, and cell invasion. It was also observed that *TGF- β* plays a key role in the induction of cell metastasis [31].

The stimulatory role of heparin sulfate proteoglycans in the migration and the invasion of cells was explored. Human glioblastoma A-172 and fibrosarcoma HT1080 cells from the cell culture collection of vertebrates were subjected to culture using different media. Hspn90 was purified from different models and parts. Enzymatic degradation was carried out to confirm the impact of sulfated glycosaminoglycans on the migration of the cell. The optical density assisted in the measurement of cell invasion. Flow cytometry was performed on the cells that were then undergone through the Western blot analysis. It was concluded that the Hspn90-mediated cell migration was inhibited after the enzymatic degradation and decreased after de-sulfation [32].

The processes in the enhancement and action of glypican-1-positive exosomes in colorectal cancer cells were monitored. Human colon carcinoma cells from which the exosomes were isolated, purified, and identified using an electron microscope were cultured. The genes of SLIT2 and HIF-2 α were suppressed using small interfering RNA (siRNA). Amplification of the human SLIT2 gene was done using PCR. Many other protocols were performed like co-immunoprecipitation, hypoxia, the MTT assay, the colony formation assay, the nuclear isolation, western blot analysis, and immunohistochemistry. The assemblage of mice was randomly divided into five groups and was injected to extract tumors. They deduced that there was an increase in the percentage of exosomes after silencing the SLIT2 gene and the hypoxia condition of the cell. The increased exosome results in the spreading of cells and the formation of colonies by the cancer cells in the colon [33].

The function of SLIT3–ROBO4 signaling in the endoplasmic reticulum (ER) stress-induced delayed corneal epithelial and nerve regeneration was reviewed. They took male mice and injected them with ER stress agonists. The corneal epithelium was scratched out from those that were given some treatments and then those were subjected to culture. Many analytical procedures were carried out to validate the effect of TG on cell proliferation and cell migration. Immunofluorescence staining was performed that has been followed by whole-mount staining. The tissues treated with TG and without TG are then sequenced using RNA sequencing which was then set for RT-qPCR. ELISA, Western blot, and Tunnel assay were also done. They concluded

that the TG treatment has shown suppression of wound healing in the epithelium as well as a reduced number of cell production. This group has also shown a high expression of SLIT3/ROBO4 which was associated with inhibiting the effects of TG and SLIT3 on corneal epithelium and nerve regeneration [34].

The effects of heparin sulfate on the inhibition of *TGF- β* in the proliferation of cancerous lung cells and glioblastoma using the SLIT 2 model were investigated. The lung cells and glioblastoma were cultured and incubated under specific conditions. After a day, the morphology of the cultured cells was examined under a microscope. The cells were also counted. For the evaluation of changes occurring during cell proliferation, different assays were used. The cells were then subjected to RT-PCR for gene expression. With the help of Two-way ANOVA, statistical results were made. The research concluded that there was a reduced number of cells, lesser cell proliferation, and TGF was also inhibited [35].

The effects of SLIT2 on cell migration and movement in different cancer types were explored. The samples affected by tumors were obtained from various parts, i.e., breast cancer cells, lung cancer cells, ovarian cancer cells, colorectal cancer cells, and other accompanying cancerous cells. Cell migration and motility were evaluated using the MDA-MB231 breast cancer cell line. Various chemotaxis assays were used to assess the cell migration and motility. The tissues were stained with various markers such as CXCR4, SLIT2, and ROBO1 to assess their expression. Among the three gene markers, SLIT2 has shown an increase in motility and migration of cancerous cells especially in ovarian cells [36].

The genetics and developmental biology of zebrafish were studied. The role of SLIT3, ROBO1, and SLIT1a has been studied in cell migration and signaling. The live zebrafish were maintained and bred under controlled conditions for experiments at the genetic level. A mutation in SLIT3, ROBO1, and SLIT1a was induced with the help of CRISPR-Cas9 and TALEN. The effects of morpholinos and inhibitors were used to examine the abnormalities by injecting them into the embryo of zebrafish. Various microscopy techniques were applied to analyze the development of zebrafish and to track the cell migration. The level and pattern of gene expression have been studied using PCR. HaCaT cells were cultured with ROBO1 expression to measure Sphingosine-1-Phosphate (S1P) production. It was deduced that zebrafish carry

antigens for SLIT proteins. Also, the synthesis and release of SIP were related to the production of S1P in eukaryotes [37].

The genetics of the *Caenorhabditis elegans*, focusing on the mutations in two genes were studied. The genes selected for this study were genes *mum-1/rib-1* and *mum-3/rib-2*. The main focus of the study was to investigate the effects of these genes on the nervous and physiological mechanisms. The mutated genes were outcrossed several times to obtain the genetic stability. With the help of various marker genes used for visualization of neuroanatomical structures, defects were observed. For the location of the *mum1/rib-1* gene, the mapping was conducted. The role and expression of genes *rib-1* and *rib-2* were observed and examining them till the protein level. The genes *rib-1* and *rib-2* have been associated with the expression of heparin sulfate. Any mutation in these genes causes suppression in the production of heparin sulfate thus affecting neuronal cell migration. It has been demonstrated that the axonal guidance was misled [38].

The impact of SLIT proteins in axonal guidance was monitored. Many genetic fly models of *Drosophila* were used to study the deficiency in genes like *SLIT*, *ROBO*, *KUZ*, and *COMM*. The *PTC* mutant was allowed to express the UAS-SLIT gene under controlled conditions for the determination of its restoring ability. The expression of the SLIT and ROBO genes was continuously monitored. It was concluded that the axonal tracts are directly linked to the loss of SLIT expression. Both SLIT and ROBO were observed to be involved in maintaining the position of the axonal tract [39].

Various cell types and techniques are used to study the behavior of cells affected by electrophoresis and gene loss. Different cell types used in this research included fNPCs (fetal neural progenitor cells), astrocytes (derived from fNPCs), and BTICs (brain tumor-initiating cells). The cell tracking was performed using the galvanotaxis technique while the protein localization and distribution were performed using the confocal immunofluorescence technique. To study the impact of glycosaminoglycan in galvanotaxis, the cells were treated with different enzymes. The genes were knocked down using nanoparticles, and the expression was then assessed using qRT-PCR. The role of ROBO1 in cell behavior was monitored by knocking down ROBO1 in fNPCs. This research is directed toward a novel mechanism that facilitates the SLIT/ROBO signaling pathway [40].

A detailed research on husbandry, genetic mutagenesis, and experimental study on the cell migration and signaling of zebrafish was carried out. For the zebrafish husbandry, various zebrafish lines were maintained under controlled conditions. With the help of genetic mapping, the mutation in the SLIT3 gene was identified. Mutagenesis among three genes, i.e. SLIT3, ROBO1, and SLIT1a, was successfully introduced. Some specifically unwanted genes were silenced using morpholino. The cell movement and migration were observed using MATLAB scripts and 3D imaging software. Various proteins have been analyzed using the immunofluorescence technique. The cloning of human ROBO1 cDNA was performed to create various copies for the further assessment of the production of sphingosine kinase (*SphK*) and *S1P*. Thus, the results highlight the importance of the SLIT/ROBO signaling pathway with *S1P* production in regulating the morphogenesis and alignment of cells [41].

The effect of the mutation in the *mmyslm* gene of *Drosophila* was investigated. The mutation was identified at the genetic level and was allowed to recover and cause deposition at the Bloomington *Drosophila* Stock Center. Various transgenic lines and GAL-4 were used to determine the improvement in the axon guidance by restoring the *mmyslm* gene. The distribution of SLIT protein and ROBO1 receptors proved the experiment to be successful. The mutation in *mmyslm* gene was also monitored using various assays. The *mmyslm* mutants have shown clear defects in SLIT secretion and ROBO1 expression [42].

CHAPTER 3

METHODOLOGY

3.1. Cell Culture Maintenance and Conditioning

The lung cancer cells (HepG2) were kept in DMEM (Gibco-12800-017). As a supplement, 10% FBS was added, along with 1% pen/strep. The incubation of the culture was performed under standard culture techniques, including a temperature of 37° C and 5% carbon dioxide to maintain humidified conditions. The culture of lung cancer cells was supplemented with the addition of 1ng/ml *TGF- β* (Invitrogen™ PHG9204) on the next day of culturing. After the cells had reached 70% of confluency, the conditions of media were shifted to 1ng/ml Human recombinant SLIT2 protein (Abcam-ab82131) and heparin (Fisher Scientific BP2524100). After this, SLIT2 and Heparin were added, and the cells were retrieved after 24 hours for analysis.

3.2. Morphology Analysis

The analysis of cells was done after the 24-hour exposure of the SLIT2 protein and heparin. The analysis was carried out by using a Nikon Eclipse TS100 microscope with a Nikon ELWD 0.3/OD75 camera system to observe any variation in morphology. With the help of a hemocytometer, the cell numbers were counted by doing Tryptane blue staining.

3.3. Cell Proliferation Assays

The cells were then cultured in 96 well plates with a density of 1x10³ cells/well. *TGF- β* was induced into the cell culture, and the culture was then allowed to be exposed to SLIT2 and heparin. Various assays were performed to investigate any change in the cell proliferation, cell viability, and metabolic activity of the cell.

3.4. MTT Assay

For the determination of cell metabolic activity, an MTT assay was done. For this assay, 0.1mg/ml MTT salt (Thermo fisher Scientific- M6494) in PBS was added to cells followed by incubation of 3 hours. Absorbance was analyzed by BioTek ELx808 absorbance reader at 570 nm after the addition of de-staining solution (DMSO).

3.5. Neutral Red Assay

For the determination of cell viability, the Neutral assay was performed. The cells were then incubated with a final concentration of 40µg/ml neutral red (Sigma Aldrich-N4638) and diluted in DMEM. The culture was then incubated for an hour. The absorbance was studied at 570 nm after de-staining (with a solution of 50%: absolute ethanol, 49%: deionized water, and 1%: Glacial acetic acid).

3.6. Gene Expression Analysis By RT-PCR

One prominent feature of metastasis is cell migration. For this validation, the generation of cell adhesion proteins, including mesenchymal and epithelial indicators, was evaluated. The materials were maintained at -80°C in 1 mL of TRIzol reagent prior to RNA isolation. The manufacturer's instructions for extracting RNA from the aqueous phase were followed in order to isolate the RNA with the use of the Trizol solution technique. RNA that had been extracted was quantified. After the RNA was extracted, cDNA was synthesized using the pico-drop and DNAase treatment. The synthesis of cDNA was done using 1 µg purified RNA using a cDNA synthesis kit (Thermo-K1622). The cDNA was then set for real-time PCR (RT-PCR). The forward and reverse primers used in the RT-PCR were 5µM. The data from the test genes' Δ CT values and melting curve analysis were contrasted with those of the housekeeping genes (GUS B). The outcomes were displayed graphically.

CHAPTER 4

RESULTS

4.1. Reduction In Cell Number By Heparin-Amplified SLIT2

It has been noticed that *TGF- β* has increased the number of cancer cells in HepG2 after being induced for 24 hours. However, in the presence of SLIT2, a severe decrease has been noted in the cell number. The growth of cancerous cells was down-regulated in the presence of a combination of SLIT2 with heparin.

4.2. Heparin Facilitates Reduction In Cell Proliferation By SLIT2

All the cells treated by *TGF- β* treatment have shown high metabolic activity of cell and cell proliferation. Yet, after the addition of SLIT2 in the cells treated by *TGF- β* , a reduction in the metabolic activity of cells was observed. As the heparin was added with the SLIT2 in the *TGF- β* treated cells, a noticeable decrease in the cell proliferation was witnessed. However, no effect has been monitored on the cell viability after the addition of SLIT2 and heparin collectively. These findings have been clearly explained with the help of the MTT assay and the Neutral Red assay (Figures 4.1 & 4.2).

4.2.1. MTT Assay

In the MTT assay, the addition of SLIT2 to the control showed a reduction in the viability of the cells. As the *TGF- β* was added, the cells showed greater proliferation and viability of cells. As the *TGF- β* was introduced with SLIT2, a reduction in cell production relative to the control was observed. The effect of *TGF- β* , SLIT2, and Heparin collectively showed a little decrease in the cell proliferation (Figure 4.1).

MTT Assay

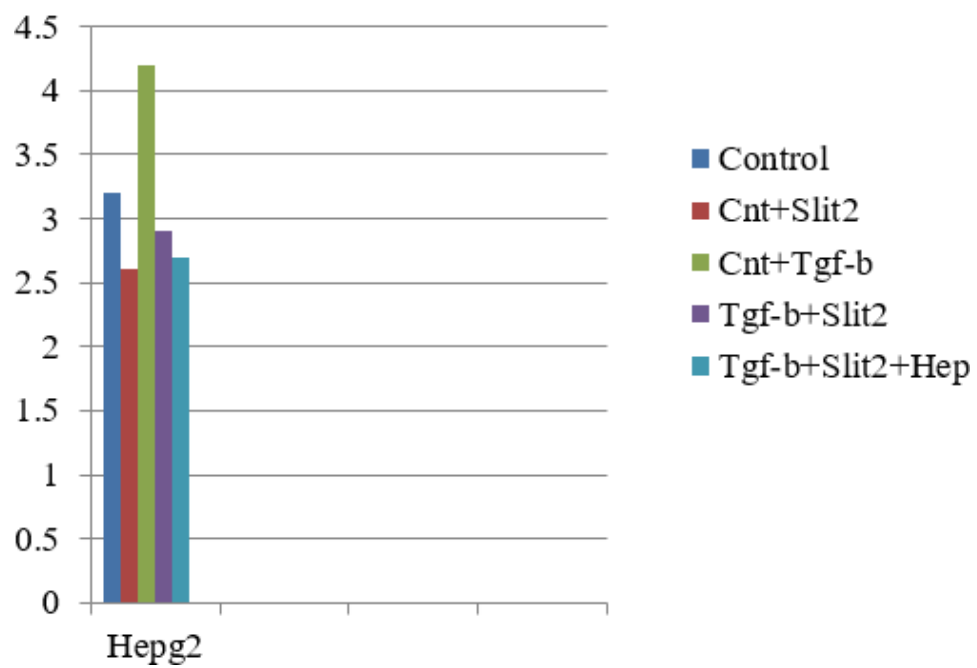


Figure 4.1. MTT assay in HepG2 cells

4.2. Neutral Red Assay

In the Neutral Red assay, the addition of SLIT2 to the control showed a reduction in the viability of the cells. As the *TGF- β* was added, the cells showed better proliferation of cells. As the *TGF- β* was introduced with SLIT2, an increase in cell production relative to the control was observed. The effect of *TGF- β* , SLIT2, and Heparin altogether showed no effect on the cell viability (Figure 4.2.2).

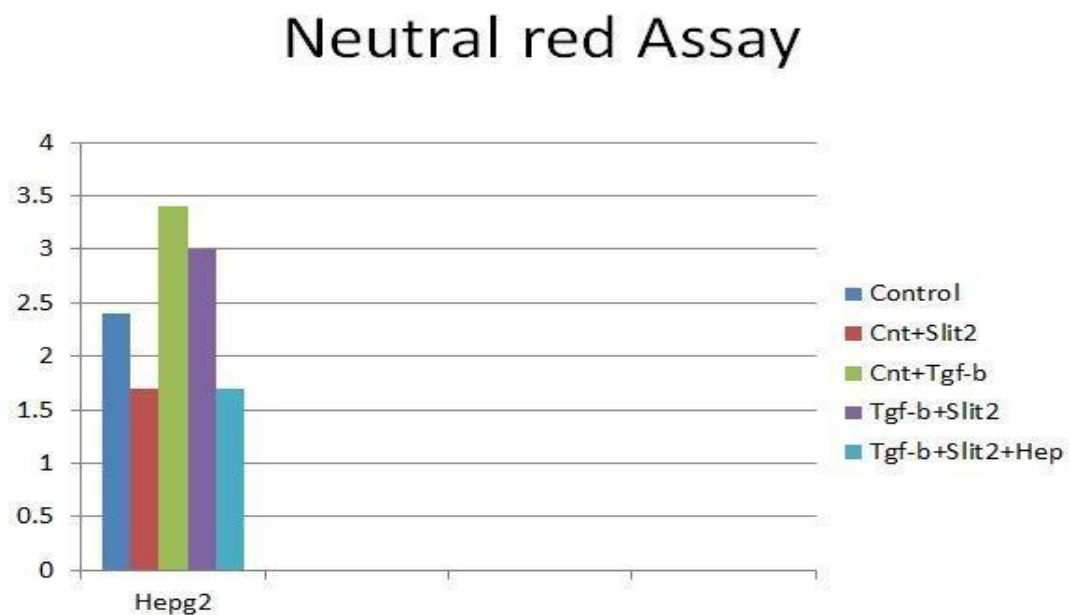


Figure 4.2. Neutral red assay in HepG2

4.3. SLIT2 Reversed *TGF- β* Initiated Up-Regulation In Mesenchymal Gene Expression

An up-regulated expression of N-CAD, Vimentin, and Integrin has been observed after the treatment of cells with *TGF- β* . The addition of SLIT2 and heparin resulted in the up-regulation of E-CAD expression, while a reduction in the regulation of cancer cells has been observed in the mesenchymal gene markers. The expression of integrin beta 1 has been increased after the addition of *TGF- β* , but it was not affected after the addition of SLIT2 (Figure 3).

4.3.1. E-Cadherin

The expression of E-Cadherin was analysed by RT-PCR, the results indicated no change in E-Cadherin expression by addition of SLIT2 only. With addition of *TGF-β* the expression of E-Cadherin was increased slightly as compared to the control group, with adding *TGF-β* along with SLIT2, the expression of E-Cadherin increased many folds which then again downregulated when heparin was added in presence of SLIT2 and *TGF-β*. As E Cadherin is epithelial marker gene, so the increase in its expression with heparin along with SLIT 2 indicates reduction in cancer progression even in presence of *TGF-β*.

4.3.2. N-Cadherin

The expression of N-Cadherin was analyzed and the results indicated expressions increased by adding SLIT-2. With the addition of *TGF-β* it is elevated many folds, after the addition of SLIT-2 in *TGF-β* it become downregulated. When heparin was added in *TGF-β* and SLIT-2 the expression become slightly down regulated. As N-Cadherin is mesenchymal marker and its elevation is associated with cancer metastasis, so the reduction in *TGF-β* induced elevation in its expression with addition of SLIT2 and heparin indicate the anticancer potential of the SLIT2 in combination of heparin.

4.3.3. Vimentin

The expression of Vimentin was analyzed it showed no change in expression of Vimentin as compared to control group when SLIT-2 was added alone, but with the addition of *TGF-β* the expression of vimentin showed elevation. When we added *TGF-β* with SLIT-2 the expressions became downregulated. When heparin was added with *TGF-β* and SLIT-2 expressions become slightly decreased again. As vimentin is mesenchymal marker and its elevation is associated with cancer metastasis, so the reduction in *TGF-β* induced elevation in its expression with addition of SLIT2 and heparin indicate the anticancer potential of the SLIT2 in combination of heparin.

4.3.4. Integrin

The expression of Integrin was analyzed in our protocol the results indicated the slightly increased expression by adding SLIT-2. When we added *TGF-β* expression goes many folds upregulated. With the addition of *TGF-β* and SLIT-2 it becomes down

regulated. When heparin was added along with *TGF-β* and SLIT-2 it becomes slightly up again.

RT-PCR

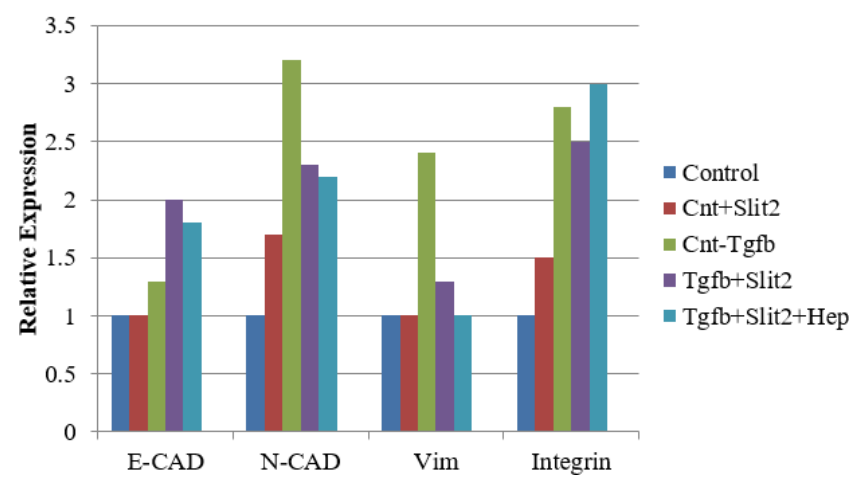


Figure 4.3. Expression analysis of cell adhesion genes in HepG2

CHAPTER 5

DISCUSSION

The role of the Roundabout receptor as a tumor endothelial marker has been stated and observed in the vascular network of diverse tumors. A total of four roundabout proteins (ROBO 1-4) have been discovered and documented in mammals. They are integral receptors for SLITs that are associated with the axon guidance molecules [35]. The SLITs have now been depicted to perform two very vital functions. They act as chemorepellents either by inhibiting or promoting the chemokine-induced migration of the mature white blood cells [43]. The SLIT-ROBO mechanism has been used in combination to deal with cancer management. The double role of the SLIT-ROBO signaling pathway is evident from its impact on cancer management. It not only possesses the tumorigenic effect, but also displays its role in the anti-tumor properties depending on the particular type of cancer [35].

It has been noticed that the binding ability of SLIT molecules to ROBO and Heparin sulphate is due to the presence of leucine rich domain. This region supports the binding of SLIT to both the molecules efficiently, thus enabling them to be bind together as receptor-ligand interactions. It has been observed that the function of SLIT-ROBO interactions has been greatly affected by the presence or absence of heparin sulfate. Heparin sulfate is a member of the family of polysaccharides, glycosaminoglycans, that play a crucial role in regulating various biological phenomena. They interact with numerous protein ligands and cause variations in cell growth, cell differentiation, metabolism of lipids, coagulation and clotting of blood, the interactions between two cells or a cell and matrix, and cancer metastasis [35].

Heparin sulfate is also significant for the signaling of various cytokines. The development of heparin sulfate with the specific ligand proteins can be proved beneficial in both the fields of diagnosis and therapeutics [35]. Chemokines have been studied extensively to depict the inflammatory response during the process of migration of cells [44].

E-cadherin is a very significant protein that has played an important role in the adhesion of one cell to the other. It has also played a vital role in maintaining the polarity of epithelial cells [45]. The present study shows the relative expression of E-

cadherin, which shows an up-regulation in HepG2 cancer cells. A similar study shows the importance of E-cadherin in the spread of cancer cells. The previous study reported that the absence or loss of E-cadherin may result in tumor formation [44]. On the contrary, a study has shown a reduction in the expression of E-cadherin during the spread of cancer [46]. The experiment was assessed by giving some protocols to E-Cadherins We analyzed in RT-PCR which showed no expressions with SLIT-2 but with the addition of *TGF- β* expressions increased slightly. When SLIT-2 along with *TGF- β* is added it goes many folds. We know that Cadherins are marker genes so expression with heparin decreased when SLIT-2 along with *TGF- β* is added.

N-cadherin has been considered an important mesenchymal protein that is associated with the transition between epithelial to mesenchymal during different types of cancers. The present study stated that the expression of N-cadherin has increased in HepG2 cancerous cells. Similar results have been mentioned by a previous study that stated the expression of N-cadherin in prostate cancer [47]. In N-Cadherins we did experiments and we analyzed that with SLIT-2 expression increased. With the addition of *TGF- β* along with SLIT-2 it become downregulated. When heparin was added with *TGF- β* and SLIT-2 it indicates anticancer potentials and gives positive results.

Vimentin is crucial and has a key role in various normal processes of the cells. It is involved in spreading and propagating the malignant cells to the distant parts of the body. The present study mentions the role of vimentin in the progression of HepG2 cancerous cells. In contrast, a previous study has shown that the absence of vimentin is related to the progression of cancer [48]. Recent studies have shown a new insight where vimentin has been used to target the cancerous cells for the treatment. A previous study has shown that vimentin can be proven as an emerging marker to detect malignancies as well as for their treatment [49]. When we are dealing with Vimentin in our experiment Vimentin showed no change in expressions with SLIT-2. When *TGF- β* along with SLIT-2 is added expressions become decreased. When heparin was added with *TGF- β* and SLIT-2 it decreased again. As Vimentin is mesenchymal marker and its elevation is associated with cancer metastasis, so the reduction in *TGF- β* induced elevation in its expression with addition of SLIT2 and heparin indicate the anticancer potential of the SLIT2 in combination of heparin.

Integrins are the proteins that play an important part in the initiation, migration, and spread of malignant cells. This has provided an insight into targeting the cancer cells and suggesting the treatment [49]. The present study indicates that the integrins are directly related to the up-regulated expression of malignant cells of HepG2. It is the hour of need to control its expression with the help of various therapies. A previous study has demonstrated similar results that integrins are involved in the transmission and spread of malignant cells. The study also promotes the innovative treatment of integrins in causing malignancy [50]. When we are doing experiment of Integrin with *TGF- β* , SLIT-2, and heparin, we had different expressions. The expression of Integrin was analyzed in our protocol, the results indicated a slightly increased expression by adding SLIT-2. When we added *TGF- β* , expression goes many folds upregulated. With the addition of *TGF- β* and SLIT-2 it becomes down regulated. When heparin was added along with *TGF- β* and SLIT-2 it becomes slightly up again.

The present study aimed to determine the role of various proteins in the proliferation and progression of malignancy in HepG2 cells. It has been evident that the above-mentioned proteins are vital for understanding the mechanism of cancer progression. Although their expressions in the proliferation of cancer cells vary but they can provide a developing insight into the treatment.

CONCLUSION

Cancer has been considered one of the major and leading causes of mortality across the world. The malignant cells not only show their progression to the development of cancer but also spread it to other viable and vital cells of the body. In the context of controlling the progression of cancer, several proteins have been observed that play a role in preventing its spread to other parts of the body. Many proteins, such as E-cadherin, N-cadherin, Vimentin, and integrin, are known to play an integral part in the progression and proliferation of cancerous cells. A thorough and complete understanding of SLIT/ROBO mechanism and its role against cancer has provided a new insight into treating cancers. The emergence and development of SLIT/ROBO signaling pathway with Heparin sulfate has proved to be a blessing. In the future, it might assist mankind in treating them against such deadly diseases.

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