CHAPTER 1

INTRODUCTION
1.1 Cancer

Despite recent advancement in diagnostic tools and medicines that are able to detect and cure diseases, Cancer continues to be second most leading cause of mortality, after heart disease. However recently, cancer has ousted the heart disease and has become the major cause of death in Hispanic Americans [1]. Cancer can be defined as uncontrolled division of cells due to defects in normal regulatory signalling pathways. The development of cancer involves complex, dynamic changes in the genome that initially lead to the generation of pre-neoplastic lesions and eventually gives rise to clonal variants that proliferate into tumors, usually over many decades. Completion of human genome project has further assisted in better understanding of genes that are involved in cancer initiation and progression [2]. There are three major classes of genes which are involved in maintenance of cellular physiology in the normal cells namely (1) tumor suppressors are the gene which are mainly involved in cell cycle check points but upon mutation these gene lose their ability to control cell cycle progression e.g. Retinoblastoma (RB) TP53, APC etc. Each cell in the body except for germ cells has two copies of a particular gene. The mutation in the tumor suppressor genes are recessive in nature i.e. mutation in one copy of the gene is not sufficient (haploinsufficiency) to overcome its ability to control its function; hence it requires mutation in both the copy of the gene. Mutation in both the copy of the tumor suppressor gene is analogues to a non-functional brake of an automobile. Driver of the vehicle with dysfunctional brake fails to stop it even after applying brake; similarly mutation in tumor suppressor gene cannot withhold the cells from continuous division. (2) Proto-oncogenes are the normal genes which are involved in cell cycle progression, inhibition of cell differentiation and apoptosis but upon mutation even in a single copy of
gene makes it constitutively active (dominant) as an oncogene e.g. rat sarcoma (RAS), MYC, B-RAF etc. Mutation in proto-oncogene is analogous to automobile with stuck accelerator; even if the driver tries to de-accelerate he fails. Similarly, mutation in proto-oncogenes cannot prevent the cells from dividing. (3) Stability or caretaker genes are responsible for repairing the DNA damage which occurs during exposure to mutagens or while repairing the error during normal DNA replication. Thus stability genes control the rate of mutation. Mutation in these genes leads to the impairment in DNA repair and therefore, increases the frequency of mutation e.g. BRCA1/2, ATM, MLH1 etc. [3].

Any qualitative or quantitative alterations in these three classes of genes lead to breakdown of their normal functioning. These genetic alterations are known to occur by point mutation, deletion, gene amplification, chromosomal translocation or other mechanisms. Besides, qualitative or quantitative changes in the genes, structural alterations in gene can also occur by epigenetic mechanisms. Methylation of cytosine bases in DNA or modification in the expression of genes by changes in histone profile are the common epigenetic changes that also play vital role in tumor progression. For instance, there is change in global DNA methylation pattern during cancer progression leading to genomic instability [4]. Deregulated expression of microRNAs (miRNAs) is another emerging epigenetic mechanism involved in carcinogenesis. Several of miRNAs are found to be upregulated or downregulated in numerous cancers [5].

It is predicted that during the process of tumorigenesis, cells must acquire a minimum of six mutations to become abnormal or malignant [6]. These mutations are included in those genes that make the cells malignant. The Six basic properties required for malignant transformation are,
1. **Self-sufficiency in growth signals**: Normal cells require growth factors for their growth and proliferation. However, tumor cells are able to synthesize their own growth factors or they amplify the growth factor signalling by overexpressing growth factor receptors such as HER-2/neu in stomach and breast cancer; epidermal growth factor receptor, erbB in stomach, brain and breast tumors [7].

2. **Insensitive to growth-inhibitory signals**: In order to maintain the tissue homeostasis many anti proliferative or anti-growth signals operate within the normal tissue. However, tumor cells evade these anti proliferative signals by downregulating the receptors through which these signals transmit. For instance, TGFβ signalling is an important anti proliferative signal, to avoid such signalling tumor cells downregulate TGFβ receptors [8] or they expresses mutant or dysfunctional receptors [9].

3. **Extensive replication potential**: According to Hayflick, normal cells have definite replicative potential, after that cells show senescence which is due to loss of 50-100 bp telomeric DNA from ends of each chromosome after every cycle of cell division. Tumor cells overcome the problem of replicative senescence by overexpressing the enzyme telomerase which prevents the loss of telomeric DNA by maintaining the ends of chromosomes [10].

4. **Ability to avoid apoptosis or programmed cell death**: Expansion of tumor cells depend not only on their rate of proliferation but also on the rate of cell attrition. Apoptosis is leading cause of cell attrition and it is regulated by Bcl2 family of proteins which has both pro and anti-apoptotic functions. Tumor cells evade the apoptosis by down regulating the expression of death receptor proteins, for instance
reduced expression of CD95 has been reported in neuroblastoma and lymphoma, or by overexpression of inhibitor of apoptotic proteins, for example survivin, its overexpression has been reported in several cancers [11].

5. **Sustained angiogenesis:** In normal tissues the process of angiogenesis is regulated by the balance between angiogenesis inducer and their countervailing inhibitors. However, in tumor cells the balance is shifted towards angiogenic switches. At the primary site tumors do not grow beyond the size of 2 mm in diameter due to lack of nutrients and oxygen. Hypoxic condition within the tumor, induces the expression of hypoxia inducible factor (HIF) which in turn, initiates the process of angiogenesis by regulating the expression of molecules like Vascular Endothelial Growth Factor (VEGF) which is involved in endothelial cell proliferation [12].

6. **Competent to invade and produce distant tumors:** The tumors that remain confined to the boundaries of an organ are called benign tumors and can generally be taken care of by clinical intervention. However, tumors often attain the property to invade the surrounding normal tissue, breach the organ basement membrane and disseminate to distant organ sites via lymphatics or blood circulation and form tumors at these sites. This property of dissemination is referred to as metastasis. Tumors that attain this property of invasion and metastasis are called malignant and it is the major cause of mortality in cancer patients. More than 90% of the patients die because of the invasive and metastatic cancers rather than the primary tumors. Although important, this is the least understood aspect of tumor biology because of the complexity and multiple step nature of this process [13].
All these six critical features are hailed as the hallmarks of cancer development [6]. Over the years the ability of tumor cells to evade host immune defence mechanisms has also been recognised and researched [14]. **Evasion of immune response** is now also considered to be an additional hallmark of cancer. Cancer cells are highly proliferating cells and they have increase demand of energy for the generation of new cells. Normal cells depend on glycolysis, Krebs cycle and mitochondrial oxidative phosphorylation pathway for glucose metabolism. However, cancer cells can reprogram the glucose metabolism mainly to glycolysis even in presence of oxygen. Cancer cells have devised several strategies to fulfil the energy demand. For instance, they show increased expression of glucose transporter (Glut1 and Glut3) which facilitate the import of glucose into cytoplasm. The glycolytic pathway intermediates are utilized by the tumor cells for the generation of nucleosides and amino acids which in turn help in biosynthesis of macromolecules and organelles required for assembling new cancer cells [15]. **Reprogramming of energy metabolism is now considered to be an additional hallmark to the existing list** [16]. Besides these genetic alterations, various factors in the host microenvironment are also believed to influence malignant cell growth. Although, invasion and metastasis are the major cause of mortality in cancer patients, the molecular mechanisms underlying these processes are still poorly understood [17].

### 1.2 Metastasis

Metastasis is defined as the dissemination of tumor cells from the primary site of origin to non-contiguous organ sites and formation of secondary metastatic foci [18]. It is believed to be a complex multistep process. Clinical observations provided initial clues
about the possible mechanisms involved in metastasis. Pathological examinations showed that tumors that are contained within the boundaries of basement membrane (carcinoma in situ) have a better prognosis as compared to those that breach the basement membrane (invasive). The latter generally metastasize to lymph nodes and even distant organs. Similarly, the tumors that metastasize were found to be well vascularised. Clinical observations also showed that some tumors metastasize only regionally to lymph nodes or to organs in the anatomic vicinity. It also revealed that highly invasive and aggressive tumors are highly metastatic, although, some like gliomas aggressively invade the surrounding normal tissues but do not metastasize to other organ sites. These studies very clearly showed that invasion and metastasis are the most lethal aspects of malignancy and provided clues about the possible steps required for a tumor to be metastatic [19].

It was realized that for continuous growth the tumor cells require both oxygen and nutrients and thus they do not grow beyond a certain size unless well vascularised. For tumors to metastasize, the first requirement would be to detach from the primary and invade the surrounding normal tissue towards the source of oxygen and nutrients. The tumors thus need to be motile and be able to degrade surrounding matrix and basement membrane (BM). The newly formed blood vessels are poorly formed, the endothelial lining is sometimes discontinuous and they often lack BM. This offers an easy escape route for their entry into circulation, referred to as intravasation. Invasive tumor cells have the ability to actively intravasate by degrading the vascular basement membrane and displacing endothelium. Not all tumor cells are equipped to survive the high shear forces in circulation and the host immune defences. However, many tumor cells have evolved mechanisms to survive in circulation. The next step is the organ colonization. Several tumors metastasize
only to the organs in the anatomic vicinity like the regional lymph nodes or the organs receiving the afferent blood vessel from the primary. However, many tumors bypass several organs in the blood flow path and very specifically colonize distinct organ sites [20]. Although clinical studies provided insight about the different steps, it did not provide clues to the possible mechanism or the molecules that are involved at each step.

To overcome this, several in vivo and in vitro experimental models and cell lines with specific metastatic characteristic were developed for each step of metastasis and each step was studied in isolation [21, 22]. This not only helped in confirming the involvement of steps in metastasis but also resulted in identification of large number of host and tumor derived molecules that participate in these step of metastasis [23].

1.3 Metastatic cascade

The sequence of events which are involved in dissemination of tumor cells from primary site to a new distant organ site is often referred to as metastatic cascade.

1.3.1 Detachment of tumor cells from primary tumor

Normally cells are held in place by their interactions with the neighbouring cells and with the underlying matrix or basement membrane. Cell-cell interactions are mediated by tight junctions, adherens junctions and the desmosomes. Claudins, occludins and junctional adhesion molecules (JAMs) are the transmembrane proteins involved in the formation of **tight junctions** that are connected to the actin cytoskeleton via adaptor proteins like zona occludins (ZO-1, 2 or 3) proteins and several others. The classical and non-classical cadherins are involved in the formation of **adherens junctions** and
desmosomes, respectively. E-cadherin mediates cell-cell adhesion by mediating homophilic interactions between E-cadherins on neighbouring cells. The adhesive state is maintained by the interaction of cadherins to the actin based cytoskeleton via the adaptor proteins referred to as catenins. Defects in any one of them results in non-adhesive state. Tumor cells are known to often downregulate cadherins and modulate the functions of catenins like β-catenins by phosphorylating it. The non-classical cadherins like desmogleins and desmocollins form desmosomal complexes by homophilic interactions between them on the neighbouring cells. They are connected to intermediate filaments via adaptor proteins like plectins [24]. Alteration in any of these components impairs cell-cell adhesion.

Cell to matrix interactions are primarily via two types of structures, focal adhesions and hemidesmosomes. The dense BM that surrounds the organs and blood vessels prevents passive movement of cells across it and the extracellular matrix (ECM) has very similar composition. They consist of collagenous proteins of several different types like Collagen-I (ECM) and Collagen-IV (BM), non-collagenous glycoproteins like fibronectin (ECM) and laminin (BM), glycosaminoglycans (GAGs) and proteoglycans [25, 26]. The ECM and BM may differ in the types of these components and the proportions of each to provide its unique property and specificities. Integrins and cell surface proteoglycans are the major receptors that participate in cellular adhesion and movement.

Both focal adhesion and hemidesmosomal junctions are formed by integrin receptors. The laminin receptor, integrin α6β4 is the key component of hemidesmosomes. The long cytoplasmic tail of β4 integrin appears to aid the interaction of this integrin receptor with the underlying intermediate filaments formed by keratins via the adaptor proteins. These interactions with underlying BM provide stability to the epithelial cells.
Altered expression of these receptors, adaptor proteins, or changes in cytoskeletal intermediate filament proteins, results into changes in adhesive state. There are 18α and 8 β integrin subunits which combine variously to form 24 different heterodimeric integrin receptors that together are able to recognise all the major collagen and non-collagenous glycoprotein components of ECM and BM. Integrins are connected to actin based cytoskeleton (microfilaments) via adaptor proteins like talin, vinculin and so on. Tumor cells modulate the expression of these receptors, adaptor proteins or the organization of the cytoskeleton to achieve altered adhesive and motile state [27, 28].

Recent studies have highlighted that detachment of cells during metastasis is further augmented by a process similar to that seen in embryonic cells termed as Epithelial to Mesenchymal Transition (EMT). During EMT, cancer cells express various transcription factors such as snail, slug, twist, ZEB1 and ZEB2 which directly or indirectly regulate E-cadherin expression. E-cadherin is the major molecule of adherens junction, with the loss of E-cadherin there is concomitant loss of apical and basal polarity of cells. The adherent epithelial morphology of the cells changes to a more motile mesenchymal phenotype with the gain of mesenchymal marker like N-cadherin and vimentin [29]. The transition of epithelial cells into mesenchymal state is crucial initial step in the cascade of metastatic events.

1.3.2 Invasion into the surrounding normal tissue

The cells that have broken free from the primary tumor need to be motile and are able to create space for movement. The surrounding matrix is used as traction for the movement of cells. Integrin receptors together cover all the major collagen and non-
collagenous glycoproteins and thus serve as a major class of molecules involved in movement of cells. Modulation of the integrin receptor expression, post translational modifications (PTMs) on them or their association with membrane microdomains all regulate cellular movement. Tumor cells utilize all these mechanisms to achieve a motile state. The cell surface proteoglycans may also have an important role in mediating movement on GAGs and proteoglycan components of the matrix. CD44 the hyaluronate receptor is a good example of receptors that mediate interactions with the GAGs and play a key role in cancer metastasis. Multiple splice variants of CD44 exist, and some of these variants have been shown to be specific for the metastatic phenotype.

Tumor cells must create space for movement. Tumor cells secrete a whole range of matrix degrading enzymes; however, as the same matrix is used as traction for movement, the degradation is highly regulated. Most of the enzymes are secreted in zymogenic form and their activation occurs in a cascade in a space and time dependent manner. Urokinase plasminogen activator (uPA) that converts plasminogen into plasmin is one of the key enzymes that control such regulation. Plasmin converts most of the proenzymes like Pro-MMPs into active MMPs whose substrates include the protein core of proteoglycans, collagenous protein and non-collagenous glycoprotein components of ECM and BM. The enzymes uPA gets associated with the cell surface via its receptor uPAR which often associates with the receptors involved in motility. The uPA/uPAR system and the membrane tethered forms of MMPs (MT1-MMP) that get activated during transport to the cell surface and associated with motility receptors together with uPAR are the major regulators that couple matrix degradation and cellular movement. The other components that participate in tumor cell invasion include the lysosomal enzymes cathepsins, the
ADAM (A Disintegrin and Matrix metalloproteinase) family of proteins, glycosaminoglycanases like heparanases and several others [30]. Each of these molecules helps tumor cells in their invasion and dissemination.

1.3.3 Intravasation

The process of entry of tumor cells into the lumen of lymphatic or blood vessels is termed as intravasation. Intact vascular BM lining the blood vessels acts as obstacle for the entry of tumor cells in the circulation. For intravasation metastatic cells either produces degradative enzymes that disrupt the vascular BM or induces the process of angiogenesis. Tumors do not grow beyond a certain size unless well vascularized. Metastatic tumors are generally highly angiogenic. The newly formed blood vessels around tumors however, are poorly formed. They lack BM and even the endothelial lining is often discontinuous. This offers an easy escape route for tumor cells to get into circulation [31]. The process of intravasation can also be facilitated by the molecular alteration that supports the ability of the tumor cell to cross the endothelial barrier and pericyte. Recently, it has been shown that notch signalling can contribute to tumor cell intravasation. Tumor cells expressing notch receptors binds to its ligand jagged1 in the endothelial cells of tumor associated blood vessels and thus induces intravasation. The transcriptional regulator, amino terminal enhancer of split (Aes) has been shown to inhibit notch signalling in primary colon carcinoma and thus notch mediated tumor cell intravasation but in invasive colon carcinoma tumor cell downregulate Aes and thus promote intravasation [32]. Intravasation of breast carcinoma has been shown to be enhanced by the activation of the TGF-β receptors, probably by promoting the expression of Angiopoietin-like 4 (ANGPTL4). Its
expression in turn disrupts the vascular endothelial cell junction and thus increases the permeability of cancer cells to the micro vessels wall and thus augments local invasion [33]. Intravasation of the breast carcinoma cells has also shown to be enhanced by the perivascular tumor associated macrophages [34].

1.3.4 Survival in circulation

Once the tumor cells have successfully intravasated into the lumen of blood vessels, they can easily disseminate through venous and arterial circulation. During the circulation tumor cells must survive variety of stresses in order to reach distant sites. For e.g. anoikis, a form of apoptosis induced in absence of adhesion to substratum, they also seem to be deprived of integrin ECM interaction dependent survival signals, to overcome that tumor cells show increased expression of Tyrosine receptor Kinase (Trk receptor) expression that prevents anoikis and promotes their survival [35]. Tumor cells also must overcome the damage sustained by shear forces of blood flow, predation by natural killer cells, a component of innate immune system, and also the toxicity induced by high level of oxygen. In order to overcome these, tumor cells form large emboli by their interaction with platelets. Cell surface carbohydrates in the form of Lewis antigens and integrin receptors for fibrinogen on tumor cells and P-selectin and fibrinogen receptor integrin αIIβ3 on platelets have been implicated in tumor cell embolization. This not only aids in evading immune surveillance but also acts as shock absorber [36]. It helps tumor cells to lodge mechanically into fine vasculature of the secondary organ and platelet derived growth factors aid its sustained growth.

1.3.5 Organ colonization
Once in circulation tumor cells are able to reach almost all organ sites. However, some tumors metastasize only regionally either to the lymph nodes or to the organs in the anatomic vicinity, while several others metastasize to very specific distant organ sites. Most of the regional metastasis can be explained based on the lymphatic or blood flow patterns. Tumors often metastasize to the draining lymph nodes or to the organs receiving afferent blood vessels from the primary. Liver receives maximum number of colon cancer cells via portal vein from colon that drains into liver, and thus chances of colon cancers metastasizing to liver is very high. Similarly, prostate cancers are also believed to colonize vertebral bones through vertebral venous plexus of spine [37]. However this is not the exclusive mechanism of prostate cancer metastasis to bone. Tumors that are either released into circulation as multicellular emboli or are able to form heterocellular emboli with platelets of leucocytes while in circulation also get trapped in the first vasculature that they encounter and thus metastasize in the regional vicinity. This is often referred to as Anatomical or Mechanical mode of organ colonization proposed by Dr. J. Ewing and is also referred to as Ewing’s hypothesis [38].

However, several tumors metastasize to very specific distant organ sites bypassing several organs during the course of their journey from the primary. Breast cancer cells metastasize only to lung, liver and bones, and some to the brain. Some of the melanoma cells metastasize to brain. Prostate cancer metastasizes mainly to bone and less frequently to other organs. Uveal melanoma metastasizes most frequently to liver [23]. The phenomenon of Organ specific metastasis has intrigued researchers for over a century. Dr. Paget, as early as 1889 proposed Seed and Soil hypothesis, based on autopsy study of almost 735 breast cancer patients. He compared cancer cells to the seeds and the target
organ as the soil, and proposed that like seeds which get dispersed in all directions when the tree comes to fruition but are able to grow only on the soil congenial for its growth [39]. Similarly, once the cells reach circulation they are able to reach almost all the organs but are able to grow and give rise to secondary metastasis only in the organs that support their growth. By late 1970’s Prof. Fidler brought organ specific metastasis again into focus by developing several animal models and cell lines that metastasize in an organ specific manner. He showed that apart from organ microenvironment, adhesive interactions between molecules on tumor cells and those on the target organ are also important in Organ Specific Metastasis [40]. Over the years the chemokines expressed by the target organ and their receptors on tumor cells have also been shown to play a key role in organ specific metastasis of some cancers [41].

Irrespective of the mode of organ colonization, the first barrier for colonizing an organ is the organ endothelium. The arrested tumor cells would need to extravasate out of the circulation and adapt to the new growth environment in the target organ. For extravasation, the tumor cells need to interact with the endothelial cells to retract it. The next step is to degrade the exposed vascular basement membrane. Tumor cells utilize cell surface receptors to interact with basement membrane components and the matrix degrading enzymes to breach it to get into the organ parenchyma. The next major hurdle is the ability of the tumor cells to adapt to the organ environment.

However, very few tumor cells appear to be able to adopt the new organ microenvironment to form metastatic foci. As tumor cell population is highly heterogeneous; all the cells are not competent enough to form colonies and thus may remain dormant. For instance, breast carcinoma cells remain latent for several years to
decades, whereas lung adenocarcinomas cells remain dormant for months; however few
cells known as tumor initiating cells are competent to grow. Initial growth of the tumor
cells depends on both the autocrine and paracrine factors but latter on tumor cells become independent of any cytokine and growth factors [42]. For a cell to be metastatic it must be competent in all the above steps of metastasis. The cells fail to metastasize even if they are defective in any one of these steps of the metastatic cascade. This is termed as “metastatic inefficiency”.

Invasion appears to be the major event in negotiating all the major steps of the metastatic cascade. Tumor cells defective in this property are unable to metastasize, and thus understanding the molecular mechanisms involved in the invasion process would yield information to tackle metastasis.

1.4 Tumor cell invasion

Tumor cell invasion is the hallmark of all malignant tumors. Invasion in itself is a complex and poorly understood process. The biochemical mechanism of tumor cell invasion appears to be identical, to the one used by the normal non-malignant cells, like endothelial cell during angiogenesis and trophoblast cells during implantation of embryo. A three step hypothesis has been proposed to illustrate the cascade of biochemical events during tumor cell invasion [43]. The initial step in cancer cell invasion is the modulation of tumor cell adhesion to extracellular matrix followed by proteolytic degradation of the ECM/BM components and migration of cells using the matrix components as traction for movement or their proteolytic degraded products as chemoattractants. However, all these steps are linked and interdependent.
A large number of both host and tumor derived molecules participate in this process. Previous studies have clearly demonstrated that invasive and metastatic tumor cells show distinct changes in the repertoire of cell surface molecules as compared to less invasive and non-metastatic cells. These changes are in the form of alteration in expression of, i) molecules involved in cellular adhesion like cadherins, integrins and their associated proteins, ii) molecules involved in proteolytic degradation like MMPs and other proteases, iii) molecules involved in motility like CD44, integrins and their associated proteins. However, all these processes are very finely regulated in spatio-temporal manner. The molecules involved in modulating cellular adhesion and motility are similar; therefore their roles in regulating adhesion and motility have been discussed together after matrix degradation.

1.4.1 Matrix degradation

Once the tumor cells get detached from the surrounding neighbouring cells, they need to gain access to blood circulation, for that tumor cells need to degrade the surrounding ECM and BM. Basement membrane forms the major barrier whose degradation would be a prerequisite for tumor cell dissemination. For degrading these matrices, tumor cells along with surrounding stromal cells produce several classes of proteases which aid in matrix degradation.

1.4.2 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are one such class of proteases which are Ca$^{+2}$ and Zn$^{+2}$ ion dependent endopeptidase. These are involved in various physiological processes such as
tissue remodelling, in the inflammatory responses, organ development and in various diseases like cancer. There are about 23 known human MMPs [44]. Each MMP consists of mainly four domains: the signal peptide domain (pre-domain), pro-peptide domain, catalytic and c-terminal hemopexin domain (except for MMP-7, MMP-23 and MMP-26 which lack hemopexin domain). Besides, these domains few MMPs also have transmembrane (TM) and short cytoplasmic tail domain. Based on the presence or absence of TM domain MMPs are grouped into secreted and membrane anchored type proteases. Secreted MMPs are in catalytically inactive form (zymogen) due to interaction of the cysteine residue of the pro-domain with Zinc ion of catalytic site. Activation of MMP involves disruption of interaction between cysteine residue in the pro-domain and Zinc ion in the catalytic domain, a mechanism termed as ‘Cysteine Switch’.

Illustration 1: Depicting the structural domains of matrix metalloproteinases (MMPs)
Depending on the substrate they cleave, secreted MMPs are further categorized into matrilysins, stromelysins, collagenase and gelatinases. MMP-2 and MMP-9 are major gelatinases, presence of collagen binding domain (CBD) within their catalytic domain, distinguish them from other MMPs. Gelatinases are overexpressed in several malignant tumors and their expression correlates with tumor aggressiveness and poor prognosis. Besides the secreted gelatinases recently, they are also shown to be associated with membrane proteins such as integrins, CD44 and Ku proteins via hemopexin or CBD domain. Their interaction with membrane proteins probably aids invasive cells in the focalized degradation of matrix [45-47]. The activity of the MMPs are regulated by their endogenous inhibitors knows as Tissue Inhibitor of Matrix Metalloproteinase (TIMPs) there are four TIMPs have been reported so far, TIMP1, 2, 3 and 4. TIMPs reversibly inhibit MMPs in1:1 stoichiometric ratio. Their overexpression or inhibition markedly inhibited or increased the metastatic and invasive potential of several cancer cell lines, respectively [48].

Membrane tethered MMPs are attached to the cell membrane either by glycosylphosphatidylinositol (GPI) anchored or transmembrane domain. MT1-MMP is the major protease which has been shown to be localized in the invadopodia. Like other MMPs MT1-MMP is also synthesised in zymogenic form and their activation involves removal of prodomain by the action of Golgi resident pro protein convertase furin, during its trafficking to the cell membrane. Activation of MT1-MMP is very essential because it is one of the key molecules involved in the cascade of MMP activation. Increased expression of MT1-MMP correlates with the invasive potential of several cancers [49]. Manipulation of their expression either by downregulation or overexpression concomitantly decreased or
increased the invasive potential of cell lines [50, 51]. As MT-MMPs exist in the already activated form on the cell surface, they promote invasion either by degrading the matrix directly or by activating other MMPs like MMP-2 in the vicinity of the cells, thus highlighting their importance in overall tumor cell invasion.

1.4.3 uPA/uPAR Proteolytic system

Urokinase-type plasminogen activator (uPA) is 45-55 kDa serine protease secreted in inactive form (pro-uPA). Activation of pro-uPA occurs by the binding to its receptor, urokinase Plasminogen Activator Receptor (uPAR). Upon activation uPA converts plasminogen into plasmin, a broad substrate specificity protease which can degrade several extracellular matrix proteins. Plasmin can also directly activate the zymogenic form of MMPs into active MMPs. uPAR is a glycosylphosphatidylinositol (GPI) anchored membrane protein, enhanced expression of uPAR towards invasive front has been reported in several cancers such as gastric carcinoma [52]. Increased expression of uPAR towards invasive front may facilitate invasion by the localized degradation of matrix through the activation of plasmin and MMPs. Moreover, uPAR in association with integrins also regulates tumor cell adhesion and motility. Recently, it has been shown that downregulation of uPAR decreases the tumor cell invasion by modulating their adhesion and migration. Not only the expression but the localization of uPAR in lipid rafts has also been shown to play an important role in regulating migration and invasion [53].

1.4.4 Cathepsins

Cathepsins are a group of lysosomal cysteine and aspartic proteinases namely cathepsin B, L and cathepsin D present in almost all mammalian cells. Cathepsins are
involved in the degradation of intracellular or endocytosed proteins. During the process of carcinogenesis cathepsins may be secreted outside by the tumor cells or translocated to the cell membrane for degrading the components of ECM and basement membrane. In order to prevent indiscriminate degradation of matrices, cathepsins may be secreted as procathepsins and can be converted into active form by other proteases. Activity of cathepsins can be regulated by their natural inhibitors known as cystatins, a, 10-13 kDa protein. Besides their direct role in matrix degradation they can directly activate MMPs and uPA, and thus facilitate matrix degradation [54]. Expressions of several cathepsins have been reported to be associated with malignant transformation. For instance, cathepsin D expression has been shown to be associated with the aggressiveness of breast and prostate cancer. Cathepsin B activity has been shown to be elevated in metastatic variant of murine melanoma cells. Inhibition of both intracellular and extracellular cathepsins has been shown to inhibit the invasive ability of cancer cells, indicating that both the secretion of enzyme and intracellular degradation pathways are important for invasion [55].

1.4.5 Proteoglycanase

Proteoglycans are the major constituent of the ECM and BM. In order to invade through these matrices tumor cell produces several enzymes that are capable of degrading them. Hyaluronidase, a hyaluronic acids degrading enzyme, whose expression has been shown to be enhanced in breast cancer [56]. Recently, overexpression of hyaluronidase expression in breast cancer cell line has shown to increase the invasive potential of these cell lines [57].
Heparanase is another proteoglycanase capable of cleaving the carbohydrate chain of heparan sulphate (HS) proteoglycan. As the secreted growth factors remain anchored to the proteoglycans of the ECM, heparanase mediated remodelling of the ECM by the cleavage of HS results in the release of GAG anchored growth factors that play an important role in tumor cell growth and angiogenesis. Increased expression of this enzyme has been reported in several cancers [58, 59] and has also shown to be localized towards the invasive front in esophageal carcinoma [60].

1.4.6 A Disintegrin And Metalloproteinases (ADAMs)

ADAMs are a family of proteins characterized by presence of a prodomain, metalloproteinase domain, a disintegrin domain having integrin receptor binding activities, cysteine rich domain, EGF like domain, transmembrane domain and cytoplasmic domain. They are involved in shedding of cell membrane proteins as well as degradation of extracellular matrices. Expression of ADAMs have shown to be involved in several steps of cancer cell progression and they also serve as a biomarker for several cancers [61, 62]. Increased expression of ADAM17 has been shown to be involved in hypoxia induced invasiveness of glioma cell lines. shRNA mediated inhibition of ADAM17 inhibited the ability of glioma cells to invade [63]. Closely related proteases to ADAMs are ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs) unlike ADAMs which are membrane bound (except for variant form of ADAM 12 and 28) ADAMTS is secreted. Overexpression of some ADAMTS likes ADAMTS-4 and 5 have been reported in glioblastoma, these ADAMTS may contribute to invasiveness of glioblastoma by the cleavage of brevican, a brain specific proteoglycan [64].
Degradation of ECM and BM components by the above protease paved the way for the migration of the tumor cells.

1.4.7 Modulation of cellular adhesion and motility

Cell motility is one of the crucial steps in cancer cell invasion and it requires dynamic interaction between tumor cell and the substratum on which it adheres and moves. Though, matrix degradation is an important event during tumor cell invasion, it is highly regulated and generally occurs towards the invading front of the cell. The degraded products of ECM generated as a result of matrix lysis serve as chemoattractant for directional movement of the tumor cells (Chemotaxis). The regulated degradation is important as the same matrix is used by the tumor cells as traction for forward motility (Haptotaxis) [65]. ECM is highly heterogeneous and consists of proteoglycans, collagens and non-collagenous glycoproteins. Integrins participate in motility by serving as receptors for all the major collagens and non-collagenous glycoproteins. The interactions of cell surface proteoglycans with GAGs and proteoglycans on the ECM/BM play a key role in the motility. Hyaluronate receptor CD44 is one of the examples of these interactions which play a key role in metastasis of certain cancers.

1.4.8 Hyaluronate receptor - CD44

CD44 is the major receptor for the hyaluronic acids (HA), one of the key constituent of ECM. CD44 has been shown to mediate both cell to cell as well as cell to matrix interaction. In order to be metastatic and invasive, tumor cells have been shown to modulate the expression of CD44. CD44 pre-mRNA consists of 20 exons, due to alternative splicing of introns it exist in several isoforms (CD44v6-v10, v = splice variants).
CD44s is the standard CD44 isoform formed by the splicing of introns between fifth and sixteenth exon. In the metastatic prostate cancer, tumor cells downregulate CD44s as compared to their benign counterpart or they overexpress other isoforms (CD44v7-10) [66]. Increased expression of CD44v6 isoform has been reported in several aggressive cancers such pancreatic adenocarcinoma, head and neck squamous cell carcinoma and breast cancer. Interaction of HA with CD44 has been shown to regulate the cancer cell motility in ovarian carcinoma cell lines, interaction of CD44 with HA promotes their motility. Inhibition HA binding site on CD44 by monoclonal antibody against CD44 prevents the HA mediated increased cell motility of human ovarian carcinoma cell lines [67]. The extracellular domain of CD44 is also extensively modified by N-glycans, O-glycans which has been shown to regulate HA binding [68]. In addition to these CD44 is also modified by the addition of proteoglycans like chondroitin sulphate and heparin sulphate which adds in its interaction with growth factors [69]. Besides CD44, integrins are the major receptor of large components of ECM and BM and their interaction with these components have been shown to play an important role in regulating cancer cell adhesion and motility.

1.4.9 Integrins

Integrins are obligate heterodimeric transmembrane glycoprotein receptors composed of non-covalently associated α and β subunits. There are about 18α and 8β subunits known so far which combine variously to form 24 different heterodimeric integrin receptors [70]. Integrins are the major receptors for both collagens and non-collagenous glycoproteins. Each integrin receptor has distinct substrate specificity but often they have overlapping specificity with varied affinity for ligands (Illustration: 2). For instance, α5β1
is the major fibronectin receptor; besides this fibronectin also serve as ligand for several integrin heterodimers.

Each integrin subunit has one large N-terminal extracellular domain (approx. 800 amino acids), a transmembrane domain (approx. 20 amino acids) and except for β4 integrin which has large cytoplasmic tail (approx. 1000 amino acids) all others have a short cytoplasmic tail (approx. 13-17 amino acids). The cytoplasmic tail of integrin connects to actin cytoskeleton by several adaptor proteins, except for β4 integrin whose cytoplasmic tail is connected to intermediate filament proteins via adaptor proteins like plectins. The extracellular region of both α and β subunits contain distinct subdomains. The alpha subunit comprises of β propeller head domain, a thigh and two calf domains. Beta propeller domain of α subunits contains an additional inserted (I) domain of about 200 amino acids. The extracellular domain of β subunits also contains αI domain, a PSI (plexin/semaphorin/integrin) domain, a hybrid domain, four EGF repeats and membrane proximal β tail domain (βTD). I domain of integrin subunit is crucial for ligand binding [71].

Depending on the ligand binding, integrin exists in three different conformations such as bent, active and clustered. When integrins are not bound to the ligand they are called as inactive, or in rested/bent conformation. In the rested conformation, transmembrane domains of both the subunits are in close proximity to their cytoplasmic domain. In active conformation integrins are bound to ligand, which results in extension of extracellular domain and separation of cytoplasmic and transmembrane domain. Upon ligand binding when many activated integrins are clustered together at the plasma membrane they are said to be in clustered conformation [71]. Clustering results in the
formation of focal adhesion, which is essential for actin cytoskeleton assembly and activation of downstream signalling for several cellular functions. When integrins are bound to ligand they are capable of both inside out and outside in (bidirectional) signalling across the plasma membrane and thus regulate processes such as differentiation, survival, adhesion and motility [70].

Illustration 2: (A) Different types of integrin receptors. (B) Heterodimers formed by β1 integrin and their substrate specificity.
Integrins play an important role in regulating adhesion and protrusions in migrating normal as well as tumor cells. Cellular migration requires discrete steps: polarization i.e., formation of distinct front and rear end, cellular protrusion, transmembrane connection of cytoskeleton to ECM for the generation of traction for forward propulsion of the cells and retraction from the rear end [73]. Several signalling molecules are involved in these processes. Downstream Focal Adhesion Kinase (FAK) signalling is very crucial for integrin mediated directional cell movement. FAK serves as a scaffold to recruit src, a tyrosine-protein kinase, to focal adhesion, directing several pathways to promote cell migration. Continuous rearrangement of actin cytoskeleton regulates cell spreading which is a key requirement for cell movement. Actin cytoskeletal reorganization is mainly mediated by Rho GTPase family of proteins. Tumor cells can achieve optimum adhesion required for invasion and movement by either upregulating or downregulating certain integrin receptors. In malignant melanomas increased expression of αVβ3 integrin has been reported towards invasive front as compared to pre neoplastic tumors [74]. Besides, the alteration in expression of integrins during the process of invasion, metastatic tumor cells show increased level of activated β1 integrin in contrary to primary tumor which show lowered level of activated β1 integrin [75].

Integrins are highly glycosylated molecules; each subunit contains several N-glycosylation sites. Glycosylation on integrin has been shown to affect integrin structure, dimerization, affinity, clustering and stability of different integrin receptors [76-80]. Besides alteration in their expression, altered glycosylation of integrins also plays an important role in regulating its functions, probably by influencing its interaction with ECM
or other membrane proteins in its vicinity. The motility regulating functions of integrins may be modulated by another family of cell surface proteins known as tetraspanins.

1.4.10 Tetraspanins

Tetraspanins are the transmembrane proteins that transverse the membrane four times. Tetraspanins associate among themselves or with proteins from other families like integrins, growth factor receptors and proteases to form a membrane microdomain called Tetraspanin Enriched Microdomains (TEMs) [81]. TEMs are quite distinct from the rafts in terms of their disruption by Triton X-100 at 4°C, solubility because of palmitolylation in non-ionic detergent, insensitivity to cholesterol depletion [82]. Tetraspanins modulate the function of their associated proteins such as immunoglobulin superfamily proteins (IgSF), growth factor receptors and integrins [83]. Tetraspanins modulate the function of integrin receptors by regulating their compartmentalization or localization on the cell membrane, modulating their signalling and trafficking [84]. Tetraspanins are a family of 33 known human proteins of 25-50 kDa in size and made up of about 230 amino acids, it has certain characteristic residues such as CCG motif in outer large extracellular loop, disulphide bridge, and polar residues in transmembrane domains (Illustration 3). Majority of animal cells contain several tetraspanins. While tetraspanin CD81 is widely expressed, few tetraspanins like CD151 have very limited distribution which is found mainly in epithelial, endothelial, neuronal and fibroblastic cells. However, some tetraspanins like RDS/peripherin and uroplakin are expressed only in outer segment of rod cells in retina and in urethra, respectively.
Most of the tetraspanins are posttranslationally modified by glycosylation or palmitoylation. Glycosylation is mainly present on the large extracellular domain of tetraspanins, except for CD9 which has glycosylation on small extracellular domain and CD81 which lacks glycosylation. Palmitoylation of tetraspanins on the other hand, occurs at the membrane proximal cysteine residues towards cytoplasmic leaflet. Tetraspanins are known to play an important role in wide variety of biological processes such as fertilization, viral and protozoan infection, cell proliferation, immune cell activation, tumorigenesis, cell motility and invasion [85].

**Illustration 3: Depicting the structural representation of tetraspanins**

Invasive and metastatic tumors downregulate most tetraspanins except for CD151 and CO-029, in the process of tumorigenesis, tetraspanins such as CD82 and CD9 act as tumor suppressor proteins [86]. Over expression of these tetraspanins in invasive cell lines inhibits their invasion and metastasis by regulating adhesion, motility and matrix degradation. Expression of CD82 in metastatic prostate cancer cell lines inhibits cellular...
protrusions and retraction crucial for cell movement by attenuating actin reorganization [87]. Tetraspanins such as CO-029 and CD151 promotes invasion and metastasis by regulating cell migration and matrix degradation. CD151 is the major laminin receptor associated tetraspanin. Knockdown of CD151 in highly invasive breast cancer cell line MDA MB231 inhibits cellular migration. CD151 has been shown to increase MMP-9 secretion by their homophilic interaction [88]. Thus tetraspanins can perform both pro as well as antimetastatic functions [89].

Microdomains formed by the tetraspanin CD151 and CD82 modulate the function of laminin and fibronectin receptors respectively. Glycosylation on both integrin and tetraspanin have been shown to regulate motility [90]. Recently, it has been found that tetraspanin can modulate the integrin function by regulating its glycosylation [91]. Besides, these alterations in expression of molecules involved in the processes of invasion, tumor cells also have been found to exhibit several membrane modifications in the form of altered glycosylation.

1.5 Altered cell surface glycosylation associated with invasion and metastasis

1.5.1 Glycosylation

Glycosylation is the most abundant posttranslational modification. More than 50% of all the known proteins and about 80% of secreted and membrane proteins are glycosylated. About 1-2% of the human genome encodes for the enzymes such as glycosyltransferases (enzymes which can add sugars) or glycosidases (enzymes which can remove sugars) [92]. Glycosylation involves covalent addition of oligosaccharides to the
lipids or proteins forming glycoconjugates which include **glycolipids, proteoglycans and
glycoproteins**.

**Glycolipids**

Glycolipids are the molecules containing one or more monosaccharide units attached to ceramide by a glycosidic linkage. In glycosphingolipid, the monosaccharide unit is glucose which is attached to the terminal primary hydroxyl group of lipid moiety. Based on the oligosaccharides composition the glycolipids are divided into seven families these are lacto, neolacto, globo, isoglobo and ganglio series in vertebrates; mollu and artho series in invertebrates [93]. Altered expression of gangliosides has also been observed in several cancers for instance, in melanoma increased expression of gangliosides such as GD3, GM3 have been observed, while GD2 in neuroblastoma [94]. Recently increased expression of GD2 and GD3 has been reported in breast cancer stem cells as compared to non-cancer stem cells [95].

Glycolipids are an important constituent of membrane microdomains called Rafts. These form a platform for sorting and signalling involved in cancer cell adhesion and migration [96].

**Proteoglycans**

Proteoglycans comprise of a core protein and one or more covalently attached glycosaminoglycan (GAGs). GAGs are composed of repeating units of disaccharides where one sugar is usually uronic acid (D-glucuronic acid or L-Iduronic acid) and other sugar is N-acetyl glucosamine (GlcNAc) or N-acetyl galactosamine (GalNAc). Depending on the
type of GAGs proteoglycans are mainly of five types these are chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and hyaluronic acid. Proteoglycans are either secreted into the extracellular matrix to form hydrated gels, which support the tissues to withstand compressional forces or localize on the cell membrane. The cell membrane associated proteoglycan serve as co-receptor to help the cells in binding to the extracellular matrix or affect the growth factor signalling. Changes in the expression of heparan sulphate proteoglycans (HSPG) such as syndecans and glypicans have been shown to be altered in several cancers. For instance, syndecan-1, a membrane anchored HSPG has been shown to be elevated in stroma of breast cancer cells and its expression correlates with poor prognosis [97]. Similar, overexpression of glypican-1, a GPI anchored HSPG has been observed in gliomas and breast cancer and their expression has been shown to affect metastasis by modulating growth factor signalling [98, 99].

**Glycoproteins**

These are another type of glycoconjugates where oligosaccharides are covalently attached to the proteins. In glycoproteins glycosylation can be ‘O’ linked if sugars are attached to the hydroxyl group of serine or threonine or ‘N’ linked if sugars are attached to the amide group of asparagine residues having a consensus sequence (N-X-S/T) where X is any amino acid except for proline or aspartic acids.

**O-Linked oligosaccharides or O-Glycans**

Formation of O-linked glycans occurs by the covalent addition of the N-acetylglactosamine (GalNAc) to the hydroxyl group of the serine and threonine residues. Biosynthesis of O-glycans occurs in the lumen of Golgi apparatus and it involves sequential
addition of monosaccharides to the growing polypeptide chain by the action of glycosyltransferase, the resulting product becomes the acceptor substrate for the subsequent glycosyltransferase. Besides, addition of GalNAc, less commonly other sugars (galactose, mannose and xylose) can also be added to OH-group of ser/thr residues of polypeptide chain forming different types of O-glycans. Mucins are the extensively O glycosylated proteins. Polypeptide sequence of mucins has variable number of tandem repeat (VNTR) region rich in serine, threonine and proline. The presence of proline residues within VNTR region facilitates formation of O-glycans. These are further elongated by the action of other glycosyltransferases resulting in the formation of elongated, branched structures known as core I, II, III and IV. Elongated mucin type O-glycans are found in normal epithelium of gastrointestinal, genitourinary and respiratory tract. Expression of altered mucin type of O-glycans has been reported in several cancers. For instance, in carcinoma of breast and colon mucin has truncated (incompletely glycosylated) O-glycan structure T/Tn antigen [100]. Recently, it has been shown that O-glycans have both metastatic and antimetastatic role [101]. O-glycans with core 2 structure have metastasis promoting function in several cancers, such as bladder cancer. Bladder cancer cells expressing enzyme which catalyses core 2 structures (C2GnT) forms more lung metastatic foci as compared to non-expressing cells. O-glycans with core2 structure in bladder cancer cells prevent NK cell mediated tumor killing. However, expression of core3 synthesizing enzyme (core3 synthase) in prostate cancer cell lines decreased their metastatic ability by regulating integrin expression and thus tumor cell invasion and motility [102].

**N-Linked oligosaccharides or N-Glycans**
The biosynthesis of N-linked oligosaccharides occurs in lumen of endoplasmic reticulum and continued in Golgi by the sequential action of several resident glycosyltransferases and glycosidases. The N-glycan biosynthesis involves several discrete steps, these are [103]-

1. Formation of N-glycan precursor molecule and its addition to Dolichol pyrophosphate.

Synthesis of N-glycan precursor begins in the cytosolic face of the ER by the transfer of GlcNAc-P from UDP-GlcNAc to the dolichol phosphate (DP) to form dolichol pyrophosphate N-acetylg glucosamine (GlcNAc-DPP). Dolichol is a polyisoprenol lipid carrier consisting of five isoprene units attached linearly in head to tail manner. Sequential addition of another GlcNAc and five mannose residues from UDP-GlcNAc and GDP-Man, respectively, to the GlcNAc-DPP generates (Man$_5$GlcNAc$_2$ DPP). The Man$_5$GlcNAc$_2$-DPP residues translocate across the lipid bilayer from cytosolic side of ER by the action of flippase, so that glycan can be exposed to the luminal face of ER. The fourteen sugar precursor molecule (Glc$_3$Man$_9$GlcNAc$_2$-DPP) is formed by the further addition of four mannose and three glucose residues to the Man$_5$GlcNAc$_2$-DPP [103].

2. Transfer of precursor molecule from dolichol pyrophosphate to growing polypeptide chain.

The Glc$_3$Man$_9$GlcNAc$_2$ is transferred en bloc from DPP on to the Asn residue of Asn-X-Ser/Thr consensus sequence of the nascent growing polypeptide chain in the lumen of ER by the action of multisubunit protein complex, oligosaccharyl-transferase [103].
3. Processing of N-glycans

It involves sequential removal of glucose molecules by the action of glucosidase I and II. Further processing of N-glycans is mediated by ER resident α-mannosidase I which removes one mannose. Glycoproteins with this type of sugars (Man₈GlcNAc₂) are referred as **high mannose type**. Subsequent removal of three mannose residues from Man₈GlcNAc₂ in the Golgi by the action of Golgi resident α-mannosidase-IA, B, C results in the formation of Man₅GlcNAc₂. The Man₅GlcNAc₂ is the common molecule for the formation of hybrid and complex type of oligosaccharides. Addition of N-acetylglucosamine residue to the C-2 of the mannose α1,3 in the core of Man₅GlcNAc₂ by the action of N-acetyl glucosaminyl transferase (GnT)-I, form GlcNAc-Man₅GlcNAc₂. Glycoproteins with this type of sugars are referred to as **hybrid type**. Removal of two mannose form GlcNAcMan₅GlcNAc₂ by the action of Golgi resident α-mannosidase II results in the formation of GlcNAcMan₃GlcNAc₂. By the subsequent action of GnT-II on C2 of the mannose α1,6 in the core of GlcNAcMan₃GlcNAc₂, form the precursor molecule for biantennary complex type of sugars. Tri and tetra antennary structure are formed by the action of GnT-IV and GnT-V, respectively. Another Golgi resident enzyme GnT-III, adds N acetylglucosamine to the β mannose of the core resulting in the formation of bisected hybrid and complex type of sugars [103].

4. Maturation of N-glycan

It involves addition of sugars a) mainly fucose to the to the core (first GlcNAc attached to the asparagine residue b) elongation of the hybrid and complex N-glycan by the addition of β linked galactose to the terminal GlcNAc of each antennae resulting in the formation of N
acetyl-lactosamine (LacNAc) structure c) Maturation of N-glycans by the addition of terminal sugars like sialic acids, fucose, galactose, N-acetyllactosamine and sulphate to antennary structures known as capping or decoration of sugars.

Thus, all N-Glycans have a common pentasaccharides core (Man$_3$GlcNAc$_2$). Depending on the action of specific enzymes, which act in proper order and time and their expression in respective Golgi compartment, N-linked glycoproteins formed are of three types, namely high mannose, hybrid and complex type [103].

Several observations and experimental evidences over the past few decades have generated the current opinion that N-glycans play very crucial role in progression and metastasis of cancer. By the comparative analysis of the surface glycoproteins from the virus transformed and non-transformed cells, Warren and Glick observed that glycans from the transformed cells were highly branched and sialylated [104, 105]. This phenomenon was established as “Warren and Glick phenomena”. Later on it was found that the enzyme N-acetylglucosaminyl transferases-V (GnT-V) was responsible for the formation of highly branched N glycans on polyoma virus transformed baby hamster kidney (BHK) cells [106]. GnT-V is a Golgi resident enzyme, encoded by the Mgat5 gene, catalyses the formation of highly branched complex N-linked oligosaccharides (Illustration 4) and its increased expression has been observed in several cancers.

1.5.2 Beta1,6 branched N-linked oligosaccharides and cancer metastasis
Neoplastic transformations have been associated with several changes in glycan profile.

Illustration 4: Typical structure of N-linked oligosaccharides (bi, tri and tetra antennary)

Significance of increased expression of the β1,6 branched N-oligosaccharides in metastatic progression has emerged from several clinical evidences observed in human cancers. Increased expression of β1,6 branched N-oligosaccharides has been reported in the atypical hyperplasia and breast carcinoma as compared to normal and benign tissues. Expression of these oligosaccharides in human colorectal carcinoma serves as an independent prognostic marker for tumor recurrence and patient survival [107]. Moreover, enhanced expression of these oligosaccharides has been also demonstrated in gliomas, melanomas, esophageal, mucinous tumor of ovary, gastric carcinomas and endometrial cancer [108-111].

Clinical observations showing the association between expression of these oligosaccharides and human cancers, was further supported by numerous experimental evidences from human and murine cancer cell lines. Transformation of Rat2 fibroblast cells
with oncogene like T24H-ras or v-fps have been shown to increase both, the GnT-V activity and metastatic potential of these cells [112]. Similarly, transformation of NIH3T3 cells by expression of her2/neu oncogene induces the expression and activity of GnT-V enzyme which in turn increased the expression of β1,6 branched N-oligosaccharides on specific sets of proteins [113]. Glycosylation mutant of highly metastatic MDAY-D2 cell line was associated with their loss in their metastatic potential due to loss in the activity of GnT-V [114]. Inhibition of the formation of these oligosaccharides in metastatic cell lines by N-glycosylation inhibitor Swainsonine, which results in the formation of hybrid instead of complex N-glycans showed significant reduction in metastatic potential [115]. By manipulating the expression of these oligosaccharides in the cell lines either by overexpression or downregulation of GnT-V showed concomitant increase and decrease in their metastatic potential. Expression of GnT-V in colon cancer cell lines increased their ability to metastasize to distant organ by promoting the ability of these cells to attach to vascular endothelium [116]. Expression of GnT-III, a competitive inhibitor of GnT-V in B16F10 cells inhibited the ability of these cells to form metastatic colonies in the lung [117].

Besides, exemplifying the association of these oligosaccharides and metastasis in cell lines, their association with metastasis had been also demonstrated in mgat5 knockout mice. Polyoma middle T antigen induced experimental mammary cancer and lungs metastasis was significantly inhibited in mgat5 (-/-) mice as compared to mgat5 (+/+ ) counterparts [118].

Thus, evidences obtained from the increased association of β1,6 branched N-linked oligosaccharides in several human cancers, from their chemical inhibition or glycosylation
mutant and also from genetic manipulation of their expression conclusively established that β1,6 branched N-oligosaccharides play an important role in metastasis. Expression of these oligosaccharides was not only associated with progression of several cancers but also they have been shown to play a pivotal role in organ specific metastasis.

1.5.3 Beta1,6 branched N-linked oligosaccharides and organ specific metastasis

Organ specific metastasis involves specific interaction between the tumor cells and vascular endothelium of the target organ, extravasation and adaption of the tumor cells in new microenvironment. Beta1,6 branched N-oligosaccharides is the preferred site for the addition of various terminal sugars like sialic acids (SA), fucose or poly-N-acetyllactosamine (polylacNAc) (Illustration 5). These substitutions serve as novel ligands for the several endogenous lectins expressed on organ vascular endothelium. For instance, sialyl Lewis X antigen (sLe^x) substituted β1,6 branched N-oligosaccharides promotes the metastasis of melanoma to the liver by facilitating their attachment to the liver vascular endothelium via E-selectins which serve as ligand for sLe^x antigen [116].
Illustration 5: Depicting the β1,6 branched N-linked oligosaccharides and probable associated terminal sugars.

Recently, it has been shown that the presence of poly-N-acetyllactosamine substitutions on β1,6 branched N-oligosaccharides facilitate the lung colonization of B16 murine melanoma cells. Galectin-3, a beta galactoside binding lectin has been shown to be expressed in highest amounts on the lungs of mice. It was shown to be expressed on all the compartment of the lungs, including the surface of lung vascular endothelium. Expression on the vascular endothelium serves as a ligand for the polylacNAc substituted β1,6 branched N-oligosaccharides and which in turn helps in mediating lung specific metastasis [119, 120]. Besides, their role in mediating initial interaction these oligosaccharides also participate in all the steps of extravasation and possibly survival and proliferation of cancer cells in the secondary site. Several growth factor receptors have been shown to be modified...
by the β1,6 branched N-linked oligosaccharides their contribution in the growth of the tumor cells as the secondary metastatic site could not be rule out. [121] The expression of these oligosaccharides appears to influence majority of the steps of metastasis and thus may also have a role in regulating tumor cell invasion.

1.5.4 Beta1,6 branched N-linked oligosaccharides and invasion

Expression of β1,6 branched N-linked oligosaccharides have been shown to be strongly associated with invasive normal as well as metastatic cancer cells. Endothelial cells express these oligosaccharides when they need to be invasive during angiogenesis [122]. Cells of the immune system like activated granulocytes, macrophages and lymphocytes express them to extravasate and reach the inflamed site [123]. Even trophoblast express these oligosaccharides during implantation of embryo into uterus [124]. Increased expression of these oligosaccharides is reported in several invasive cancers such as gliomas [125] and towards invading front in esophageal carcinoma [126]. Increased expression of β1,6 branched N-oligosaccharides correlates with invasive potential of metastatic sublines as compared to non-metastatic murine mammary carcinoma cell line SP1 [127]. Expression of the enzyme GnT-V in non-invasive human fibrosarcoma cells and murine fibroblast cells made them invasive [128]. Moreover, downregulation of the GnT-V or overexpression of its competitive inhibitor GnT-III in highly invasive cancer cell lines significantly decreased their ability to invade [129].

1.5.5 Possible mechanisms by which β1,6 branched N-oligosaccharides affect cancer cell invasion
Expression of these oligosaccharides possibly regulates processes critical for invasion like adhesion, matrix degradation and motility. Invasive tumor cells mediate all these process either by the modification of the proteins carrying them or the signalling mediated by them. Expression of these oligosaccharides on cell adhesion molecules like integrins, cadherins CD44 or ECM components like laminin regulates cell to cell or cell to ECM interaction. Expression of such oligosaccharides has been shown to regulate adhesion to both ECM and BM components. Expression of GnT-V in an immortalized lung epithelial cell line, Mv1Lu and HT1080 inhibits adhesion to Col-IV and FN possibly by altering the glycosylation of integrin receptors [79, 130]. E-cadherin is the one of the molecule that serves as substrate for both GnT-V and GnT-III. Addition of bisecting N-glycans on E-cadherin by GnT-III expression has been shown to stabilize adherens junction and thus cell to cell adhesion possibly by preventing its endocytosis. However, addition of β1,6 branched N-oligosaccharides on E-cadherin alters its localization and thus it promotes invasion by reducing cell to cell adhesion [131].

In addition to regulating adhesion, presence of these oligosaccharides on proteins involved in matrix degradation like matriptase has been shown to regulate the invasive ability of prostate cancer cell lines by regulating its activity [132], and stability of matriptase in human gastric cancer cell line MKN45 [133]. Inhibition of the expression of these oligosaccharides has been shown to inhibit matrix degradation by enhancing the expression of Tissue Inhibitor of Matrix metalloproteinase-1 (TIMP-1) [134]. MMP-2 and 9 are the major collagenases which are involved in the matrix degradation. Recently, it has been shown that presence of such oligosaccharides on TIMP-1 negatively modulates its ability to inhibit MMP-9 activity [135].
Besides regulating adhesion and matrix degradation, expression of these oligosaccharides also regulates cancer cell motility. Increased expression of these oligosaccharides has been shown to be involved in melanoma progression by up regulating cell motility [136]. Beta1,6 branched oligosaccharides served as high affinity ligand for galectin-3. The interaction between galectin-3 and such oligosaccharides on α5β1 integrin causes its activation and promote motility of mammary carcinoma cells on fibronectin [137]. Similarly, expression of GnT-III and GnT-V in MKN45 cell line concomitantly decreased and increased the motility of these cells on laminin-5 by modulating the glycosylation of the α3 integrin [138]. Recently, it has been shown that downregulation of GnT-V gene in gastric carcinoma cell line BGC823 inhibited invasion and metastasis by inhibiting EGFR signalling mediated EMT and MMP-9 secretion [139].

Thus, β1,6 branched N-oligosaccharides appears to influence metastasis by affecting the most crucial aspects in metastasis that is invasion. Despite considerable amount of research, the mechanism by which these oligosaccharides regulate invasion is not very clear and it requires further investigation.

1.6 RATIONALE OF THE STUDY

Metastasis is the major cause of cancer related mortality and invasion is involved in majority of the steps in metastasis cascade. Cancer cell invasion thus, is the hallmark of metastasis. Cell surface molecules play an important role in negotiating most steps in invasion and metastasis. Tumor cells show several surface modifications associated with metastasis and invasive phenotype. One such consistently observed cell surface modification is the expression of β1,6 branched N-linked oligosaccharides. Expression of
such oligosaccharides correlates positively with both invasive cancer cells as well as the normal cells involved in invasive functions.

B16 murine melanoma variant B16BL6, selected specifically for the invasive characteristics, and its parent cell line B16F10 have been used as a model system to investigate the mechanism by which these oligosaccharides regulate invasion (illustration 6). Previous work in lab has explored their role in invasion by comparing invasive variants B16BL6 and B16F10 which differ in the expression of these oligosaccharides, by using N-glycosylation inhibitor Swainsonine (SW) and by using antisense to the enzyme GnT-V. It was shown that their expression i) correlates positively with adhesion to most ECM and BM components and regulates chemotaxis (motility in response to soluble chemoattractant) positively on both ECM (fibronectin as one representative) and BM (matrigel) components however, ii) it regulates haptotaxis (motility in response to substratum bound chemoattractant) in a complex manner, enhances it on fibronectin but attenuates it on matrigel iii) although, the expression of these oligosaccharides has no effect on the secretion of MMPs, it always correlated with invasiveness [140].
Illustration 6: The B16 murine melanoma model. (A) Generation of B16 murine melanoma cell lines. (B) Experimental metastasis assay. (C) Spontaneous metastasis assay

Above studies raise several key questions,

- Expression of β1,6 branched oligosaccharides are always associated with invasion and with increased adhesion on different matrix components, however, there are reports where their expression correlates negatively with adhesion. **How do these oligosaccharides regulate adhesion both positively and negatively? Is it because of differences in terminal substitutions on them?**
Although, the expression of these oligosaccharides correlates with the invasiveness of melanoma cells, it does not correlate with the secretion of MMPs. Degradation of matrix is a key component of invasion process, it is important to investigate the mechanism by which these oligosaccharides regulate matrix degradation. How do these oligosaccharides regulate matrix degradation?

The expression of these oligosaccharides regulates motility differentially on ECM and BM components. What is the mechanism and its significance in terms of invasion?

1.6.1 AIMS AND OBJECTIVE OF THE STUDY

To investigate the role of terminal substitutions on β1,6 branched N-linked oligosaccharides in regulating cellular adhesion and thus invasion.

To investigate the role of β1,6 branched N-linked oligosaccharides in regulating matrix degradation.

To investigate the role of these oligosaccharides in regulating the motility of cells on ECM and basement membrane components.