Chapter – 1

Introduction

1.1 Cancer

1.1.1 Overview

Cancer has remained a conundrum that is still unsolved by the interrogative scientific minds. Cancer is one of the leading cause of death worldwide. In 2012 cancer caused 8.2 million deaths and 14 million new cases were reported (Stewart and Wild). International Agency for Research on Cancer (IARC) has stated that the number of deaths due to cancer will increase to 13.2 million and new cases reported to 21.4 million, by 2030. According to the National Cancer Registry Programme (NCRP) in India, cancer causes 0.3 million death each year and the total cancer cases are projected to rise from 9,79,786 in the year 2010 to 1,148,757 cases by 2020 (Takiar, Nadayil, and Nandakumar; Ali, Wani, and Saleem).

Cancer is a class of disease exemplified by immoderate proliferation of abnormal cells in the body. Normally, the cell division is under strict supervision of intricate network of signals which directs the cells to divide as and when required. But sometimes the cell division goes out of control, allowing the cells to divide and producing new cells even when they are not required. This uncontrolled cell division results in the mass of tissue, called as tumor, neoplasm or new growth. This mass of tissue may compress, invade, extirpate the normal tissues nearby and can also metastasise to the other parts of the body (Hunter; Pawson and Nash). This deregulated cell division/growth due to the flustered signalling pathway is the defining feature of all cancers.

The network signals controlling the cell growth and division in-turn are regulated by intricate genetic control systems. These genetic control systems respond to various stimuli such as growth signals, growth-inhibiting signals and apoptotic signals. The deregulated cell growth that gives birth to majority or all the cases of cancer are caused by genetic damage (Lodish et al.). Mutations mainly in two broad classes of genes have been incriminated for the onset of cancer viz., proto-oncogenes and tumor suppressor genes. Proto-oncogenes usually code for proteins that are generally involved in cell growth and differentiation. These genes upon mutation forms activated oncogenes which direct the cells destined for apoptosis, to proliferate. Tumor suppressor genes normally slow down cell growth, repairs the DNA damage and directs the cell when to undergo apoptosis. Hence, damage to them allows uncontrolled growth (Strahm and Capra).
The vast majority of the cancers are apparently sporadic in nature and results due to lifetime of exposure to carcinogens such as certain chemicals and UV radiation or by errors during replication or during repair of genes (Cairns). Normally cancer causing mutations occur in somatic cells rather than germ line cells and are not inherited. However, recently inherited mutations have been implicated in cancers such as: breast, liver and lung cancer (Travis et al.). But, the term “hereditary cancer” is a misnomer as in this case instead of cancer, only the predisposition to cancer is inherited. In spite of it being a genetic disease, cancer has two major differences from other inherited diseases viz., majority of cancers are caused by somatic mutations while other inherited diseases are caused entirely by germ line mutations; secondly, cancer occurs due to accumulation of several mutations and not from a single mutation (Vogelstein et al.).

The tumors are divided basically into two types viz., benign and metastatic tumor. Benign tumor are restricted to the area where it grows as the cell adhesion molecules hold the cells together keeping it localised to the area where it grows. Whereas, cells of malignant tumor grow and divide more rapidly than benign tumor, do not respond to apoptotic signals and even infiltrate the nearby tissue. Along with the progression of malignant tumor, the invading cells enter the circulatory system eventually forming secondary tumors, this process is called as metastasis (Cooper).

Cancer cells can be differentiated from normal cells by histopathological examination. Unlike normal cells they are less differentiated and they exhibit the typical characteristics of rapidly growing cells viz., enlarged nucleus, scanty cytoplasm and relatively less specialized structure (Rashed and Bekele). There are several characteristic of tumor cells that distinguish them from normal cells known as hallmarks of cancer.

1.1.2 Hallmarks of cancer

The genetic changes that cause oncogenesis metamorphose the fundamental properties of the cell permitting the cells to grow vehemently. Hanahan and Weinberg in January 2000, underlined six key features (hallmarks of cancer) of cancerous cells viz., sustained proliferative signalling, unresponsive to antigrowth signals, escaping apoptosis, maintaining replicative immortality, angiogenesis and tissue invasion/metastasis; these feature can be seen as largely responsible for driving malignant behaviour (D Hanahan and Weinberg). Recently, they added two additional hallmarks (deregulating energy metabolism and evading immune destruction) and two enabling characteristics (genomic instability/mutation and tumor conducive
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inflammation) (Douglas Hanahan and Weinberg) (Figure 1.1). The role played by each factor is describe briefly below.

![Figure 1.1 Hallmarks of cancer.](image)

1.1.2 Sustained proliferative signalling

Normally cells require a plethora of intricately controlled signals for it to shift from dormant stage to a proliferative state. The signals are relayed into the cells on binding of various ligands viz., growth factors, cell-cell adhesion and extracellular matrix components to variety of trans membrane receptors via various intermediary messengers. This process is delicately controlled in normal cells. However, in the cancerous cells this control is disturbed and they proliferate uncontrollably (Harrington). Cancer cells achieve this limitless proliferative ability via three main strategies: self-production and release of growth factors, altering the structure, number or function of the receptor on their surface and by deregulating the downstream signalling (D Hanahan and Weinberg).

1.1.2.2 Unresponsive to antigrowth signals

Normally the cell division is kept under check as and when required by anti-proliferative signals and thus maintaining tissue homeostasis (D Hanahan and Weinberg). These signalling pathways mainly control the cell cycle clock and is controlled by cyclins, cyclin-dependent
kinases (CDK), cyclin-dependent kinase inhibitors and retinoblastoma protein (Rb) (Hannon and Beach; Datto et al.). Hence, mutation in anti-proliferative signalling pathways is commonly seen in cancer and plays a significant role in cancer progression (Harrington).

1.1.2.3 Escaping apoptosis

Tumor cell proliferation not only depends on the rate of proliferation but also on rate of cell erosion. Cell erosion occurs mainly by programmed cell death or apoptosis. Normal cells are in a continuous control of anti-apoptotic and pro-apoptotic signals that they receive (Hanahan and Weinberg). Contradictingly, aberration in apoptotic pathway signalling is commonly seen in cancer. Two of the best-known cancer associated genes viz., p53 (tumor suppression gene) and bel-2 (oncogene) are involved in apoptosis. Bel-2 has been shown to overexpress in several cancer (Vaux, Cory, and Adams) whereas, p53 is inactivated in various cancer (Symonds et al.). Hence, anti-cancer treatments targeting apoptotic pathway have faced failures due to drug resistance.

1.1.2.4 Maintaining replicative immortality

Normal somatic cells divide for a limited number of times (60-70), known as Hayflick limit. Later, the cellular growth is permanently arrested, known as replicative senescence (Hayflick). This process occurs due to gradual shortening of the telomere at the end of the chromosome after each cyle and eventually causes chromosome fusion. The affected cells undergo senescence and die (Counter et al.). Unlike normal cells, stem cells and cancerous cells have the ability to maintain the length of their telomere. In most cancers, this occurs mainly by two ways viz., by upregulation of the telomerase enzyme which continuously repairs the ends of telomeric DNA (Bryan and Cech) or by recombination based interchromosomal exchanges of sequence information (Bryan et al.). This allow limitless proliferation of the daughter cells (Harrington).

1.1.2.5 Angiogenesis

Normally angiogenesis in tissues is under strict control by pro-angiogeneic and anti-angiogenic signals. The proliferation of the tumor depends directly on its capacity to secure blood supply. A tumor can grow till it attains the size of 60-100 µm by obtaining nutrition and oxygen by diffusion, beyond this, the growing tumor needs to acquire its own dedicated blood supply (Harrington). This in turn is facilitated by disturbing the balance between pro- and anti-angiogenic factors. Vascular endothelial growth factor (VEGF; pro-angiogenic) is known to be
overexpressed in variety of cancers (Niu and Chen) whereas, thrombospondin-1 (anti-angiogenic) is downregulated (Dameron et al.).

1.1.2.6 Tissue Invasion/Metastasis

The process of detachment of cells from primary tumor mass and its invasion into adjacent tissues followed by transportation to distant parts of the body via blood circulation eventually establishing secondary tumor sites, is called as metastasis. Metastasis is responsible for 90% of cancer related deaths (Sporn). Invasion followed by metastasis involves synchronisation and orchestration of a series of complex biological processes. However, the genetics and the entire biochemical process taking place behind this event is poorly understood. Several classes of molecules are now known to be recognised which facilitates the process viz., integrins, cell-cell adhesion molecules (CAMs) and extracellular proteases (Johnson). Additionally, irrespective of the organs to which tumor metastasize (e.g. lung cancer to adrenal gland and brain; breast cancer to bone, liver and brain) they follow the same particular pattern and is driven by various chemokines which communicate with the cancerous cells by binding to chemokine receptors expressed by tumour cells eventually guiding them to a suitable environment where they can establish a colony (Harrington).

1.1.2.7 Reprogramming energy metabolism

In the latest review by Hanahan and Weinberg they have incorporated reprogramming of energy metabolism as a hallmark of cancer (Douglas Hanahan and Weinberg). According to which, cancer cells requires a tailoring of the glucose metabolism for their limitless proliferation. Normal cells in oxygenated conditions metabolize glucose by normal oxidative phosphorylation, to yield pyruvate which is finally metabolized in mitochondria to generate carbon dioxide and water. However cancerous cells, even under aerobic conditions, tend to switch their metabolism to aerobic glycolysis (Warburg effect) (Warburg). This change has been shown to be associated with various activated oncogenes (viz., RAS, MYC) and aberrant tumor suppressor genes (viz., TP53) (DeBerardinis et al.; Jones and Thompson). This change allows the cancerous cells to deflect the glycolytic intermediates into various biosynthetic pathways which, eventually increases the biosynthesis of the macromolecules and organelles required for rapidly dividing cells.

1.1.2.8 Evading immune destruction

The role of immune system in progression of cancer is still poorly understood. The immune system keeps a constant vigil of the aberrant cells as per the theory of immune
surveillance. The most relevant evidence implicating the role of immune system in carcinogenesis comes from the fact that prolonged immunosuppression is associated with increased incidence of cancer (Vajdic and van Leeuwen) Additionally, several reports suggest that tumor grows more vigorously and frequently in immunosuppressed mice as compared to normal mice. Thus carcinogenesis can be attributed to the failure of the immune system to spot and destroy the aberrant cells. In few tumors, cancer cells through immuno-editing become less immunogenic by altering the antigenic profile on its surface, thereby evading the immune system and allowing the cancers to develop and metastasize (Mougiakakos et al.)

1.1.2.9 Genomic instability

Genomic instability is one of the two enabling characteristics defined by Hanahan and Weinberg. Genomic instability is defined as a state where the genome loses its integrity allowing a number of mutations which eventually alters it constitutively and directs it toward the hallmarks of cancer explained earlier (Douglas Hanahan and Weinberg).

1.1.2.10 Inflammation

Inflammation is the second of the two enabling characteristics defined by Hanahan and Weinberg. Inflammation is the beginning of any disease. In cancer, due to inflammation various inflammatory mediators are recruited which ultimately promotes carcinogenesis by creating an environment conducive for tumor growth (Douglas Hanahan and Weinberg).

1.2 Cellular heterogeneity in cancer

Initially tumor was believed to be a mass of homogenous cells possessing abnormal and deregulated growth characteristics. But advancement in the understanding of cancer cell biology annulled this initial theory as the tumor was found to be composed of a mass of heterogenous population (Reya et al.; Alison, Islam, and Wright; Friedman and Gillespie). This tumor heterogeneity lies at several levels viz., cell size, morphology, membrane receptor expression, their ability to differentiate, proliferate, metastasize and sensitivity to chemotherapy. Tumor heterogeneity creates many problems in deciding the treatment strategy as the tumor specimen collected after biopsy for analysis, may not represent the whole tumor (Beerenwinkel, Greenman, and Lagergren). Unravelling the molecular events leading to this heterogeneity could lead to a major breakthrough for cancer treatment.

The intratumor heterogeneity is explained by two major theories viz., Classical model (Clonal evolution model) and Cancer stem cell model.
1.2.1 Clonal evolution model

This model describes carcinogenesis to be an event involving sequential accumulation of mutations and then gradual selection of these clones (Figure 1.2) (Caldas; Fulda and Pervaiz). Several pieces of evidence indicate, that tumorigenesis in humans is attributed to the stepwise accumulation of multiple mutational ‘assaults’ on a cell. These assaults induces genetic instability ultimately deregulating the essential components of cellular growth balance and drives the progressive transformation of normal human cells into highly malignant derivatives (Nowell).

Figure 1.2 Schematic representation of tumor heterogeneity by clonal evolution model.

The first mutation in a cell would result in limited expansion of the progeny of a single cell. Later, one of these cells might acquire a second mutation to form a small benign tumor. One cell in this benign tumor would then acquire a third mutation, generate its sister cells and form a more advanced tumor composed of generation of cells with three mutations. If eventually, the cell accumulates a sufficient number of hits to become malignant, it would invade surrounding tissues and metastasize to other organs (Rajagopalan et al.). Majority of the therapies were prepared keeping clonal evolution model in mind, which eventually led to their failures (Clarke and Becker).

1.2.2 Cancer stem cell model

Due to the failure of many therapies to treat cancer, a new hypothesis was proposed that there has to be a certain population of cell within the tumor capable of self-renewal, growth and
metastasis. The tumor initiating property and its further maintenance amongst the mass of this heterogenous population lies in very few cells which were called as Cancer Stem Cells (CSCs) (Clarke and Becker; Greaves; Jordan, Guzman, and Noble). The self-renewal and differentiation results in the production of various cell types, leading to tumor heterogeneity. Whereas, other cells forming the bulk of the tumor do not possess unlimited differentiation capacity and cannot divide to form different cell types. The cardinal characteristics of CSCs are given below (Clarke and Becker),

- Self-renewal.
- Heterogeneity (ability to produce multiple cells).
- Resistance to apoptosis.

1.2.2.1 Origin of CSCs

CSCs are produced from normal stem/progenitor cells/differentiated cell of an organ. Normal stem cells/progenitor cell/normal cell can be transformed to CSCs after undergoing mutations in oncogenes, DNA repair genes, tumor suppressor genes and epigenetic mutations (abnormal methylation and histone modifications) (Bonnet and Dick; Huntly and Gilliland; S.C. Li et al.). They constitute a very small fraction of the tumor cellular population. Metastasis of these cells is the main cause for the formation of secondary tumors at distal sites. Tumor recurrence is also the result of high drug resistance shown by these cells to chemotherapy (Latifi et al.). CSC model is explained in Figure 1.3.

![Cancer Stem Cell Model](image)

**Figure 1.3** Schematic representation of tumor heterogeneity by cancer stem cell model.
Rudolf Virchow and Julius Conheim gave the first hypothesis about the existence of CSCs in the 19th century as they observed histological similarity in normal stem cells and CSCs. Furthermore, with the advent of fluorescent activated cell sorting (FACS) it was possible to separate the cells based on its surface properties. In 1997, Bonnet and Dick isolated a subpopulation of cells from acute myeloid leukemia (AML) by the presence of a surface marker CD34 (CD34+) and absence of marker CD38 (CD38-) (Bonnet and Dick; Shaikh, Kala, and Nivsarkar). A small fraction of this subpopulation of cells was able to initiate tumor in non-obese/severe combined immunodeficient (NOD/SCID) mice (Visvader and Lindeman). The discovery of the CD34+/CD38- cell subpopulation gave the proof of existence of CSCs and the beginning of the extensive research on the existence of CSCs in AML and other solid tumors.

1.2.2.2 CSC markers for isolation and characterisation

The CSC population constitutes a small fraction of cells amongst the innumerable tumor cellular population as well as stromal cells. Thus it is necessary to utilise specific markers to selectively isolate CSCs. CSCs currently are isolated based on their difference of the surface properties between CSCs and other tumor cells. Currently many markers such as EpCAM, CD24, CD90, CD133, CD44 and Aldehyde Dehydrogenase1 (ALDH1) (Singh et al.; Ricci-Vitiani et al.; O’Brien et al.; Al-Hajj et al.; J. Zhou et al.; Eramo et al.) are being used for isolation of CSCs. Beginning with the initial work in AML, CSCs now have been characterized in many solid tumors such as liver cancer, lung cancer, breast cancer, brain tumors, pancreatic cancer and melanomas (Fulda and Pervaiz). Some markers used to isolate CSCs along with their brief description are mentioned below.

1.2.2.2.1 CD44

CD44 was originally known as a leukocyte-homing receptor. It constitutes a family of various glycoproteins transcribed by a single gene of various sizes. It serves several functions (pleiotropic factor) viz., cell to extracellular matrix adhesion, tissue remodeling, and cell migration. Hyaluronan (hyaluronic acid), a glycosaminoglycan and a major component of extracellular matrix is a strong ligand for CD44 (Stern). CD44 is being used as a CSC marker not only for AML but also for a variety of cancers viz., pancreas, breast, prostrate, head/neck and bone sarcomas (Marhaba et al.).

1.2.2.2.2 CD133

CD133 is 120 kDa, five trans-membrane antigens implicated in several cancers. Yin et al. first discovered CD133 as a novel marker for human haematopoietic stem and progenitor cells (Yin et al.). Recent studies have shown CD133 to be a specific marker for isolation of
CSCs from a wide range of malignant tumors viz., brain, pancreas, colorectal, breast, ovarian and prostate cancers (Yang and Chang; Visvader and Lindeman; Ahn, Goode, and Simpson; Klonisch et al.). It is expressed in several tissue viz., central nervous system stem cells (Uchida et al.), human lymphatic/vascular endothelial precursor cells (Salven), human trophoblasts (Pötgens et al.), and human prostatic epithelial stem cells (Richardson et al.). In spite of the ongoing research its function still has to be deciphered. It is believed to have a role in stem cell activation pathways and cell-cell adhesion (Yu et al.; Taïeb et al.).

1.2.2.3 Epithelial cell adhesion molecule (EpCAM)

EpCAM over-expression was first found in human colon carcinoma, three decades ago (Herlyn et al.). EpCAM a glycosylated, 30- to 40-kDa membrane protein, is expressed in variety of tissues viz., epithelial tissues, cancers, progenitor and stem cells. Further analysis showed it to be expressed on all major human adenocarcinoma, few squamous cell carcinoma, hepatocellular carcinoma and retinoblastoma (Baueuerle and Gires; Trzpis et al.). EpCAM is also implicated to be a marker for isolation of CSCs in solid tumors as well as normal stem cells and progenitor cells (Gires, Klein, and Baueuerle; Visvader and Lindeman). Investigation into mechanical role of EpCAM in cancer progression, implicated it to be involved in proliferation, metastasis and altered signal transduction (Münz et al.; Osta et al.).

1.2.2.4 Aldehyde dehydrogenase

Aldehyde dehydrogenase (ALDH) enzyme superfamily embodies a variety of enzymes, metabolizing a wide spectrum of exogenous and endogenous aldehydes. ALDH enzyme is responsible for the oxidation of retinol to retinoic acid, which has been implicated in cell proliferation, cell differentiation and stem cell proliferation (Huang et al.). It is found to be expressed in human and murine haematopoietic stem cells (Jones et al.; Armstrong et al.), normal and cancerous human mammary stem cells (Ginestier et al.), murine neural stem cells (Corti et al.), and normal and malignant human colorectal stem cells (Huang et al.; Dylla et al.). These observations strongly propel ALDH expression as a marker for CSCs.

Earlier, ALDH activity was measured by classical methods such as substrate-oxidizing activity of the cell lysate and by using immunoreaction such as Western blot or immunohistochemical analysis. But after the advent of Aldefluor assay, which employs an ALDH activated fluorescent substrate as a marker for isolation of ALDH expressing cells, the isolation and estimation of ALDH expressing cells has been simplified. This assay has been used for isolation and estimation of CSCs form variety of tumors with high ALDH activity (Armstrong et al.; Corti et al.; Dylla et al.; Jiang et al.)
1.2.2.5 OCT4 and NANOG

Pluripotency and self-renewal capacity is the defining feature of any stem cell. OCT4 and NANOG are known to play an important role in the maintenance of these processes for embryonic stem cells (ESCs) (Boiani and Schöler; Nichols et al.). OCT4 is a constituent of Pit-Oct-Unc (POU) transcription factor family, and is expressed normally in totipotent or pluripotent stem cells (Boiani and Schöler; Pesce et al.). NANOG is a downstream target of OCT4 and it helps in determining the cell fate of the pluripotent cell mass during embryonic development (Chambers et al.). NANOG requires the presence of OCT4 for it to function (Cavaleri and Schöler). Recently, OCT4 and NANOG expression has been shown in various cancers viz., breast, testicular, pancreatic and bladder cancer (Gidekel et al.; Jin et al.; Monk and Holding; P. Wang et al.; Wen et al.). OCT4 over-expression is shown in breast CSCs implicating it in self-renewal and tumorigenesis (Ponti et al.). NANOG over-expression was found in colorectal tumor and was associated with lymph node metastasis and poor prognosis (Meng et al.). Recently, there is a growing body of evidence indicating crosstalk between stemness pathway, metastasis and tumor progression. However, its functional and mechanical significance still remains to be understood.

1.2.2.6 Side population

Initially, hoechst 33342 dye-effluxing side population (SP) cells from mice bone marrow were found to be haematopoietic stem cells (Goodell et al.). Since then, SP cells possessing stem cell-like properties and capabilities have been discovered in variety of human haematologic and solid malignancies. These cells show characteristic features of CSCs viz., self-renewal activity, ability to produce differentiated progeny, tumorigenicity, expression of CSC markers and genes (Wu and Alman). Thus, the growing evidence implicates SP cells to be CSCs. Additionally, SP cells are resistant to chemotherapy and associated with tumor recurrence (Wu and Alman; Hirschmann-Jax, Foster, Wulf, Nuchtern, et al.; Hirschmann-Jax, Foster, Wulf, Goodell, et al.). ABCG2 gene, a constituent of the ATP binding cassette (ABC) transporter superfamily, is overexpressed in SP cells and could serve as a marker for CSCs (S. Zhou et al.). This subpopulation is generally isolated by FACS using an ultraviolet (UV) laser.

However, Wu and Alman have pointed out several problems in using the SP phenotype as a CSC marker (Wu and Alman):

- Cells that are naturally resistant to Hoechst dye toxicity will not consist only of stem-like cells
• Many, variables *viz.*, difference in staining times, dye concentration, and cellular density vastly affects the SP percentage and property
• Gating strategies are not well defined to provide with consistent results.

These problems would potentially cause cross contamination of the SP with non-SP cells, eventually resulting in ambiguity in data generated. Hence more stringent gating strategies along with the other methods could give better results.

1.2.2.7 *In-vitro* and *in-vivo* assays

Several in-vitro assays have been used for isolation and characterisation of CSCs *viz.*, sphere formation assay, colony formation assay and label retention assay (Clarke *et al.*; Yang and Chang). However, these assay face limitations such as selection pressure that the testing environment exerts on the cells allowing only those cells with particular characteristic to survive and limitation of determining the clonality (single-cell origin) (Clarke *et al.*). Hence, these assays needs to be combined with assays such as *in-vivo* tumor xenograft which is considered as a gold standard for testing CSCs (Schatton and Frank).

1.2.2.8 Newer strategies

Surface based isolation requires costly antibodies and sophisticated instrumentation. Recently new approaches are being developed to isolate/enrich CSC subpopulation, wherein gradual increasing dose/ single dose of chemotherapeutic agents are employed to destroy the bulk of the cancer cells only allowing drug-resistant cancer stem-like cells to survive (DCSLCs) (Hamilton and Olszewski). This approach exploits the drug resistant property of CSCs to selectively enrich them. This was shown in chemotherapy based enriched/selected cells of lung cancer cell lines which displayed CSC features such as spheroid formation, stem cell specific markers, self-renewal and differentiation capacity, high tumorigenicity and metastatic potential (Barr *et al.*)

1.3 Conventional cancer therapies

In spite of the threat that cancer poses, the results showed by conventional treatment strategies is rather poor. Conventional therapies currently used are surgery, chemotherapy and radiotherapy. These strategies have met with several roadblocks such as resistance, metastasis, tumor recurrence and innumerable side effects. Moreover, surgery could only be used for solid tumors, hence for remaining cancers the only viable options left are chemotherapy and radiation therapy. Additionally, chemotherapy and radiotherapy only act on rapidly dividing cells including the normal cells which causes many side effects. Due to their non-specificity they
result in significant morbidity in patients undergoing chemo and radiotherapy (Freeman and Mayhew; Joshi et al.).

Various molecules such as doxorubicin (DOX), paclitaxel (PTX), vincristine, cisplatin etc. are currently being used for the treatment of various cancers. These molecules show their activity through various pathways such as intercalation in the DNA, DNA methylation and tubulin stabilization. In spite of the promising activity shown by some of these molecules, their non-specific activity and developed resistance greatly limits their use. They show side effects like immunosuppression, nausea, vomiting, alopecia and stomatitis (Burish and Tope).

In radiotherapy, ionizing radiation interacts with the biological components of the cells viz., lipids, proteins, and nucleic acids. Radiation energy breaks the chemical bonds holding the molecules together especially the DNA molecules, wherein they elicit a series of DNA double stranded breaks. Consequently, unrepaired DNA double strand breaks can cause chromosomal anomaly, loss of genetic material eventually causing cell death (Prise and O’Sullivan). Even this therapy lacks specificity and affects both normal and cancerous cells. Major side effects seen with radiation therapy are vascular stenosis, pneumonitis, delayed wound healing, induration of the skin, hair loss, bowel problems, myocarditis and pulmonary fibrosis.

Surgical resection involves careful excision of the cancerous tissue followed by chemo or radiation therapy, hence eliminating the cancer completely. This procedure too faces the limitation of relapse as complete removal of the affected tissue is difficult (Uramoto and Tanaka). Hence, the unresected tissue or cell can grow again when conducive conditions are restored, causing relapse. Additionally, the patient also has to undergo physical and mental trauma due to surgery which causes distress and depression (Golden-Kreutz and Andersen). Recently, studies showing correlation between surgical resection and tumor recurrence along with aggressive metastasis have been published (Freedman and Fowble).

In light of these observations and studies there is a need for advanced and targeted therapies which could circumvent these side effects, target the culprit cells only, reduce the stress/morbidity associated and also prevent relapse and recurrence. Recent studies have implicated the CSCs for majority of the problems faced during cancer treatment. Current studies indicate that the CSCs survive the standard chemo and radiation treatment and persist later on (Dean, Fojo, and Bates; X. Li et al.). CSCs are also known to be more tumorigenic and highly invasive and thus implicated for tumor recurrence as well as metastasis (LaBarge). Therefore, eliminating CSCs could be an efficient strategy for cancer treatment (Lacerda, Pusztai, and Woodward; LaBarge; Z. Wang et al.).
1.4 CSC targeted therapies

CSCs are distinct subpopulation in tumor cell mass possessing distinct characteristics such as self-renewal, differentiation capacity, tumorigenicity, drug resistance and high invasive capacities (Chen, Huang, and Chen). Failure of conventional therapies have been largely attributed to presence of CSCs and a need to generate therapies targeting them is emphasized (Lacerda, Pusztai, and Woodward; Chen, Huang, and Chen).

Extensive research is going on to design specific therapies for elimination of CSCs. Along with the identification of specific CSC characteristics, questions are being raised on the viability of these characteristics to be exploited as potential targets. There has been a paradigm shift in the research of CSCs where the current focus is to identify the features which make them different from bulk of the tumors. Therapies such as those targeting the key signalling pathways, differentiation therapy, immunotherapy, targeting the tumor microenvironment, exploiting the metabolic differences and developing nanocarriers that would deliver the drug load directly to the CSCs are being investigated. These strategies are explained briefly below (Figure 1.4):

![Figure 1.4 Venn diagram representing various CSC targeting strategies.](image)

1.4.1 Targeting signalling pathways

Several key signalling cascades are involved in conferring the characteristic properties to the CSCs. Hence, targeting them could be an efficient and specific strategy. Several pathways
such as Wnt, Hedgehog (Hh) and Notch signalling pathways play a key role in self renewal capacity of CSCs.

Wnt signalling pathway has been shown as an essential component in maintenance of CSCs of tumors such as melanoma, breast, colon, liver and lung cancers. β-Catenin a key mediator in Wnt signalling is known to play a key role in cell-cell adhesion, epithelial-mesenchymal transition (EMT) and metastasis (Zhao et al.). Cells possessing activated Wnt pathway demonstrate similarity to embryonic stem cells (ESCs) and thus directly linked to stemness (Kalluri and Weinberg). Molecules such as nonsteroidal anti-inflammatory drugs (NSAIDs) and vitamin A/D are known to inhibit this pathway, whereas several new molecules and short interfering RNAs (si RNA) are in clinical trials (Takahashi-Yanaga and Kahn; Hu and Fu).

Hh pathway was first described in drosophila and was shown to regulate the differentiation, proliferation and migration of the target cells (Tabata, Eaton, and Kornberg; Nüsslein-Volhard and Wieschaus). Many studies implicating the role of Hh in regulating the CSCs in breast, prostrate, chronic myeloid leukaemia (CML) and glioblastoma have been demonstrated (Hu and Fu). Hh pathway conveys the intracellular signals via smoothended. The discovery of cyclopamine as an Hh pathway inhibitor has led to the investigation of several lead compound which are in clinical trials (Hu and Fu).

Another pathway namely Notch signalling pathway is highly conserved pathway, and plays a key role in maintenance of CSCs in breast cancers, glioblastoma and some other cancers. In a study, down-regulating Notch pathway led to the reduction in CSCs as well as increased sensitivity to radiation (Hovinga et al.). Another exciting strategy could be targeting the drug efflux transporters (ABC transorters) either by directly inhibiting them or by targeted therapies.

1.4.2 Differentiation therapy

Differentiation therapy is another approach specifically targeting CSCs. CSCs generally are locked into undifferentiated state, their self-renewal capacity and differentiation potential makes them highly tumorigenic (Chen et al.). Hence, removing this block and forcing them back to differentiated rapidly dividing cells, in combination with chemotherapy could eliminate them and also reduce the chance of tumor recurrence (Chen et al.). The first success using this strategy was seen when all-trans retinoic acid (ATRA) was used in the treatment for acute promyelocytic leukaemia. ATRA caused leukaemic promyelocytes to differentiate and generate rapidly dividing graulocytes (Pattabiraman and Weinberg). The success of this strategy has led
to the hypothesis that differentiation therapy could be used to treat other cancers. In case of CSCs, these therapy would induce their exit from the CSC state into more differentiated or epithelial state of other cancerous cells. Tang et al was the first to show that bone morphogenic protein (BMP) forces CD133+ cells to undergo differentiation resulting into non-CSCs (Tang, Ang, and Pervaiz).

1.4.3 Immunotherapy

Last decade has seen a renewed interest in the concept of directing immune system of the affected patient against the cancerous cell using tumor associated antigen. Two main strategies viz., use of target specific peptides isolated from tumor antigens as a cancer vaccine and blocking the immune evasive mechanism inherent to cancer cells that obstructs the anti-tumor immune responses. These strategies could add another dimension in targeting CSCs by exploiting the cell surface antigens specifically expressed by CSCs. As CSCs are known to express specific markers, this characteristic feature could be exploited to survey tumor surface antigens that are exclusively expressed on the CSCs as compared to the majority of cancer cells to preferentially design stem cell-specific immunotherapies (Pattabiraman and Weinberg).

1.4.4 Targeting tumor microenvironment

Targeting CSCs directly is one major strategy for destroying these cells and eventually the tumor they support. However, alternative strategies based on the fervently growing knowledge of the tumor microenvironment and its role in activation of EMT program in cancerous cells transforming them to stem cell state could be an interesting approach (Pattabiraman and Weinberg). Heterotypic signals emitted from the stroma is responsible for this transformation. Fibroblasts, adipocytes, myofibroblasts, mesenchymal stem cells (MSCs) and infiltrating immune cells are the chief signal emitting cells. Several studies supporting this hypothesis have been carried out. The findings suggest secretion of cytokines (IL-6 and PGE₂) and growth factors by these cells activate pathways such as Wnt signalling pathway and JAK-STAT pathway which helps in maintaining the stem cell or by conferring them with stem-like properties (Pattabiraman and Weinberg; Marotta et al.; Yang et al.). This strategy is still in nascent stage and researchers are working on finding new targets and developing new molecules for the same.

1.4.5 Nanoparticle platforms for targeted delivery to CSCs

Nanoparticulate drug delivery system offers several advantages over conventional drug delivery such as improved bioavailability, ability to bypass first-pass metabolism, provide
sustained release thereby avoiding peaks and troughs seen with multiple dosing, reduced toxicity, efficient tailoring of the drugs with narrow therapeutic window, efficient targeting and improved patient compliance (Kayser, Lemke, and Hernández-Trejo). Additionally, the new generation of nanoparticles (NPs) are multifunctional i.e. they are loaded with the active compound and attached with targeting moieties, in several cases they even carry imaging agents that permit the visualization using various technologies (X-rays). These “theranostic” (therapeutic and diagnostic) devices promise to transform the cancer treatment completely (Burke et al.).

The targeted delivery systems can target the tumor site using two strategies viz., passive targeting and active targeting (Figure 1.5). The passive targeting occurs by drainage of NPs at the tumor site through the leaky vasculature and localisation for longer period due to inefficient drainage. This effect is called as Enhanced Permeability Retention (EPR) effect (Danhier et al.). Whereas, for active targeting the nanosystem is attached to a targeting moiety (antibodies, ligands, aptamers) specifically targeted to the receptors expressed selectively on the targeted cell (Thorpe; Molema, Meijer, and De Leij).

CSCs possess a distinct signature of the surface receptors that could be exploited by attaching antibodies or ligands complementary to receptors on the nanocarriers. Alshaer et al described a DOX loaded CD44 conjugated liposome targeting aggressive CSCs of hepatocellular carcinoma (HCC). The delivery system resulted in higher drug accumulation in
the cells in comparison to free drug (Alshaer et al.). In another study NIR-absorbing gold plated single-walled carbon nanotubes conjugated to CD44/ folic acid showed rapid uptake by circulating CSCs, which could be destroyed by irradiation (Galanzha et al.; Kim et al.).

Since, these approaches are costly as it employs costly antibodies, there is a need to identify cheaper alternatives. Research should be focused on identification of novel targets, which could be targeted by cheaper ligands. Recently, glucose transporter 1 (GLUT1) was found to be overexpressed on CSCs (Keith and Simon). GLUT1 is coded by the SLC2A1 gene (Mueckler et al.). Its main function is to transport glucose across the cell membrane (Olson and Pessin). Glucosamine (GLN) is a strong ligand for GLUT1 receptor (Zhao and Keating). It is cheap and easily available and could be used for CSC targeting.

In spite of several advantages, these nanosystems however come with new set of problems not encountered previously, viz., mitochondrial damage, DNA damage, cardiovascular effects, and platelet aggregation (De Jong and Borm; Alkilany and Murphy). The NPs in contact with the biological microenvironment tend to absorb proteins, which could alter their physicochemical properties and behave differently in the *in-vivo* environment (Figure 1.6). NPs could also cause protein aggregation and amyloid formation which is the basis of many diseases (Cedervall et al.).

![Figure 1.6 Schematic representation of the difference in the stability of nanosystems between in-vitro testing environment and biological milieu.](image)

Nanotoxicology, overall has been an unexplored area, but some studies underlining the toxicity caused by the NPs have been reported. Platelet aggregation was reported due to the use
of carbon nanotubes (Radomski et al.). Furthermore, NPs (cerium oxide particles, polymeric NPs, and quantum dots) were reported to assist in the nucleation process preceding amyloid formation (Cedervall et al.; Sayes et al.). In light of these studies, there is a need for more stringent in-vivo testing for the nanosystems formulated (Scientific Committee on Emerging and Newly-Identified Health Risks).

1.5 Hypothesis

Cancer is one of the leading causes of death seen in mankind. The complexity owing to which cancer has largely remained unconquered could be explained using various models amongst which CSC model has managed to answer all the questions asked. Targeting CSCs could be an answer to the various problems associated with cancer treatment. However, isolating CSCs is another major problem. Enriching DCSLCs by gradually increasing the dose of the chemotherapy could kill all the other cells leaving DCSLCs behind. These, DCSLCs could be used for further studies. GLUT1 receptor is known to overexpress in CSCs, which could be targeted using GLN decorated poly (lactic-co-glycolic acid) (PLGA) NPs. These NPs would destroy the DCSLCs thereby reducing or alleviating the problem of multi drug resistance, relapse and metastasis, which is largely attributed to the CSCs.

1.6 Objectives of the study

- Preparation and characterization of glucosamine coated doxorubicin loaded PLGA nanoparticles (GLN-DOX-PLGA-NPs) and glucosamine coated paclitaxel loaded PLGA nanoparticles (GLN-PTX-PLGA-NPs).
- Enrichment of DCSLCs using gradually increasing dose of DOX.
- Characterization of enriched DCSLCs using various assays used to identify CSCs
- In-vitro and in-vivo activity of the formulations on the enriched DCSLCs.

1.7 Significance of the study

CSCs have been found to be responsible for the continuous generation of the cancerous cells and hence the therapies which target the cancer cells and not the CSCs sub population have proven to be largely ineffective. Side effects caused by the anticancer drugs, is another major problem faced by chemotherapy. Hence, there is a need to develop a delivery system which not only delivers the drug locally at the tumor site but also possess the property of interaction with the cancer cells and cancer stem cells. The possible outcomes of this study would be:
Enrichment of DCSLCs using chemotherapy could give a cheap and an easy strategy to isolate/enrich DCSLCs that could be used for further analysis.

GLN conjugated NPs would reduce the DCSLCs population, which may lead to an efficient strategy for treating cancer.

The targeted drug delivery system is actively targeting the tumor cells specifically and hence may reduce the interaction of the drug-loaded NPs with the normal cells reducing the adverse effects of the chemotherapy.

This delivery system may lay a strong foundation for designing therapies targeting CSCs.

1.8 Proposed plan of work

The research work principally involved was the preparation of GLN-DOX-PLGA-NPs and GLN-PTX-PLGA-NPs using Box-Behnken Design (BBD) their characterization by dynamic light scattering (DLS), fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and transmission electron microscopy (TEM). DCSLCs were enriched by gradually increasing the dose of DOX over a period of four months and were characterized using sphere formation assay, soft agar colony formation assay, quantitative gene expression using polymerase chain reaction (PCR), FACS analysis and tumor xenograft development. The enriched cells were subjected to in-vitro cell viability assay and cell uptake assay. The mechanistic study of the cell uptake was also undertaken. Finally, in-vivo study of the formulation was done in the tumor xenograft model and in-vivo biodistribution was also carried out. A brief outline of the work undertaken is mentioned below:

1) Formulation development
   a) Preparation and optimization of doxorubicin loaded PLGA nanoparticles (DOX-PLGA-NPs).
   b) Preparation and optimization of paclitaxel loaded PLGA nanoparticles (PTX-PLGA-NPs).
   c) Covalent attachment of GLN on the surface of NPs and its characterization.
   d) Development of ex-vivo stability model.

2) In-vitro work
   a) DOX mediated enrichment and characterization of DCSLCs from two cell lines (A549 and HepG2).
   b) Characterization of DCSLCs for cancer stem-like property.
   c) In-vitro activity in DCSLCs and cell uptake study.
3) *In-vivo* work
   
   a) Development of tumor xenograft model.
   
   b) *In-vivo* activity and biodistribution study of the developed formulation.

### 1.9 Outline of the thesis

The thesis comprises of 5 chapters including introduction (chapter 1), review of literature (Chapter 2), materials and methods (Chapter 3), results and discussion (Chapter 4) and summary and conclusion (Chapter 5). Chapter 1 and 2 discusses the reviewed literature about isolation of CSCs, targeted delivery systems available for CSCs, development of tumor xenograft model and ex-vivo stability study model. The chapter 3 summarizes the materials and methods of the experimentation undertaken during the research work. The results of the experiments are discussed in chapter 4. The summary of the research work and conclusions drawn are mentioned in the chapter 5.