General Introduction
Cancer: An overview

Tumorigenesis is a multistep process, and these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (Hanahan and Weinberg, 2000). In response to environmental cues, the normal cells in a multi-cellular organism are genetically programmed to grow, divide, and differentiate to maintain the tissue homeostasis and eventually die over time. In contrast to this cancer cells acquire successive genetic mutations which make them disrupt all the cell-cycle quality controls, and undergo limitless proliferation. A human body that is composed more than $10^{14}$ cells; billions of cells experience mutation every day and potentially disrupts all such cell-cycle checkpoint controls. Therefore, mutation of somatic cells may offer a selective advantage, allowing it to divide more vigorously than its neighbors and to become the founder of the mutant clone. Repeated rounds of mutation, competition and natural selection of somatic cells cause matters to go from bad to worse (Sottoriva et al., 2015). Cancer cells have two defined heritable properties: - i) limitless cell proliferation, a relentlessly growing mass of abnormal cells that disregard normal restraints of cell division and ii) invasion and colonize into the territories that is normally reserved for other normal cells (Fidler, 2003). The limitless proliferation potential of mutant cells break normal cell division check-points to become derailed and subsequently establishes a tumor or neoplasm. The tumor is said to be benign where neoplastic cells remain clustered together in a single mass. At this stage, complete cure can be possible by removing the mass surgically. A tumor is said to be cancerous when it becomes malignant. The abnormal mutant cells metastasize through the blood-stream and lymphatic tissue and eventually spread to invade surrounding tissues. The tissue micro-environment in a developing tumor is comprised of proliferating tumor cells, the stromal tissue, infiltrating immune cells and a variety of other tissue cells (Figure-3). A unique micro-environment emerges in the course of tumor progression as a result of its interactions with the host. Immune cells present in the tumor include those mediating adaptive immunity, T lymphocytes, dendritic cells (DC) and occasional B cells, as well as effectors of innate immunity, macrophages, polymorphonuclear leukocytes and natural killer (NK) cells (Whiteside, 2008). Malignancy
refers to a condition where proteins are uncontrollably over-expressed or structurally destabilized, e.g., p53. The tumor suppressor p53 functions as a key regulator of cell-cycle machinery, which is found to be mutated in 50% of reported human tumors. p53, the guardian of the genome, executes its tumor suppressor function through maintenance of the genetic integrity, cell-cycle machinery, apoptosis and DNA repair. To check genetic errors, p53 accumulates in the nucleus in response to cellular stress like DNA damage, hypoxia, and nucleotide deprivation. Once p53 is transported into the nucleus, it trans-activates its target genes, involved in either in cell-cycle arrest (e.g., p21, 14-3-3) or apoptosis (e.g., BAX, PUMA, NOXA). Single point mutations in p53 mostly cluster within the DNA-Binding domain (DBD) of p53. Mutations that affect p53 3D-structure often show altered sub-cellular localization or have a tendency to aggregate within the cytoplasm. Dominant-negative activity and gain-of-function of structurally destabilized mutant p53 arises from their increased aggregation propensity. Six hallmarks of cancer together constitute an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases. Next, we will focus on both the conventional hallmark features of malignant cells as well as the upcoming theories on emerging traits of cancer (Figure-1).

**Hallmarks of cancer:**

Recently it was suggested that malignant cells share some characteristic traits (hallmarks) which are responsible for the transformation of normal cells into cancer cells (Hanahan and Weinberg, 2011). These distinctive and complementary traits contribute to tumor growth and metastatic dissemination. The hallmarks of cancer comprise six biological capabilities acquired during multi-step development of human tumors.
Figure-1: The six hallmarks of cancer. The six hallmarks proposed by Hanahan and Weinberg in the first decade of cancer research (Hanahan and Weinberg, 2000). Distinctive and complementary capabilities that enable tumor growth and metastatic dissemination—continue to provide a solid foundation for understanding the biology of cancer.

They include the following:

1. **Sustaining proliferative signaling:**

   The chronic proliferation of malignant cells is the most prominent of all the fundamental traits associated with cancer. Normal cells have a fine-tuned mechanism for production and release of growth promoting signals which monitor the progression of cells through their cell growth and division cycles. Cell cycle checkpoints and regulatory proteins, cyclins and CDKs (cyclin-dependent kinases) are responsible for maintaining the timely division of normal tissues. This ensures a homeostasis of cell number and thus maintains normal tissue architecture and function. Cancer cells become the master of their destinies through deregulation of these signals (Hanahan and Weinberg, 2011). These sustaining signals are conveyed through the growth factors that bind cell-surface receptors containing tyrosine kinase activity, which results in proliferation of cancer cells relentlessly (Lemmon and Schlessinger, 2010). This process can be initiated by alternative mechanisms:

   - Cancer cells can acquire the ability to produce the growth factors themselves.
   - Cancer cells might send signals to the neighboring tumor-associated stroma, which in turn reciprocate through the supply of growth factors to the cancer cells.
2. Evading growth suppressors:

In addition to inducing and sustaining growth stimulatory signals, cancer cells also adopt strategies to avoid programs that negatively regulate cell proliferation. Several tumor suppressor genes have been identified that are responsible for limiting cell growth and proliferation. The two most crucial tumor suppressor proteins are RB and TP53 that control cell proliferation, apoptosis, and senescence program. These two proteins operate as central control nodes which govern the decisions of cells whether to proliferate, or go for either senescence or apoptosis. Abnormalities in tumor suppressor genes enable cancer cell to proliferate limitlessly (Burkhart and Sage, 2008; Deshpande et al., 2005).

3. Resisting cell death:

Programmed cell death or apoptosis serves as a natural barrier toward the development of malignant growth. The early work by Hayflick suggested that a normal cell can undergo a finite number of cell-division cycles, i.e. it has a finite replicative potential (Hayflick, 1997). Once such populations of normal cells exceed a certain number of cell division cycles, they enter a quiescent stage called ‘senescence.’ The cells no longer divide once senescence ensues. Fas ligands/receptors of the extrinsic apoptotic pathway and downstream proteases (caspases) of intrinsic apoptotic pathway compose the apoptotic machinery. The apoptotic trigger that sends signals to regulators and effectors of apoptotic machinery, works by counterbalancing pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins. Tumor cells exploit several strategies to resist cell death. Most common is the loss of TP53 function that eliminates the critical damage sensors from the apoptosis-inducing circuitry.

4. Enabling replicative immortality:

Cancer cells require unlimited replicative potential to form macroscopic tumors. In contrast to normal cell lineages in the body, the cancer cells in the tumor mass undergo an unlimited number of successive cell growth and division cycles. In normal tissues, this limitation in cell-cycle progression is achieved through two distinct barriers, senescence- an irreversible entrance of cells into non-proliferative but viable condition, and programmed cell death. Most of the established cancer cell lines possess this characteristic trait by their capability to proliferate in culture without proceeding either to senescence or apoptosis.
Multiple lines of evidence suggest that the telomeres protecting the ends of the chromosomes are directly linked with the ability of cancer cells to attain unlimited replicative potential. Telomerase, the specialized DNA polymerase, which adds hexanucleotide repeats at the chromosome ends, remains almost absent in non-immortalized cells. On the contrary, cancer cells express significantly higher levels of telomerase, which enables the cancer cells to encounter progressive telomeric erosion, hence inducing immortalization.

5. Inducing angiogenesis:

Tumor tissues have high demands of nutrients and oxygen to sustain continuous neoplastic growth. To support this, endothelial cells proliferate and assemble into tubes (vasculogenesis), forming tumor-associated neovasculature by the process of angiogenesis. In addition to this, the sprouting of new blood vessels from the existing ones also accelerates the blood supply to the tumor tissues, meeting their high metabolic demand. During tumor progression ‘angiogenic switch’ remains always turned on, causing normally quiescent blood vasculature to continuously sprout new vessels to maintain malignant growths (Hanahan and Folkman, 1996). It has been suggested that angiogenic switch is governed by countervailing factors that either induce or oppose angiogenesis. Vascular endothelial growth factors (VEGFs) play the central role in inducing angiogenesis. VEGF gene expression is up-regulated by both hypoxia and oncogenic signaling. Other pro-angiogenic proteins, such as members of the fibroblast growth factor (FGF) also contribute to tumor angiogenesis. Interaction of these growth factors with their respective receptors leads to activation of pro-angiogenic and pro-invasive matrix degrading enzymes, e.g. MMP-9.

6. Activating invasion and metastasis:

Carcinomas arising from epithelial tissues progressed to higher pathological grades, often show local invasion and distant metastasis. The metastasis process begins with local invasion, followed by intravasation of cancer cells into the nearby blood and lymphatic vessels, then the transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lamina of such vessels into the parenchyma of distant tissues (extravasation). These lead to the formation of small nodules of cancer cells
(micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors. This last step is often being termed as “colonization”. During the progression of cancer, a developmental regulatory program, referred to as the “epithelial-mesenchymal transition” (EMT) often took place which transforms epithelial cells with mesenchymal features. These transformed cells then acquire the abilities to invade, to resist apoptosis, and to disseminate or metastasize (Klymkowsky and Savagner, 2009; Polyak and Weinberg, 2009). This is associated with the alterations in the shape of the cancer cells and their attachment to the neighboring cells and ECM. Loss of E-cadherin, a key cell-to-cell adhesion molecule, in cancer cells is the best-characterized alteration contributing to invasion and metastasis. Cancer therapeutics that is designed to target adhesion receptors and proteases has not been proven to be successful in slowing tumor progression in clinical trials. The reason is being that cancer cells adopt diverse strategies to modify their migration mechanisms in response to different conditions.

7. **Conceptual progress of hallmarks capabilities:**

Although these above discussed six hallmarks of cancer have been found to be an integral part associated with most forms of cancer, a conceptual progress of understanding the same has added more complexity in the last decade. An increasing body of research suggests that two additional hallmarks of cancer are intricately associated in the pathogenesis of some and perhaps all cancers (Figure-2). One involves the capability to reprogram cellular metabolism to most effectively support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. Additionally, two consequential characteristics of malignancy facilitate the acquisition of both core and emerging hallmarks, i.e. genomic instability and tumor-promoting inflammation. Next, we will focus on the emerging hallmark, genomic instability, and mutation, so as to decipher the contribution of p53 structure and function in the progression of cancer.

8. **An enabling characteristic: Genome instability and mutation**

Genomic instability and thus mutability endow cancer cells with genetic alterations that drive tumor progression. Genome maintenance systems detect and resolve DNA defects to ensure that the rates of spontaneous mutations remain low during each cell division cycle.
Cancer cells often accelerate the rates of mutation which further orchestrates tumor development. Genomic instability in cancer cells contributes to random mutations including chromosomal re-arrangement; among these there are rare genetic changes which orchestrate hallmark capabilities. This enhanced mutability is achieved through breakdown of one or more components of genomic maintenance machinery or increased sensitivity to mutagenic agents.

**Figure-2:** The emerging and enabling hallmarks of cancer, proposed by Hanahan and Weinberg in the first decade of cancer research (Hanahan and Weinberg, 2011).

Accumulation of successive mutations are also accelerated through compromising the surveillance systems that continuously monitor the genomic integrity and induce genetically damaged cells for either senescence or apoptosis. A diverse array of defects affecting various components of the DNA-maintenance machinery– often referred to as the “caretakers”– have been documented (Kinzler and Vogelstein, 1997). The catalog of defects in these caretaker genes includes those whose products are involved in (1) detecting DNA damage and activating the DNA repair machinery, (2) directly repairing damaged DNA, and (3) inactivating or intercepting mutagenic molecules before they have damaged the DNA (Negrini et al., 2010). TP53 gene here plays the central role, which is often referred to the ‘guardian of genome’ (Lane, 1992). Single point mutations in p53 DNA-binding domain lead to alteration of 3D-structure of p53 as well as its wild-type function. Co-aggregation of
mutant p53 with other tumor suppressors (p63 and p73) in the cytosolic environment results in ‘Gain of oncogenic function’ with ‘loss of wild-type tumor suppressor function’.

**The tumor suppressor p53-the ‘Guardian of genome’**

Tumorigenesis includes a process of acquiring successive genetic mutations that transform a normal cell into a malignant one. During tumorigenesis, deregulated expression of transcription factors promotes proliferation and differentiation of the neoplastic population. Relative expression of these proteins is found to be altered in cancer compared to that of the normal cells. Tumor suppressor protein p53, as discussed earlier, acts as a cell cycle regulator, involved in the maintenance of the genetic integrity. Controlling the cell cycle machinery, apoptosis and DNA repair are the critical activities of the tumor suppressor that elicit an anti-cancer response. Activation of p53 is triggered by events like DNA damage, hypoxia, heat shock and various other stress signals. Depending on the nature of the stress it decides the specific cellular outcome in order to restrict any anomaly at the genetic level. Once activated, conformation of p53 undergoes extensive modifications at both N- and C-terminal regions Post-translational modification of p53 includes (Phosphorylation at serine 6, 9, 15, 18, 20, 33, 37, 46, 315, 376 and at threonine 81, 150, 155; acetylation at Lysine 120, 320, 382; deacetylation at Lysine 370, 372, 373, 381, 382, 386; ubiquitination at Lysine 386). Once p53 is transported into the nucleus, it trans-activates its target genes, involved either in cell-cycle arrest (e.g., p21, 14-3-3) or apoptosis (e.g., BAX, PUMA, NOXA). In unstressed cells, the activity of p53 is blocked by its negative regulator MDM2, which upon binding to N-terminus of p53 targets it for proteasomal degradation. In response to diverse stress stimuli, this inhibition by MDM2 is relieved, functional p53 tetramers are stabilized, transported into the nucleus and trans-activate genes like p21, 14-3-3, MDM2, BAX, PUMA, and NOXA. Under basal conditions, p53 can direct pre-initiation complex assembly to its target genes. Through the recruitment of histone acetyl-transferases (HATs), histone methyl-transferases (HMTs) and other co-regulators of transcription in the Response Elements (REs) of target genes, p53 can unwind closed chromatin and start transcription. Mutations affecting the three-dimensional structure of p53 have been reported to cause aberrant nucleo-cytoplasmic shuttling, cytoplasmic retention or mislocalization, thereby resulting in loss of its tumor suppressor functions. In recent years, p53 was found to have great influence in processes such as cellular differentiation, self-renewal, and plasticity, ensuring a balance between genome stability
and plasticity in normal stem cells. Cancer stem cells (CSCs) arise from the accumulation of genetic insults in normal stem cells or progenitor cells or by dedifferentiation of existing differentiated cells. These CSCs were reported in some human hematological and solid tumors and had been defending by their capability to seed new tumors. It is also to mention that drug resistance and cancer occurrence are largely mediated by cancer stem cells expressing mutant p53 (Shetzer et al., 2014).

**Cancer as an aggregating disease characterized by mutant p53 co-aggregation: upcoming theories on cancer**

New theories are emerging on the development of cancer. During malignancy, proteins are unusually uncontrollably over-expressed or structurally affected due to genetic mutations, ultimately resulting in changes in activity and protein-protein interaction in cancer microenvironment. Detailed molecular investigation of cancer micro-environment has marked cancer as an aggregating disease. The tumor suppressor p53, as mentioned before, found to be mutated in over 50% of all reported human tumors and found to form cytosolic aggregates (Figure-3), making it as an excellent target for anti-cancer therapy. It has been reported that the DNA-binding domain of p53 is conformationally unstable and the majority of the hot-spot mutations (Y220C, R273H, R175H, R282W, R248Q, and R249S), further destabilize the protein structure. Moreover, a proportion of these mutants are at least partially destabilized, often referred to as ‘structural mutants’. According to upcoming reports on mutant p53 co-aggregation, for structurally destabilized p53 mutants, these effects result from mutant-induced coaggregation of wild-type p53 and its paralogs p63 and p73, thereby also inducing a heat-shock response. Reports also suggest that aggregation of mutant p53 arises from self-assembly of aggregation-nucleating sequence, present within the hydrophobic core of p53. In the p53 germline mutation database, tumors carrying aggregation-prone p53 mutations have a significantly lower frequency of wild-type allele loss as compared to tumors harboring non-aggregating mutations, suggesting a difference in clonal selection of aggregating mutants. In the course of mutation, this aggregation-prone hydrophobic sequence gets exposed to the cytosolic environment. The ‘dominant-negative’ and ‘Gain-of-function’ activities stem from the increased aggregation propensity of structural mutants (Xu et al., 2011). Upon its mutation, structurally destabilized mutants not only induces misfolding and co-aggregation of wild-type p53, but also of its paralogs, p63, and p73 into cellular inclusions, causing inefficient transcription of
its target genes that are essential for cell growth control and apoptosis (Xu et al., 2011). Overall, recent theories in cancer study reveal a novel disease mechanism for a mutant p53 gain of function and suggest that, at least in some respects, cancer could be considered an aggregation-associated disease.

**Figure-3**: Cells of the tumor micro-environment, (a) consisting cancer stem cells (CSCs), cancer cells (CCs), cancer-associated fibroblasts (CAF) and Immune-inflammatory cells (upper panel). The distinctive micro-environments of tumors (middle panel). (b) Enabling hallmark of cancer involves genome instability and mutation, which is reflected by cytoplasmic aggregation of p53 structural mutants (lower panel).

**Switching of the character of p53: an enabling hallmark of cancer**

Rousseau and Schymkowitz have discussed in their paper that certain mutations in p53 cause the protein to misfold in a way that the proteins start to aggregate. Hot-spot mutations cause p53 to assume a completely different factor in tumor scenario. From being
an anti-tumor factor, mutant p53 changes its signaling to speed up malignancy. As already discussed, mutant p53 forms aggregates with other tumor suppressors, p63 and p73 in the cancer cell, causing deficient transcription of the target genes involved in cell growth control and apoptosis. Trans-activating isoforms of these two genes have been demonstrated to inhibit tumor metastasis and increase the sensitivity for radiochemotherapy. Though this mechanism involves tetramer-dependent interactions between the DNA-binding domain of p53 and its paralogs, the exact mechanism of interaction remains unexplained. Tumor cells with mutant p53 status hence acquire ‘Gain-of-oncogenic function’ with ‘Loss-of anti-tumorigenic function’. This switch of the character of mutant p53 appears as another enabling hallmark of cancer cells.