HYPOTHESIS

Quercetin may inhibit EGF-mediated signaling molecules involved in cell survival, proliferation, migration and invasion of prostate cancer cells.

AIM OF THE STUDY

To investigate the effect of quercetin on EGF-mediated signaling molecules in human androgen independent Prostate cancer cell line by In vitro studies:

- Molecules involved in cell survival and proliferation, particularly EGFR, PI3K, PDK1, mTOR, AKT, Ras, Raf, ERK1/2, GSK3-β, Cyclin D1, FOXO-1, NFκB, PCNA, Bcl-2, Bax and Caspase-3.

- Molecules involved in EMT, Migration and Invasion mainly E-cadherin, N-cadherin, vimentin, Snail, Slug, Twist, ICAM-1, VCAM-1, P-selectin, E-selectin, MMP-2 and MMP-9.

- To check the effect of quercetin on EGF-induced angiogenesis/capillary tube formation by using HUVEC cells.
2.1.1 GROWTH FACTORS

Mammalian tissue development and regeneration take place within a milieu of growth factors. These affect many features of cell development, such as survival, proliferation, differentiation, and certain aspects of cell behaviour. The responding cell type, the concentration of factor, and the presence of other stimuli determine the precise effect of any given factor, such that some growth factors may fulfil a variety of functions under different circumstances.

Classically, a growth factor stimulus is transmitted into the cell \textit{via} activation of specific, transmembrane receptors that modify key regulatory proteins in the cytoplasm. These in turn affect the decisions controlling proliferation and differentiation, including changes in gene expression and reactivity to other factors. These are indications that some factors may function both extra- and intra-cellularly and that this characteristic is correlate with potential oncogenicity. The relatively low transforming ability of extracellular factors alone is probably attributable to the limitations imposed by down-regulation of their cell surface receptors. Aberrant production of secreted growth factors can however, play decisive roles in tumorigenesis by increasing the proliferation rate and degree of cellular autonomy and extending the area available for tumor expansion (Steiner, 1995).

Growth factors use autocrine or paracrine pathways to signal stromal and epithelial cells in the microenvironment. Growth factors play a significant role in the regulation and growth of normal, hyperplastic and malignant prostatic epithelium. The important ones are the epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), the transforming growth factor-beta (TGFβ), Nerve
growth factor (NGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) family (Steiner, 1995; Hellawell and Brewste, 2002). EGF plays a critical role in PCa progression especially during the transformation of androgen dependent PCa into more aggressive androgen independent PCa. EGF also plays a major role in cancer metastasis. Hence, the role of EGF on PCa was investigated in the present study.

2.1.2 EPIDERMAL GROWTH FACTOR

In 1975, epidermal growth factor (EGF) was found to bind to a cell surface receptor termed the epidermal growth factor receptor, abbreviated as EGFR (Carpenter and Cohen, 1979). TGF-alpha, amphiregulins, heparin-binding EGF is a family of EGF like growth factors. Three similar sequences to EGFR were discovered in the 1980’s by screening cDNA libraries and termed the human epidermal growth factor receptors: HER2, HER3, and HER4 (Gschwind et al., 2004). EGFR, HER2, HER3, and HER4 comprise the epidermal growth factor receptor family.

The epidermal growth factor receptor family members are transmembrane glycoproteins containing an extracellular domain, a transmembrane domain, and a cytoplasmic region containing the juxtamembrane region, the tyrosine kinase domain, and the C-terminal region with tyrosine phosphorylation sites (Figure 1) (Gschwind et al., 2004). EGFR are usually found on epithelial cells and have access to their growth factor ligands from nearby extracellular matrix (Normanno et al., 2006) or through the bloodstream (Ramsauer et al., 2006; Bessman and Lemmon, 2012).
The epidermal growth factor receptors play a central role in development. In worms and flies, a single epidermal growth factor receptor regulates specific events in organogenesis (Bublil and Yarden, 2007). Additional EGFR in vertebrates allow more combinatorial interactions to specify increased signal diversification (Marmor et al., 2004). Gene inactivation in mammals shows that EGFR is need for normal skin, kidney, and GI tract (Bublil and Yarden, 2007). HER2, HER3 and HER4 are involved in heart and brain formation in mice (Zhou and Carpenter, 2002). All EGFR are involved in mammary gland development (Bublil and Yarden, 2007). In adults, EGFR are part of homeostatic systems that control proliferation and differentiation of epithelial tissues (Zhou and Carpenter, 2002).
The epidermal growth factor receptor family is referred to widely as the ErbB/EGFR family due to the similarity of the EGFR protein sequence to that of an avian erythroblastosis viral oncogene v-ErbB. This finding suggested that mutations in the EGFR could transform it into an oncogene (Gschwind et al., 2004). Due to the fundamental role of EGFR/ErbBs in controlling cellular processes, EGFR/ErbB dysregulation can promote cancer. Overexpression of EGFR is found in cancers of the breast, head and neck, lung, brain, prostate (Marmor et al., 2004), and in advanced colorectal cancer (Toffoli et al., 2007). HER2 overexpression is associated with many cancer types, including breast, lung, ovarian, prostate, gastric, oral, kidney, pancreatic, cervical and endometrial cancer (Rabindran, 2005). HER3 is overexpressed in multiple types of cancers and in almost one third of invasive breast carcinomas (Karamouzis and Argiris, 2007). The role of HER4 in cancer is not clear. HER4 overexpression has been observed in some cancers (Zhou and Carpenter, 2002), but the presence of HER4 also correlates with reduced primary breast tumor progression and improved patient prognosis (Vidal et al., 2007).

### 2.1.3 EGFR ACTIVATION

In normal development, EGFR/ErbB family member interactions specify development of many systems in the body. Investigation of the behaviour of deregulated EGFR/ErbB family members due to overexpression or mutation has shown that their interactions promote cancer (Stern, 2003). Early research established that EGF ligand activation of EGFR kinase function results in increased glycolysis, enhanced cell proliferation and cell morphological changes (Carpenter and Cohen, 1979). Overexpression of EGFR in the A431 epidermoid cancer cell line suggested...
that, there might be a link between EGFR activation and the increased cell proliferation, which occurs in cancer cells.

Experiments revealed that activated EGFR correlated with increased phosphorylation (Carpenter and Cohen, 1979). EGFR purified from A431 cells could phosphorylate tyrosine residues in response to EGF stimulation (Ushiro and Cohen, 1980). When EGF stimulated EGFR, EGFR itself became phosphorylated (Yarden and Schlessinger, 1987). Thus, it appeared that activated EGFR could both phosphorylate other proteins and phosphorylate itself.

EGFR activation correlates with its dimerization. EGFR exists predominantly as a dimer when stimulated. EGFR was observed bound to EGF, with the majority present as dimers (Yarden and Schlessinger, 1987). Much research has probed the structural details of receptor dimerization. The EGFR extracellular domain may be sufficient to promote dimerization. EGFR extracellular domain was incubated with radiolabeled EGF and cross-linked, when analyzed by autoradiography, dimers were present (Hurwitz et al., 1991). Dimerization may be important in restraining the potent biological activity of EGFR by requiring the receptor to find a partner prior to signal transduction. Furthermore, an activated receptor may not be able to phosphorylate itself directly. There is evidence that dimer partners transphosphorylate each other on particular C-terminal tyrosine residues (Honegger et al., 1989).

EGFR activation depends on EGF binding, dimerization of EGFR, and EGFR kinase function, resulting in phosphorylation. These three events are targets for EGFR therapeutic interventions. EGFR could dimerize with other ErbBs, not only with itself. EGFR/HER2 dimers was found by treating the human breast carcinoma SKBR3 cell
line with radiolabeled EGF and cross-linking reagent (Goldman et al., 1990). Characterization of the properties of the individual ErbBs revealed differences in structure and function, influencing their ultimate signal transduction capabilities. These differences impact how therapeutics need to be designed to inhibit the individual ErbBs. Unlike the other ErbBs, HER2 does not have a growth factor ligand. HER2 could not be tyrosine phosphorylated in response to a panel of ErbB ligands (Klapper et al., 1999).

A unique attribute of HER3 compared to the other ErbBs is its impaired kinase activity. HER3’s weak kinase activity was observed in ErbB infected Sf9 insect cells (Guy et al., 1994). Supporting a role for HER3 in cancer, data show that the HER2/HER3 dimer pair is prevalent in HER2 overexpressing N87 gastric carcinoma cells (Tzahar et al., 1996), and the breast cancer SKBR3 and BT474 cell lines (Sergina et al., 2007). Another unique characteristic of HER2 is its potent mitogenicity. This finding from basic research is confirmed clinically, in that many cancers overexpress HER2 (Rabindran, 2005). The mitogenicity of HER2 suggests that this ErbB is an important cancer therapeutic target.

The presence of HER2 correlates with mitogenicity of growth factor ligand activated Ba/F3 mouse pro-B-lymphocyte cells. EGFR, HER2, HER3, and HER4 were transfected singly or in pairs into these cells, and growth factor ligand was added to induce tyrosine phosphorylation. Based on trypan blue staining, lines containing HER2 alone, or HER2 with EGFR, HER3, or HER4, survived in the absence of IL-3 (Riese et al., 1995).
2.1.4 EGFR STRUCTURE

The extracellular portion of EGFR was characterized as containing four domains: I (residues 1-165), II (residues 166-309), III (residues 310-481), and IV (residues 482-618) (Ferguson et al., 2003). In the crystal structure containing EGFR extracellular domain bound to EGF, a 2:2 complex was formed in which the two EGF ligands were located on opposite sides of the receptor dimer. EGF bound to Domains I and III within each monomer. The two-receptor molecules were bound through Domain II regions with hand-like structures that made contact in the dimer. The dimerization “arm” encompassed 20 amino acids protruding from Domain II with the seven-residue “hand” at the tip of the arm making contact with the hand of the other dimer partner (Ogiso et al., 2002). A similar crystal structure was obtained with a complex of truncated human EGFR extracellular domain and the ligand human transforming growth factor α, indicating that EGFR binds different ligands using the same mechanism (Garrett et al., 2003). This suggests that a therapeutic agent that blocks ligand binding could be effective against multiple ligands.

In addition to dimerization of extracellular domains, transmembrane and cytoplasmic regions of EGFR may also dimerize, and these additional dimeric contacts may be necessary for the induction of kinase activity. Therefore, therapeutics that inhibits dimerization of transmembrane and cytoplasmic regions may also be an effective way to prevent signal transduction. Since ligand binding appears necessary to induce conformational changes that permit extracellular domains to dimerize, the existence of unliganded dimers is unexpected. Analysis by single wavelength fluorescence cross-correlation spectroscopy and Forster resonance energy transfer (FRET) revealed that preformed unliganded EGFR homodimers, HER2 homodimers,
or EGFR/HER2 heterodimers were present on the cell surface in transfected CHO cells (Liu et al., 2007a). Using FRET and image correlation microscopy, EGFR was found on the surface of A431 cells as clusters of unliganded oligomers (Clayton et al., 2007).

Full length EGFR could form an unliganded homodimer mediated by cytoplasmic contacts. EGFR dimer complexes were detected in EGFR transfected cells that had no EGF stimulation (Yu et al., 2002). In support of kinase interactions promoting dimerization, unliganded EGFR oligomers dispersed when A431 cells were subjected to a tyrosine kinase inhibitor (Clayton et al., 2007).

Although it appears that transmembrane and cytoplasmic domain contacts occur in dimerized unliganded receptors, ligand binding to the extracellular domain is pivotal for signal transduction. Mutants of EGFR extracellular domain dimer interface residues transfected into CHO cells showed reduced phosphorylation of EGFR in response to EGF (Ogiso et al., 2002). Stimulation with EGF was necessary for tyrosine phosphorylation of unliganded EGFR homodimers (Yu et al., 2002). Structures of HER3 and HER4 are similar to that of EGFR (Cho and Leahy, 2002).

In contrast to the crystal structures of EGFR, HER3, and HER4, a structure of HER2 was strikingly different, possible explaining the extremely mitogenic character of HER2. A crystal structure of the human HER2 extracellular domain showed that Domains I and III formed a stable interface, similar to that of the EGFR crystal in which EGF was bound. Domains II and IV did not contact each other, and the Domain II binding loop was exposed constitutively. The fixed open structure of HER2 may explain why it does not require ligand to become active (Cho et al., 2003). Amino acid differences in Domain I and Domain III of HER2 compared to EGFR are probably...
responsible for the inability of HER2 to bind ligand (Tzahar et al., 1996; Garrett et al., 2003).

The possibility that ErbBs may interact with receptors outside their own family opens up a new area for research. In breast cancer cells, HER2 forms a dimer with a receptor tyrosine kinase from a different family. In SKBR3 breast cancer cells, data from immunoprecipitation experiments showed an association between HER2 and the insulin-like growth factor-1 receptor (IGF-1R), suggesting that the two receptors could form a heterodimer (Nahta et al., 2005). It is not known whether this is a feature unique to these abnormal cells. Although the crystal structure of an IGF-1R extracellular domain shows similarities to the EGFR structure (Ward and Garrett, 2004), it is unclear how the two receptors could interact to form a dimer.

2.1.5 EGFR DOWNSTREAM ACTIVATION

EGF exerts effects on cell cycle progression and survival pathways (Marmor et al., 2004). Receptor activation results in cytoplasmic signaling that conducts signal transduction to the nucleus (Mass, 2004), leading to gene transcription and cellular responses (Mendelsohn and Baselga, 2006). Ultimately, it is deregulation of these downstream pathways that drives cancer progression (Hirata et al., 2002).

EGFR has been shown to bind a variety of ligands including EGF, transforming growth factor-α and amphiregulin (Yarden and Sliwkowski, 2001). Ligand binding triggers receptor dimerization, promoting the autophosphorylation of specific tyrosine residues within the intracellular domain, creating binding sites for transduction proteins containing Src homology 2 or phosphotyrosine-binding domains (Hynes and Lane, 2005). Several downstream signaling pathways are subsequently activated including
the Ras/mitogen-activated protein kinase cascade, phosphatidylinositol-3 kinase/Akt, protein kinase C and the signal transducers and activators of transcription factors (Marmor et al., 2004). These pathways promote cell proliferation, prevent apoptosis, and increase cell migration. The receptors and ligands of the EGFR family mediate complex interactions between tumor cells and the neoplastic environment that ultimately results in enhanced tumor growth and progression. On the other hand, the ectodomain shedding of EGFR ligands including epidermal growth factor (EGF), transforming growth factor-a (TGF-a), heparin binding EGF-like growth factor (HB-EGF), and amphiregulin (AR), act in an autocrine or paracrine fashion and activate EGFR via a ligand-dependent mechanism (Hynes and Lane, 2005). These growth factors and their receptors have been reported to be overexpressed in advanced PCa including EGF, TGF-α and β, FGF and IGF (Culig et al., 1996).

2.1.6 PI3K-AKT PATHWAY

AKT/PKB (protein kinase B) kinases mediate signaling pathways downstream of activated tyrosine kinases and phosphatidylinositol 3-kinase. AKT kinases regulate diverse cellular processes including cell proliferation, survival, cell size and response to nutrient availability, tissue invasion and angiogenesis.

PI3K is a heterodimeric enzyme composed of 110-kDa catalytic subunit and 85-kDa regulatory subunit which serves as a major signaling component downstream of growth factor receptor tyrosine kinases (Luo et al., 2003). PI3K catalyzes the production of the lipid secondary messenger phosphatidylinositol-3,4,5-triphosphate, which in turn activates a wide range of downstream targets, including the serine/threonine kinase Akt (Luo et al., 2003). Full activation of Akt/PKB is PI3K
dependent and requires both recruitment to the plasma membrane and phosphorylation on two key residues, Thr 308 and Ser 473 (Lawlor and Alessi, 2001).

The AKT/PKB kinases, which include AKT1, AKT2, and AKT3, are key intermediates of signaling pathways that regulate cellular processes controlling cell size/growth, proliferation, survival, glucose metabolism, genome stability, and neo-vascularization. The biochemical mechanisms leading to AKT activation are well defined (Scheid and Woodgett, 2003; Brazil et al., 2004; Bellacosa et al., 2005).

A large body of literature has documented frequent hyperactivation of AKT kinases in a wide assortment of human solid tumors and hematological malignancies (Bellacosa et al., 2005; Patel, 2013). Moreover, genetically modified mice have been used as in vivo models to demonstrate that aberrant AKT signaling can contribute to malignancy, either alone or in cooperation with other genetic alterations (Luo et al., 2003; Bjornstian and Houghton, 2004). Since the AKT signaling cascade is frequently deregulated in many types of cancer and, in some malignancies, has implications with regard to tumor aggressiveness (Mitsiades et al., 2004), there is potential utility in molecularly targeting components of the AKT pathway for cancer therapy and, possibly, cancer prevention.

Akt is now known to be a central node in a signaling pathway consisting of many components that have been implicated in tumorigenesis, including upstream PI3K, PTEN (Phosphatase and Tensin homologue deleted on chromosome Ten), NF1 and LKB1, and downstream tuberous sclerosis complex 2 (TSC2), Forkhead Box Class O (FOXO) and eukaryotic initiation factor 4E (eIF4E). Several of these proteins (AKT, eIF4E, and both the p110a catalytic and p85a regulatory subunits of PI3K) can behave
as oncoproteins when activated or overexpressed, while others (PTEN, FOXO, LKB1, TSC2/TSC1, NF1, and VHL) are tumor suppressors. Somatic genetic and/or epigenetic changes involving genes encoding these AKT pathway components have been reported in various sporadic cancers (Johannessen et al., 2005).

Moreover, germline mutations in PTEN, LKB1, TSC2/TSC1, NF1, and VHL are linked with five different dominantly inherited cancer syndromes characterized by numerous scattered hamartomas, which are benign tumors with normal differentiation but disrupted architecture, and predisposition to certain malignancies (Eng, 2003; Kwiatkowski, 2003; Johannessen et al., 2005). Each of these tumor suppressors is a negative regulator of the AKT-mTOR pathway, which, when deregulated, results in altered translation of cancer-related mRNAs that regulate cellular processes such as cell cycle progression, autocrine growth stimulation, cell survival, invasion, and communication with the extracellular environment (Mamane et al., 2004).

Akt phosphorylates and inactivates the FOXO transcription factors, which mediate the expression of genes critical for apoptosis, such as the Fas ligand gene. AKT also activates IkB kinase (IKK), a positive regulator of NF-κB, which results in the transcription of anti-apoptotic genes (Pommier et al., 2004; Zhong et al., 2010). In another mechanism to thwart apoptosis, Akt promotes the phosphorylation and translocation of Mdm2 into the nucleus, where it downregulates p53 and thereby antagonizes p53-mediated cell cycle checkpoints (Mayo and Donner, 2002; Zhou and Hung, 2002).

Akt, a major downstream effector of EGF-mediated cell survival inhibits apoptosis induced by a variety of apoptotic stimuli. Akt inhibits molecular events that
precede cytochrome C release (Gottlob et al., 2001; Parone et al., 2002), suggesting that Akt inhibits apoptosis by maintaining the integrity of mitochondria. It has been demonstrated that members of the Bcl-2 protein family are critical regulators of mitochondrial integrity. An apoptotic stimulus may activate one or more of the “BH3-only containing” pro-apoptotic members of the Bcl-2 family, which proceed to directly or indirectly activate one or both of the terminal Bcl-2 family death effectors Bax and Bak at the mitochondria (Scorrano and Korsmeyer, 2003). The anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL, directly antagonize the activity of the BH3-only proteins. Akt has shown to negatively regulates, the activity of several pro-apoptotic members of the Bcl-2 family such as Bax and Bak. Akt elevates mitochondrial hexokinase (mtHK). Hexokinase (HK) catalyzes the phosphorylation of glucose to yield glucose-6-phosphate (G-6-P), which constitutes the first committed step of glucose metabolism (Gottlob et al., 2001). HKI and HKII bind to the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane thus inhibiting the mitochondrial binding of Bax (Pastorino et al., 2002). Therefore, Akt regulates mitochondrion-associated proteins, such as HKs, which may antagonize the activation of Bax and Bak at the mitochondria following an apoptotic insult, thereby maintaining mitochondrial integrity and preventing the release of apoptogenic factors. Akt prevents apoptosis by phosphorylating and inactivating caspase-9, and the pro-apoptotic Bcl-2 family member, Bad (Cardone et al., 1998; Datta et al., 1999).

Apart from regulating apoptosis, Akt also promotes cell proliferation. Progression through the cell cycle is controlled by the activity of protein kinase complexes consisting of cyclins and cyclin dependent kinases (cdks) and associated regulatory proteins (Malumbres and Barbacid, 2001). Cyclin dependent kinases (cdks)
are activated by binding to specific cyclin proteins that are synthesized periodically during the cell cycle. Progression from G1 to S phase of the cell cycle requires formation of complexes between cdk4 or cdk6 and D-type cyclins during early to mid-G1, followed by formation of complexes of cdk2 and cyclin E during late G1. Accumulation of the D-type cyclins in G1 is required for cell cycle entry and is regulated by extracellular growth factors (Sherr and Roberts, 1999). During G1/S transition, Akt regulates the level of cyclin D, c-myc, p27kip1 and p21waf1 by preventing their proteosomal degradation.

GSK-3β phosphorylates cyclin D1 at Thr 286, which promotes its degradation via ubiquitin-mediated pathway (Diehl et al., 1998). Thus, Akt phosphorylating and inactivating its substrate GSK-3β prevents the degradation of cyclin D1, which facilitates the G1/S progression. Akt can also inactivate the cdk inhibitor proteins p21 and p27, thereby promoting cdk activity and cell cycle progression (Liang and Slingerland, 2003). PI3K/Akt pathway provides major survival signals to prostate and many other cancer cells (Datta et al., 1999; Kandasamy and Srivastava, 2002; Downward, 2004). Constitutive activation of Akt is frequently described in many types of human cancers (Khwaja, 1999). Increase of p-Akt expression particularly at serine 473, has been shown to correlate with higher Gleason score and is an excellent predictor of poor clinical outcome in PCa patients (Kreisberg et al., 2004).

Development of hormone-insensitivity in patients who have been on long-term androgen ablation therapy for PCa is associated with reinforcement of the PI3K-Akt pathway (Pfeil et al., 2004). Activation of PI3K/Akt promotes cell survival, cell migration, proliferation and cytoskeletal rearrangement (Nicholson and Anderson, 2002).
2.1.7 Ras/Raf/MEK/ERK PATHWAY

The mitogen-activated protein kinase (MAPK) cascade is a key signaling pathway that regulates diverse cellular functions including cell proliferation, survival, differentiation, angiogenesis, and migration. Classical activation is initiated by ligand binding to receptor tyrosine kinases (RTK) at the cell surface and via Ras, then Raf, then MEK (mitogen-activated protein kinase kinase), culminates in the regulation of gene transcription in the nucleus by the last pathway component, extracellular signal regulated kinase (ERK). Since the pathway regulates many cellular functions that are classic hallmarks of cancer, it is deregulated in numerous cancer types, including PCa (Chang et al., 2003; Chung and Kondo, 2011; Ward et al., 2012). Ras protein family members belong to a class of protein called small GTPase, and are involved in transmitting signals within cells (cellular signal transduction). The name 'Ras' is an abbreviation of 'Rat sarcoma', reflecting the way the first members of the protein family were discovered.

The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression. This pathway is often activated in certain tumors by chromosomal translocations such as BCR-ABL, mutations in cytokine receptors such as Flt-3, Kit, Fms or over expression of wild type or mutated receptors, e.g., EGFR (Mc Cubrey et al., 2007). This pathway has diverse effects, which can regulate cell cycle progression, apoptosis or differentiation (Steelman et al., 2004). This pathway consists of a small G-protein of the Ras family and three-tiered kinase cascade Raf/ MEK/ERK.
The Ras protein is anchored to the cell membrane. The activated receptors assemble the multimeric proteins at the cell membrane that contain guanosine exchange nucleotide factor (GEF) and Son of sevenless (SOS) proteins. GEFs promote Ras to release GDP and bind to GTP. The resulting conformational change Ras/GTP to interact with the effector molecules such as the Raf. This leads to the translocation of Raf to the cell membrane where it is activated. The activation of Raf occurs via a multimeric step. The mammalian Raf gene family consists of A-Raf, B-Raf and Raf-1 (C-Raf). Raf is a serine/threonine kinase and is normally activated by a complex series of events including: (i) recruitment to the plasma membrane mediated by an interaction with Ras (Yan et al., 1998); (ii) Dimerization of Raf proteins (Luo et al., 1996); (iii) phosphorylation/dephosphorylation on different domains (Fabian et al., 1993); (iv) disassociation from the Raf kinase inhibitory protein (RKIP) (Dhillon et al., 2002) and (v) association with scaffolding complexes (e.g., kinase suppressor of Ras, (KSR) (Chang et al., 2003). There are at least thirteen regulatory phosphorylation sites on Raf-1 (Steelman et al., 2004). Some of these sites e.g., S43, S259 and S621 are phosphorylated when Raf-1 is inactive. This allows 14-3-3 to bind Raf-1 and confer a configuration which is inactive. Upon cell stimulation, S621 becomes transiently dephosphorylated by an unidentified phosphatase. Phosphatases such as protein phosphatase 2A (PP2A) dephosphorylate S259 (Dhillon et al., 2002). 14-3-3 then disassociates from Raf-1. This allows Raf-1 to be phosphorylated at S338, Y340, and Y341, rendering Raf-1 active. The S338 residue present in Raf-1 is conserved among the three Raf isoforms. S338 phosphorylation on Raf-1 is stimulated by Ras. The scaffolding protein RKIP has been shown to inhibit Raf-1 activation and downstream signaling (Corbit et al., 2003). RKIP is a member of the phosphatidylethanolamine-
binding protein (PEBP) family. Interestingly RKIP can bind either Raf or MEK/ERK but not to Raf, MEK and ERK all together.

Activated Raf phosphorylates the MEK which in turn activates the ERK protein. MEK1 (multi-gene family is evolutionarily mitogen-activated protein kinase/ERK kinase protein kinase) is a tyrosine (Y-) and S/T-dual specificity (Alessi et al., 1994). Its activity is positively regulated by Raf phosphorylation on S residues in the catalytic domain. All three Raf family members are able to phosphorylate and activate MEK. MEK1 and MEK2 will later phosphorylate and activate extracellular-signal-regulated kinases 1,2 (ERK or MAPK), which are S/T kinases. ERKs can directly phosphorylate many transcription factors including Ets-1, c-Jun and c-Myc. ERK can also phosphorylate and activate the 90 kDa ribosomal S6 kinase (p90Rsk).
2.1.8 EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells.

**Cadherins**

Cadherins are the major cell adhesion molecules. They are calcium-dependent adhesion molecules and play a crucial role in the spatial segregation of cell types and
organisation of different tissues during embryonic development (Gumbiner, 2005; Brasch et al., 2012). Cadherins interact with other cadherins on adjacent cells by a complex of proteins called catenins. The catenins bind to the actin cytoskeleton of the cell. The cadherin-catenin complex forms the classic adherans junctions, which integrate the epithelial cells in a mechanical unit. Cadherins join cells together by homophillic binding, binds to the same type of cadherin on another cell. Cell adhesion by cadherins is mediate by both the homophillic binding of extra cellular domains and binding of cytoplasmic domain of cadherin with actin cytoskeleton. Homophillic binding between the extracellular domains of cadherins is initiated and stabilized by binding of Ca2+ (Troyanovsky, 2005; Troyanovsky et al., 2007).

E-cadherin, also known as uvomorulin, is expressed on all the early embryonic cells of mammals. Later its expression is restricted to epithelial cells. Mesenchymal cells, which are less polarized and more motile than epithelial cells, express N-cadherin

**Fig.3.** Overview of EMT pathway (Brasch et al., 2012)
(neural cadherin) and various other cadherins such as R-cadherin and cadherin-11 (Tran et al., 2002; Chu et al., 2008). E-cadherin is expressed specifically by endothelial cells at the junctional complex. Endothelial cells also express N-cadherin whose function is unknown, as they are not expressed at the junctions. E-cadherin is expressed by epithelial cells where it provides the mechanical strength to the tissue, however many epithelium-derived cancer cells loose the expression of E-cadherin (Battle et al., 2000; Comijn et al., 2001; Taniuchi et al., 2005; Fan et al., 2012). Enzymatic activity is not found in classical cadherins and catenins but in adherens junctions, they can associate with kinase and phosphatase enzymes such as Fer and PTP1B (Arregui et al., 2000; Weiner and Jontes, 2013). Adhesion of E-cadherin activates PI3-K and Akt/protein kinase B (Kovacs et al., 2002).

N-cadherin is typically expressed by mesenchymal cells, which are more motile in nature than epithelial cells. Studies have reported unusual expression of N-cadherin in epithelium derived tumors and this upregulation of N-cadherin promotes cell motility and invasiveness (Hazan et al., 2000; Gravdal et al., 2007). This shift in the expression of cadherins from E-cadherin to N-cadherin occur during gastrulation where it affects the phenotype of participating cells and helps in the separation of different types of cells, for example, a shift in expression from E-cadherin to N-cadherin helps the segregation of neural tube from the epithelium (Cavallaro et al., 2002; Christofori, 2003).

**Cadherin switching and its role in prostate cancer metastasis**

Cadherin switching usually refers to shifting of E-cadherin expression to N-cadherin expression but also involves conditions where N-cadherin expression is
upregulated without a significant change in expression of E-cadherin and also situations where other cadherins like R-cadherin, P-cadherin, T-cadherin and cadherin-11 etc, are co expressed with E-cadherin (Tomita et al., 2000; Derycke and Bracke, 2004). Cadherin switching is an important event occurring during metastatic progression of a tumor by enhancing the invasiveness of the tumor cells (Riou et al., 2006; Hulit et al., 2007). Decrease in expression of E-cadherin and increase in N-cadherin expression has been observed in various metastatic tumors. Upregulation of N-cadherin expression mediates a homotypic adhesion between PCa cells and stromal fibroblasts and facilitate metastasis (Tran et al., 1999; Barr et al., 2008).

Prostate cancer invasion proceeds through the surrounding stroma, migration to the perineural space and finally penetrate the capsule to escape from the primary location (Villers et al., 1989; McNeal et al., 1990). In addition to facilitating the escape from prostate gland, N-cadherin expression also aids the invasion of local blood vessels by the tumor cells. As endothelial cells also express N-cadherin in extra-junctional spaces, with an unclear role (Novarro et al., 1998), a homotypic interaction between PCa cells and endothelial cells promote metastasis by allowing access to the blood vascular system, possibly involving the IL-6-TGF-β-MMP-9 pathway, as demonstrated by ex vivo cell culture experiments (Wang et al., 2013).

Cadherin switching, decreased expression of E-cadherin and increased expression of N-cadherin, was observed in LNCaP-19 tumor cells, as the tumor progressed towards a stage of androgen independency suggestive of a correlation between cadherin switching, invasiveness and androgen independency in PCa (Jennbacken et al., 2006). Studies have shown that apoptosis is induced in both normal and cancer cells, when cadherin adhesion is disrupted (Graff et al., 2000; Nightingale
et al., 2003). In most of the epithelial malignancies, a key step in metastasis of carcinomas of breast and prostate is the transcriptional repression of E-cadherin gene (Peinado et al., 2004). Although the mechanisms which regulate the abnormal expression of N-cadherin in carcinoma progression are yet unknown, it has been shown that N-cadherin expression during epithelial-mesenchymal transition is induced by TGFβ1 through GTPase RhoA signalling (Guo et al., 1998) while at the later stages, PCa cells are resistant to this growth factor (Bhowmick et al., 2001). A basic helix-loop-helix transcription factor Twist-1 which regulates the expression of E-cadherin and increased expression of mesenchymal genes during morphogenesis has been shown to be up-regulated in breast and prostate carcinomas (Yang et al., 2004).

A study on the role of Twist-1 in regulating N-cadherin expression has shown that increased accumulation of Twist-1 in nucleus results in β1 integrin mediated cell adhesion. Twist-1 directly binds to an E-box cis-element located in the first intron of the human N-cadherin gene and initiates the transcription of N-cadherin (Alexander et al., 2006). N-cadherin also plays dual functional roles in homophillic cell-cell adhesion and regulation of apoptosis. Studies involving PC3 cell lines have shown that homophillic adhesion of N-cadherin is linked to Akt signalling and inhibition of mitochondrial apoptotic pathway. Homophillic adhesion between extracellular domains of N-cadherin provides specific signals that regulate the levels of Bcl-2 by recruitment and activation of PI3-kinase and phosphorylation of Akt, which leads to phosphorylation of Bad at Ser-136 and stabilizes Bcl-2 (Tran et al., 2002).
Vimentin

Vimentin, a 57-kDa protein, is one of the most widely expressed and highly conserved proteins of the type III intermediate filament protein family (Franke et al., 1982; Cochard and Paulin, 1984; Evans, 1998; Larsson et al., 2004). Vimentin has gained much importance as a canonical marker of EMT (Thiery, 2002), a cellular reprogramming process in which the epithelial cells acquire a mesenchymal phenotype that renders the cells to dramatically alter their shape and exhibit increased motility. This EMT is characterized by the expression of vimentin IFs in epithelial cells, which normally express only keratin IFs. Accordingly, during the reverse process of EMT, known as mesenchymal-epithelial transition (MET), the cells start acquiring epithelial phenotype and show a decreased vimentin expression with lower motility rates (Chaffer et al., 2006). Increased vimentin expression has been reported in various tumor cell lines and tissues including prostate cancer, breast cancer, endometrial cancer, CNS tumors, malignant melanoma and gastrointestinal tumors including pancreatic, colorectal and hepatic cancers.

Vimentin IFs are found in the cytoplasm of mesenchymal cells, where it functions to maintain the cyto-architecture and tissue integrity (Franke et al., 1982). Vimentin is known to interact with a large number of proteins and participates in various cellular functions. Further, vimentin is also involved in a number of other processes that involve formation of complexes with several cell-signaling molecules and other adaptor proteins. For example, vimentin was shown to interact with phosphorylated Erk (pErk), a MAP kinase and protect it from dephosphorylation (Perlson et al., 2006). Using biochemical and molecular modeling approaches, it was observed that pErk binding was localized to the second coil-coiled domain of vimentin.
and this binding was calcium dependent. From these observations, it was suggested that vimentin is stabilizing pErk by protecting it from dephosphorylation by calcium-dependent steric hindrance thereby enabling long distance transport of phosphorylated Erk within the cell (Perlson et al., 2006). AKT1 kinase was shown to bind phosphorylated vimentin and protect it from caspase-induced proteolysis that leads to increased cell motility and invasion of soft-tissue sarcoma cells (Zhu et al., 2011). 14-3-3 proteins participate in a multitude of cell signaling and cell cycle processes. Phosphorylated vimentin was shown to interact with 14-3-3 protein and prevent the assembly of Raf-14-3-3 and other such complexes, thereby suggesting that vimentin regulates 14-3-3 complexes and controls various intracellular signaling and cell cycle control pathways by modifying 14-3-3 availability (Tzivion et al., 2000). Scrib, a protein involved in cell migration, is protecte from proteasomal degradation upon interaction with vimentin, suggesting a possibility that vimentin upregulation during EMT leads to stabilization of Scrib to promote directed cell migration to increase the invasive capacity of cells (Phua et al., 2009).

Vimentin functions as a regulator of Annexin-1 (Ax-1) and enhances cell migration by inducing Axl. Further, Slug- and Ras-induced EMT changes were shown to be dependent on the up-regulation of vimentin (Vuoriluoto et al., 2011).

**Vimentin in Prostate cancer**

In prostate cancer, vimentin expression was mainly detected in the poorly differentiated cancers and bone metastases and was nearly undetectable in well differentiated tumors or in moderately differentiated tumors (Lang et al., 2002; Zhao et al., 2008). Further, vimentin expression was associated with motile PCa cell lines.
(Singh et al., 2003) and its down-regulation in PC-3 cells led to a significant decrease in tumor cell motility and invasive activity (Lang et al., 2002). Vimentin was over expressed in PCa cell line and after experimentally abrogating the expression of vimentin; there was a significant decrease in the invasiveness of the tumor cell line (Singh et al., 2003). Interestingly, there was no change in the invasiveness of LNCaP cells after forced expression of vimentin. The authors speculated a possibility that vimentin's expression contributes to the development of an invasive phenotype in conjunction with other yet to be discovered proteins or at a later stage of the cancer development. In another study, vimentin was shown to be over expressed in highly metastatic human prostate epithelial cancer cell line PC-3M-1E8 and its role in modulating the invasiveness was attributed for its ability to regulate E-Cadherin/β-catenin complex via C-src regulation (Wei et al., 2008). Several other studies also supported the view that vimentin is over expressed in PCa and contributes to their invasive and metastatic potentials (Wu et al., 2007; Sethi et al., 2010).

**Cell adhesion molecules (ICAM, VCAM) and Selectins**

Metastatic spread of cancer cells is a key event in tumor progression and in determining the prognosis of patients with malignant disease. Malignant cells detached from the primary tumor must penetrate into blood or lymph vessels, survive in the circulation and then arrest in the capillary endothelium of distant organs, extravasate, and grow as secondary lesions (Fidler, 2002). Therefore, adhesion interactions between endothelial and cancer cells seem to be crucial for the successful development of metastasis. These interactions are modulated by specific cell surface receptors including the selectins, the integrins, and the immunoglobulin like family of cell adhesion molecules (Ohene-Abuakwa and Pignatelli, 2008).
It is now increasingly apparent that certain members of these families of cell adhesion molecules are involved in tumor progression. E-selectin (also known as endothelial leukocyte adhesion molecule–1) is expressed on activated endothelial cells and may bind cells expressing specific carbohydrate ligands containing sialyl-Lewis residues (Ramphal et al., 1996; Wu et al., 2012; Coupland and Parish, 2014).

Selectins mediate binding of leukocytes to the microvascular endothelium, and studies have suggested that the efficiency of the E-selectin–mediated binding of certain cancer cell lines to endothelial cells correlates with their metastatic potential (Moss et al., 2000; Coupland and Parish, 2014). Intercellular cell adhesion molecule–1 (ICAM-1) and vascular cell adhesion molecule–1 (VCAM-1) are both members of the immunoglobulin superfamily of adhesion molecules. ICAM-1 is constitutively expressed by endothelial cells and by some leukocytes, and it serves as a ligand for the leukocyte 2 integrin receptors LFA-1 and Mac-1. VCAM-1 is found mainly on activated endothelial cells and provides a ligand for the 41-integrin receptor VLA-4. Both ICAM-1 and VCAM-1 are involved in firm leukocyte-endothelial adhesion, facilitating leukocyte transmigration through the vascular wall. TNF-α upregulates intercellular adhesion molecule (ICAM)-1 expression on the RPE, which allows lymphocyte function-associated antigen-1 (LFA-1) to bind on leukocytes that contribute to leukocyte adhesion at sites of inflammation (Thichanpiang et al., 2014). Cannabinoid-induced upregulation of ICAM-1 on lung cancer cells is responsible for increased cancer cell susceptibility to LAK cell-mediated cytolysis (Haustein et al., 2014).

E-selectin, ICAM-1, and VCAM-1 expression have been demonstrated on the endothelial cells of small vessels at the invasive margin of tumors, suggesting possible
interactions between endothelial and tumor cells involved in metastatic spread (Sawada et al., 1994; Nelson et al., 1994; Coupland and Parish, 2014). The soluble forms of E-selectin, ICAM-1, and VCAM-1 have previously been recognized (Gearing et al., 1992). They have been detected in the supernatants from cytokine-activated cultured endothelial cells (Pigott et al., 1992) and in the serum of healthy subjects, as well as in patients with gastric, hepatobiliary, breast, and colonic cancer, where high circulating levels are generally associated with more advanced and metastatic disease (Banks et al., 1993; Alexiou et al., 2001). Previous studies have shown increased concentrations of ICAM-1 and VCAM-1 in gastric cancer patients when compared with age-matched controls, with a correlation between these increased markers (and elevated preoperative E-selectin levels) and the presence of metastatic disease or intraperitoneal spread (Velikova et al., 1997; Benekli et al., 1998). Selectins mediate small cell lung cancer systemic metastasis (Heidemann et al., 2014). Aberrant presentation of HPA-reactive carbohydrates implies Selectin-independent metastasis formation in human PCa (Lange et al., 2014)

2.1.9 MATRIX METALLOPROTEINASES (MMPs)

Matrix metalloproteinases (MMPs) are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for the tissue remodeling and degradation of the extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan. Matrix metalloproteinases are excreted by a variety of connective tissue and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes.
The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes present in both normal and pathological tissues in which matrix remodelling is involved, including embryonic development, wound healing, arthritis, angiogenesis, and tumour invasion and metastasis (Liotta et al., 1991). The enzymes contain a zinc atom at their active site and depend on calcium for their activity. The MMPs degrade the components of the extracellular matrix, with MMP1 degrading fibrillar collagen and the gelatinases (MMP2 and MMP9) being important in degrading the basement membrane. MMPs play a role in the invasion of normal tissues by tumors and their subsequent metastatic spread. The local production of MMP-9 and other proteases, such as plasminogen activator, by PCa cells or stroma facilitates the degradation of the extracellular matrix and results in tumor invasion and subsequent metastasis (Festuccia et al., 2005).

The proteolytic effect of MMPs facilitates the migration of endothelial cells through the altered extracellular matrix toward the source of the angiogenic stimulus. Hence MMPs are an integral component for the angiogenic process. It has been reported that there is a link between EGFR function and MMPs expression that may contribute to the invasive phenotype. In human bladder tumour, cell lines RT112, EGF at 10 or 50ng/ml induced the MMP-9 expression (Nutt et al., 2003). In nonmalignant (S1) and malignant (T4-2) human breast epithelial cells, it was reported that MMP-9 was regulated by Raf/MEK/ERK signaling in 3D cultures. Suppression of either MEK or MMP-9 using shRNAs allowed T4-2 cells to form quiescent structures (Weaver, 2002).

2.1.10 CASPASES
Caspases or cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases are a family of cysteine proteases that play essential roles in apoptosis, necrosis, and inflammation (Strasser et al., 2000). Caspases, totaling 14 family members to date, are synthesized as inactive zymogens, which must be proteolytically cleaved at two (or three in some cases) aspartate residues to generate the active mature enzyme. The generations of active caspases interact with specific adapter molecules to facilitate their own auto processing. Active initiator caspases in turn cleave and activate the downstream “executioner” caspases. These, then cleave their target substrates to orchestrate the proteolytic dismantling of the cell (Henson et al., 2001; Slee, 2001; Green and Evan, 2002). Not all caspases are involved in apoptosis. The caspases that have been well described are caspases-3, -6, -7, -8, and -9 (Thornberry and Lazebnik, 1998; Mancini et al., 1998).

The intrinsic and extrinsic apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribonuclease, and the caspase-activated deoxyribonuclease becomes active leading to nuclear apoptosis. The upstream caspases that converge to caspase-3 are caspases-9 and -8 in the intrinsic and extrinsic pathways, respectively. The downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, inhibitory subunits of endonucleases, and finally destruction of “housekeeping” cellular functions. Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing (Mancini et al., 1998).

2.2.1 PROSTATE GLAND
The prostate gland is a compound tubuloalveolar, male accessory sex organ, and it is present below the urinary bladder, opening into urethra. The word prostate is derived from Greek word, literally meaning "one who stands before", "protector", "guardian". A fully developed gland is pyramidal in shape with the base directed towards the neck of the bladder and apex resting on the urogenital diaphragm (Marker et al., 2003). Prostate gland is made up of several glandular and non-glandular components that are tightly fused together with in a common capsule (Mc Neal, 1988).

The glands of the prostate are derived from the endoderm of the urogenital sinus, while the fibrotic and muscular tissues are derived from the mesoderm (Coffey, 1993). The prostate undergoes significant growth during fetal development, puberty, and in most men, during late middle age. Prostate growth and development are dependent on androgen production by the fetal testes, which begins at about the eight week of gestation (Coffey, 1993; Habert et al., 2001). The differentiation of the urogenital sinus is dependent on dihydrotestosterone (DHT), which is essential for the mediation of growth and development of the prostate from the pelvic portion of the urogenital sinus. The fetal testis (Leydig cells) produces testosterone from day 12.5-post conception (dpc) onwards in the mouse, by 15 dpc in the rat and by 9 weeks of gestation in humans (Habert et al., 2001). UGS arises in humans at about 7 weeks of gestation and in male and female mice at approximately 13dpc. The male and female UGS are morphologically undistinguished until about 17.5 dpc in mouse, 18-19 dpc in rats and 10-12 weeks of gestation in humans. At this stage prostatic morphogenesis is initiated by circulating androgens produced by the fetal testes (Marker et al., 2003). At the end of puberty, the prostate reaches approximately 26 g and is maintain at that weight unless benign prostatic hyperplasia (BPH) develops. The average weight of the
prostate with histological confirmed BPH at the time of autopsy is 33 ± 16 g (Berry et al., 1984).

Prostatic morphogenesis starts as an outgrowth of solid buds from the UGS epithelium (UGE) into the surrounding UGS mesenchyme (UGM). The prostatic buds are solid cords of epithelial cells that grow into the UGM in a precise spatial pattern to form the lobular sub divisions of the prostate (Cunha et al., 1987; Timms et al., 1994). In human fetus, the prostatic buds originate from different parts of prostatic urethra. The first epithelial outgrowth arises from the prostatic urethra around the tenth week of human gestation and by 13 weeks, there are approximately seventy primary ducts (Cunha et al., 1987).

Anatomy

The largest part of the prostate is the anterior or ventral fibromuscular and non-glandular region, which forms the ventral surface of the gland and which constitutes about one-third of the entire prostate, which can be divided into lobes or zones. The division made by McNeal is currently the one most applied (McNeal, 1988). The glandular prostate can be subdivided into four zones as follows:
Fig. 4. Zones of the prostate gland (McNeal, 1988)

I. A peripheral zone that represents about 70% of the glandular part of the prostate. This zone forms the lateral and posterior or dorsal part of the organ. The ducts of the peripheral zone open into the distal prostatic urethra.

II. A central zone that comprises about 25% of the glandular prostate. This zone is edge shaped and surrounds the ejaculatory ducts. The central zone is surrounded by the peripheral zone, at least in its distal part, and its ducts open into the prostatic part of the urethra, in close proximity to the ejaculatory ducts.

III. A transitional zone that is the smallest glandular part and it comprises only about 5-10% of the prostate. This zone consists of two independent small lobes whose ducts leave the posterolateral recesses of the urethral wall at a single point, just proximal to the point of urethral angulation and at the lower border of the preprostatic sphincter.
The last zone known as anterior fibro-muscular zone or stroma, doesn't contain any glandular parts but consists of muscles and tissues.

In rodent, the prostate shows a lobular anatomy, the organized and encapsulated individual lobes arise from the urogenital sinus, located in specific positions around the urethra. In mouse, the prostate can be divided anatomically into distinct lobe pairs, which are not encased by abundant stroma and a capsule into a single gland as in the human prostate. The individual lobes are defined, according to their position relative to the bladder, as the ventral, dorsal, lateral and anterior (also known as the coagulating gland) prostate lobes (VP,DP,LP,AP). The DP and LP are often grouped together as Dorsolateral prostate (DLP).

All lobes are responsive to estrogen and androgens, but varying degrees; the VP is more sensitive to androgen and the AP is more sensitive to estrogens (Prins and Birch, 1995; Risbridger et al., 2001). The proportion between prostate epithelial and stromal compartments differs from species to species. In adult rodents, the epithelial to stromal ratio is approximately 5:1. In contrast, normal prostate of human and other primates demonstrates approximately equal numbers of stromal and epithelial cells (DeKlerk and Coffey, 1978; Bartsch and Rohr, 1980).

The prostate is a compound tubuloacinar gland. Within the acini and tubules, the epithelium forms complex folds and papillae supported by a thin highly vascularised loose connective tissue. The secretory epithelium is mainly pseudostratified, comprising tall columnar cells and basal cells which are supported by a fibroelastic stroma containing randomly orientated smooth muscle bundles. The epithelium contains scattered neuroendocrine cells, which partly control release and
expulsion of prostatic secretions during ejaculation. The secretory components of the
gland are divided into three concentric layers. The innermost area is comprised of
mucosal glands which are concentrated around and secrete into the upper region of the
prostatic urethra. The middle or internal area contains submucosal glands which secrete
via short ducts into the urethral sinuses. The outer or peripheral area constitutes the
majority of the gland and secretes via long ducts into the urethral sinuses. The anterior
isthmus is an area of the gland ventral to the urethra, relatively free of glands and rich
in fibromuscular tissue.

Mature prostatic ducts contain three major cell types, luminal secretory
epithelial cells, basal epithelial cells and stromal smooth muscle cells that can be
distinguished by their patterns of differentiation marker expression (Wang et al.,
2001). A thin layer of connective tissue forms the “true” capsule in the periphery of the
prostate, outside of which the pelvic fascia forms the “false” capsule (Dixon et al.,
1999).

The human prostate gland receives dual autonomic innervation from both
parasympathetic (cholinergic) and sympathetic (noradrenergic) nerves in the prostatic
nerve plexus, a part of the pelvic autonomic plexus that lies adjacent to the prostate
gland. The autonomic nerves arising from the pelvic plexus escort the vascular supply.
Both cholinergic and noradrenergic fibres innervate the prostate stroma, and
cholinergic nerves innervate the smooth muscle of the capsule and the space around the
blood vessels and are responsible for the secretory function of the epithelial part. The
sympathetic nerves control the prostatic musculature, and their excitation closes the
bladder neck during ejaculation of the seminal fluid into the urethra (Dixon et al.,
1999).
A prostatic ductal system is defined as a single prostatic functional unit in which all glandular structures share a single drainage duct into the urethra. Each ductal system is made-up of three segments, proximal, intermediate and distal regions. These regions consist of three types of cells, epithelial, stromal and neuroendocrine cells. The epithelial and stromal compartments of the prostate ductal system enjoy a dynamic coexistence characterized by an active dialogue of cell-to-cell signaling, which influences proliferation, differentiation and apoptosis. The human prostate consists of more than 30 such ductal systems (Mc-Neal, 1988). They are present in all regions of the prostate at birth, but rapidly disappear from the peripheral regions after birth and then reappear at puberty (Cohen et al., 1993). The neuroendocrine cells contain neurosecretory granules rich in various peptide hormones and biogenic amines. These include thyrotropin-releasing hormone (TRH), TRH like peptide, neuron-specific enolase (NSE), Chromogranin A (CgA), Serotonin (5-HT), Thyroid stimulating hormone (TSH) like peptide, Calcitonin (CT), Somatostatin (ST), and Parathyroid hormone (PTH) related peptide gene product (Abrahamsson and di Sant'Agnese, 1993).

The axis of the ductal system in the rat prostate is divided in to three regions (Lee, 1997). Owing to the distance from the urethral orifice of the duct, the entire length of the prostatic ductal system can be designated as proximal, intermediate and distal regions (Martikainen et al., 1991). The prostatic stromal cells are a group of pleomorphic mesenchymal cell types. One of the most prominent features of prostate stroma is the multiple layers of smooth muscle cells surrounding the proximal region (Nemeth and Lee, 1996; Lee, 1997). Functional cytodifferentiation of luminal epithelial cells occur with the expression of the prostate-specific secretory proteins. In human
prostate, secretory activity is detectable during fetal half-life (13th week of gestation) (Xia et al., 1990), presumably due to the action of fetal testicular androgens.

**Secretions**

The prostate is to secrete prostatic fluid, a slightly alkaline fluid, milky or white in appearance, that usually constitutes 50–75% of the volume of the semen along with spermatozoa and seminal vesicle fluid. Prostatic fluid facilitates the capacity of sperm fertilization. Prostatic fluid is the mixture of complex and heterogeneous, organic and inorganic compounds like zinc, magnesium, calcium, fructose and citrate, which are derived from the seminal fluid (Mann and Lutwak Mann, 1981). Prostatic fluid is slightly acidic with pH about 6.5 (Huggins, 1947). It also contains number of proteolytic enzymes like PSA (Prostate specific antigen), PAcP (Prostatic acid phosphatase) and human kallikrein-2 and nitrogenous compounds like phosphoryl choline, polyamines like putresine, spermine, spermidine (Beyler and Zaneveld, 1982). Up to 57 major protein groups, of which 27 is non-serum proteins (i.e. presumably exuded by the epithelial cells) have been identified. Major prostatic-specific proteins are prostatic acid phosphatase (PAP), prostate specific antigen (PSA) and prostate binding protein (PBP), which are expressed at pubertal and adult ages (Neal et al., 1992, Dixon et al., 1999).

The proteolytic enzymes liquefy the semen after ejaculation and the phosphatases and salts modify the vaginal environment to enhance sperm survival. Proteolysis is the major function of prostate secretion, being rich in exopeptidase and endopeptidase. The most extensively studied protease is PSA, also known as seminin, seminal protease or chymotrypsin-like protease (Neal et al. 1992, Dixon et al., 1999).
The clotting enzymes play a role in the action of fibrinolysin, contributed by the seminal vesicles to coagulate semen shortly before ejaculation. Fibrinolysin functions in the prostate by balancing the clotting enzymes, providing motility to the sperm.

**Prostate-specific antigen (PSA)**

PSA, also known as gamma-semnoprotein or kallikrein-3 (KLK3), is a glycoprotein enzyme encoded in humans by the KLK3 gene. PSA is a member of the kallikrein-related peptidase family and is secreted by the epithelial cells of the prostate gland. PSA is a serine protease regulated by androgen and member of the tissue kallikrein family of proteases (Yousuf and Diamandis, 2001). It is produced primarily by prostate ductal and acinar epithelium and is secreted into the lumen. Its function is to cleave semenogelin I and II in the seminal coagulum (Balk et al., 2003). PSA is a 33-kDa molecular weight peptide contains 237 amino acids and is regulated by androgens (Stamey et al., 1987; Partin et al., 1996; Arcangeli et al., 1998; Catalona et al., 1998). The majority of PSA (70-80%) that enters into the peripheral blood is intact and circulates as an 80-90 kDa complex with protease inhibitor α1-antichymotrypsin (Lilja et al., 1991; Stenman et al., 1991). Minor amounts are complexes with other prostate inhibitors including α2-macroglobulin and α1-antitrypsin. Pro PSA and the various truncated forms of pro PSA identified in prostate and serum (Mikolajczyk et al., 1997).

As a screening tool, the increase in serum concentration of PSA is used as a marker for PCa, which is more sensitive than digital rectal examination and prostatic acid phosphatase (PAcP) (Stamey et al., 1987). PSA has been shown to degrade insulin like growth factor binding proteins (IGFBP-3), proteolysis releasing insulin like
growth factor (IGF), the rise in the available IGF results in the stimulation of cellular proliferation (Peehl et al., 1996).

**Prostatic Acid Phosphatase (PAcP)**

Prostatic acid phosphatase (PAcP), is a glycoprotein synthesized by the prostate gland. It is a member of a diverse group of isoenzymes, the acid phosphatases, which are capable of hydrolyzing phosphate esters in acidic medium. They are classified on the basis of their electrophoretic mobilities (Moul et al., 1998). Human PACP is a prostate epithelium-specific differentiation antigen. Two forms of PACP have been identified: one stays intracellular; while the other is secreted. Its molecular weight is 102 kDa and contains 7% by weight of carbohydrate. This is the major form of secretory protein. The cellular enzyme activity and mRNA level is decreased in prostate carcinomas, compared with normal or benign prostatic hypertrophic cells (Meng and Lin, 1998). The cellular form of PACP could function as Protein tyrosine phosphatases (PTPases) in cells. PACP exhibits PTPase activity and dephosphorylates protein tyrosine-phosphorylated protein (PTP) (Lin and Clinton, 1986). (Reference value: \(< \text{or} =2.1 \text{ ng/mL}\))

**2.2.2 HORMONAL REGULATION OF PROSTATE GLAND**

**Androgen**

The prostate gland depends on androgens for its development and maintenance of its structural and functional integrity. The necessity of androgen action is illustrated by the minimal or no development of the prostate gland caused by congenital AR dysfunction or deficiency of 5α-reductase in human males (Griffin, 1992). Following castration, the rodent prostate undergoes rapid involution as a result of programmed
cell death, or apoptosis, in glandular epithelium and endothelium (Kyprianou and Isaacs, 1998). Within 2-3 weeks following castration, a majority of glandular epithelial cells are lost in both human and rodent castrates (Staack et al., 2003).

Testosterone is not the major androgen responsible for growth of the prostate. Testosterone is converted in target cells to dihydrotestosterone (DHT) by the 5α-reductase enzyme, which is expressed in two isoforms. In the prostate type 2 5α-reductase is the isoform primarily responsible for DHT formation (Steers, 2001). It has been demonstrated that stromal cells express both isoforms, whereas epithelial cells preferentially express the less active type 1 isoform (Iehle et al., 1999; Steers, 2001).

Testosterone and DHT both bind to the androgen receptor, but yet exert biologically distinct effects. These differences are considered due to kinetic differences of binding of androgens to the receptor. DHT binds with a greater affinity to the androgen receptor than does testosterone. This results in potential differences in function of the hormone response element, DNA activation, and subsequent messenger RNA production (Avila et al., 1998).

AR is a member of the steroid hormone receptor family of genes. As the AR gene is located on the X chromosome it is single-copy in males, allowing for the phenotypic manifestation of mutations without the influence of a wild-type co-dominant allele. More spontaneous mutations of human AR have been identified than of any other gene, partly because AR is not essential to the formation of a viable human organism. Complete loss of AR function in genetic males (XY) results in the complete androgen insensitivity syndrome (CAIS). The main phenotypic characteristics of individuals with CAIS are female external genitalia, a short, blind ending vagina, the
absence of Wolffian duct derived structures, the absence of a prostate, development of
gynecomastia and the absence of pubic and axillary hair.

**Estrogen**

A role for estrogens has long been implicated in prostate physiology and
pathophysiology. The expression of both known estrogen receptor subtypes in adult
human and rodent prostate is now well established, with expression of ERα described
primarily in a subset of stromal cells and ER-β restricted to the ductal epithelium
(Cooke *et al.*, 1991; Makela *et al.*, 2000). ER-β shares many of the functional
characteristics of ER-α, the molecular mechanisms regulating the transcriptional
activity of ER- β may be distinct from those of ER- α. In human prostate, the growth
effects of estrogens during fetal development are mediated primarily by ER-β, which
can be immunodetected in the nuclei of nearly 100% of epithelial and in the majority of
stromal cells throughout gestation (Adams *et al.*, 2002).

Interestingly, the growing incidence of BPH with increasing age coincides with
a shift in the androgen/estrogen ratio in favour of estrogens, not restricted to serum
hormone values, but also seen in the prostate itself (Krieg *et al.*, 1993). Exogenous
estrogen administration in adult rodents leads to squamous metaplasia (SQM) of the
anterior prostate lobe (Andersson and Tisel, 1982; Risbridger *et al.*, 2001). Neonatal
exposure of rodents to high doses of estrogen is known to permanently imprint the
growth and function of the prostate and predispose the gland to hyperplasia and severe
dysplasia analogous to prostatic intraepithelial neoplasia with aging (Prins *et al.*, 2001).
**Prolactin**

Prolactin is a major physiological regulator of prostatic epithelial cell function and metabolism (Costello and Franklin, 1994). Prolactin receptor has been identified in prostate (Aragona and Friesen, 1975). Grayhack and Lebowitz (1967) first reported that prolactin increased the citrate content of rat lateral prostate without any corresponding effect on ventral prostate. Prolactin stimulates mitochondrial aspartate aminotransferase (mAAT) and pyruvate dehydrogenase (PDH) activities, the two key regulatory enzymes involved in citrate synthesis by prostate epithelial cells (Costello and Franklin, 1994). Prolactin and androgen synergistically regulate the prostate epithelial cell proliferation (Liu *et al.*, 1995; Lissoni *et al.*, 2005). Studies carried out in the human and animals *in vivo* and *in vitro* revealed presence of prolactin receptors in normal, benign and malignant prostate tissues (Wylot *et al.*, 2006).

**Thyroid hormone**

The thyroid hormones, triiodothyronine (T$_3$) and its prohormone, thyroxine (T$_4$), are important role in the regulation of cell growth and differentiation. T$_3$ is an important regulator of prostate tissue (Esquenet *et al.*, 1995). Optimum levels of thyroid hormones are essential for the maintenance of normal structure and metabolic integrity of prostate. Thyroid hormone especially triiodothyronine (T$_3$) is an important regulator of many cell types promote cell growth and differentiation (Esquenet *et al.*, 1995). Thyroid hormone mediates action through binding with receptor; it is a member of steroid receptor superfamily. Thyroid hormones differentially regulate prostatic glycoprotein metabolism. Prostatic-$\beta$-glucosidase, $\beta$-Galactosidase and $\beta$-N-acetyl glucosaminidase activities increased uniformaly in hyperthyroid and decreased in
thyroidectomised adult rats (Maran et al., 1998). $T_3$ modulates proliferation, secretory function and AR concentration in androgen dependent (LNCaP) cell line (Esquenet et al., 1995). Exposure of LNCaP cells to $T_3$ for 6 days, stimulated PSA secretion by 2-3 folds. $T_3$ stimulates the PSA protein in the presence of androgens in LNCaP cells without affecting AR expression (Zhang et al., 1999).

**Oxytocin**

Oxytocin is produced by mammalian prostate, stimulates the 5-$\alpha$ reductase activity (Nicholson, 1996; Assinder, 2008), which converts testosterone to DHT. DHT in turn stimulates the growth of prostate. Oxytocin also stimulates muscular contractions during ejaculation (Bodanszky et al., 1992). The localization of the oxytocin receptor within the plasma membrane modulates oxytocin's proliferative response in the prostate (Whittington et al., 2007; Sendemir et al., 2008; Zhong et al., 2010).

**Gonadotropin-releasing hormone (GnRH)**

GnRH is a trophic peptide hormone responsible for the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. GnRH is synthesized and released from neurons within the hypothalamus. It constitutes the initial step in the hypothalamic–pituitary–gonadal axis (Neill, 2002). GnRH-R expression was found in breast, prostate, endometrial cells, ovarian, pancreatic and hepatoma cells (Imai and Tamaya, 2000; Finch et al., 2008).

**Luteinizing hormone (LH)**

LH stimulates testicular steroidogenesis and also acts directly on the prostate gland (Sriraman et al., 2001). LHRH is expressed in PCa cells together with receptor
and negatively regulates cell proliferation through the activation of inhibitory G protein (Gi)-cAMP (Cyclic adenosine monophosphate) intracellular signaling pathway (Moretti et al., 2003). High incidence of LHRH receptor gene expression was identified in human PCas (Halmos et al., 2000).

**Growth Hormone (GH)**

Growth hormone is a 191-amino acid; it is a type of mitogen and is responsible for postnatal growth of the prostate gland (Ruan et al., 1999; Westley and May, 1995). GHRH itself acts as an autocrine/paracrine growth factor in human cancers, including prostate (Chopin and Herington, 2001). GH receptor (GHR) is potentially involved in PCa through stimulating IGF-I production in prostate epithelium (Wang et al., 2005; McKay et al., 2007). It has been reported that human GH and IGF-1 induces LNCaP cell proliferation (Bidosee et al., 2011).

**Insulin like growth factors (IGF) family**

IGFs are mitogens that play a pivotal role in regulating cell proliferation, differentiation, and apoptosis (Muta and Krantz, 1993; Harrington et al., 1994; Sell et al., 1995; Kulik et al., 1997). Six IGF-binding proteins (IGFBPs) can inhibit or enhance the actions of IGFs (Peehl et al., 1995). Normal prostate cell expresses IGFBP-2, -3 and -4, but not IGFBP-1. It requires IGF-I and II for proliferation (Cohen et al., 1991). IGFs are produced primarily by stromal cells and activate IGF-I receptor on prostatic epithelial cells (Cohen et al., 1991; Goda et al., 2008; Yamada and Lee, 2009).
**Epidermal growth factor (EGF)**

Human EGF is a 60 kDa protein that stimulates cell growth, proliferation, and differentiation by binding to its receptor EGFR (Massague, 1990). EGF is the first peptide growth factors characterized from the mouse submaxillary gland (Carpenter and Cohen, 1990). TGF-alpha, amphiregulins, heparin-binding EGF is a family of EGF like growth factors. Early growth response-1 (Egr-1) increased the transcription of EGF, amphiregulin and epiregulin, resulting in autocrine activation of the EGFR and downstream MEK/ERK cascade of PCa cells (Sauer *et al*., 2010; Boccaccio *et al*., 2014; Patane, 2014). EGF and TGF-α are 53, 50 aminoacids polypeptide, they have similar biological properties. An EGF family member in normal prostate is localized in the secretory epithelium and EGFR present on the luminal surfaces of the prostate epithelial cells (Sherwood and Lee, 1995; Parums, 2014). TGF alpha is preferentially over expressed than EGF during periods of prenatal and neonatal prostate epithelial development (Taylor and Ramsdell, 1993; Boccaccio *et al*., 2014).

**Transforming growth factor -β (TGF-β)**

TGF-β family is important for inducing differentiation and inhibiting prostate epithelial cell proliferation and for maintaining normal prostate homeostasis (Burchardt *et al*., 1999; Guo and Kyprianou, 1999; Untergasser *et al*., 2003). TGF-β 1- 5 differentially enhance the expression of N-cadherin, cell adhesion molecules, fibronectin, and tenascin in precartilage condensations, suggesting that TGF-β isoforms play an important role in the establishment of cell-cell and cell-extra cellular matrix interactions during precartilage condensations (Chimal-Monroy and Diaz de Leon, 1999; Kondaiah *et al*., 2000; Goswami *et al*., 2003). The TGF-βs have an inhibitory
role within the normal prostate, controlling proliferation and inducing apoptosis in epithelial cells (Martikainen et al., 1991).

Two types of TGF-β receptors namely type I and II (TβRI and TβRII) are involved in signal transduction (Wrana et al., 1994). TGF-β is overexpressed in advanced PCa and exerts diverse functions in stromal cells via both SMAD-dependent and SMAD-independent signaling pathways (Coffey et al., 1986; Roberts et al., 1986; Derynck and Zhang, 2003; Zhu and Kyprianou, 2005). Loss of both TβRI and TβRII correlates with tumor stage, survival and recurrence rate (Zhang et al., 2005; Zhu and Kyprianou, 2005). Recovery of TβRII function in human PCa cell line inhibits the growth of xenograft tumors through the induction of apoptosis and inhibition of cell proliferation (Guo and Kyprianou, 1999; Song et al., 2006). TGF-beta1 suppresses IL-6-induced STAT3 activation through regulation of Jak2 expression in prostate epithelial cells (Starsichova et al., 2010).

**Platelet-derived growth factor (PDGF)**

Platelet-derived growth factor (PDGF) is a dimeric glycoprotein that regulate cell growth and division. In particular, it plays a significant role in angiogenesis (Rosenkranz and Kazlauskas, 1999; Yu et al., 2003). Both α- and β- PDGFRs mediate strong mitogenic signals, studies suggest that β-PDGFR induces more potent transforming signals than α-PDGFR (Bejcek et al., 1992; Yu et al., 2000). PDGF-D promotes epithelial-mesenchymal transition (EMT), which in turn increases prostate tumor growth (Kong et al., 2008).
Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein with a molecular weight of approximately 45 kDa. VEGF promotes cell proliferation; survival and migration also regulate embryonic vasculogenesis, angiogenesis, and permeability in numerous physiological and pathological conditions in the prostate gland (Kitagawa et al., 2005; Cohen, 2006; Shibuya and Claesson-Welsh, 2006). VEGF is the key mediator of angiogenesis in cancer, in which it is up regulated by oncogene expression, a variety of growth factors and also hypoxia. Angiogenesis is essential for cancer development and growth (Carmeliet, 2005).

VEGF-A and VEGF-B promote vascular angiogenesis predominantly by activating their receptors VEGFR-1 (Flt1) and VEGFR-2 (Flk and KDR). VEGF-C (Joukov et al., 1996) and VEGF-D, also known as c-fos-induced growth factor (Orlandini et al., 1996), have been identified as important mediators of lymph-angiogenesis and tumor metastasis by activating receptors VEGFR-2 and VEGFR-3 (Achen et al., 1998). Mature, bioactive VEGF-C and VEGF-D bind to their cognate receptor VEGFR-2 and VEGFR-3 via tyrosine phosphorylation and results in the induction of angiogenic and lymph-angiogenic signals (Jussila and Alitalo, 2002). Normal prostate epithelium expresses low levels of VEGF expression, which is additionally increased in prostate adenocarcinoma and lymphnode metastasis (Mazzucchelli et al., 2000; Zeng et al., 2004; 2006). Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers (Smith et al., 2010).
Nerve growth factor

NGF and other neurotrophins are also produced in the prostatic stroma and exert a paracrine regulatory effect on epithelial cell growth. Increased expression of nerve growth factor, in cancer cells, activation of the tyrosine kinase, as opposed to the p75NTR receptor, are all potential mechanisms for nerve growth factor promotion of PCa growth (Djakiew, 2000).

Hepatocyte growth factor

Hepatocyte growth factor is produced in the stromal cells of the prostate, increases cancer cell proliferation and invasiveness. The protein product of the c-met proto-oncogene is hepatocyte growth factor receptor. Quantitative expression of c-met increases progressively in human tissue with benign prostatic hyperplasia, prostatic intraepithelial neoplasia, localized cancer and metastases (Myers and Grizzle, 1996).

Fibroblast growth factor (FGF)

FGF plays an important role in the normal prostate (Jacobs et al., 1988; Mydlo et al., 1998). Another important growth factor KGF (Keratinocyte Growth Factor) is a member of basic FGF, produced from stromal cells and even this has mitogenic effect on epithelial cells (Peehl et al., 1995). FGF signaling promotes development, tissue homoeostasis and tumorigenesis of prostate (Lin and Wang, 2010).

2.2.3 DISORDERS OF PROSTATE

Prostatitis

Prostatitis is a frequently painful condition that involves inflammation of the prostate and sometimes the areas around the prostate.
Scientists have identified four types of prostatitis:

- Chronic prostatitis/chronic pelvic pain syndrome
- Acute bacterial prostatitis
- Chronic bacterial prostatitis
- Asymptomatic inflammatory prostatitis

Men with asymptomatic inflammatory prostatitis do not have symptoms. A health care provider may diagnose asymptomatic inflammatory prostatitis when testing for other urinary tract or reproductive tract disorders. This type of prostatitis does not cause complications and does not need treatment (Blumenfeld et al., 1992).

**Benign Prostatic Hyperplasia (BPH)**

BPH is a benign increase in size of the prostate due to the hyperplasia of prostatic stromal and epithelial cells. About 80% of men eventually develop enlarged prostates, but only some experience significant symptoms. BPH is a normal condition and is not life-threatening. BPH occurs in the inner zone of the prostate, while cancer tends to develop in the outer area (Berry et al., 1984).

**2.2.4 PROSTATE CANCER**

Prostate cancer is the second most common cancer in men worldwide. Two third cases of PCa are diagnosed in more developed regions of the world (Jemal et al., 2011). More than 1.1 million cases of PCa were recorded in 2012, accounting for around 8 per cent of all new cancer cases and 15 per cent in men (Siegel et al., 2013). Age-adjusted incidence rates of PCa have increased dramatically and this is largely because of the increased availability of screening for prostate-specific antigen (PSA) in men without symptoms of the disease. This test leads to detection of many PCAs that is
small and/or would otherwise remain unrecognised, and which may or may not develop further into higher stage disease.

Incidence rates vary by more than 25-fold worldwide, with the highest rates recorded primarily in the developed countries of Oceania, Europe, and North America. Males of African descent in the Caribbean region have the highest PCa mortality rates in the world, which is thought to reflect partly due to the difference in genetic susceptibility (Miller et al., 2003; Bock et al., 2009). In contrast to the trends in Western countries, incidence and mortality rates are rising in several Asian and Central and Eastern European countries, such as Japan (Baade et al., 2009; Bray et al., 2010).

Prostate cancer mostly diagnosed in African, Americans (116/1000000 persons/year), intermediate incidence rates are found in Caucasians (71/1000000) and lowest rate among Asians (Japanese, 39/1000000; Chinese 28/1000000; India, 8/1000000) (Gronberg, 2003). Average annual cancer incidence rates in India ranged from 5.0 to 9.1 per 100000/year (Hebert et al., 2006). PCa incidence in Indian major cities Bangalore 2.4%, Chennai 4.7%, Delhi 3.1% and Mumbai 0.8% per 1 lac population (Yeole, 2008, Mathur et al., 2010). Swaminathan et al., (2011) also showed cancer trends in Chennai and predicted the future cancer burden in Chennai and Tamil Nadu state, India, using data on 89 357 incident cancers from the Chennai registry during 1982-2006. Among men, a 21% decline in the incidence of oesophageal cancer by 2016 contrasts with the 42% predicted increase in PCa.

Most prostate cancers are slow growing; however, some grow relatively fast. The cancer cells may spread from the prostate to other parts of the body, particularly the bones and lymph nodes. Initially PCa is androgen dependent, however in the later
stages it becomes androgen independent. Prostate cancer transformation is a three-stage phenomenon of initiation, promotion and progression (Goldsworthy et al., 1990). Initiation occurs when a carcinogen induces general or specific changes to DNA, these are transient and readily eliminated by DNA repair mechanism (Leadon, 1990). If the genomic insult is extensive or of a chronic nature, or if DNA repair mechanism are compromised, unrepaired lesion are fixed as mutation in the target cell population via cell proliferation (Coleman and Tsongalis, 1995). Replication of the initiated cells, which often have gained selective growth advantages into focal aggregates marks the beginning of promotion, while progression may take many years, it can also be accelerated by general or specific endogenous or exogenous factors. When it occurs, several sequential and parallel events are evident in the advancing neoplasm. These include physiological properties that enhance invasiveness, morbidity and dissemination an escape from immune surveillance, and the emergence of new growth regulatory mechanism in distantly metastatic sites (Pilot, 1993; Cheng et al., 1994).

Risk Factors

The risk factors of PCa can be classified as exogenous and endogenous factors.

Exogenous factors

Life style, dietary factor, specific environmental and geographical factors are the exogenous determining factors affecting the rate of tumor promotion and progression (Henderson et al., 1982; Carter et al., 1990; Henderson et al., 1991; Giovannucci et al, 1993).
Life Style

There are several biologically plausible mechanisms suggesting that increased exposure to carcinogenic compounds in cigarettes, such as polycyclic aromatic hydrocarbons, heterocyclic aromatic amines, and nitrosamines promote prostate carcinogenesis (Hecht, 2006). Important hormonal factors may also be influenced by smoking. It is suggested that male smokers have elevated circulating levels of testosterone, androstenedione, and dihydrotestosterone (DHT) (Shiels et al., 2009), compared to non-smokers. Vasectomy and physical activity are the other factors, which induce the risk of PCa (Gronberg, 2003).

Dietary Factors

Dietary factors like calcium have been associated with risk of PCa. The increased calcium levels in the circulation inhibit the 1α, 25-dihydroxy vitamin D3 synthesis. Vitamin D3 involves normal cell, differentiation and also inhibits PCa cell growth and development. Vitamin D3 can protect oxidative stress in non-malignant human prostate epithelial cell lines (Bao et al., 2008). Calcium intake increases risk of PCa among Singapore and Chinese men (Butler et al., 2010). Dietary fat has the most significant association with PCa (Wittemore et al., 1995; Giovannucci, 1995). Animal fat especially from red meat is associated with the highest risk of PCa (Mettlin et al., 1989). Calcitriol was found to inhibit MNU and testosterone induced PCa and may be an effective therapy for the treatment of early PCa in vivo model (Senthilkumar et al., 2006).
Obesity

Obesity is more consistently related to aggressive prostate tumors and that abdominal obesity may be associated with an increased risk of PCa even in relatively lean men (Hsing et al., 2001; Hubbard et al., 2004). Higher serum levels of leptin, the product of obesity gene, Ob have been linked to larger tumor volume (Gade-Andavolu et al., 2006). Leptin, an adipocyte-derived cytokine that is closely associated with obesity, has associated with the motility and migration of human PCa cells and expression of αvβ3 integrin on these cells (Huang et al., 2011).

Environmental Factors

An epidemiological study suggests that increased cadmium exposure is correlated with increased PCa incidence (Piscator, 1981; Sanchez et al., 1992; Waalkes and Rehm, 1994). In, in vitro studies cadmium causes malignant transformation of rat ventral prostatic epithelial cells (Ghatak et al., 1996). While exposure to cultured human prostatic epithelium, induces cell proliferation (Webber, 1985). Cadmium down-regulates expression of the X-linked inhibitor of apoptosis protein (XIAP) in PCa cells (Golovine et al., 2010). Arsenic metal ions induces significantly, increased the risk of PCa (Barthel, 1981). Prostate stem cells have a survival selection advantage during arsenic exposure that favors their accumulation and facilitates their malignant transformation. Exposure to environmental estrogen agonists such as DDT, PCB, Methoxychlor and other herbicides and fungicides may significantly contribute PCa risk (Barthel, 1981; Thomas and Colborn, 1992; Prins, 2008).
**Geographical Pattern**

Prostate cancer incidence is high in the Northern European countries, United States and Canada. Southern Europe and South Americans, African observed in moderate incidence while, Asian countries have the lowest incidence (Zaridze *et al.*, 1984). The rate of PCa increases at least by 1% every year in Asian countries as the people adapting to western life style. The prostate cancer incidence in India, ranks 5th in mortality rate (Quinn and Babb, 2002).

**ENDOGENOUS FACTORS**

The endogenous risk factors are genetic factors and endocrine factors.

**Age**

Prostate cancer incidence escalates dramatically with increasing age. More than 65% of all PCas are diagnosed in men over the age of 65. The registration rate by age cohort in England and Wales increased from eight per thousand population in men aged 50 to 56 years to 68 per thousand in men aged 60 to 64 years; 260 per thousand in men aged 70 to 74 years, and peaked at 406 per thousand in men aged 75 to 79 years (Colloca and Venturino, 2011). At all ages, incidence of PCa in blacks exceeds those of whites (Partin *et al.*, 1991).

**Family History**

Men who have first-degree family members with PCa appear to have double the risk of getting the disease compared to men without PCa in the family. Approximately 15% of men with a diagnosis of PCa will be found to have a first-degree male relative
(e.g., brother, father) with PCa, compared with approximately 8% of the U.S. population (Cannon et al., 1982; Steinberg et al., 1990; Wittemore et al., 1995).

Genetic Factors

Scientists have found several inherited gene changes that seem to raise PCa risk (Bostwick, 1996). AR gene mutations in the steroid binding domain were found in metastatic PCa patients (Culig et al., 1993). The frequency of mutation generally appears higher in hormone refractory metastatic tumor compared with untreated lower grade primary tumors (Marcelli et al., 2000). Androgen receptor germline sequence variants cause PCa risk (Lindstrom et al., 2007). AR gene amplification may also contribute to the development of androgens independent growth of PCa (Visakorpi et al., 1995).

The expression of estrogen receptor is commonly involved in the progression of PCa. The genetic polymorphism of genes in the estrogen metabolism significantly increases with familial PCa risk. Moreover, polymorphism in codon 10 of ER-α and variants of the GGGA polymorphism from the α-gene might be associated with increased risk of PCa (Cancel-Tassin et al., 2003; Talcott et al., 2003). Whereas, ER-β decreases during malignancy progression, owing to methylation of CpG dinucleotides in the promoter gene, this suggests that ER-β involved in the tumor suppression function (Leav et al., 2001; Zhu et al., 2004; Ji et al., 2005).

Epigenetic changes including CpG methylation and histone acetylation play important roles in the regulation of AR pathway signaling. Hypermethylation of the AR gene is more frequent in CRPC tissues (29%) compared with untreated primary tissues (10%) suggesting that hypermethylation may contribute to the development of a
castrate-resistant phenotype (Nakayama et al., 2000; Bayraktar, 2010; Albany et al., 2011).

**Oncogenes and Tumor suppressor genes**

Oncogenes and tumor suppressor genes are involved in neoplastic transformation of the prostate (Bostwick, 1996). Ras oncogene activated by point mutations in codon 6 causing missense mutations was identified in PCa sample (Suzuki et al., 1994). The c-myc gene is a cellular proto-oncogene and is a nuclear phosphor protein (Kato et al., 1992). Studies have shown that the frequency of c-myc alterations, is associated with increased risk of PCa (Buttyan et al., 1987; Funa et al., 1991). Over expression of c-myc oncogene is seen in PCa (Hawksworth et al., 2010).

pRb (Retinoblastoma protein) gene located on the short arm of chromosome 13, functions in regulating the cell cycle progression. Mutations of the Rb gene have been identified in various human neoplasms. Several other groups have evaluated genetic alterations in the region of the Rb gene using polymorphic markers (Ittmann and Weiczorek, 1996; Jarrard et al., 2002).

p53 gene negatively regulates cell growth (Levine et al., 1991). Inactivation of the gene, contributes to the genesis and progression of numerous cancers. The frequency of p53 mutation in primary PCa ranges from 1-42% (Effert et al., 1993).

PTEN (Protein tyrosine phosphates) is a tumor suppressor gene. This can suppress tumor cell growth by antagonism of protein tyrosine kinases, which may regulate tumor cell invasion and metastases through interaction at focal adhesions (Li et al., 1997). Inactivation of PTEN gene by homozygous deletions occurs in approximately 13% of primary localized PCa (Wang et al., 1998).
Metastases suppressor genes (MSGs)

E-cadherins is a MSGs located on chromosome 16q22.1. The gene product is involved in developmental morphogenesis and maintenance of the epithelial phenotype is mediatory epithelial cell-cell recognition and adhesion processes (Takeichi, 199; Takeichi, 1995). Studies using frozen PCa samples have revealed a strong correlation between decreased E-cadherins staining and increased histological grade advanced clinical stage and the presence of metastasis at diagnosis (Umbas et al., 2005).

kAI1 gene located on human chromosome 11p11.2 has been shown to suppress the tumor metastasis when introduced in to the highly metastatic dunning R-3327 rat PCa cell line AT6.1. In addition, expression of the gene is reduced in human cell line derived from metastatic PCa specimen (Gao et al., 1997). Another one important MSG is located near kAI1 is CD44 (Dong et al., 1995). The CD 44 gene 11p13 encodes an integral membrane glycoprotein that was initially discovered to be a lymphocyte homing molecule. Its expression was upregulated in PCa samples and it is proved to be involved in PCa invasion (Iczkowski et al., 2003). Polymorphisms in p21 gene are overexpressed in PCa (Facher et al., 1997).

Endocrine Factors

The development of the prostate is dependent upon the secretion of dihydrotosterone (DHT) by the fetal testis. Testosterone causes normal virilization of the Wolffian duct structures and internal genitalia and is acted upon by the enzyme 5-alpha-reductase (5AR) to form DHT (Ross et al., 1986). Serum androgen levels tend to be elevated in high-risk populations, such as African, American; however, androgen levels are found to be normal in Japanese men than in Caucasians. There are multiple
evidences suggesting that estrogens are involved in prostate carcinogenesis. In worldwide the African-Americans have the higher risk of PCa with elevated levels of serum estrone (E₁) and estradiol (E₂) levels even in healthy young men (Srinivasan et al., 1986). More epidemiological data support that early neoplastic epithelial cells develop in an environment of rising estrogenic stimulation and decreasing androgenic influence. CCDC62/ERAP75 is a co-activator of ER and this protein is mainly present in the nucleus and widely expressed in many PCa cell lines (PC-3, DU145, LNCaP, 22Rv1) than in the normal prostate epithelial cells (BPH-1). CCDC62/ERAP75 which is preferentially expressed in PCa cells enhances the ER-β transactivation of, target genes expression and E₂-mediated LNCaP cell growth (Chen et al., 2009).

SCREENING AND DIAGNOSIS

The PSA blood test is widely available for screening men for PCa. Digital rectal examination (DRE), a physician palpates the prostate in order to feel lumps or masses. Biopsy, if preliminary tests raise the suspicion of cancer, physicians will perform a biopsy. Biopsy is used to diagnose PCa, and is a very accurate method for predicting the severity of an existing cancer. Bone Scans and X-Rays are revealed, whether the cancer has invaded the bones. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI), scans can further pinpoint the location of cancer that has spread beyond the prostate.

Symptoms

Prostate cancer usually causes no symptoms in the early stages. As the malignancy spreads, it may constrict the urethra and cause urinary problems. Later-stage urinary symptoms typically include, weak urinary stream, inability to urinate,
blood in the urine, interruption of urinary stream (stopping and starting), and pain or burning sensation during urination.

**Treatment**

**Hormonal therapy**

Androgen deprivation therapy (ADT), with gonadotrophin releasing hormone analogues or surgical orchidectomy, is standard treatment for advanced PCa (Wilt et al., 2005) and antiandrogen, LHRH agonists, such as leuprolide also used for PCa treatment.

**Surgery**

Radical prostatectomy or cryosurgery removes or destroys the prostate gland. The vessels that carry semen to the surrounding tissue may also be removed. With cancer that has spread beyond the prostate, the pelvic lymph nodes should be removed.

**Radiation**

Radiation is used to destroy tumors. Complications include damage to adjacent organs such as the gastro-intestinal tract and bladder incontinence and impotence.

**Common side effects of androgen suppression drugs**

Osteoporosis is a disease, loss of bone density. This risk is higher with orchidectomy than with androgen suppressants. Some androgen suppressants, such as bicalutamide, may cause less bone loss. The use of selective estrogens receptor modulators (SERMs) may actually be bone protective. A number of medications are available to help prevent or reduce bone loss. Bisphosphonates are used for preventing the bone loss. Other common side effects include, diarrhoea, loss of muscle mass,
psychological disturbances, fatigue, loss of sexual drive and sexual dysfunction, swelling of the breasts (gynecomastia), nausea and vomiting, hair loss and anemia (Daniell et al., 2000; Debruyne, 2002).

**Types of cancer:**

There are two types of prostate cancer

A. Androgen-dependent cancer

B. Androgen-independent cancer

**A. Androgen-dependent cancer**

Prostate gland functions as regulated by androgen. Initially PCa is androgen-dependent and a leading cause of cancer morbidity and mortality in men (Bubley and Balk, 1996). The majorities of PCa tissues express AR, for their growth, and initially respond to androgen ablation therapy (Huggins and Hodges, 1972). Epidemiological data further suggest a role for increased AR activity in stimulating PCa development, as higher testosterone levels and lower levels of sex steroid binding globulin are associated with an increased risk of PCa (Gann et al., 1996).

**B. Androgen-Independent Cancer**

In advanced disease, PCa usually returns within about 18 months after antiandrogen treatments. In such cases, the condition is referred to as androgen-independent, and the tumors are not responsive to antiandrogen therapy. The transition to androgen independence is a multifaceted process that involves selection and outgrowth of pre-existing clones of androgen-independent cells (clonal selection) as
well as adaptive up-regulation of genes that help the cancer cells survive and grow after androgen ablation (adaptation).

Prostate cancers are heterogeneous tumors composed of various subpopulations of cells that respond differently to androgen withdrawal therapy. Indeed, this tumor heterogeneity may reflect either a multifocal origin, adaptation to environmental stimuli, and/or genetic instability of the initial cancer; this heterogeneity, in turn, provides the basic requirements for androgen-independent progression to occur because of clonal expansion or adaptive mechanisms in different subpopulations of cells. PCa is initially dependent on androgens for growth. Almost every patient develops recurrent androgen independent PCa which has a very poor prognosis because no effective treatment is currently available.

Prostate carcinoma is a leading hormone-dependent malignancy associated with a high incidence of morbidity and mortality due to its ability to develop metastatic lesions in different organs. Initiating as a less aggressive hormone-responsive type, PCa gradually progresses to a highly invasive hormone insensitive phenotype associated with the loss of functional AR due to AR gene silencing, mutations in the AR gene, or interference in hormone receptor signaling pathways (Grossmann et al., 2001). Over time, cancer cells become refractory to any kind of hormonal treatment, one of the few therapeutic strategies currently available for treating patients with PCa (Denmeade and Isaccs, 2002).

2.2.5 FLAVONOIDS

Flavonoids (or bioflavonoids) (from the Latin word *flavus* meaning yellow) are a class of plant secondary metabolites. Flavonoids belong to the polyphenol family.
Flavonoids can be visualized as two benzene rings which are joined together with a short three carbon chain. One of the carbons of the short chain is always connected to a carbon of one of the benzene rings, either directly or through an oxygen bridge, thereby forming a third middle ring, which can be five or six-membered. The flavonoids consist of 6 major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids (Harborne and Williams, 2000). Together with carotenes, flavonoids are also responsible for the coloring of fruits, vegetables and herbs.

Flavonoids are potent antioxidants *in vitro* and *in vivo*. They have beneficial effects against cancer, cardiovascular disease, inflammation and neurodegenerative disorders (Middleton *et al*., 2000; Williams *et al*., 2004). Green and black tea contains about 25% percent flavonoids. Other important sources of flavonoids are apple (quercetin), citrus fruits (rutin and hesperidin).

Flavonoids are ingested daily in human diet; much attention has been paid to their antioxidant properties and to their inhibitory role in various stages of tumor development in animal studies (Hertog, 1996). They are known to act against several pathological conditions, owing mainly to their antioxidant activity, and their possible role as chemopreventive factors. It can protect the organism against ROS and present multiple biological effects, including liver protection, antithrombotic, anticancer, and immunostimulant activities (Hertog *et al*., 1996; Santos *et al*., 1998; Van Acker *et al*., 1998).

### 2.2.6 QUERCETIN

Quercetin was discovered by Albert Szent-Gyorgi, in 1930, who also discovered vitamin C (The Nobel Prize in Physiology or Medicine 1937). Quercetin (3,5,7,3′,4′-
pentahydroxyflavone) is an important dietary flavonoid, present in different vegetables, fruits, seeds, nuts, tea and red wine. The average daily intake of quercetin can reach 30 mg in most western countries. It has been shown to have diverse biological activities, including anti-proliferative and apoptotic effects (Choi et al., 2001). It also prevents oxidant injury and cell death by several mechanisms including scavenging oxygen radicals, protecting against lipid peroxidation, and chelating metal ions (Ishige et al., 2001; Wang et al., 2104). Quercetin is able to protect retinal pigment epithelial cells from oxidative damage and cellular senescence in vitro. Quercetin has also received greater attention as pro-apoptotic flavonoid with a specific and almost exclusive activity on tumor cell lines rather than normal, non-transformed cells (Kook et al., 2008).

Chemical structure -- molecular mass 338 g/mol - molecular formula C$_{15}$H$_{10}$O$_{7}$

Quercetin belongs to a family of naturally occurring, plant compounds known as polyphenols, flavonoids, flavonols and bioflavonoids, and it appears to have both anti-inflammatory and antioxidant properties. Quercetin exerts multiple pharmacological effects. Quercetin arrests the cell cycle and induces apoptosis (Wei et al., 1994; Bischoff, 2008). It is also known that quercetin inhibits heat shock protein expression (Wei et al., 1994). Quercetin (3, 3’, 4’,5,7 penta hydroxyl flavanone) which possesses a wide spectrum of pharmacological properties (Aviram and Fuhrman, 2002)
is found in many kinds of food. Quercetin inhibits the proliferation of cancer cells (Choi et al., 2001; Kuo et al., 2004; Ong et al., 2004) and also inhibits breast cancer cell growth and induces apoptosis (Choi et al., 2009). Quercetin inhibits hepatocyte growth factor induced medulloblastoma cancer cells (Labbe et al., 2009). Quercetin also enhances TRAIL-induced apoptosis in PCa cells via increased protein stability of death receptor 5 (Jung et al., 2010).

Our earlier studies showed that quercetin induces cell cycle arrest and apoptosis in PCa cells (Vijayababu et al., 2005). Quercetin decreased secretion of IGF-I, IGF-II and increased IGFBP-3 secretions in PC-3 cells (Vijayababu et al., 2006). Senthilkumar et al., (2010, 2011) demonstrated that quercetin induces apoptosis (intrinsic and extrinsic mechanisms) and regulates uPA/uPAR mRNA expression in PCa cells. Sharmila et al., (2013, 2014) showed that quercetin acts as a chemopreventive agent in chemically induced PCa in rats.

However, the role of quercetin on EGF- mediated signaling in cell survival, proliferation, migration and invasion of PCa cells is not known. So the present study is aimed to investigate the effect of quercetin on EGF- mediated signaling pathways in human androgen independent PC-3 cell line.

**Absorption of quercetin**

Most animal and human trials of oral dosages of quercetin aglycone show absorption in the vicinity of 20 percent. Rats eating a diet supplemented with 0.2-percent quercetin for three weeks attained a serum concentration of 133 μM, mainly in sulfated and glucuronidated forms (Morand et al., 1998). Humans fed fried onions containing quercetin glucosides equivalent to 64 mg of the aglycone form reached a
maximum serum concentration of 196 ng/ml (0.6 μM) 2.9 h after ingestion. The half-life of this dose was 16.8 hrs, and significant serum levels were noted up to 48 h post ingestion. Elimination half-life was measured at 25 h (Hollman et al., 1995). Hollman et al. (1997) fed nine healthy subjects with quercetin glucosides equivalent to 64 mg aglycone from onions, glycosides equivalent to 100 mg aglycone from apples, and pure rutinosides equivalent to 100 mg aglycone. Peak plasma levels of 225 ng/ml (0.8 μM) were reached after the onion meal, 90 ng/ml for the apples, and 80 ng/ml for the rutinoside. Half-life was again found to be about 25 h. Thus, it can be determined that absorption of dietary quercetin is reasonably generous (Ross and Kasum, 2002).

Recent studies in our laboratory demonstrated that quercetin and its metabolites were detected in the serum by HPLC. Quercetin can be absorbed in the intestine after luminal hydrolysis by Phlorizin hydrolase (LPH), an enzyme at brush border membrane of intestinal cells, involved in the in vivo intestinal uptake of quercetin sugars. HPLC analysis demonstrated detectable levels of quercetin and its conjugated metabolites in the serum indicating sufficient intestinal absorption of quercetin (Firdous et al., 2014).

2.2.7 MECHANISMS OF ACTION

Anti-Oxidant and Pro-Oxidant Properties

Quercetin is an excellent free radical scavenging antioxidant owing to the high number of hydroxyl groups and conjugated π orbitals by which quercetin can donate electrons or hydrogen, and scavenge H₂O₂ and superoxide anion (•O₂−) (Heijnen et al., 2001). The reaction of quercetin with •O₂− leads to the generation of the semiquinone
radical and \( \text{H}_2\text{O}_2 \) (Metodiewa, et al., 1999). It also reacts with \( \text{H}_2\text{O}_2 \) in the presence of peroxidases, and thus it decreases \( \text{H}_2\text{O}_2 \) levels and protects cells against \( \text{H}_2\text{O}_2 \) damage.

**Cell Cycle as a Possible Target**

Apart from scavenging ROS, another important effect of quercetin is to regulate cell cycle by modulating several molecular targets, including p21, cyclin B, p27, cyclin-dependent kinases and topoisomerase II. Depending on the cell type and tumor origin, quercetin is able to block the cell cycle at G2/M or at the G1/S transition. In particular, quercetin causes G2/M arrest in human esophageal squamous cell carcinoma cell line through up-regulation of p73 and p21waf1 and subsequent down-regulation of cyclin B1, both at the mRNA and protein levels (Zhang et al., 2005). In human breast carcinoma cell lines such as SKBr3, MDA-MB-453 and MDA-MB-231 cells, low doses of quercetin inhibit proliferation. Cell-cycle arrest occurs at the G1 phase through the induction of p21 and through the concomitant decrease of phosphorylation of the retinoblastoma protein (pRb). Quercetin downregulates the cyclin B1 and cyclin-dependent kinase (CDK) 1, which are essential in the progression to the G2/M phases of the cell cycle (Jeong et al., 2009).

In human lung cancer cells NCI-H209, quercetin glucuronides induce cell-cycle arrest at G2/M phase by increasing the expressions of proteins such as cyclin B, Cdc25c-ser-216-p and Wee1 (Yang et al., 2006). A similar antiproliferative effect has also been observed both for highly or moderately aggressive PCa cell lines, whereas no effect has been found for poorly aggressive PCa cells (Nair et al., 2004). In HepG2 human hepatoma cells, quercetin blocks cell-cycle progression at the G1 phase, and exerts this effect through the increase of p21 and p27 and p53 (Mu et al., 2007).
Similar effects on the cell cycle have also been reported in SW872 cells (Robaszkiewicz et al., 2007). Topoisomerase II (TopoII) is another potential and delicate target of quercetin (Bandele et al., 2008).

**Tyrosine kinase inhibition**

Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the transduction of growth factor signals to the nucleus. In patients with advanced cancers, intravenous administration of quercetin (dosages 60-1700 mg/m²) led to inhibition of lymphocyte tyrosine kinase at one hour in nine of eleven cases. This inhibition was seen as late as 16 hours post-administration (Ferry et al., 1996). Tyrosine kinase expression is thought to be involved in oncogenesis via an ability to override normal regulatory growth control (Boutin, 1994; Ferry et al., 1996). Drugs targeting tyrosine kinase activity (tyrphostins) are envisioned as possible anti tumor agents without the cytotoxic side-effects seen with conventional chemotherapy (Klohs et al., 1997). Quercetin was the first tyrosine kinase inhibited compound tested in a human phase I trial (Ferry et al., 1996).

**Estrogen receptor binding capacity**

The role of the type II estrogen receptor (ER II) *in vivo* is not entirely clear (Markaverich et al., 1988). Quercetin has been shown to induce ER II expression in both type I estrogen receptor positive (ER+) and type I estrogen receptor negative (ER-) human breast cancer cells. The induction of ER II allows for greater growth inhibition of ER- cells with quercetin treatment (Scambia et al., 1993). In cultured human melanoma cells, quercetin was found to bind ER II sites with an affinity similar to tamoxifen and diethylstilbestrol. The concentration required for 50-percent growth
inhibition for one cell line was lower for quercetin (7 nM) than for tamoxifen (9 nM), otherwise the growth inhibitory activity of the two compounds was similar (Piantelli et al., 1995). ER II sites are found in normal tissue and on many different human tumor types, including breast, ovarian, colorectal, meningeal, leukemic, and melanoma (Piantelli et al., 1995). ER II expression is independent of estrogen-receptor (type I) status.

**Inhibition of heat shock proteins**

HSPs are a group of evolutionarily, highly conserved chaperone proteins (Lindquist and Craig, 1988) that are found in all organisms and in all cell types. Quercetin has been found to inhibit production of heat shock proteins in several malignant cell lines, including breast cancer (Hansen et al., 1997), leukemia (Elia et al., 1996) and colon cancer (Koishi et al., 1992). Quercetin inhibits the induction of heat shock proteins (HSPs) and thermo tolerance without affecting the synthesis of other proteins (Koishi et al., 1992; Scambia et al., 1993). Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses (low circulation, fever, etc.), and are associated with shorter disease free survival (Ciocca et al., 1993) and chemotherapy drug resistance (Oesterreich et al., 1993) in breast cancer. Quercetin also suppresses HeLa cell viability by inhibition of HSP70 (Jung et al., 2010a).

**Direct Pro-Apoptotic Effects of Quercetin**

The proapoptotic effects of quercetin may result from multiple pathways. First, in MDA-MB-231 cells, quercetin treatment increases cytosolic Ca\(^{2+}\) levels and reduces
the mitochondrial membrane potential, thus promoting activation of caspase-3, -8 and -9 (Chien et al., 2009). The capability of quercetin to induce apoptosis via mitochondrial pathway has been confirmed in U937 cell line (Ferraresi et al., 2005). Quercetin inhibits cell growth and induces apoptosis by down-regulating the transcriptional activity of β-catenin/Tcf signaling, with the consequent down-regulation of cyclin D1 and survivin (Ma et al., 2005; Shan et al., 2009). Quercetin likely triggers apoptosis through the generation of ROS and the subsequent activation of AMPKα1 and ASK1 which in turn, accompanied by p38 activation and recruitment of caspases (Lee et al., 2008). The antiproliferative and pro-apoptotic effects could be related to the capability of quercetin to directly bind tubulin, provoking the depolymerization of cellular microtubules (Gupta and Panda, 2002). Quercetin is a potent enhancer of TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, through the induction of the expression of death receptor (DR)-5, a phenomenon that specifically occurs in PCa cells (Jung et al., 2010b). The enhancement of TRAIL-induced apoptosis by quercetin also occurs through the inhibition of the expression of survivin in the ERK-MSK1 signal pathway (Kim et al., 2008).

Thus, the capability of quercetin to induce apoptosis in cancer cells (via both the intrinsic and extrinsic pathways) undoubtedly renders this molecule an interesting tool in the oncology field.

**Use of quercetin with standard oncologic therapeutics**

**Radiotherapy**

An *in vitro* study showed a significant but mild enhancement of the cytotoxic effect of radiation on rat hepatoma cells when quercetin was added to the medium (van Rijn and van den Berg, 1997).
Chemotherapy

Quercetin has been shown to increase the therapeutic efficacy of cisplatin both \textit{in vivo} and \textit{in vitro}. In mice bearing human tumor xenografts, intraperitoneal treatment with a combination of 20 mg/kg quercetin and 3 mg/kg cisplatin led to a significantly reduced tumor growth compared to treatment with either drug alone (Hofmann \textit{et al.}, 1990). In contrast to the experiment using various doses of quercetin on pharyngeal cancer xenografts in mice (Castillo \textit{et al.}, 1989) treatment with 20 mg/kg quercetin was not found to be an effective single agent therapy. An \textit{in vitro} study showed quercetin worked synergistically with busulphan against human leukemia cell lines. Quercetin/busulphan concentrations in 1:1 and 3:1 ratios led to demonstration of much smaller cytotoxic doses of busulphan (Hofman \textit{et al.}, 1990). Addition of 1-10 \textmu M quercetin to adriamycin treatment led to a dose-dependent increase in cytotoxicity compared with chemotherapy treatment alone in cultured multidrug-resistant human breast cancer cell lines (Scambia \textit{et al.}, 1993). Quercetin has also been shown to decrease resistance to gemcitabine and topotecan (Sliutz \textit{et al.}, 1996). Simultaneous liposomal delivery of quercetin and vincristine enhances estrogen-receptor-negative breast cancer cells (Wong and Chiu, 2010).

This finding is consistent with the fact that many flavonoids have been shown to decrease resistance to chemotherapy in multidrug-resistant tumor cell lines. Our earlier studies showed that quercetin induces cell cycle arrest and apoptosis by decreasing antiapoptotic Bcl-2 and increasing Bax and p21 expression in PC-3 cells (Vijayababu
et al., 2005). Quercetin decreased secretion of IGF-I, IGF-II and increased IGFBP-3 secretions (Vijayababu et al., 2006). Senthilkumar et al., (2010; 2011) reported that quercetin induced apoptosis and decreased uPA/uPAR expression in PCa cells.

However, the role of quercetin on EGF – mediated signaling pathways in PCa cells is not known. So the present study is aimed to investigate the effect of quercetin on EGF-mediated signaling involved in PCa cell survival, proliferation, invasion and migration in human androgen independent PC-3 cell line.
2.2.8 **SCOPE OF THE PRESENT STUDY**

Epidermal growth factor plays a critical role in cancer progression to advanced stages. Prostate cancer becomes more dependent on growth promoting actions of EGF during androgen withdrawal. Increased production of EGF during cancer progression helps in cancer metastasis. The discovery of the drugs for the treatment of PCa is important, since it is the fourth most common cancer in worldwide, and its incidence is rising in India. Recently, it was reported that PCa is leading cancer prevalence in USA. Prostate cancers typically begin as androgen-sensitive lesions but frequently develop into androgen-insensitive lesions with the progression to advanced stages. Plant based dietary flavonoids has been shown to reduce the incidence as well as progression of PCa. Flavonoids are widely distributed in plants, fulfilling many functions. Flavonoids are polyphenolic compounds and they possess anticancer effects on several cancer models. Quercetin, a bio-flavonoid is the most abundant flavonoid present in onions, many vegetables and fruits. Its anticancer effects have been investigated in many tumors and cancer cell lines. Quercetin has been demonstrated to induce cell death and prevent PCa in cell lines and in rat model. Prostate cancer progression is mediated by activation of survival, proliferation and inhibition of apoptosis signaling. However, the role of quercetin on EGF - mediated signaling pathways in cancer progression remains unknown. Hence, the present study was designed to investigate the effect of quercetin
on EGF-mediated signaling molecules involved in cell survival, proliferation, migration and invasion of human androgen independent PCa cell line.

**Hence the objectives of the present study are:**

To investigate the effect of quercetin on EGF-mediated signaling molecules involved in cell survival and proliferation of PC-3 cell line.

To evaluate the role of quercetin on EGF–induced epithelial to mesenchymal transition, migration and invasion *via* EGFR/PI3K/Akt pathway in PC-3 cell line.

To evaluate the effect of quercetin on EGF–induced capillary tube formation (angiogenesis) in HUVEC cells.