Science is not to be regarded merely as a storehouse of facts to be used for material purposes, but as one of the great human endeavors to be ranked with arts and religion as the guide and expression of man's fearless quest for truth.

Sir Richard Arman Gregory
3. INTRODUCTION AND REVIEW OF LITERATURE

3.1 Cancer

Carcinogenesis is a process of multistep genetic changes resulting in a progressive transformation of normal cells into highly malignant phenotype. Two hypotheses have been proposed for the generation of cancer (1). First is stochastic model in which self renewal and differentiation are random and all the cells have equal but low probability of extensive proliferation. Only cells with self renewal capacity can sustain tumor growth (Fig. 1). Second is hierarchy model in which distinct classes of cells exist within a tumor (2, 3). Recent development on clinical studies on reoccurrence of cancer on chemotherapy treated patient supported the hierarchy model and advocate that only a small definable subset, the ‘cancer stem cells’ can initiate tumor growth (Ref. 4-6 and Fig. 2). Recent advancement in cancer research suggests that most of human tumors share the hallmarks of cancer and these are: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, genome instability, reprogramming energy metabolism,

![Figure 1: Schematic representation of proposed model for tumorigenesis. A. represents Stochastic model in which each cell has equal probability to form tumor whereas B represents Hierarchy model in which only a small subset of cells have the capacity to initiate tumor. (Adapted from Dick JE. Nat Biotechnol. 2009;27:44-46)
evading immune destruction and tumor microenvironment (7).

3.2 Breast Cancer

Cancer of the breast is a deregulated process of growth. About 1% of breast cancers occur in men, even though breast cancer is a disease of women (8). Cancer of the breast is the second leading cause of death in women after lung cancer. Approximately 1.38 million new cases with 0.45 million deaths due to breast cancer have been reported annually worldwide (8-9).

3.2.1 Breast Cancer Symptoms and Signs

Most breast cancers are discovered before symptoms appear by feeling a breast lump or through abnormality on mammography. However, breast discharge, nipple inversion and changes in the skin overlying the breast are also considered as breast cancer symptoms (10).

Figure 2: Schematic representation of proposed model for acquired resistance against chemotherapeutic agents. This observation further strengthens the hierarchy model of tumor initiation and highlighted that importance of targeting cancer stem cells rather than non-cancer stem cells. (Adapted from Reya et al. Nature 2001; 414:105-111)
3.2.2 Causes of Breast Cancer

Although age is another crucial factor, it may occur at any stages of life and the probability of breast cancer occurrence increases with advancement in age (10).

3.2.2.1 Genetic Causes

Breast cancer as a result of genetic alteration is believed to be hereditary that comprises about 5%-10% of total breast cancers (11). First-degree relatives (daughter, sister, and mother) are at highest risk. Several second-degree relatives (grandmother, aunt) with breast cancer may also increase risk. Even breast cancer in a male increases the risk to his close female relatives (12).

3.2.2.2 Hormonal Factor

Women who start their periods at an early age (12 or younger) or experience a late menopause (55 or older) have a relatively higher risk of occurrence (13). Using oral contraceptive pills in woman increases the risk of breast cancer than women who have never used them (14).

3.2.2.3 Lifestyle and Dietary Agents

Obese or overweight or high fat intakes are associated with high risk for breast cancer, particularly in postmenopausal women (15-17). Studies are also showing that regular exercise may reduce the risk of developing cancer of the breast.

3.2.2.4 Environmental Factor

Person with radiation treatment for one or other disease increases the risk of developing breast cancer over a period of time (18). As reported, women who received radiation therapy to the upper parts before 30 years of age have a significantly higher risk of developing breast cancer than the normal individual.

3.2.3 Stages of Breast Cancer

Staging is basically a classification of tumors based on the tumor size, lymph node involvement, and metastasis (TNM). Cancer of the breast is staged from 0 to IV by TNM staging system (19). Stage 0 is noninvasive breast cancer, which has zero metastasis and has no effect on lymph nodes. Stage I is breast tumor with the size of less than 2 cm in diameter which has not metastasized. Stage II is tumor that has spreaded to lymph nodes and is relatively small in size or tumor is somewhat larger but has not spread to lymph nodes. Stage III is breast tumor with size more than 5 cm and with greater spread to lymph nodes, or of
the inflammatory type. **Stage IV** is a metastatic breast tumor irrespective of tumor size (19-20).

### 3.2.4 Medical Treatment

Surgery is the important aspect for the therapy of breast cancer. Many breast cancer patients undergo treatment in addition to surgery, which may include radiation therapy, chemotherapy or hormonal therapy. The decision about which additional treatments are needed is based upon the stage and type of cancer, the presence of hormonal and/or HER-2/neu receptors, and patient health and preferences. Chemotherapy consists of the administration of medications that kill cancer cells or stop them from growing. Tamoxifen, Fulvestrant, Toremifene, Aromatase inhibitor and Lapatinib has been the most commonly prescribed to ER positive breast cancer patient (21-26). The other drugs anastrozole, exemestane, and letrozole are also being used for the treatment of breast cancer (27-30). Trastuzumab (Herceptin) is an antibody against the HER-2 protein, a protein responsible for cancer cell growth in women with breast cancer (about 15%-25% of breast cancers) (31). Another monoclonal antibody, Bevacizumab (Avastin) has been shown to have activity in the treatment of breast cancer and is used in combination with chemotherapy (32).

### 3.3 Melanoma

Melanoma or malignancies of melanocytic tissues is a kind of skin cancer that has been identified as one of the most malignant cancer in the United States and around the world (33). More than 68,130 new cases of melanoma and 8,700 deaths have been reported in the United States in the year, 2010 (33).

#### 3.3.1 Causes of Melanoma

Excess exposure to sun causes normal skin cells to grow abnormal which attack the tissue surrounding it (34). Melanoma is believed to be hereditary which tends to run in families (35). Having many atypical moles in individual increases the risk of melanoma (36-37).

#### 3.3.2 Symptoms of Melanoma

The important symptom of melanoma is a change in the shape, size, or color of mole or birth mark. Melanoma can be found anywhere on the body. Mostly, it is on the upper back in men and women as well as on the legs of women. Melanomas usually have an irregular or asymmetrical shape (37).
3.3.3 Treatment

Surgery is the most common treatment to remove the melanoma (38). However, Dacarbazine is being used as chemotherapy to the melanoma, even then 80% of the patient does not respond to it (39).

3.4 Osteopontin

Osteopontin (OPN) is a secreted, non collagenous, sialic acid rich, chemokine like ECM-associated phosphoglyco-protein, and is a member of SIBLING (Small Integrin Binding Ligand N-linked Glycoprotein) family which plays crucial role in determining oncogenic potential of various cancer (40-44). OPN was discovered as a transformation-specific secreted phosphoprotein by Senger et al. (45). OPN was found to be expressed in various tissues and cell types including bone, brain, kidney, vascular smooth muscle cells and many cells of epithelial origin (42, 43). Several investigators have reported the role of OPN in several physiological and pathological processes such as bone remodeling and calcification, atherosclerosis, wound healing, tissue injuries, metastasis and angiogenesis as well as in certain diseases such as arterial neointimal hyperplasia, myocardial necrosis, restenosis, formation of kidney stones, renal tubulointerstitial fibrosis and autoimmune diseases (43). OPN can protect cells or inhibit apoptosis against apoptotic agent by activating and transducing cell survival signal. This anti-apoptotic effect of OPN depends on phosphorylation status of OPN (46). Recently, Courter et al. have demonstrated that the RGD domain of OPN protects cells against apoptosis under stress and enhances tumor growth and metastasis (47). The enhanced levels of OPN in various aggressive cancer cells indicate that it may play important role in tumorigenesis (43). Moreover, transformation of mouse epidermal cells by TPA is associated with the induction of OPN expression (48). Expression of OPN is increased in a variety of cancers and is correlated with enhanced tumor progression, angiogenesis and metastasis (Ref 49-51 and Fig. 3).

3.4.1 Location of OPN in Genome

Human OPN gene is present as a single copy with approximate length of 11.1 kb (52) that contains 7 exons, six of which contain coding sequence (53). The exact location of human OPN gene at long arm of chromosome 4 is 4q22.1 (54). However, OPN gene is positioned at chromosome 5 in mice and at chromosome 8 in pig genome (54). Response element of several transcription factors like Sp1, AP-1, AP-2, PEA-3, AP-4, AP-5, CTF/NF-
1, Oct1/2, C/EBPa/AML-1 and vitamin D response element are found in the promoter region of OPN gene (55-58).

Figure 3: OPN determines the oncogenic potential of various cancers. (Adapted from Chakraborty et al. Current Molecular Medicine. 6; 819-30: 2006)

3.4.2 Splice Variants and Structural features of OPN

Kiefer et al. have shown an alternative spliced form of OPN, in which a 42-nucleotide sequence coding for 14 amino acids beginning at residue 58 is removed in human bone and decidual cells (52). Later, Saitoh et al. have reported three splice variants that are expressed in human glioma tumors (59). Moreover, He et al. have reported that besides full length OPN transcript (OPN-a), two splice variants of OPN in which OPN-b lacks exon 5 and OPN-c lacking exon 4 are expressed in MDA-MB-231 cells as shown in Fig. 4 (53). Recently, Tilli et al. have provided new insights into early steps of prostate carcinogenesis and demonstrated that OPN-c splicing isoform acts as biomarker contributing to improve PCa diagnosis and prognosis (60).

Native human OPN gene encodes a protein containing 314 amino acid residue with molecular mass of 34 kDa. However, OPN has diverse biological function and its molecular
mass varies from 25-98 kDa depending upon the degree of posttranslational modification or polymerization or fragmentation by certain protease (40-42). N-terminal of OPN is a highly acidic region consisting of nine consecutive aspartic acid residues and an arginine-glycine-aspartate (RGD) cell adhesion sequence (40). This RGD amino acid sequence binds to cell-surface integrins (e.g. αvβ3) and regulates cell migration and proliferation (61). The degree of phosphorylation and glycosylation of OPN determines the integrin receptor to be interacted to transduce signaling (43). The GRGDS sequence is flanked by several highly conserved sequences including the thrombin and MMPs cleavage site (Ref. 42 and Fig. 4). The conserved nature in the C-terminal region of OPN across the species suggests an important functional role of the region (41). The C-terminal of OPN binds to CD44 cell surface receptor and regulates various cellular events independent of posttranslational modification (41). Between the GRGD motif and the CD44-binding domain is the recognition site for β1 integrin (SVVYGLR), which is exposed only upon thrombin cleavage for interaction (62).

OPN is modified post-transcriptionally through extensive phosphorylation at tyrosine-serine/threonine, sulphation at tyrosine and glycosylation. Sorensen et al. have identified 27 different phosphorylation sites in bovine milk OPN (63). Phosphorylation is mostly catalyzed by casein kinases mainly at serine/threonine residues of OPN. In addition to single N-linked glycosylation site, there are 26 O-linked glycosylation sites in bovine OPN (64). The tyrosines in OPN do not exist within sequences recognized by sulphotransferase enzymes, although tyrosine can be sulphated. Therefore sulphation occurs mostly in highly phosphorylated form of OPN in the sialylated oligosaccharide side-chains (40). OPN plays crucial role in ECM remodeling by acting as a substrate for liver transglutaminase as well as the plasma transglutaminase factor XIIIa (65, 66). Moreover, it also serves as a novel substrate for matrix metalloproteinases MMP-3 and MMP-7 and the resulting fragments binds to β1 integrin receptor leading to enhanced adhesion and migration (67). Sialic acid residues in the OPN molecule are essential for interaction with the receptor resulting in enhanced invasive behavior of the OPN expressing cancer cells (68).
Figure 4: Schematic representation of functional domain organization of OPN and its splice variants. Upper panel: It has various MMPs and thrombin target site. GRGD domain of OPN interacts with various integrin whereas C-terminal interacts with CD44 variants to transduce cell survival signal in cancer cells. Lower panel: Two different splice variants OPN-b and OPN-c are shown along with OPN-a. Alternate splicing of OPN excludes Exon 5 in OPN-b and Exon 4 in OPN-c. (Adapted from Ahmed et al. Expert Opin Ther Targets 2011;15:1113-26)

3.4.3 OPN in Tumor Biology

3.4.3.1 Breast Cancer

The impact of OPN in human physiological and pathophysiological condition particularly in cancer has been major area of research for last decades. Several studies have clearly indicated that OPN acts as a key regulator in cancer growth and metastasis (Fig. 5). The expression profile of OPN varies from cell to cell types and tumor types as well. This variability in OPN expression among tumors has triggered interest in possible implications as a marker of tumor aggressiveness and patient prognosis (69-72). Das et al. have shown that OPN stimulates breast cancer cell motility and NF-κB mediated secretion of uPA through PI3-Kinase/Akt signaling pathways (73). Moreover, Ahmed et al. has revealed that OPN
selectively regulates p70S6K/mTOR phosphorylation leading to NF-κB dependent AP-1-mediated ICAM-1 expression in breast cancer cells (74).

**Figure 5: OPN regulates signaling cascades in tumor cells.** OPN triggers multiple intracellular pathways leading to tumor progression, metastasis and angiogenesis through binding to αvβ3 integrin and CD44 mediated pathways. OPN interacts with integrin to induce NIK-dependent NF-κB activation through both IKK/ERK-mediated pathways, and regulates the negative crosstalk between NIK/ERK and MEKK1/JNK1 pathways that stimulates uPA-dependent MMP-9 activation in melanoma cells. The binding of OPN with CD44 receptors induces p38 MAPK mediated furin expression leading to cell survival and motility in cervical cancer cells. OPN induces PI3-kinase/Akt-dependent NF-κB activation and c-Src-dependent EGFR transactivation, ERK phosphorylation, and AP-1 activation that resulted in uPA secretion in breast cancer cells. The enhanced levels of downstream effector molecules (COX-2, furin, PGE2, MMP-2, MMP-9, VEGF and uPA) regulate tumor growth, metastasis and angiogenesis. *(Adapted from Ahmed et al. Expert Opin Ther Targets 2011;15:1113-26)*

Recently, Allan et al. have indicated that OPN is a key molecular player involved in lymphatic metastasis of breast cancer and by enhancing the establishment/persistence of tumor cells in the lymphatics through RGD-mediated adhesive interactions (75). More recently, Mi et al. have proposed that tumor-derived OPN promotes tumor progression via the transformation of mesenchymal stromal cells (MSC) into cancer associated fibroblast (CAF) by regulating CCL5 expression in MSC (76). Moreover, Behera et al. have shown that
OPN promotes breast tumor growth by activating STAT3 through αvβ3 integrin-mediated JAK2 phosphorylation (77). Furthermore, Chakraborty et al. have revealed that OPN regulates VEGF-dependent breast tumor growth and angiogenesis via autocrine and paracrine mechanisms and further suggested that knocking down of OPN could effectively curb breast cancer progression (78, 79).

3.4.3.2 OPN in Melanoma Cancer

As melanoma is one of the most aggressive cancers affecting humans. Zhou et al. have documented the role of OPN in melanoma cell migration and invasion (80). Recent advancement in the melanoma research highlighted that melanoma growth is associated with enhanced OPN expression. However, Geissinger et al. have demonstrated that growth factor receptor-induced secretion of OPN could promote antiapoptotic signaling through appropriate interactions with the extracellular matrix in an autocrine manner in melanocytes (81). OPN controls cell motility, invasiveness and tumor growth either by inducing MAPK/κBα kinase-dependent NF-κB or NIK/MEKK1-dependent AP1-mediated promatrix MMP-9 activation in murine melanoma system (82, 83). Philip et al. have shown that OPN induces cell migration, invasion, and tumor growth and regulates MMP-2 activation through NFκB-mediated induction of MT1-MMP (84). Further they revealed that curcumin could downregulate OPN induced NF-κB-mediated promatrix MMP-2 activation (85). More recently, Das et al. have demonstrated that GLI1 a downstream transcription factor of hedgehog pathway promotes melanoma growth by upregulating OPN expression (86).

3.4.4 OPN in Angiogenesis and Metastasis

Metastasis is a process by which cancer spreads from primary tumor site to distant location. Angiogenesis is the process of growth of vasculature from the existing blood vessels. OPN along with VEGF have been shown to be associated with angiogenesis, tumor growth and metastasis in several tumor models (Ref. 42 and Fig. 6). Recently, Dai et al. have revealed that OPN enhances angiogenesis directly through PI3K/Akt- and ERK-mediated pathways where VEGF acts as a positive feedback signal in endothelial cells (87). Moreover, Liu et al. demonstrated that Tiam1-regulated OPN in senescent fibroblasts regulates the migration and invasion of associated epithelial cells (88). Epithelial to mesenchymal transition is very important phenomena associated with the metastatic cancer cells. Bhattacharya et al. for the first time revealed that OPN regulates epithelial mesenchymal...
transition-associated growth of hepatocellular cancer in a mouse xenograft model (89). Overexpression of OPN and \( \alpha v \) integrin in laryngeal and hypopharyngeal carcinomas associated with differentiation and metastasis (90). Moreover, Shojaei et al. have shown that OPN induces growth of metastatic tumors in a preclinical model of non-small lung cancer (91). Interestingly, Hedley et al. have shown that downregulation of OPN expression could suppress metastasis by attenuating breast cancer metastasis suppressor 1 (BRMS1) expression (92).

3.4.5 Stromal OPN

The significance of factors expressed by stromal cells and its communication with microenvironment during cancer initiation and progression has been the area of intense investigation in recent years. Stroma-tumor interactions play an important role in the regulation of tumor development and progression (Ref. 93-96 and Fig. 7 & 8). Koro et al.
demonstrated that OPN derived from bone microenvironment enhances breast cancer cell migration (97). Ohyama et al. have shown that OPN-deficiency suppresses growth of B16 melanoma cells implanted in bone and osteoclastogenesis in co-culture conditions (98). Recently, Chakraborty et al. using OPN-knockout mice revealed the importance of host/stroma derived OPN in regulation of breast tumor growth (99). Moreover, Hayashi et al. demonstrated that serum derived from OPN wild type mice could promote melanoma cell motility but not from the serum of OPN-KO mice and they further revealed that enhanced melanoma migration is associated with upregulation of ERK signaling (100).

Figure 7: Schematic representation of OPN as an instigator in regulation of tumor growth. OPN secreted from the highly aggressive tumor cells (Instigator tumor) stimulates stromal factors in the bone marrow leading to the activation of tumor growth (responder tumor) at secondary site. Adapted from Mc Allister et al. Cell. 2008;133(6):994-1005.

Pazolli et al. suggested that stromal-derived OPN support tumorigenesis by stimulating preneoplastic cell proliferation leading to its expansion in early lesions (101). Moreover, Luo et al. have revealed that OPN derived from senescent stromal cells promote the growth of preneoplastic cells through activation of MAPK pathway (102).
Several recent reports have highlighted the importance of OPN in hematopoietic niche in regulation of one or other types of cells. Recently, Chung et al. have shown that absence of intrinsic OPN expression did not affect NK cell development, whereas the absence of OPN in the microenvironment caused a significant reduction in NK cell population (103). Iwata et al. have showed that activated monocytes secretes OPN in the marrow microenvironment which down-regulates Notch1 gene expression in CD34+ cells (104). Moreover, other groups have simultaneously revealed that OPN is a hematopoietic niche component that acts as a negative regulator of stem cell pool size (105, 106). However, Sumitomo et al. have demonstrated that OPN controlled by MED1 transcription factor positively regulates HSPCs size (107).

3.4.6 OPN a Potential Target for Cancer Therapy

OPN has been implicated as an important mediator of progression in melanoma and breast cancer angiogenesis and metastasis. Therefore it has been intensively investigated for

Figure 8: Schematic representation of role of OPN in stroma-tumor interaction. The figure illustrates that secreted OPN from tumor interacts with stromal cells leading to the induction of EMT followed by metastasis. (Adopted from Ahmed et al. Expert Opin Ther Targets 2011;15:1113-26)
use as a potential therapeutic target in the treatment of cancer. Recently, Zhang et al. have demonstrated that OPN enhances the expression and activity of MMP-2 via the SDF-1/CXCR4 axis that regulates invasion in hepatocellular carcinoma cell lines (108). Moreover, Ravindranath et al. have suggested that Tcf-4 can act as a repressor or activator of breast cancer progression by regulating OPN expression in a Wnt-dependent manner and that Tcf-4 and OPN together may be a novel prognostic indicator for breast cancer progression (109). Recently, Shevde et al. have shown that knocking down OPN suppresses tumorigenicity of human metastatic MDA-MB-435 cells (110).

Figure 9: Diagrammatic representation of OPN and its receptor targeted therapeutic approaches in cancer. (Adapted from Ahmed et al. Expert Opin Ther Targets 2011;15:1113-26).

By using mammalian two-hybrid system and co-immunoprecipitation experiment Jin et al. have isolated apolipoprotein D as a novel interacting protein of OPN which could inhibit the OPN-induced preneoplastic transformation (123). This was further confirmed by Mason et al. by using Agelastatin A and demonstrated that inhibiting OPN may be the better approach to treat neoplastic cells (111). Moreover, using Aptamer technology for targeting
OPN inhibits growth and metastasis of MDA-MB-231 breast cancer cells (124). More recently, Dai et al. have shown that humanized anti-OPN antibody could inhibit breast cancer growth and metastasis in vivo (125). Other group have shown Nectin-like molecule 1 (NECL1) or 5-aza-dC could inhibits migration and invasion in glioblastoma cells by targeting OPN expressions (111-112). Therefore targeting OPN with natural or synthetic inhibitor could be better approach for the management of cancer (Fig. 9 & Table 1).

<table>
<thead>
<tr>
<th>Cancer Model</th>
<th>Inhibitors</th>
<th>Target molecule(s)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>Agelastatin A</td>
<td>Beta-catenin and Tcf-4 signaling</td>
<td>inhibition of OPN-mediated malignant cell invasion, adhesion and colony formation in vitro (113).</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Parecoxib</td>
<td>blockade of NR4A2 and Wnt signaling</td>
<td>increases Survival and Reduces Intestinal Polyp Burden of ApcΔ14/+ Mice by down regulating OPN expression (114).</td>
</tr>
<tr>
<td>Prostate</td>
<td>Celecoxib</td>
<td>PGE2</td>
<td>suppressed OPN-induced tumor growth (115)</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>TricostatinA</td>
<td>HDAC inhibitor</td>
<td>Inhibit PMA induced tumor growth by down regulating OPN expression (116).</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Triterpene lupeol</td>
<td>Wnt/β-catenin signaling</td>
<td>Mel 928-derived tumor growth inhibition was Associated with a decrease in the expression OPN and cyclinD1 (117).</td>
</tr>
<tr>
<td>Liver</td>
<td>OPN-siRNA</td>
<td>OPN</td>
<td>Down-regulation of OPN inhibits metastasis of hepatocellular carcinoma cells via a mechanism involving MMP-2 and uPA (118).</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>anti-αvβ3 integrin monoclonal antibody</td>
<td>Blocking OPN-Αvβ3 interaction</td>
<td>Abrogation of the interaction between OPN and alphavbeta3 integrin reduces tumor growth of human lung cancer cells in mice (119).</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>OPN RNAi and anti-OPN Ab</td>
<td>Silence or block OPN activity</td>
<td>remarkable inhibitory effects against liver metastasis by the pancreatic cancer cell line (120).</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>LY294002</td>
<td>PI3K inhibitor</td>
<td>Suppresses OPN-dependent pro-tumorigenic signal by inhibiting PI3K/Akt signaling pathway (121).</td>
</tr>
<tr>
<td></td>
<td>Simvastatin,</td>
<td>Inhibit 3-hydroxy-3-methylglutaryl coenzyme A</td>
<td>induces apoptosis and cell growth arrest by reducing OPNexpression in ovarian clear cell carcinoma (122).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMG-CoA reductase</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Synopsis of therapeutic potential of targeting OPN regulated downstream kinases, transcription factor and effector oncogenic molecules. (Adapted from Ahmed et al., Expert Opin Ther Targets 2011;15:1113-26)

3.5 Andrographolide

Andrographolide (Andro) is a diterpene lactone isolated from the plant “Andrographis paniculata” (126). This plant grows widely in many east and south Asian countries such as India, China, Sri Lanka, Thailand and Myanmar and has a therapeutic usage in Ayurvedic and traditional Chinese medicine (TCM) (127-128). According to TCM
Andrographis paniculata a cold property herb and used to liberate body heat as well as to expel toxins. The plant is particularly known as king of bitters and has been used usually as a therapy against dysentery, liver diseases, tonsillitis, inflammation, herpes, diarrhoea and fever (127-131). Andro is chemically designated as (3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5, 8-adimethyl-2-methylene-1-napthalenyl] ethylidene] dihydro- 4-hydroxy-2(3H)-furanone) (132). Andro exhibits vast range of biological activities by regulating various target genes (Fig. 10). In recent past, the compound is shown to possess anti-tumor, anti-viral and cardio-protective properties (127-133). However, it possesses weak anti-microbial activity targeting bacteria and viruses (134). Several reports have suggested the cytotoxicity of Andro on cancer cells. Moreover, it has been shown to inhibit cell cycle progression in variety of cancer cell line.

Recent study demonstrated that Andro increases in G0/G1 phase of cell cycle and at the same time significantly decreases at S and G2/M phase of cell cycle in HL-60 cells (135). It has been further demonstrated that Andro regulates the expression of cell cycle related proteins and thereby inhibits cell cycle progression (135). Recently, Andro has shown to induce apoptosis by activating the extrinsic death receptor pathway in certain human cancer cell types (135, 136). In certain cell types, the activated caspase-8 is sufficient to activate the effector caspases (caspase 3/7) in response to Andro. However, in majority of cell types, signal is amplified through mitochondria to activate the effector caspases (127, 137).

3.6 Apoptosis

Apoptosis is a mechanism of programmed cell death (PCD) involved in the regulation of tissue homeostasis. Series of biochemical events occur in apoptotic cells leading to specific characteristics such as blebbing, loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (138). Extrinsic (Fas and other TNFR superfamily members and ligands) and the intrinsic (mitochondria-associated) pathways are two major pathways of apoptosis where caspases are involved (139). Recent advancement in this field suggested a mechanism of apoptosis involving apoptosis inducing factor (AIF) and is caspases independent (140). Recent studies showed that ER as a third subcellular compartment implicated in apoptotic execution by alterations in Ca^{2+} homeostasis and accumulation of misfolded proteins in the
ER resulting in ER stress (141). Moreover, prolonged ER stress can lead in the activation of BAD and/or caspase-12, and execute apoptosis (141).

3.7 Phosphatidylinositol 3-kinases

Phosphatidylinositol 3-kinases (PI3-kinases) are a family of related intracellular signal transducer enzymes capable of phosphorylating inositol at the 3 position hydroxyl group of phosphatidylinositol. PI-kinases are enzymes that promote various cancers by regulating cellular functions such as cell survival, proliferation, differentiation, motility and intracellular trafficking (142, 143). Based on its regulation, in vitro lipid substrate specificity and primary structure PI-kinases are divided into three different classes: Class I, Class II, and Class III (144). Class I PI-kinases are heterodimeric molecules which are further classified

**Figure 10**: Typical diagram representing the major target molecules and its functional implication on physiological status of cancer cells. *(Adapted from, eCAM 2011; DOI:10.1093/ecam/nep135)*
into IA and IB depending on the sequence similarity. Class IA PI-kinases is composed of a heterodimer between a regulatory subunit (p85) and a catalytic subunit (p110). Five different variants of the p85, designated p85α, p55α, p50α, p85β, or p55γ and three variants of the p110 designated as p110α, β, or δ catalytic subunit has been reported so far (144-147).

P110α is mutated in many cancers and some of these mutations results in the permanent activation of the kinase. Moreover, PTEN which acts as a tumor suppressor antagonizes PI3-kinase signaling provides significant contribution to cellular transformation and the development of cancer (148). The effects of PI3Ks are thought to be mediated by a downstream effector Akt. Akt1, Akt2, and Akt3 are three members of the Akt gene family which are located on different chromosomes in humans (149). Moreover, Akt is overexpressed in a number of cancers, including pancreatic, ovarian, colon and steroid hormone-insensitive breast cancers (150-154).

3.8 Nuclear Factor kappa B (NF-κB)

NF-κB is a family of homo/heterodimeric transcription factors. NF-κB is shown to be activated by various external stimuli such as UV, microbial or fungal or viral infection as well as multiple proinflammatory cytokines, ECM proteins, various growth factors, oxidative stress, and other physiological and pathophysiological conditions (155-158). Activated NF-κB target diverse genes such as: genes involved in immunoregulatory and inflammatory functions, genes responsible for cell survival (anti-apoptotic genes), genes that positively regulate cell proliferation and that encode negative regulators of NF-κB, and all of the four categories controls different physiological function leading to tumorigenesis (159-162). The activation of NF-κB not only enables malignant transformation and tumor progression, but also provides a mechanism by which tumor cells escape immune surveillance and resist therapy (163-166). Constitutive activation of NF-κB has been reported in various aggressive cancers including lymphomas, melanomas and breast cancers (167-170). Moreover, enhanced level of NF-κB was detected in ER -ve breast cancer as compared to ER +ve breast cancers (171). However, it has also been reported that blockade of the constitutive NF-κB activity in metastatic human prostate cancer cells resulted in suppression of angiogenesis, invasion and metastasis (113). Inhibition of NF-κB in head and neck squamous cell carcinoma attenuates cell survival and tumor growth (172, 173). NF-κB plays an important role in the generation of metastatic phenotype of cancer cells by regulating the expression of
various cell adhesion molecules such as ICAM-1, VCAM-1, matrix metalloproteinases such as MMP-9, chemokine receptors such as CXCR4, urokinase type plasminogen activator (uPA) and cycloxygenase-2 (COX-2) (42, 43, 74, 174). NF-κB has known to stimulate angiogenesis and metastasis by inducing the expression of VEGF (79). Thus, NFκB plays pivotal role in the regulation of OPN induced cell proliferation, migration and cell survival and neoplastic transformation (175).

3.9 AP-1 transcription factor

Activator protein-1 (AP-1) is one of the first mammalian sequence-specific transcription factors recognized, belongs to the dimeric basic region-leucine zipper (bZIP) protein group composed of Jun, Fos, and activating transcription factor (ATF) protein family members (176-178). AP-1 is composed of different combinations of hetero- or homodimers and the nature of its composition determine the genes that it regulates (176-178).

3.9.1 c-Jun

c-Jun is the best characterized component of AP-1. It is a nuclear protein and is expressed in many cell types at low levels, but its expression is upregulated in the presence of various growth factors, cytokines, and UV irradiation (177). The human c-Jun protein is composed of 334 amino acids. It has three main domains that are particularly well conserved among the different Jun and Fos family members; the leucine zipper (bZIP) domain (hydrophobic interaction and dimerization), the basic region (DNA binding region) and the transactivation domain (177-179).

3.9.2 c-Fos

c-fos is an immediate-early proto-oncogene with rapid and transient transcriptional activation following mitogenic stimuli (179), and was originally identified in the FBJ and FBR murine sarcoma viruses by Curran et al. (180). It also involved in cellular proliferation, differentiation, transformation, and apoptosis (176). c-Fos protein is of 381 amino acids containing a hydrophobic bZIP for protein-protein interactions, and a basic region for DNA binding. c-Fos contains a DEF-motif in the C-terminus which acts as a ERK-docking site (181).
3.9.3 Activation and regulation of AP-1

AP-1 complex may be a homodimers of c-Jun, or heterodimers with other Jun, Fos or ATF proteins. Jun-Fos heterodimers are more stable than Jun-Jun homodimers of activation protein (AP-1) complex (182). Jun-ATF dimers bind preferentially to the CRE (183) whereas Jun-Fos dimers bind with the highest affinity to the TRE element and with slightly lower affinity to cAMP response element (CRE) (184). c-Jun activity is regulated by its phosphorylation at Ser 63 and 73 and Thr 91 and 93 within transactivation domain (185, 186).

3.10 ERK MAPKs

ERKs were the first MAPKs to be identified as ERK1 and ERK2 (187). ERKs are widely expressed and activated by growth factors, cytokines, viral infections and ligands for G-protein coupled receptors, transforming agents, and carcinogens leading to the cellular proliferation (188). The ERK activation involves receptor tyrosine kinases (RTKs) signaling through the small guanosine triphosphate (GTP) binding protein Ras. The GTP form of Ras binds to c-Raf-1 at the plasma membrane and this activated c-Raf-1 phosphorylates MEK1 and MEK2 and stimulates their ability to activate ERK1 and ERK2 by phosphorylation (189). Scaffold protein, MEK partner-1 (MP1) functions as a regulator of MAP kinase signaling by binding to MEK1 and ERKs (190). Nuclear ERKs phosphorylate and activate transcription factors including activator protein-1 (AP-1), Elk-1, switch-activating protein-1a (SAP-1a), estrogen receptor, and STAT proteins (191-193). Other forms of ERKs has been reported such as ERK3, ERK4, and ERK5. Recent evidence suggests that MEKK3 activate ERK5 via MEK5 (194). Cell cycle transition depends on the regulation of cyclin-dependent kinase (CDK) expression and activation. Hence sustained activation of ERKs is necessary for cell cycle progression. Recently, c-Fos a component of AP-1 has been shown to act as a molecular sensor of ERK signaling. Moreover, several reports have demonstrated that OPN regulates ERK signaling and its downstream transcription factor AP-1 leading to the regulation of several cellular processes (175).

3.11 ABCG2 Transporter

ATP-binding cassettes (ABC) are transmembrane proteins that transport specific molecules across the membrane against the concentration gradient by utilizing energy (195, 196). Broadly ABC transporters are divided into full and half transporters. Full transporter
contains two ABC domains and two transmembrane domains (TMD) whereas half transporters contain one each ABC and TMD on a single peptide. Half transporters form hetero- or homo-dimer to become fully functional. Based on domain organization and sequence similarity eukaryotic ABC transporters have been subdivided into 7 subgroups, A-G (197-199). Seven sub-family of ABC superfamily are ABC1, ALD, GCN20, MDR/TAP, MRP, OABP, and White. ABCG2 a half-transporter is a member of White subfamily. Alternatively it referred as ABC15; ABCP; BCRP; BCRP1; BMDP; CD338; CDw338; EST157481; GOUT1; MRX; MXR; MXR1 and UAQTL1 (197). ABCG2 acts as a xenobiotic transporter which may play a crucial role in multi-drug resistance to chemotherapeutic agents. Significant expression of ABCG2 has been observed in placenta, apical membrane of intestine, blood-testis barrier, blood-brain barrier, stem cells, hematopoietic progenitor cell and cancer stem cells. Moreover, it also expresses on other organs such as apical membrane of kidney, liver and in lactating mammary gland (200-202). ABCG2 has been shown to be overexpressed in many other tumor cells that are associated with resistance to a number of drugs including doxorubicin, bisantrene, mitoxantrone, methotrexate and topotecan (203-206).

3.12 Side Population

Side population (SP) was originally defined by Goodell et al. as a Hoechst exclusion in flow cytometry. Hoechst 33342 is a fluorescent dye which enters cells through simple diffusion and binds preferentially to AT-rich region of the minor groove of DNA as well as RNA and gets effluxed through ABC transporters utilizing ATP as a source of energy (207-208). A dichoric mirror (LP635 nm) is used to split the emitted fluorescence. Two distinct channels on the flowcytometer are used to detect the emitted fluorescence when excited by UV light. These are Hoechst Blue 450/50 nm band pass filter and Hoechst Red 675/20 nm long pass filter channels. Hoechst red channel is more sensitive and therefore small change in dye concentration results in distinct tail of cells often referred to as Side Population (207, 209). Several reports have suggested that hematopoietic stem cells as well as progenitor cells exhibit this phenomenon. Emerging studies have revealed that cancer cells do exhibit SP phenotype and its percentage varies from cell to cell type depending on the degree of ABCG2 expression.