Chapter 1

Review of Literature
1.1 Introduction: Ovarian Cancer

Ovarian cancer (OC) is a highly aggressive malignant disease that is the leading cause of gynecologic mortality and fourth most common cause of cancer related deaths in women (Jemal et al. 2007). OC exhibits minimal, nonspecific, or no symptoms at early stage; hence, majority of cases are clinically diagnosed at advanced stage resulting in poor prognosis that makes it a significant threat to women worldwide. At early stage, neoplastic-lesions are limited to ovaries, and can be cured in 90% of patients (Ye et al. 2007). Although, response of ovarian carcinoma with standard surgery followed by platinum-taxane chemotherapy appears promising in 70% of cases, less than one-fifth of cases can be treated at advanced stage malignancy (stage III and IV). Moreover, frequencies of platinum resistance in approximately 25% of patients within six months (Miller et al. 2009) of diagnosis make its management more difficult and further reduces the five-year survival to 31% (Jemal et al. 2009).

Detection at preclinical state has been suggested as a promising approach in clinical management of the disease that may also improve survival (Bast et al. 2002). In clinical practice, conventional chemo-therapeutic approaches are still considered as most favorable treatment strategy against OC. Contemporarily in chemotherapeutic approach, a combination of platinum-derived drugs viz. cis- and carboplatin is the most efficient treatment for OC patients. It has been reported that 90% of OC are clonal and arise from the single cells progeny instead of multifocal origin (Jacob et al.1991; Mok et al,1992). OC exhibits substantial cellular and molecular heterogeneity among ovarian cancers tumors collected from different patients.
1.2 Epithelial ovarian cancer and progression of the disease

Ovarian tissue is histologically derived from the coelomic layer during embryonic development and is covered by a single-cell layer of peritoneal mesothelium. The clinical histo-pathological observations lead to the categorization of OCs into three types (Fig. 1.1A), based on the source of cell types they derived from:

i) Epithelial, which arise from ovarian surface epithelia (OSE) and is of most common type;

ii) Germ cell, originates from the cells that are destined to form eggs within the ovaries;

iii) Sex cord-stromal cell or connective cells, which hold the ovaries together and produce female hormones

Primary finding suggested that OC is derived from the OSE or postovulatory inclusion cysts that underwent constant exposure to hormones or chemokines (Auersperg et al, 2001).

Among the three OC subtypes, epithelial ovarian carcinoma (EOC) make up 90% of all ovarian malignant tumors, and are known to be highly heterogeneous in nature. Based on cellular differentiation EOC can be further classified into serous, endometrioid, clear cell, mucinous and Brenner (transitional) differentiated tumor types (Fig.1.1B) (Seidman et al. 2002; Scully et al.)
1999a; Scully et al. 1999b). Tumors in these categories can also be further divided into benign, borderline and intermediate types. Among all above EOC sub-types, serous ovarian adenocarcinoma accounts for approximately 70% of reported cases and therefore is the most common type (Bast et al. 2004).

1.2.1 OSE model of epithelial ovarian cancer progression

In general, malignant disease follows a multi-step progression scheme, wherein it undergoes a series of phenotypic alterations from adenoma to adenocarcinoma followed by metastasis (Vogelstein, 1993). This progression scheme has been replicated in vitro with high degree of fidelity in colon carcinoma (McCance et al. 1988; Rader et al. 1990). It has been suggested earlier that epithelial ovarian cancer progresses through a multistep process (Link et al. 1996). Although in case of EOC, low-grade carcinomas are believed to follow such scheme, it is debated for high-grade EOC progression; since precursor lesions of the disease have not been defined yet (Kurman et al. 2008). Thus, origin of high-grade malignant disease as yet was considered a debated issue. Extensive peritoneal metastasis from the surface of ovary which is evidently seen in ovarian cancer suggested OSE as primary site of origin. In ovary, it has been suggested that specific bodies of OSE viz. inclusion cyst and cleft are representative of the earliest stage of cellular transformation which leads to formation of primary tumors (Auersperg et al. 2002). Dissemination of cells into the peritoneal cavity is suggested to be a consequence of the exfoliation from the primary site (Fig.1.2). In peritoneal cavity, these cells either form ascites containing spheroids of tumor cells or migrate to distant sites/serosa and form secondary tumors.

Further analysis of genetic alterations categorized EOC into type I and II tumors (Kurma et al. 2008). Type I tumors represent benign-to-borderline phenotype with relatively stable genotypes, while type II are of metastatic and advanced stage tumors that are substantially genetically
unstable. At molecular level, analysis of gene expression patterns has allowed distinction between OC sub-types (Schwartz et al. 2002) or between low-grade and high-grade metastatic tumors (Meinhold-Heerlein et al. 2005; Bonome et al. 2005; Ouellet et al. 2005).

Figure 1.2: Schematic model of EOC progression from the surface of ovary. (Adapted and modified from Burleson et al, 2004).
1.2.2 Fallopian tube epithelial (FTE) model of epithelial ovarian cancer progression

In contrary to earlier findings, increasing evidences over the last 6 years have suggested that early incidences of neoplastic transformation takes place at fallopian tube epithelium (FTE) and thus proposed FTE a source of EOC instead of ovary (Kurman et al. 2010; Crum et al. 2007; Kim et al. 2011). These reports not only suggested fallopian tube as a site of origin but, also showed that FTE can advance de novo metastatic progression of high-grade serous adenocarcinomas (Kim et al. 2011).

FTE theory suggests that frequent inflammatory response associated with ovulation process could be a potential event to initiate cellular transformation (Ness et al. 1993). During ovulatory cycle, FTE cells are subjected to higher levels of cytokines and chemotactic agents, which cause double-strand DNA breakage (DSB) and contribute to an early “p53 signature” precursor lesions (Ness et al. 1993). In agreement to this, women taking anti-inflammatory medications (NSAIDS, ASA) are at low risk of ovarian cancer (Altinoz et al. 2004). Accumulation of subsequent genetic mutations further leads to serous tubal intraepithelial carcinoma that eventually forms advance stage serous carcinoma (Fig.1.3).
Figure 1.3: Schematic model showing fimbrial plica and progression of normal fallopian tube epithelium to invasive serous carcinoma. (Derived and modified from Karst et al, 2010).

**1.2.3 Molecular heterogeneity defined model of epithelial ovarian cancer progression**

More recently, some researchers have characterized EOC based on molecular heterogeneity or somatic genetic alterations which were occur in a subtype-specific manner (Singer et al. 2005; Wilner et al. 2007; Ahmed et al. 2010), (Table 1.1).

**Table 1.1:** Classification of epithelial ovarian tumor sub-types derived from different histologies and details of precursor lesions during tumor progression.

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<tr>
<th>EOC sub-types</th>
<th>Tissue of origin</th>
<th>Precursor lesions and progression</th>
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| Serous        | Fallopian tube epithelium | **Precursor:** serous cystadenoma  
**Progression to:** invasive, aggressive high-grade serous carcinoma |
| Mucinous      | Endocervical epithelium  | **Precursor:** mucinous cystadenoma;  
**Progression to:** intraepithelial carcinoma then to invasive mucinous carcinoma |
| Endometrioid  | Proliferative epithelium | **Precursor:** endometriosis,  
**Progression to:** intraepithelial carcinoma then to invasive endometrioid carcinoma |
| Clear cell    | Gestation epithelium    | **Precursor:** endometriosis, clear cell adenofibroma;  
**Progression to:** intraepithelial carcinoma then to invasive clear cell carcinoma |
| Brenner (Transitional) Tumor | not clear | **Precursor:** Brenner tumor;  
**Progression to:** malignant Brenner (transitional cell) carcinoma |

**1.3 Cellular transformation of epithelial ovarian cell**

Process of cellular transformation is known to be associated with changes in genetic/epigenetic and protein profiles that disrupt diverse cellular pathways. Such molecular alterations leads to
the functional aberrations and eventually promote cell to progressively acquire hallmarks of
cancer (Fig.1.4) (Holliday et al. 1996; Russo et al. 1988; 1996; 1998). Such functional aberrations
are consequences of change in expression profile which may involve functional remodeling of
proto-oncogenes & tumor-suppressors with response to several biological and/or environmental
factors (Briand et al. 1987; Band et al. 1990; Bartek et al. 1990; Soule et al. 1990; Tait et al.
1990; Garcia et al. 1991). Recent report suggests that existence of higher cellular heterogeneity
in epithelial ovarian cancer (EOC) could be associated with the transition amongst epithelial and
mesenchymal properties (Strauss et al. 2011). These evidences are derived from clinical as well
as experimental surveillances made over the last three decades, which have shown that histo-
pathological changes are associated with the change in cellular phenotype over time. Some
researchers believed that such alterations represent a precursor state of disease which usually
referred to as pre-neoplastic, (Nettesheim et al. 1981; Sporn et al. 1976).

OSE neoplastic transformation is indicative of mullerian differentiation resulting in appearance of
new epithelial properties and reduced mesenchymal features as an early event (Auersperg et al.
2001). Such transformation events include alteration of epithelial features, including altered
phenotype (Roskelley et al. 2002), stimulated epithelial e-cadherin and membrane antigen
levels, junction complexes remodeling (Auersperg et al. 2001; Inque et al. 1992; Davies et al.
1998). It also involves increased levels of secretory markers such as mucins (mucin1-4) and
CA125 (Auersperg et al. 2001; Roskelley et al. 2002). Inactivation of p53 and pRb tumor
suppressor genes (Flesken-Nikitin et al. 2003), upregulation of specific oncogenes, or
combination of both (Orsulic et al. 2002) have been noted as an early event in EOC
transformation. It also has been observed that expression of epithelial marker of transformed
cells such as e-cadherin expression alters as cells de-differentiate (Inque et al. 1992; Davies et
al. 1998; Veatch et al. 1994) and progressively acquire a more aggressive mesenchymal
phenotype.
Figure 1.4: Schematic demonstration showing hallmarks of cancer (Derived and modified from Hanahan et al, 2011).

Gregoire in 2001, first demonstrated malignant transformation and formation of tumor in HPV-16 E6/E7 gene expressing human OSE (Gregoire et al. 2001). Further, an in vitro approach demonstrated transformation of epithelial ovarian cell using SV40T antigen with catalytic subunit of hTERT telomerase on alternative transfection with specific oncogenes e.g. KRAS, HRAS and c-erbB-2 (Kusakari et al. 2003). It has also been observed that expression of prostaglandin E (PGE) is associated with EOC transformation and progression (Rask et al. 2006). Later, immortalization with hTERT, cyclin D1 and cdk4 demonstrated development of xenograft in KRAS^{+} and p53^{+} expressing primary epithelial ovarian cells (Sasaki et al. 2009). Although, such inducible transformation models with ectopically expressed oncogenes described in vitro transformation, lack of suitable spontaneous cellular transformation model became major
limitation. Such models establish as a result of progressive accumulation of genetic mutations and would be capable of imitating apparent molecular events associated with aggressive disease.

1.4 Cancer progression model system

Lack of suitable in vitro or in vivo model system has become the major limitation to understand etiology of the complex disease, exploring which may offer an insight into the molecular alteration in a stage specific manner. Molecular expression profiling of such progression models may provide a comprehensive approach towards understanding the gradual expansion of disease and acquisition of hallmarks of cancer. In the first such initiatives, Vogelstein and colleagues suggested a schematic model of colorectal cancer progression, wherein a normal colonic epithelium gives rise to small adenomatous polyps on progressive mutagenesis (Vogelstein et al. 1988; Vogelstein et al. 1993) that further increases severity of neoplasia and develops an invasive adenocarcinoma (Shibata et al. 1993; Bos et al. 1987). Over the last two decades, this model has become a gold standard and been adapted in number of malignancies, including brain and bladder (Fearon et al. 1990; Vogelstein et al. 1988; Sidransky et al. 1992; Dalbagni et al. 1993). Protein expression profiling of such progression model will improve understanding of underlying mechanisms, and also has implications in prevention of disease, development of prognostic protein markers and screening tests for pre-symptomatic diagnosis (Kern et al. 1989; Sidransky et al. 1992; Giardiello et al. 1993).

Studies in pancreatic cancer also exhibit stepwise progression from mild dysplasia to severe dysplasia with progressive accumulation of mutations (Kozuka et al. 1979; Furukawa et al. 1994; Kloppel et al. 1980). During the process, duct epithelia developed into infiltrating carcinoma via a series of histologically defined pancreatic intra-epithelial (PanINs) precursors.
At gene level, induced HER-2/neu expression, point mutations in K-ras followed by inactivation of p16 and loss of p53, DPC4, BRCA2 function occurred at early, transitional and late stage respectively. Head and neck squamous cell carcinoma (HNSCC) also has been suggested to progress through a series of well-defined clinical and histopathological stages (Califano et al. 1996). Concomitant methylation of RASSF1A and SFRP1 genes also has been considered an early event in mammary cell transformation (Dumont et al. 2009). In order to catalogue such molecular alterations, establishment of experimental cell models is essential to reconstruct sequential molecular events associated with progression of disease. Some researchers have undertaken this concept to establish an experimental cell system of prostate cancer progression (Thalmann et al. 1994) which, further confirmed change in molecular profiles between LNCaP (non-metastatic, androgen dependent) and C4-2 (metastatic, androgen independent) cell types (Xie et al. 2011).

As mentioned earlier, in EOC existence of several sub-types and cellular heterogeneity become a limitation to establish a suitable progression model and thus, make the situation more complex. Roberts et al in 2005, took an initiative and derived a syngeneic mouse-OSE (MOSE) model system of premalignant non-tumorigenic early (MOSE–E), transitional or borderline phenotype intermediate (MOSE–I) and highly metastatic malignant late passage (MOSE–L). In the development of this progression model system, MOSE underwent spontaneous transformation in the course of continuous passaging (Roberts et al. 2005). Gene expression analysis further delineated modulation of cytoskeleton organization in MOSE neoplastic transformation (Creekmore et al. 2011). It has been observed that the process of migration and establishment at secondary sites requires reorganization of the actin cytoskeletal network (at sites of focal adhesion complexes) and changes in the expression levels of cellular adhesion molecules, growth factor receptors and intracellular signaling kinases (Bast et al. 1993; Cruet et al. 1999).
In another attempt to establish a transgenic model, avian retroviral gene transfer technique in somatic ovarian cells of adult mice led to the derivation of ovarian carcinoma mouse model and demonstrated that p53 deficiency enables EOC progression (Orsulic et al. 2002). Despite establishment of such mouse model systems, it is difficult to identify precise molecular events associated with malignant transformation in context of human disease. Thus, profiling of concomitant molecular changes in human model system is essential to understand the mechanism of cellular transformation. Absence of representative early stage molecular or diagnostic protein markers has made disease prognosis relatively asymptomatic. Therefore, identification of the EOC protein profiles of early precursor stage and advanced stage(s) could provide new molecular insights into major pathways associated with disease progression.

1.5 Establishment of epithelial ovarian transformation model system

In 2005, an \textit{in vitro} progression model of serous ovarian adenocarcinoma (SeOvCa) transformation was established in our lab (Bapat et al. 2005) (Figure 1.5). Briefly, one of the 19 spontaneously immortalized single cell clones isolated from the malignant multi-layered ascites of the grade IV SeOvCa patient developed into a progression model system of immortal (i) pre-transformed (A4-P) cells showing slow-cycling property and inability to produce xenograft in immunocompromised mice, and (ii) transformed A4 (A4-T) cells, which demonstrated aggressive, metastatic and tumorigenic capacity.
Figure 1.5: Schematic demonstration showing establishment of A4 progression model of SeOvCa transformation.

Further, clonogenic and spheroid generation assays confirmed aggressiveness and growth potential of A4-T cells. The A4-P and A4-T model system exhibited phenotypes of early and advanced stage disease respectively. Establishment of SeOvCa progression model provided a suitable in vitro system to profile altered molecular expression of proteins associated with transformation process that are indicative of the mechanisms underlying development of malignancy and may also have implications in prevention of disease.

1.6 Protein profiling of progressive serous ovarian carcinoma and identification of altered molecular expressions

Protein expression profiling of such a progression model would essentially identify molecular alterations over time in the progression of disease. Advancement of high throughput technology and improved computational analysis in recent times has made it feasible to sense minor qualitative and quantitative change in protein expressions (Qu et al. 2002; Li et al. 2002), that
may exhibit their potential role in early diagnoses (Petricoin et al. 2002; Rosenwald et al. 2002). Profiling of differential expression patterns is preferably performed either through 2-Dimensional gel electrophoresis (2-DE) coupled with Mass Spectroscopy (MS-MS) or directly with LC-MS-MS methodology (Menon et al. 2002; Cutillas et al. 2003). Such approaches have earlier suggested upregulation of heptoglobin-α subunit (Ye et al. 2003) and inter α-trypsin inhibitor heavy chain H4 fragment (Zhang et al. 2004) in the serum of ovarian cancer patients. Reduced apolipoprotein A1 and higher level of truncated form of transthyretin also have been reported in patients serum samples (Zhang et al. 2004).

Gagne in 2005 was the first to profile proteome of an ovarian cancer tumor derived cell line viz. TOV-112D in correlation with gene expression profiles. This led to the identification of glycolysis, cellular proliferation and differentiation pathways (Gagne et al. 2005). He further carried out proteomic approach with low and high malignant tumor cells and identified upregulation of pathways associated with transcription, metabolism, cell adhesion or motility and organization of cytoskeleton in malignant cells (Gagne et al. 2007). Further, a panel of transthyretin, TTR fragment, β-hemoglobin, apolipoprotein and transferrin protein markers was identified in patients with EOC at early stage (Kozak et al. 2005), though further clinical validation failed to prove their potential for early diagnosis. Later, identification of the secretome of epithelial ovarian cancer revealed extensive shedding of membrane linked extra cellular domain proteins (Faca et al. 2008). Although, expression profiling of EOC has aided in defining the molecular functionality of the cancer cells, status of proteomic alteration during disease progression remains largely uncharacterized. Thereby, identification of differentially expressed protein in cells representing early and advanced stage of disease could be of great diagnostic potential.
Molecular profiling during disease progression is essential to reveal change in expression pattern over time that could also exhibit molecular expression in tumors (Le Page et al. 2004; Lee et al. 2004). Ouellet was the first to assess changes in the gene expression profiles between low malignant potential tumor and invasive ovarian tumors that exhibited a distinctive expression profiles (Ouellet et al. 2005). In this study, tumors with low malignant potential showed better prognosis response than the invasive tumors. Further, distinct expressions of CAS, TNF R1A, FLIP, CKS 1 and CCNE1 genes in two tumor types anticipated changes in molecular expression. Besides the identification of differential gene expression profiles upregulated levels of GPI also has been observed in ovarian tumors (Lee et al. 2004). This is found to be associated with metastatic potential of ovarian tumors (Yanagawa et al. 2004). In addition, higher levels of an oncogenic factor viz. eIF-5A2 were found to be positively correlates with clinical samples of advanced stages of ovarian cancer (Guan et al. 2004).

1.7 Transformation-associated pathways in epithelial ovarian cancer

As mentioned earlier, in the course of cellular transformation cells progressively acquire several hallmarks of cancer e.g. self-sufficiency in the growth signals, suppression of growth inhibitory signals, replicative immortality, resistance to apoptosis, invasion & metastasis, angiogenic capabilities, energy metabolism reprogramming and evading immune destruction within tumor mass in order to ensure their growth and survival (Hanahan and Weinberg, 2011) (Figure 1.2). Spontaneous mesenchymal stem cell transformation model system suggests that oncogene expression, inactivation of tumor suppressor genes, mitochondrial metabolism, DNA repair are the major events and pathways which altered during disease progression (Rubio, 2008) (Figure 1.6). Loss of cell cycle regulation has been suggested to be an early event in aggressive progression of SeOvCa (Singer et al. 2002). Such observations are further supported by high frequencies of p53 mutations that control cell cycle regulation (Pothuri et al. 2001).
Figure 1.6: Schematic representation showing causative factors and major events in the process of neoplastic transformation.

Tumor suppressors are considered major targets in neoplastic transformation. Rb as a tumor suppressor is often found to be inactivated through phosphorylation (Chau et al. 2003) that correlates with disease progression (Sherr et al. 2002). In addition to Rb, deletion of p16 locus and p53 inactivation has been detected in telomerase-immortalized human cells (Burns, 2005; Sharpless et al. 2005). In transformed cells several proteins associated with DNA repair process are found to be upregulated (Rubio et al. 2008). Together, double-strand DNA damage (DSB) repair mechanism, telomere stabilization process, and inactivation of tumor suppressor are the events essentially considered to promote transformation (Sharpless et al. 2005, Matheu et al. 2004). Several reports also have identified the involvement of retinol metabolism as an early event in EOC initiation and/or progression (Kuppumbatti et al. 2000; Roberts et al. 2002). Moreover, decreasing level of cellular retinol-binding protein-1 (CRBP1) has also been considered as being crucial to transformation.
In a separate report from the lab, a gene expression & protein interaction aided system biology network approach led to the identification of three transformation-associated functional modules of c-Myc, Rb signaling and p53/cell cycle/DNA damage repair (Bapat et al. 2010). It has been considered that p53 mediates apoptosis during DNA damage. Some other substantial processes of malignant transformation were also identified e.g. intracellular effectors mediated pathways viz. VEGF, NF-κB, cyclooxygenase-2 and nitric oxide synthase (Altinoz et al. 2004).

Altogether, functional modulation of these major cellular pathways leads to disease progression, SeOvCa is appears to involve a complex network of several dis-regulated concomitant cellular pathways.

1.8 Acquisition of resistance to apoptosis in transformed cells

Progressive acquisition of resistance to apoptosis during malignant transformation provides advantages against cancer therapeutics. Initially, it was supposed that transformed cells acquire evasion to apoptosis character either through expressing anti-apoptotic markers or by the suppression of pro-apoptotic effector proteins. In a most common mechanism, change in the p53 tumor suppressor's activity effects response of the transformed cells towards apoptosis. Loss or inactivation of a pro-apoptotic regulator network led by p53 tumor suppressor gene promotes rapid tumor growth (Symonds et al. 1994). In addition, p53 plays as a downstream effector molecule in the DNA damage signaling that may leads to apoptotic onset of sensing DSB events (Harris et al. 1996). In last decade, p53 was shown to involve in progressive cisplatin resistance via a complex regulatory network (Dempke et al. 2000). In such process cells with mutated p53, higher bcl-2 levels, or high ratios of bcl-2:bax demonstrate resistance to cancer treatment (McGill et al. 1997; Reed et al. 1996; Rudin et al. 1997). In ovarian cancer, cisplatin-induced DSBs leads to differential activation of JNK (c-JUN N-terminal protein kinase) and ERK (extracellular signal-regulated protein kinase), though blocking of either of these
sensitizes transformed cells to cisplatin treatment (Hayakawa et al. 1999). Some researchers also revealed that loss of p53 function resulting in radioresistance (Pardo et al. 1994; Gupta et al. 1996; Bristow et al. 1994).

Minor alterations in the levels of RAR/RXR nuclear receptors have been suggested to promote malignant transformation (Xu et al. 1997; Soprano et al. 2004). Retinoids are known to exert control over cellular apoptosis (Oridate et al. 1996; Lu et al. 1997; Li et al. 1998) and differentiation (Crowe et al. 1998) through stimulated RXRs signaling, however, their regulation during cellular transformation remains elusive. Recently, permissive PPARγ/RXRs heterodimer complex has been reported to coordinate cellular apoptosis in cancer cells (Shankaranarayana et al. 2009). Selective activation of RXRs leads to transcriptional activation and re-differentiation in embryonic carcinoma cells has been observed (Horn et al. 1996). Although, nuclear retinoid receptors are widely recognized to control cell death and differentiation, absence or low levels have been reported to promote disease progression (e.g. in glioma and astrocytomas (Bouterfa et al. 2000; Kleinschmidt-DeMasters et al. 1999) including lung cancer (Xu et al. 1997; Picard et al. 1999; Chakravarti et al. 2003; Brabender et al. 2005).

1.9 RXR receptors and cancer progression

The relationship of RAR/RXR with cancer has been recognized over almost two decades (Figure 1.7) (De Luca et al. 1991; Gudas et al. 1993; Moon et al. 1993). RAR/RXR receptors are known to induce differentiation and inhibit development of several malignant growths e.g., in the skin, stomach, respiratory tract and mammary gland (Aylsworth et al. 1986; Munker et al. 1987). This inhibitory effect has been observed in several aggressive transformed cells e.g. F9 teratocarcinoma, HL-60 promyelocytic leukemia and malignant melanoma cells (Breitman et al. 1980; Edward et al. 1988; Strickland et al. 1980). Activation of differentiation includes
morphological changes, reduced colony formation and saturation in soft agar assays (Wu et al. 1997). Interestingly, activation of differentiation process reverses pre-neoplastic epithelial lesions and induces myeloid cells differentiation along with few other malignancies (Muto et al. 1998; Recchia et al. 2009; Edelman et al. 2005; Yamane et al. 2009; Colombo et al. 2006).

Initially, altered expression of RA receptors was found to be associated with malignant transformation of cells. Levels of RAR/RXRs are also reported to express differentially in normal and transformed epithelia cells implying their role in disease progression (Zanardi et al. 2006). Epigenetic silencing of CRBP also has been considered as common event in human cancers (Esteller et al. 2002). In addition, RAR was also recognized as a key component of PML/RARα oncogenic complex (Zeisig et al. 2007; Zhu et al. 2007).

Figure 1.7: Association of RAR/RXR receptors with malignant transformation.

9-cis RA (CRA) exclusively activates RXRs levels which form homo or heterodimeric complexes. Actually, RXRs form heterodimers with several different receptors including RA (RAR), receptors for vitamin D [vitamin D receptor (VDR)], receptors for fatty acids [peroxisomal proliferator activated receptors (PPAR)], bile acids [farnesoid x receptor (FXR)], xenobiotics [pregnane x receptor (PXR) and oxysterols [liver x receptor (LXR)]. In heterodimer complexes,
RXRs can be an active or silent partner. In its active form, CRA ligand treatment leads to the activation of functional heterodimer. In addition to CRA, incorporation of ligand for its heterodimeric partner resulting in synergistic induction of gene transcription (Mangelsdorf et al. 1996).

It has been suggested that disruption of homotetramers to homodimers instead of monomers is sufficient to abrogate the transformation ability of RARα oncoprotein. The RXR/RARα fusion forms heterooligomeric complex that perversely recruits transcriptional co-repressors to downstream target, which has been considered an essential step in cellular transformation. Moreover, interference of RXR-dependent pathways by panRXR-agonists or RXRα shRNAs suppresses RARα fusion-mediated transformation (Zeisig et al. 2007), while silencing of RARγ causes epithelial defects (Lohnes et al. 1993). In thyroid cancer, activation of RXR receptor through specific retinoids (Schmutzler et al. 2000) has been suggested to limit re-differentiation (Brtko et al. 2007) that was also studied in neuroblastoma later (Matthay et al. 2009). Retinoids treatment has also been suggested to limit the growth of ovarian cancer growth and progression of breast cancer at early-stage (Veronesi et al. 1999). Less expression of RARs also has been reported in undifferentiated human squamous cell carcinomas (Lotan et al. 1996) and benign skin tumors (Darwiche et al. 1995). Normal and malignant prostate cells also have reported to differentially express retinoid receptors (Lotan et al. 1980). Levels of RAR and RXR type II nuclear receptors have been known to be regulated through DNA methylation and histone acetylation (Youssef et al. 2004; Cras et al. 2007). In addition to CRA; Adapalene and TTNPB are two other selective retinoids of RXR heterodimer partner i.e. RAR that also have been used to induce in vivo cellular differentiation in hormone based models (Darro et al. 1998).

Despite several investigations, precise relevance of these retinoid receptors remains largely unknown in the development and EOC progression. Recent study in skin cancer has shown a
progressive decrease in the expressions of nuclear retinoid receptors (Xu et al. 2001). It was earlier suggested that minor alteration in RXR levels may promote malignant transformation (Xu et al. 1999; Soprano et al. 2004). Low or absence of RXR-y levels are reported in glioma, astrocytomas (Bouterfa et al. 2000; Kleinschmidt-DeMasters et al. 1999) and lung cancer (Xu et al. 1997; Picard et al. 1999; Chakravarti et al. 2003; Brabender et al. 2005). In melanoma, overall survival of patients found negatively correlated with RXR-y levels (Chakravarti et al. 2007). Retinoids modulate cellular growth (Oridate et al. 1996; Lu et al. 1997; Li et al. 1998) or differentiation process (Crowe et al. 1998) via stimulated RXRs signaling however, their functional relevance during the progression of transformation remains uncharacterized.

In an interesting observation, two major pathways of cellular transformation (i.e. cellular differentiation and DSB-repair) were found to be inter-linked in human skin; where on exposure to ionizing radiation, levels of RARs and its responsive genes reduced rapidly (Wang et al. 1999). Essentially, ionizing radiation induced DSB that leads to the vitamin A deficiency in skin.

1.10 Regulation of DSB-repair pathways during disease progression

Transformed cells are essentially not DNA DSB-repair defective, and show dynamic and incessant frequency of DSB formation and subsequent repair (Figure 1.8) (Schultz et al. 2000; DiTullio et al. 2002). Thus, DSB hits are continuously being generated in human cancers, though correlation of their frequencies with the stage of malignant transformation is still undetermined. In order to evaluate such DSB-lesions, several studies have been carried out towards analyzing precancerous or pre-transformed and cancerous samples of patients. In the pre-cancerous lesions with wild-type p53, phosphorylation of signaling molecules such as ataxia telangiectasia (ATM), histone H2AX, p53 and Chk2 confirm DNA DSBs (Gorgoulis et al. 2005; Bartkova et al. 2005).
In advanced stage transformed cells, DNA DSBs are evident and incidences of compromised DNA damage checkpoint are also known. Such incidences are most frequent with p53 mutations. Loss of ATM expression and Chk2, Cdk1, Cyclin D1 checkpoint proteins have also been found loosely associated with such incidences (DiTullio et al. 2002; Gorgoulis et al. 2005; Bartkova et al. 2005). Processes of cell cycle arrest, apoptosis, or senescence are usually coordinated by p53 in normal cells that is activated by checkpoint proteins on DNA damage have been confirmed through a series of observations but these processes are repressed...
during cancer progression (Gorgoulis et al. 2005; Bartkova et al. 2005; Bartkova et al. 2006; Di Micco et al. 2006; Campisi et al. 2005).

Eukaryotic systems possess two major pathways of DSB-repair; the homologous recombination (HR) and the non-homologous end-joining (NHEJ) pathway (Figure 1.9). Mechanistically, first step of both pathways is the recognition and signaling of DNA DSBs by a protein complex containing NBS1, MRE11 and RAD50 in HR (Featherstone et al. 1998) and by KU70 and KU80 (XRCC5) in the NHEJ pathway, followed by recruitment and activation of DNA protein kinase (DNA-PK).

![Figure 1.9: Schematic illustration showing factors inducing DNA damage and HR and NHEJ pathways of DNA DSB-repair.](image-url)
Identification of two breast cancer susceptibility genes, BRCA1 and BRCA2, those are involved in the homologous recombination (HR) pathway for DSB repair (Venkitaraman et al. 2002) indicated that disease is driven by DSB-initiated events (Shen et al. 2000). BRCA1 and BRCA2 along with other three susceptible target genes (TP53, ATM and CHK2) (Khanna et al. 2001), are major determinants of DSB-repair, and are frequently induced by genotoxic therapies. Several cancer predisposition syndromes in human, such as ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS) were described by sensitivity to DSB-causative agents and chromosome instability. In modern therapeutics, diverse key markers of DNA damage-signaling and DSB-repair are found to be aberrantly expressed under genotoxic therapies which includes ionizing-radiation and chemotherapeutic modalities (Zhou and Bartek, 2004; Kastan and Bartek, 2004). Also, significant levels of several DSB components under replicative in cancer/transformed cells were reported (Gorgoulis et al. 2005; Bartkova et al. 2005a, 2006; DiMicco et al. 2006). Earlier, it was anticipated that NPM1 loss may elevate levels of H2AX phosphorylation (Colombo et al. 2005) under DNA damage state or/under UV radiation (Wu et al. 2002). Recently, recruitment of phosphorylated NPM1 downstream of RNF8 and RNF168 in HR pathway was reported by ubiquitin conjugates (Koike et al. 2010). In DSB-repair pathways, RAD50 holds central-position as of a key component of MRN complex, while NPM1 and XRCC5 contribute to HR and NHEJ pathways respectively.

Possibility of a relationship between DSBs and genetic mutations, chromosomal abnormalities with cellular transformation has been demonstrated earlier (Vamvakas et al. 1997). It also has been suggested that consequences of DSB repair defect leads to genomic instability (Halazonetis et al. 2006; Bartek et al. 2007).

1.11 DSB-repair pathway and activation of p53 signaling
Upon oxidative and genotoxic stress ATM and ATR kinases and their substrate kinases Chk2 and Chk1 activate and stabilize p53 activity thorough phosphorylation of serine residues at N-terminal (Figure 1.10) (Vousden et al. 2009; Appella et al. 2001; Banin et al. 1998; Nakagawa et al. 1999; Tibbetts et al. 1999). Phosphorylation initiates association of p53 with its co-activators and inhibits its degradation, resulting in p53-dependent transcription activation (Chehab et al. 2000; Dumaz et al. 1999; Unger et al. 1999). At this point, p53 induces or represses the transcription of a number of genes, leading to either cell cycle arrest or apoptosis (Zhao et al. 2000; Vogelstein et al. 2000). Additionally, E2F1 and c-Myc are also able to induce p53 activation through its phosphorylation by ATM/ATR kinases (Lindstrom et al. 2003). Induced ATM/ATR-dependent phosphorylation of p53 and transactivation of its target genes has accompanied by the formation of nuclear foci of phosphorylated H2AX (c-H2AX). It has been observed, oncogenic stress leads to DNA damage and ATM/ATR-dependent activation of p53 pathways, which induce apoptosis efficiently only in the presence of ARF (Pauklin et al. 2005). Therefore, disruption of either pathway predisposes the cells for transformation. At the early stage of malignant transformation, p53 regulatory network is found to be sensitive to DNA DSBs events. Kinases such as ATM were found to be performing a vital role in the immediate DSB-response through direct p53 phosphorylation (Meek et al. 2009).

In sensitive cells, cisplatin mediated genotoxic-stress found to regulates p53 transcriptional activity through ATM and ATR kinase activation (Zhao and Piwnica-Worms, 2001; Damia et al. 2001), while the transient sub-nuclear redistribution of NPM1 suggest activation and direct interaction with p53 independently of ARF (Korgaonkar et al. 2005, Colombo et al. 2002). It has been reported that p53 represses Cyclin D1 transcription (Rocha et al. 2003) and therefore enhances cisplatin sensitivity in cancer cells (Biliran et al. 2005; Yde et al. 2006). Altogether, Cdk1 is essential to control entry into and exit from mitotic state (Pan et al. 2002; Labib et al.
DSB-induced p21 inhibits Cdk1 activity in order to uphold G1/S phase cell cycle progression (Satyanarayana et al. 2007). However, Cdk1 levels reportedly increase in chronic cisplatin treatment regime in a model of lung cancer suggests acquisition of drug-resistance (Oliver et al. 2010). At onset of p53/p21 pathway, lower Bcl-2 levels may lead cells towards apoptosis.

Figure 1.10: DSB-repair pathway and activation of p53 signaling.

The p53 is ranked as frequently mutated tumor suppressor in human cancer that essentially denotes its decisive role in anti-cancer activity (Levine et al. 1997). Cells with DSB-lesions and p53 mutation survive and proliferate aberrantly that further propel progression of malignant transformation. The p53 apoptotic response has been considered as central process in genomic instable cells to avoid consequences of malignant transformation. In p53 null mice,
consequences of such defective p53 protection further conclude DNA damage-induced p53 arrest or apoptotic responses (Van Gent et al. 2001). Several model systems to support p53-mediated apoptosis is basically known to be protective mechanism against genomic threats, failing of which may leads cell to carcinogenesis (Attardi et al. 2005).

1. 12 Impaired DSB-repair pathways and genomic instability

Accumulation of endogenous and exogenous oxidative factors damages DNA strands of the double helix, which limits repair machinery to use complementary DNA strand as a template for DSB-repair. Therefore, DSB incidences can be recognized as potent trigger for chromosomal aberrations which leads to genomic instability. Loss of substantial regions of a chromosome is found associated with the inactivation of tumor suppressor genes (Lengauer et al. 1998), while amplification of such regions may promote carcinogenesis through the activation of proto-oncogenes (Lengauer et al. 1998), or induction of multidrug resistance (Ramachandran et al. 1999). In tumors, incidences of gross chromosomal aberration (i.e. translocation) are often observed that include exchange of chromosome arms. Such chromosomal rearrangements may lead to deregulation of gene expression or can facilitate fusion of two genes that then acquire oncogenic potential. Development of PML-RARα fusion protein is a classic example in acute promyelocytic leukemia (Martens et al. 2010; Villa et al. 2007). Another example comprises fusion product of BCR gene to the ABL1 gene on the Philadelphia chromosome in chronic myelocytic leukaemia patients (de Klein et al. 1982). Although, exact mechanism of such translocations has remained elusive, it was suggested that DSBs on chromosomes may facilitating such precursor lesions. Several observations performed in knockout mice have shown that cells either lacking checkpoint or DSB-repair function are subjected to genome instability (Deng et al. 2003; Artandi et al. 2000). Defective DSB-repair may provide clues
towards error-prone chromosomal segregation of unrepaired DNA that may produce genomic instability during malignant transformation (Fig. 1.11).

Figure 1.11: Model illustrating gradual acquisition of genomic instability in transformed cells through exogenous and endogenous factors.

It was suggested earlier that DSB repair defect leads to genomic instability and thus, DSB-repair process could facilitate an intrinsic barrier against disease progression (Bartek et al. 2007; Halazonetis et al. 2008). Understanding of molecular mechanism of intricate process DNA DSB-repair pathways may further shed light on acquisition of genomic instability in transformed cells.