Chapter 1

p53, Breast cancer and Chemotherapy

(A Review of literature)
1.1 Introduction

Cancer is, in essence, a genetic disease. There are at least three challenges that will occupy most of the cancer research in near future. The first is the discovery of new genes that have a causal role in neoplasia, particularly those that initiate and conclude the process. The second is the delineation of the pathways through which these genes act and the basis of varying actions in specific cell types. The third is the development of new ways to exploit this knowledge for the benefit of cancer patients (Vogelstein and Kinzler, 2004).

Breast cancer is one of the most frequently diagnosed cancers, with the lifetime risk in the more developed countries and one in eight women presenting with breast cancer. In the world, there are more than 1,000,000 cases each year, and almost half of these are in countries defined as “less developed” by the World Health Organization. However, over the last five decades, our understanding of breast cancer has progressed considerably through continued research, and its mortality has started to decline (Aapro, 2001). Though, no single genetic abnormality has yet been found for all cancers, tumor suppressor p53 is the most frequently mutated and most well characterized gene in human cancers. Depending on the study, 20-50% of sporadic breast carcinomas, including early preinvasive tumors, have mutations or alterations in the p53 gene (Hollstein et al., 1991), suggesting a critical role of p53 in breast cancer development, growth and sensitivity towards different cytotoxic therapies, for example, chemotherapy, radiotherapy, immunotherapy, or gene therapy. All the data available on tumor suppressor p53 mutation analyses of human breast carcinomas, as well as data from transgenic animal studies and experimental cell studies support an important role of p53 in mammary carcinogenesis and its chemotherapy. Molecular pathological analyses of specific components in the p53 pathway are likely to give a great impact on the diagnosis, prognostication, and selection of the right treatment for individual breast cancer patients (Borresen-Dale, 2003).
1.2 p53, the tumor suppressor

p53 was first discovered in 1979 (Lane and Crawford, 1979) at a time where oncogenic DNA viruses were a popular tool for inducing experimental malignant transformation for studying neoplasia. The cellular protein p53 was noted in close association with the oncogenic DNA virus SV40 large T protein and hence classed as an oncogene. However, isolation of complementary DNA (cDNA) from tumor cells demonstrated that p53 in these tumors contained a point mutation and led to the subsequent discovery and isolation of wild-type "normal" p53 with tumor suppressor functions. It is now estimated that a wide range of cancer types and some 50% of all human tumors have specific p53 mutations. p53, "guardian of the genome" (Lane, 1992), is regarded as a tumor suppressor gene as the tumor development is attributed either to p53 deletion, p53 mutation or aberrant p53 function (Hall and Lane, 1997). p53 is involved in distinct functions at the cellular level, namely regulation of normal cell growth and division, gene transcription, DNA repair and genomic stability. Hence, p53 is regarded as a crucial regulatory protein that integrates an array of signals in response to which it turns on a host of biochemical responses at the level of single cell and ultimately the whole organism (Hall and Johnson, 1996). p53 activation, resulting in cell cycle arrest or apoptosis would prevent the perpetuation of the genetic defects that would otherwise go unrecognised, multiplying and gradually replacing the normal cell population (Lane, 1992). Moreover, the p53 pathway is composed of a network of genes and their products that are targeted to respond to a variety of genes of intrinsic and extrinsic stress signals that impact upon cellular homeostatic mechanisms that monitor DNA replication, chromosome segregation and cell division (Vogelstein et al., 2000).

Many factors affect the cellular response of activated p53. These include the cell type, the oncogenic status of the cell, the extracellular growth and survival stimuli, and the intensity of the stress signals, the level of p53 expression and interaction with specific proteins. p53 is regulated at the level of protein stability and biochemical activities. The p53 integrates many extra- and intracellular stress signals into growth inhibitory response. By doing so, p53 engages in multinuclear complexes and signaling pathways. Among these the induction of growth
arrest/apoptosis and senescence are crucial for its role as a tumor suppressor (Sionov and Haupt, 1999).

The p53 gene is located on the short arm of chromosome 17 at 17p13.3. The protein product of the p53 gene is a phosphoprotein comprising 393 amino acid residues with at least four recognised, highly conserved "boxes" or "domains": the N-terminal (amino-terminal) transactivation domain, central DNA binding domain, a tetramerization domain, and the C-terminal negative regulatory domain. Although each domain is involved in distinct and independent functions, overall, they are interdependent in the sense that alterations within one domain can profoundly influence the functions of the other domains (Hupp et al., 2000). p53 protein in normal cells is in latent form with a low steady state levels due to the rapid rate of proteolytic degradation. Wild type p53 has a half-life of 6-20 minutes in normal cells, but the mutant form is more resistant to proteolysis, and its half-life is therefore longer (2-12 h). Unlike the wild-type protein, the mutant tends to accumulate in tumor cells allowing its detection. Loss of p53 tumor suppressor activity is associated with a failure to halt the transition from G1 to S in the cell cycle in cells that have accumulated DNA damage (Kastan et al., 1991). In normal cells the endogenous p53 in response to intracellular or extracellular stimuli results in accumulation of the stabilised, biochemically altered protein. This is mediated through post-translational modification involving phosphorylation of mainly the amino-terminus by the DNA-protein kinases (Shieh et al., 1997) or the carboxy-terminus by the cyclin-dependent kinases (Lu et al., 1997) leading to an enhanced half-life of the protein. Mutations can potentially alter the conformation of p53 leading to increased stability and hence accumulation of the protein (Hupp et al., 2000, Lane and Lain, 2002).

1.3 p53 function

p53 has been ascribed to the functions as a transcription factor, in maintaining genomic integrity and as a tumor suppressor gene mediating cell cycle arrest and/or apoptosis (Vogelstein et al., 2000; Balint and Vousden, 2001). p53 plays a role in a number of important developmental processes as well as in preventing embryonic malformations (teratogenesis) and protecting the embryo
against external “environmental” stresses (Hall and Lane, 1997). Thus p53 strikes
the balance between aging and the development of cancer (Lane and Lain, 2002).

1.3.1 p53 as a transcription factor

The cardinal feature of wild-type p53 is its ability to function as a sequence
specific transcriptional activator via DNA-binding and activation domains which
bind to specific DNA sequences of some 300 “target genes”, promoting or
suppressing their activities in response to the DNA damage (Hall and Johnson,
1996). In human cancers it is in this critical DNA binding domain (Fig.1) that the
majority of the p53 mutations occur. A number of target-genes containing p53-
binding sites both in the promoter and intron regions have been identified. Some of
the examples include p21, MDM-2 and Bax.

![Crystal structure of p53 core-domain-DNA complex. The DNA-binding region is
coloured red and include loops 2 and 3 (L2, L3) and loop-sheet-helix (LSH) motif (LIS2,

1.3.2 p53 maintaining genomic integrity

p53 appears to have a direct effect on maintaining the genomic integrity
through monitoring DNA damage by activating genes that not only facilitate but also
regulate DNA repair. p53 actively participates in various processes of DNA repair
and DNA recombination by interaction with the repair and recombination
machinery. This suggests that p53 exerts its role as the “guardian of the genome” at
two levels. In its activated form it will exert tumor suppressor activities in response
to exogenous DNA damage and cellular stress. In its non-induced form it should not
be regarded as a “silent” molecule since through inherent exonuclease activity it can
be engaged in prevention and repair of the endogenous DNA damage thus maintaining genomic integrity. There is also evidence for the binding of replication protein A (RPA) to p53 (Dutta et al., 1993). RPA is required for unwinding DNA origins, and its binding to ssDNA may be initial step in DNA replication.

1.3.3 p53 as a tumor suppressor

As a tumor suppressor, p53 plays a pivotal role in acting as the mediator between stressful stimuli and the final cellular outcome. The two main cellular responses to DNA damage mediated through p53 are growth arrest, which prevents propagation and accumulation of cells with genetic alterations, and apoptosis (programmed cell death) achieving total cell elimination.

**Growth arrest**

p53 mediates growth arrest through regulation of crucial checkpoints during both the G1 and G2 phases of cell cycle. p53-dependent G1 arrest is mediated by the transactivation of the waf1 gene that codes for the small kinase activator p21^waf1^. This protein in turn prevents entry to the "S" phase in the cell cycle by blocking the activity of the cyclin-dependent kinases (Cdk). Inhibition of this G1-phase-specific Cdk activity maintains a hypophosphorylated retinoblastoma coded protein (pRb) which in turn blocks the E2F-transcription of genes required for entry to S phase, therefore blocking cell cycle progression resulting in the accumulation of cells in G1 phase (el Deiry et al., 1993). p53- mediated G1 arrest can also be induced independent of p21 (Hengstschlager et al., 1999).

p53-dependent G2 arrest is mediated through at least two target genes, 14-3-3σ gene through sequestering phosphatases of the cyclinB/cdc2 complex and to a lesser extent the GADD45 gene which directly interacts with cdc2 and therefore disrupts the cyclinB/cdc2 complex, which is required for the G2/M transition.

**Apoptosis**

While cell cycle arrest can function to inhibit growth in normal cells it appears that cells which have acquired malignant transformation are less susceptible to growth arrest. p53-induced apoptosis correlates better with inhibiting the growth
of the transformed cells as opposed to normal cells, which indicates that the ability of p53 to induce apoptosis is integral to describing p53 as a tumor suppressor in malignant disease. The apoptotic pathway is characterized by the activation of caspases (cell death proteases) that are themselves activated by the catalytic cleavages resulting in disruption of functions of essential regulatory proteins. As a result cells are committed to enter the “cell death” pathway (Martin et al., 1995). Activation of caspases is followed by characteristic structural changes, i.e. nuclear condensation and destruction, membrane blebbing, loss of cellular volume and ultimately loss of membrane integrity. One of the most extensively studied areas in p53 research surrounds its ability to control apoptosis and first hint came from Oren and his coworkers who introduced p53 into a p53 deficient myeloid leukemia cell line (Yonish-Rouach et al., 1991). Subsequently, studies using thymocytes from p53 knockout mice showed that p53 was required for radiation-induced apoptosis (Clarke et al., 1993), together with loss of apoptosis associated with tumor progression in p53 null transgenic mice implied that apoptosis contributes to p53’s tumor suppressor activity (Symonds et al., 1994). In addition, p53 dependent apoptosis contributes to chemotherapy induced cell death was first demonstrated in studies using oncogenically transformed cells treated in vitro and in vivo (Lowe et al., 1993). The proapoptotic activity of p53 has been linked to its transactivation capabilities. Moreover, knockin mice expressing transcriptionally dead, but DNA binding proficient p53 are defective in apoptosis (Jimenez et al., 2000). The role of p53 in apoptosis can be described in the form of: transcription-dependent p53 mediated apoptosis or, transcription-independent p53 mediated apoptosis. The most intuitive link between p53-mediated transactivation and apoptosis comes from its ability to control transcription of pro-apoptotic members of Bcl-2 family. These include the multidomain Bcl-2 family member Bax (Miyashita et al., 1994), as well as the BH3-only members Puma (Nakano and Vousden, 2001), Noxa (Oda et al., 2000) and Bid (Sax et al., 2002). The induction of apoptosis, or programmed cell death, in cancer cells is thought to be fundamental to the success of treatments for cancer. Apoptosis is regulated at many levels including the initiation, transduction, amplification, execution stages and mutations that disrupt each of these stages have been detected in tumor cells. The Bcl-2 family members are intimately involved in the apoptosis, but the role of these proteins in drug-induced apoptosis has been
confusing (Gross et al., 1999). Drugs induce endogenous Bax expression through p53 dependent transcription in some cancer cell lines but not in others. In mice Bax plays no role in the most well studied examples of drug or radiation induced and p53 dependent apoptosis, involving thymocytes and intestinal epithelium. Unlike the human gene, the murine Bax gene has no p53-binding site in its promoter (Schmidt et al., 1999). The Bax protein promotes apoptosis by facilitating the release of apoptosis inducing factor (AIF) and cytochrome C from mitochondria, which in turn trigger a cascade of caspase activation. The apoptotic activity of Bax is important for tumor suppression (Yin et al., 1997). p53 is also known to not only transcriptionally activate, but also to transcriptionally repress expression of other genes such as the death-inhibiting gene Bcl-2 (Ko and Prives, 1996).

p53-induced apoptosis, independent of its transcriptional activity, occurs via a proline-rich region situated between the transactivation and the DNA binding domains (amino-acid sequence 60–90). Deletion of this region has been shown to impair the ability of p53 to induce apoptosis but not affect the induction of growth arrest. Moreover, introduction of a deletion or point mutation into the p53 sequence aimed at activating the DNA binding site but not disrupting the ability of the protein to oligomerize, has been shown to impair the ability of p53 to effectively induce apoptosis in response to DNA damage (Chen et al., 1996).

The importance of this region in mediating apoptosis independent of p53 sequence-specific transactivation has been particularly highlighted in the Li-Fraumeni cancer family patients female members of whom have a high risk of breast cancer whereby a germ line mutation within this region (proline-82) has been clearly identified and characterised (Sun et al., 1996). The cancer suppressing functions of p53 are thus attributed of growth arrest or apoptosis (Fig.2).
Two functions of p53?  
Which is critical in suppressing cancer?

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>Chemo's</th>
<th>Oncogenes</th>
<th>NOxa, PUMA, P53AIP</th>
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<td>p53</td>
<td>E1A, tTAg, c-myc</td>
<td>p21 Gadd45 others</td>
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<td>Cell Cycle Arrest</td>
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Fig. 2. (Adapted from http://www.medicine.uiowa.edu/pathology.html) p53 in growth arrest or apoptosis.

Choice between growth arrest and apoptosis

In response to DNA damage, whether the cells undergo growth arrest or apoptosis is usually dependent on more than one factor. These include the cell type and oncogenic status, the intensity and strength of the stimuli, the resting p53 levels and the degree of p53 interaction with other cellular proteins that are directly or indirectly involved in the induction of growth arrest or apoptosis. In cancer cells, key factors are the efficacy of the DNA repair mechanisms and the level of p53 expression in response to DNA damage. Whilst p53 at high levels promotes apoptosis (Chen et al., 1996), p53 expressed at low levels protects cells against it (Lassus et al., 1996). p53 remains active as long as DNA damage persists. As long as the DNA repair is rapid the period of p53 expression/activation is short and hence little p53 accumulates. When DNA damage is extensive the period of p53 expression is prolonged. In addition, the balance of the mechanisms regulating apoptosis also plays an important role: Bax (pro-apoptotic) versus Bcl-2 (anti-apoptotic), the Rb-E2F pathway that mediates p53 dependent G1 cell cycle arrest and growth/survival factor cytokines that protect cells from apoptosis. Post-translational modification of p53 involving phosphorylation at Serine-315 by the Cdks is said to alter the specific DNA binding affinities of p53 (Wang and Prives, 1995) hence its transcriptional activity. Similar effects are seen in the interaction of p53 with other proteins such as p300 that is essential for both p21 and MDM-2 induction (Avantaggiati et al., 1997) and thyroid hormone receptor B1 which upon binding to p53 inhibits Bax and
GADD45 induction (Barrera-Hernandez et al., 1998). Finally, the intensity of the DNA damaging signal along with the time period for which cells are exposed to the stressful stimuli has a major role in determining the cellular outcome. Thus, apoptosis is induced in response to higher intensity and longer period of damage inducing signals whereas the reverse is true for the induction of cellular growth arrest.

1.4 Regulation of p53 activity

Acting as the central co-ordinator between the stressful stimuli and the final outcome of the cell, p53 itself can be a subject to extensive and complex regulation (Fig.3).

Molecular mechanisms responsible for conversion of the latent p53 in normal cells to the active form are complex but comprise a series of post-translational modifications of p53, altered interaction of p53 protein with other proteins and a series of non-covalent regulators, all of which provide therapeutic opportunities (Hupp et al., 2000).
The post-translational modifications result in conformational changes within the p53 molecule to activate p53. These modifications include p53 regulation through phosphorylation sites in the N-terminal transactivation domain (residues 1-100). While the phosphorylation sites have not yet been fully characterised, “Ser-4”, “Ser-6” and “Ser-9” for the CK I (Casein Kinase I) site, and “Ser-15” and “Ser-37” for the DNA-PK (DNA-dependent Protein Kinase) site appear to be important (Meek, 1994). The interaction of p53 N-terminal domain with other proteins within the transcriptional machinery is pivotal to its role as a transcriptional activator. p53 interacting proteins belonging to the transcription machinery include MDM-2, TATA box binding protein (TBP), TBP associated factor (TAF), the p62 component of TFIH and p300/CPB (Ko and Prives, 1996; Avantaggiati et al., 1997). Amongst the key p53 N-terminal-interacting proteins in which altered expression is demonstrated in many cancers including breast cancer is the MDM-2 protein (O'Neill et al., 1998). MDM-2 protein controls the biological activity of p53 and targets p53 for destruction and therefore provides an in-built feedback mechanism whereby p53 expression is controlled at the cellular level. The p53-MDM-2 protein interaction is of physiological relevance as evidenced by over-expression of MDM-2 protein effectively inactivating wild-type p53 in soft tissue sarcomas (Hall and Lane, 1997). In addition, based on mouse studies the early embryonic lethal phenotype of an MDM-2 knockout mouse is rescued when crossed into a p53 null phenotype (Jones et al., 1995). Changes within the N-terminal domain, through interactions with other proteins, can quantitatively increase the DNA binding whereas the opposite is true for changes brought about through phosphorylation of the N-terminal sites in p53 (Shieh et al., 1997). Post translational modification of the p53 N-terminus at the position “Ser-15” by the DNA-PK has been shown to reduce the ability of p53 and MDM-2 to bind and since MDM-2 is a strong promoter of p53 degradation, this results in p53 stabilization and hence accumulation (Shieh et al., 1997). The role of MDM-2 in p53 protein stabilization is further supported by the fact that p19ARF (alternating reading frame spliced product of the murine p16INK4A locus) induces p53 stabilization through its interaction with MDM-2.
1.4.2 p53 regulation through the central core domain

Amongst the conserved regions of p53, regions within the central core DNA binding domain (residues 90–295) map the majority of the mutations observed in human cancers and have been identified. Specific DNA binding of the central core domain is essential for the role of p53 as a tumor suppressor. Mutations in the cysteine residues block p53 transactivation and tumor suppression function. Many of these residues are found to be specifically mutated in many naturally occurring human cancers.

1.4.3 p53 regulation through the tetramerisation domain

Maintaining the active p53 molecule in the form of a tetramer, enhances p53's DNA sequence specific binding properties. Altered folding and conformational structure of p53 in tumors is highlighted clearly by using antibodies specific for denatured p53 in tumor cells. Maintaining p53 in tetrameric structure is ensured by the p53 tetramerisation domain (Hupp, 1999). Alterations in this domain can reduce or indeed prevent DNA binding of p53 protein. In the Li-Fraumeni syndrome altered thermal stability of the tetramerisation domain results in ineffective MDM-2 binding to p53. The group of cellular proteins known as “heat shock proteins” (HSP) also play a pivotal role in maintenance of the conformational structure of intact p53. These proteins draw together two concepts. Heat shock proteins (mainly HSP70, HSP40 and HSP90) on the one hand have been shown to contribute to p53 inactivation and oppose apoptosis in some cell lines in relation to drug or radiation induced apoptosis (Mehlen et al., 1996; Gordon et al., 1997). This effect contributes to tumor cell survival which is further confirmed by the observed increase in HSP-mutant p53 complex in some cancers. On the other hand they are said to be involved in protecting proteins form unfolding and aim to refold denatured proteins, in addition to targeting the irreversibly “damaged” ones for destruction (Hansen et al., 1996).

1.4.4 p53 regulation through the C-terminal domain

The C-terminal regulatory domain is known to have dramatic effects on the DNA binding ability of the central core domain either through interactions with
other proteins or as the result of direct modification (Hupp et al., 2000). This domain both, acts as a negative regulatory region, controlling the DNA sequence specific binding of the central core domain, and as a separate functional domain. In addition to its role in regulation of the central core domain, the p53 C-terminus can also be regarded as a damage recognition region. The p53 C-terminus is able to bind DNA ends and strands, DNA mismatches, Holliday junctions and irradiated DNA. Moreover number of proteins involved in the DNA repair mechanisms can also interact with and regulate p53, presumably through the C-terminus. The significance of these interactions and the probable effects of these on the p53 sequence binding transactivation function are yet to be fully explored. However it is clear that the relationship between p53 and the DNA repair processes is not only through stimulation and signaling after DNA damage but also through its multiple direct interactions with the proteins involved in the DNA repair mechanisms.

1.4.5 Post translational modification of p53 involving phosphorylation

Post translational modifications involving phosphorylation effects p53 turnover and accumulation (Shieh et al., 1997) particularly within the “N” and the “C” terminal domains of the protein. The p53-signaling pathway is in standby mode under normal cellular conditions. Activation occurs in response to cellular stresses and several independent pathways of p53 activation have been identified that appear to depend on distinct upstream regulatory kinases. These include an ataxia-telangiectasia mutated (ATM)/human homologue of Rad53 (Chk2)-dependent pathway activated by DNA double strand breaks, a second pathway dependent on alternative product of the INK4 gene, p14ARF (which is activated by expression of oncogenes), and a third pathway whose activity is increased by cytotoxic antitumor agents and ultraviolet light, but is independent of other two pathways (Vogelstein et al., 2000). p53 protein can be phosphorylated by protein kinases at various sites along the length of the molecule: casein kinase (CK) I at the transactivation domain, DNA-dependent protein kinase (DNA-PK), c-Jun N-terminal kinase (JNK), mitogen-activated protein (MAP) kinase and CK II (Meek, 1994; Martinez et al., 1997). Radiation can alter phosphorylation status of at least two key regulatory sites in the p53 molecule, which in turn alter its activity as a transcription factor, and
phosphorylation of the carboxyl terminal can alter the sequence specific DNA-binding (Hupp and Lane, 1995). Thus p53 modification by phosphorylation may alter the functional balance of this key cellular regulation pathway and the relative efficacy of activation of different p53 target genes (Hupp et al., 2000).

1.5 p53 inducing or suppressing the function of other genes

Amongst some of the key p53 transactivated target genes containing p53 binding sites in which altered expression is demonstrated in many cancers are MDM-2 (O’Neill et al 1998, Jones et al 1999) and p21 (Bueso-Ramos et al., 1996; Jiang et al., 1997). MDM-2 codes for the MDM-2 protein which controls the biological activity of p53 and targets p53 for destruction (O’Neill et al., 1998). This provides an in-built mechanism whereby p53 expression is controlled at the cellular level. p21 mediates the tumor suppressing effects of p53 by inhibiting cyclin-dependent kinase (Cdk) complex activity, therefore blocking the transition from “G1” to “S” phase in cell cycle progression, mediating p53-dependent growth arrest (Harper et al., 1993).

1.5.1 MDM-2

There are at least seven different transcripts of the human MDM-2 gene, coding for the MDM-2 protein. The largest human MDM-2 protein consists of 491 amino-acids with several conserved domains: (1) the amino-terminus domain that interacts with p53 inhibiting its transcriptional activity, (2) the nuclear localization sequence (NLS), (3) nuclear export signal (NES) sequence that mediate the MDM-2 shuttling between the nucleus and the cytoplasm and vice versa, (4) the highly acidic domain mediating the MDM-2 ribosomal interaction (ribosomal protein and rRNA), (5) the zinc finger domain in addition to a further 2 zinc fingers in a ring conformation that mediate sequence specific RNA binding which may also be involved in regulation of p53 levels, (6) the caspase-3 cleavage, and (7) the DNA-PK (DNA-protein kinase) phosphorylation sites towards the carboxyl-terminal.
1.5.2 MDM-2-p53 interaction

p53 and MDM-2 forms an auto regulatory feedback loop in that p53 positively regulates MDM-2 levels whilst MDM-2 negatively regulates p53 levels and hence its activity (Fig. 4).

In response to DNA damage by irradiation there is a fall in both the MDM-2 mRNA and protein levels with subsequent rise in p53 levels. This is followed by an increase in p53’s transcriptional activity and initiation of the relevant cellular response to the DNA damage. The high levels of active p53 transcriptionally increases MDM-2 levels that subsequently results in inhibition of p53’s transcriptional activity and promotion of p53 degradation (Chen et al., 1995). Activation of p53 can occur by different molecular routes, depending on the nature of an activating signal. Central to the activation process, by whichever route, is the destabilizing of p53-MDM2 interaction. The molecular mechanisms, which activate p53, involve elements of post-translational modification, protein stabilization and protein-protein interaction (Meek, 1999).

Fig. 4. (Adapted from Oren M, J Biol Chem, (1999), Vol. 274, 36031-36034). p53 and MDM-2 interaction.
1.5.3 p21

In mammalian cells, cell cycle is positively regulated by a series of stable and unstable complexes termed as cyclin-dependent kinases and cyclins respectively. This regulation is mediated through the phosphorylation of specific substrates that are inhibited by the so-called cyclin kinase inhibitors (CKIs) which therefore negatively regulate the progression of the cell cycle (Elledge and Harper, 1994; Harper, 1997). p21 was the first CKI identified in the mammalian cells.

In ensuring the genomic integrity and prevention of damaged DNA propagation onto future generations of cells, eukaryotic cells have developed a series of “checkpoint-response pathways” that are controlled through the damage suppressor genes either acting directly or indirectly mediated through specific target genes. p21 is regarded as one of the major target genes involved in p53-mediated cell cycle growth arrest in both normal as well as in the tumour cells. Inhibition of the cell cycle progression mediated through p21 activity occurs at two main regulatory checkpoint-response pathways: G1 arrest and G2 arrest. While ectopic expression of wild type p53 can induce both G1 and G2/M arrest, in a p21 dependent manner, without apoptosis, further a p53 mutant defective in transactivation is linked to apoptosis without cell cycle arrest. These results strongly suggest that the transactivation deficient mutant is a more potent inducer of apoptosis because it has lost its proapoptotic function and retains its ability to repress antiapoptotic genes such as Bcl-2. Employing a transactivation deficient p53 mutant in gene therapy approaches or the use of drugs that convert mutant p53 to a transactivation independent mediator of apoptosis may be much more efficient therapeutic approaches than current approaches that employ wild type p53 (Kokontis et al., 2001).

1.5.4 GADD45

GADD45 is the best characterized member of growth arrest and DNA damage inducible gene family (GADD). These genes are induced by DNA damaging agents and growth arrest conditions, for example nutrient depletion or hypoxia (Fornace, Jr. et al., 1989). GADD45 is a nuclear protein, the level of which oscillates slightly during the cell cycle, being highest in G1 and lowest in S phase
Gadd45 can be transcriptionally activated by p53 via p53 consensus binding site at its third intron (Hollander et al., 1993). Although induction of GADD45 upon gamma radiation is strictly dependent on p53 (Kastan et al., 1992), p53 independent induction of GADD45 is often seen after base damaging agents such as UV radiation (Kearsey et al., 1995). Even though p53 may not necessarily be required for GADD45 induction, its disruption may reduce the extent of GADD45 induction (Zhan et al., 1996). An increased level of GADD45, either due to DNA damage or over expression of the protein, leads to G1 growth arrest of cells (Zhan et al., 1994). A role of GADD45 in DNA repair or inhibition of apoptosis has been suggested by where antisense GADD45 expression decreases DNA repair and sensitises cells to UV- and cisplatin induced apoptosis, the later function being opposite to that of p53 (Smith et al., 1996). Moreover in some experiments GADD45 has been shown to interact with PCNA, like p21 (Hall et al., 1995).

### 1.5.5 p53-dependent G1 arrest

In response to DNA damage p53 induced G1 arrest is mediated through transactivation of the WAF1 gene which codes for a small kinase inhibitor p21. By blocking the activity of the Cdk's (namely Cdk2, Cdk4, cdc2 cyclin complexes), p21 blocks the cell cycle entry to the “S” phase from G1 (el Deiry et al., 1993). Arrest of the cell cycle at G1 through inhibition of G1-specific kinases results in the maintenance of the hypophosphorylated form of the protein product of the retinoblastoma susceptibility gene “pRb”. The hypophosphorylated “pRb” blocks the E2F-transactivation of the genes which are required for the entry into “S” phase and those results in accumulation of the cells in G1 phase. Accumulation of the cells in “S” phase is also mediated through proliferating-cell nuclear antigen (PCNA). In the process of DNA replication PCNA forms a complex with replication factor C (RF-C) that together promotes recognition of a primer-template junction facilitating the uptake of DNA polymerase δ. The “trimeric” protein complex, PCNA-RF-C-polymerase δ induces DNA replication (Waga et al., 1994; Li et al., 1994). Direct binding of p21 to PCNA dissociates the PCNA-RF-C-polymerase δ complex which arrests the DNA synthesis (Waga et al., 1994). As a consequence the cell cycle stops/slow down at G2 before entering “S” phase.
1.5.6 \textit{p53-dependent G2 arrest:}

Cell cycle arrest mediated by p21 can also occur at the later stages of the cell cycle, namely the G2 phase. WAF1 mRNA upregulation as well as peaking in G1 is also seen to be transiently elevated in G2/M phase. This is said to be through p21 association with cyclin A and B complexes (Li et al., 1994). In late G2 nearly half of the Cdk2/cyclin A is complexed with p21. The inhibition of Cdk2/cyclin A can either be as the result of the direct inhibition of the activated kinase by p21, indirectly through inactivation of the Cdk-activating kinase (CAK) by p21 or, indeed through blocking the interaction of other cyclin substrates with the Cdk2/cyclin A complex.

1.6 p53 and Chemotherapy

Apoptosis is involved in the killing of cells by anticancer drugs and p53 is believed to be of principal importance in this process. However, p53 also plays a role in cell cycle arrest and DNA repair, and cellular processes that can decrease the sensitivity to chemotherapy. Therefore, p53 may play a dual role after exposure to cytotoxic treatment, activating either mechanisms that lead to apoptosis or launching processes that direct towards DNA repair and survival of the cells. The treatment of cancer relies primarily on the use of several chemotherapeutic agents and irradiation. However, the most prevalent tumors (lung, breast and colorectal cancers) are relatively resistant to these interventions. The underlying basis of cellular resistance to anticancer agents has been linked to factors like decreased drug uptake, increased drug efflux, enhanced drug inactivation or reduced drug activation, altered levels or affinity of target enzymes, repair of damaged DNA and defects in apoptotic pathways. The correlation between induction of apoptosis and chemosensitivity is mainly based on evidence from experimental systems, which indicates that the response to treatment is largely determined by genotype of the cell rather than the genotoxicity of the agents. Of the factors controlling and regulating this process, p53 is believed to be of principle importance as defects in p53-dependent functions like apoptosis, may play a significant role in resistance to cancer chemotherapy. The role of the p53-induced apoptosis in the modulation of the cytotoxicity of anticancer agents supported the possibility of p53 being a mediator of broad chemosensitivity.
The influence of p53 on the cellular sensitivity to irradiation or DNA-damaging agents is still controversial. In some systems, inactivation of p53 results in increased resistance, whereas, in other cases the disruption of wild-type p53 results in increased drug-sensitivity (Brachman et al., 1993; Fan et al., 1994). Data on the role of wt-p53 product on regulation of MDR1 is antithetical, since positive as well as negative correlation have been published (Strauss et al., 1995). Cell cycle arrest and DNA repair may be the major p53-mediated event following drug exposure. In this alternative pathway, p53 would provide both time (G1 arrest) and tools (activation of GADD45 and interaction with TFIIH protein complex) to reverse drug induced DNA damage. A study demonstrated that the inactivation of p53 was able to enhance sensitivity to multiple chemotherapeutic agents (Hawkins et al., 1996). In addition, the use of temperature-sensitive p53 mutant evidenced that non-functional p53 induced much stronger sensitization to cytotoxicity than the wild type protein (Trepel et al., 1997). The conflict in results of the in vitro data is even more pronounced when antimitotic agents, especially paclitaxel, are concerned. The presence of a functional p53 was not determinant of cytotoxicity induced by paclitaxel in ovarian cancer cell lines. However, human ovarian teratocarcinoma cells presenting p53 disruption after transfection with HPV16 E6 became 100 times more resistant to paclitaxel when compared with parental wt-p53 cell line (Wu and El Diery, 1996). All these studies certainly have helped us immensely to understand p53’s functions. However the use of different experimental models has hampered the possibility to draw definite conclusions on the role of p53 status in the cytotoxicity induced by chemotherapeutic agents. Therefore to understand the possible reasons for these conflicting data, one ought to keep in mind following considerations: 1) cancer cell lines are unstable systems prone to acquire additional genetic abnormalities; 2) most of the studies used expression systems in which either supraphysiological levels are generated or endogenous p53 or viral proteins that target p53 are also present, making the interpretation of the results very arduous; 3) cellular responses to injury may be tissue specific and some cell lines may show a clear apoptotic response to DNA damage, whereas others undergo mainly cell cycle arrest. Endogenous p53 status displayed a clear cell-type variation in the ability to predict in vitro chemosensitivity (Hawkins et al., 1996). Finally, the role of p53 in chemosensitivity may also depend on the anti-neoplastic agents used.
Recent studies have sought enhanced treatment efficacy by combining gene therapy or radiotherapy with conventional systemic chemotherapy in breast cancer (Gurani et al., 1999; Lebedeva et al., 2001). p53 gene therapy may be effective even against tumors that lack p53 mutations, because p53 may function as a growth inhibitor in a variety of gene transfer settings. Key problems with gene correction therapy include the degradation of vector by immune system and a need for higher levels of gene transduction (Obermiller et al., 2000). Technical limitations, that are preventing the gene therapy technologies to become a clinical realization and its widespread application for treatment of neoplastic diseases at present, can be overcome by the development of more efficient vectors, discovery of novel genes and the development of combined modality approaches. Radiotherapy plays an important role in the management of breast cancer. Whilst its role in achieving local control following surgery in patients with early stage cancer is well established, there is still unclear evidence to explain the factors governing radioresistance in patients who develop recurrences both in the breast and axilla (Xia and Powell, 2002; Jameel et al., 2004).

1.7 Lessons from in vivo studies of p53

As a step towards understanding the role of p53 in breast cancer, the development of mouse models have played a crucial role in defining the role of p53 as a tumour suppressor gene and in examining the effect on downstream genes. Through gene targeting technology, p53 was inactivated in the so-called “knock-out mouse” “p53−/−” disrupting the DNA-binding domain (Donehower et al., 1992; Harvey et al., 1993) or through inducing point mutations at regions mainly involving the crucial coding sequences in the p53 gene (Jacks et al., 1994). When these mice were bred to homozygosity, a high proportion showed a tendency towards rapid development of tumors by six months and almost all died or developed tumors by ten months. Heterozygous mice “p53+/−”, which model the genetics of the Li-Fraumeni syndrome, also show similar tendencies but with a slightly longer latency time to tumor development (Donehower et al., 1992; Harvey et al., 1993). The concept of tumor development by loss of one normal allele of p53, loss of heterozygosity (LOH), highlights the importance of the normal p53 function in protection against carcinogenesis.
The role of p21 as one of the major components in p53 mediated cell cycle arrest was elucidated when mice with homozygous deletion for p21WAF1 were shown to go through normal development but failed to demonstrate cell cycle arrest at the G1 checkpoint (Deng et al., 1995). Similarly, the function of MDM-2 as both a p53 transcriptionally activated gene and p53 degradation regulator gene came to light and was confirmed through studies on the mouse model. This was demonstrated through an example of “phenotype rescue” when MDM-2 deficient mice were crossed to p53-deficient mice. It was observed that though MDM-2 nullizygosity resulted in early embryonic lethality when MDM-2 heterozygous mice crossed to p53-deficient mice the double null p53 and MDM-2 mice were completely viable with no developmental abnormalities. The so-called “rescue of the embryonic lethality” highlighted the primary function of the MDM-2 in development is to inactivate the p53 activity that coincides with a high level of cell cycle progression activity. This therefore suggests that the lethality of the MDM-2 null mice was due to unregulated activity of p53 that would have otherwise been controlled by MDM-2 (Jones et al., 1995; Montes de Oca et al., 1995).

p53-/- MEFs derived from p53 deficient mice show several cellular abnormalities: the cells have shortened half life, they fail to senescence at high passages, and are able to grow at low density (Harvey et al., 1993). In addition chromosomal abnormalities and aneuploidy appear at early passage giving rise to genomic instability. Cells lacking p53 are also highly tolerant to different genotoxic impulses leading to accumulated, unrepaired DNA lesions (Lowe et al., 1993)

1.8 p53 and Breast Cancer

Breast cancer is a major global health problem, and the prevalence of this disease continues to increase steadily. It is the most common malignancy among females worldwide and more than 1000000 new cases are diagnosed every year (Parkin et al., 2001). The incidence and the mortality rate vary between different ethnically and geographically distinct populations by at least four fold with lowest incidence among Asians and highest among North Americans. It is most common malignancy in woman accounting for 30% of all female cancers in UK, with around 41000 newly diagnosed cases and 13000 deaths each year. Despite recent advances
in the management of early disease, breast cancer remains a major clinical problem, causing considerable morbidity and mortality and making considerable demands on health care resources (Barrett-Lee, 2005).

Breast cancer represents the second leading cause of cancer death among women after lung cancer. The etiology of breast cancer is still poorly understood with known breast cancer risk factors explaining only a small proportion of cases. Risk factors that modulate the development of breast cancer include: age, geographic location (country of origin), socioeconomic status, reproductive events, exogenous hormones, lifestyle risk factors (alcohol diet, obesity and physical activity), familial history of breast cancer, mammographic density, history of benign breast disease, ionizing radiation, bone density, height, IGF-1 and prolactin levels, chemopreventive agents. Breast cancer is associated with the different type of somatic and genetic alterations such as mutations in oncogenes and tumor suppressor genes. Additionally, it has been summarized that breast cancer risk associated with the following genetic factors: breast cancer susceptibility high penetrance genes (BRCA1, BRCA2, p53, PTEN, ATM, NBS1 or LKB1) and low penetrance genes such as cytochrome P450 genes (CYP1A1, CYP2D6), glutathione S-transferase family (GSTM1, GSTP1), alcohol and one carbon metabolism genes (ADH1C and MTHFR), DNA repair genes (XRCC1, XRCC3, ERCC4/xpf) and genes encoding cell signaling molecules (PR, ER, TNFα or HSP70). All these factors contribute to a better understanding of breast cancer risk. Nonetheless, in order to evaluate more accurately the overall risk of breast cancer, novel genetic and phenotypic traits need to be identified (Dumitrescu and Cotarla, 2005).

In women of western world about 10% of breast cancer cases are attributable to a hereditary predisposition to disease with mutations in the two major breast cancer susceptibility genes (BRCA1 and BRCA2) being responsible for the majority of inherited cases. Germline mutations of PTEN, LKB1, ATM, p53, chk2 and MSH1/MLH1 are also associated with breast cancer to lesser extent. However, in breast carcinomas p53 mutations occur before invasion and therefore loss of p53 function may play a role in initiating steps of tumorigenesis. Correlating with this the high frequency of p53 mutations in tumors of germline BRCA1 mutation carriers suggests that inactivation of p53 function may be required for BRCA1
tumorigenesis (Polyak, 2002). Taken together, germline mutations in high penetrance breast cancer susceptibility genes such as BRCA1, BRCA2, p53, PTEN and ATM confer a high individual risk for developing hereditary breast cancer. However, these mutations have been shown to account for only up to 5-10% of all breast cancers, probably because of their low allele frequencies in general population. Relatively common low penetrance cancer susceptibility genes, acting together with endogenous and lifestyle risk factors are likely to account for most of sporadic breast cancers, which comprise the majority of all breast cancers (Johnson-Thompson and Guthrie, 2000). Hereditary breast cancers usually arise at an early age and are often multifocal or bilateral, whereas sporadic cancers are in general unilateral and have early onset (Rebbeck, 1999).

Among high penetrance genes, p53 was the first tumor suppressor gene linked to hereditary breast cancer. Moreover germline p53 mutations have been identified in patients with Li-Fraumeni cancer susceptibility syndrome which is characterized by early onset of breast cancer among other sarcomas, carcinomas and leukemias. Affected women have an 18 fold higher risk of developing breast cancer before age of 45 as compared to the general population, and risk declines with age, with maximum before 20 (Garber et al., 1991). Breast carcinomas are also a recognized clinical feature in LFS syndrome in which germline carriage of p53 mutations predisposes to an increased incidence of several cancers although the frequency of its mutation in sporadic breast cancer is substantially lower. Specific forms of the disease appear however, to be associated with high frequency of p53 mutations (Fig.5) such as sharing pathobiology with BRCA1 and BRCA2 mutations in cancer (Gasco et al., 2003).
Fig. 5 (Adapted from Bullock and Feuer, Nat Rev Can. (2001). Vol. 1, 68-76). Frequency and distribution of p53 mutations. p53 is a 393-residue protein that contain an amino terminal transactivation domain a proline rich SH3 ligand, a core DNA binding domain, a tetramerization domain and also a carboxy terminal regulatory domain. Five boxes showing the regions of greatest sequence conservation are shown; four map to core domain. A histogram of p53 missense mutations shows that 95% of mutations occur in the core domain; six labeled residues are hot spots for mutations.

p53 protein over-expression or mutation is rare in normal breast or in benign breast conditions. While sporadic mutations in p53 occur in breast cancers, abnormalities in p53 function appear to be more common than specific p53 gene mutations (Thompson et al., 1993) unlike many other cancer types. Loss of p53 normal function as the result of loss of heterozygosity (loss of one allele) is more common than the homozygous deletion (loss of both alleles) and is seen in up to 61% of the primary breast cancers (Thompson et al., 1992) and may precede the invasive phenotype (Radford et al., 1993). In pre-malignant Ductal Carcinoma In Situ (DCIS), the highest incidence of p53 abnormalities is seen in the high grade or the comedo type (O'Malley et al., 1994), the histological types most likely to progress to invasive disease. This increased rate of p53 abnormalities in DCIS suggests p53 involvement in the early stages of the breast cancer development. For bilateral breast cancer some studies show no association with p53 (Ackerman et al., 1995) while others show a higher incidence of p53 mutations in bilateral breast cancer (Kinoshita et al., 1995). p53 gene is altered in approximately 20-40% of all
breast carcinoma cases depending on tumor size and stage of disease. The use of adjuvant hormone therapy and chemotherapy, as well as radiotherapy, have improved the survival rate, but the success of adjuvant systemic treatment depends on identification of patients at risk for developing disseminated disease. It is therefore important to identify markers that can predict tumor aggressiveness and predict the response to selected therapy. To date, most frequent sites of gene mutations are in the p53 gene with approximately 30% of the tumors having a mutation, often accompanied by loss of heterozygosity (LOH).

Two different methodologies have been used to assess p53 alterations: DNA sequencing and immunohistochemistry (IHC). Molecular pathological analyses of specific components in p53 pathway are likely to give a great impact on diagnosis, prognostication, and selection of the right treatment for individual breast cancer patients (Borresen-Dale, 2003). To examine the prognostic or extrapolative value of mutations in p53 gene, a cohort of 90 Midwestern Caucasian breast cancer patients were analyzed with methodology that detect virtually 100% of all mutations. The presence of a p53 gene mutation was by far the single most predictive indicator of recurrence and death. Analysis of p53 gene mutations may permit identification of a subset of breast cancer patients who, despite lack of conventional indicators of poor prognosis, are at high risk of early recurrence and death (Kovach et al., 1996). Observation of p53 mutation or over expression of p53 protein in up to 52% of primary breast cancer specimens (Elledge and Allred, 1994), along with increasing knowledge of the characteristics of p53, has focused the attention in recent years in 2 areas:

1. Using p53 as a potential marker for studying the relationship between mutant p53 expression and tumor development, progression, response to treatment and disease outcome and
2. Designing alternative treatment strategies specifically aiming at restoring p53 function to normal.

1.8.1 p53 as a diagnostic marker

Specific p53 mutations are only observed in 15-35% of breast cancers (Andersen and Borresen, 1995). Mutant p53 as a diagnostic marker for familial
breast cancers has been most promising in the Li-Fraumeni syndrome (Malkin et al., 1990), an autosomal dominant familial disorder characterized by the development of bone/soft tissue sarcomas, leukaemia, adrenocortical, brain and breast cancers. Typically p53 mutation in one allele is accompanied by loss of the other allele (loss of heterozygosity). Thus, somatic cells lack normal p53 function a situation that is clearly seen in the Li-Fraumeni patients who have inherited one defective copy of p53 (Malkin, 1993).

1.8.2 p53 as a predictor for treatment strategy or disease response

Genome instability in early carcinogenesis can affect key genes involved in cell survival, proliferation, invasiveness, mortality, drug resistance and other malignant characteristics. The very concept of discovering the cause of cancer seems naïve as no single genetic abnormality has yet been found for all cancers. Carcinogenesis is a multistep process characterized by genetic alterations that influences key cellular pathways involved in growth and development. Several biological pathways have emerged over the last few decades that appear to be critical to carcinogenesis (new targets for cancer drug development) and can be exploited for prognostic and therapeutic purposes (Osborne et al., 2004). Breast cancer and other malignancies result from stepwise genetic alterations of normal host cells and possibly from other epigenetic changes in the behavior of not only malignant cells but also host cells that interact with the tumor such as immune, vascular and stromal cells (Nowell, 1976). If there is single common initiating event for breast cancer, its identity remains hidden within early premalignant changes of the breast, which may even be deleted or altered during cancer progression. In case of breast cancer, loss of heterozygosity and changes in gene copy number seem to sharply increase in the transition from hyperplasia to ductal carcinoma in situ (DCIS) or higher grades of DCIS. Clonal outgrowth and evolution may explain why solid tumors possess so many genetic alterations at the time of diagnosis, hence slow progress in therapeutic applications. This can be reasoned by A) cancer related pathways are intricate and many of these intersect and interact/crosstalk; hence, our comprehension of cancer biology is still rather limited, B) available preclinical models are not reflective of accurate prediction of clinical success, C) there is considerable heterogeneity in targets and their functions among different individuals.
with seemingly similar type of cancers, D) the therapeutic targets are not totally specific to cancer cells. The very genetic alterations that induce tumorigenesis can also mediate intrinsic resistance to both physiological (growth factor withdrawal and hypoxia) and non-physiological death stimuli (drugs). As a result tumors that have never been challenged with drug can be inherently resistant to conventional chemotherapeutic agents. These observations imply that tumor genotype is the most important parameter underpinning successfully chemotherapy using current drug regimen. Nevertheless, in the last 10-15 yrs breast cancer mortality declined by 2.3% per year due to multiple factors including improvements in cancer screening and more effective novel treatment regimens (Baum et al., 2003).

Hormonal/systemic chemotherapy has been shown to significantly reduce disease relapse and prolong survival in patients with breast cancer (Early Breast Cancer Trialists Collaborative Group, 1998). Similarly the use of neo-adjuvant chemotherapy followed by surgery, radiotherapy or both may improve prognosis in patients with locally advanced breast cancers. However, it has not been possible to confidently identify the 20% of patients in whom adjuvant treatment is of benefit or those for whom such treatments ought to be avoided. In breast cancer p53 mutations are associated with worse overall and disease free survival, independent of other risk factors and have been implicated in resistance to anticancer therapies (Lowe et al., 1994). For systemic hormonal therapy, node positive patients with primary breast cancer positive for p53 mutation have been shown to have a poorer response rate to adjuvant tamoxifen treatment (Bergh et al., 1995) further supported by the reduced response to tamoxifen associated with p53 protein accumulation in cytosolic extracts (Bem s et al., 1998). Molecular evaluation of p53 status, by using sequence analysis and immunohistochemistry, are incomplete assessors of p53 functional effects. Hence the transcriptional fingerprints, which are a more definitive downstream indicator of p53 function, are emphasized. Tumors with mt and wt p53 can be readily distinguished by their expression profiles and that a 32-gene p53 signature is consistently associated with patient survival in different patient subsets, and is a superior prognostic and predictive indicator of role of p53 functionality in breast cancer, compared with p53 mutation status alone (Miller et al., 2005). The reliability of p53 as an independent marker for treatment response seems to be largely
dependent on the clinical stage of breast cancer at the time of presentation. Where p53 nuclear accumulation in early breast cancer (stage II/III), may be of significant prognostic value (Isola et al., 1992; Allred et al., 1993; Gasparini et al., 1994), in locally advanced breast cancer (stage IIIA/IIIB), on the other hand, p53 does not seem to have the same independent prognostic significance (Honkoop et al., 1998).

On the whole, p53 mutation alone does not sufficiently predict the breast cancer response to systemic therapy in clinical settings (Paradiso et al., 1996; Hazar et al., 1997). Furthermore, experimental in vitro (Wosikowski et al., 1995; Gudas et al., 1996) and in vivo studies (MacGrogan et al., 1996), have suggested that p53 function may be dissociated from drug resistance and other cellular factors are likely to play an important role. Whether p53 is a significant independent predictor for response to treatment remains unclear and is the subject of clinical trials; currently there are insufficient data to support the routine assessment of p53 status as the sole marker of response to treatment in breast cancer. Recent research has combined data on the function and status of p53, with information about other genes and products which are also known to have a role in cellular response to therapy.

1.8.3 p53 as a prognostic factor

There is a need in breast cancer management for reproducible prognostic and predictive factors to guide treatment. Prognostic factors predict a patient’s natural history independent of therapy used. These enable stratification of patients according to risk of relapse and therefore the identification of those most likely to benefit from adjuvant therapy. Predictive factors determine the likelihood of sensitivity or resistance to a particular therapy. These enable the maximization of therapeutic effect, by targeting treatment to those most likely to benefit and avoiding unnecessary toxicity to others. The whole area of prognostic and predictive factors is being revolutionized by gene-expression profiling (van de Vijver et al., 2002) for prediction of outcome in patients. So far, the strongest predictive and prognostic factor has been the number of affected lymph nodes. However, the clinical outcome remains unpredictable inspite of effective adjuvant therapy. Many investigators have therefore focused on finding molecular markers that may predict prognosis and response to therapy. In breast cancer, expression of Bcl-2 has been associated with
expression of ER and low proliferation rate; however it has not been an independently prognostic factor (Mottolese et al., 2000). At present no single predictive biological marker has been established in routine practice in breast cancer to assess clinical benefit from chemotherapy. Additionally, the mechanisms of cytotoxic drug action are complex, and involve several intracellular pathways. There is increasing recognition and clinical evidence that these involve apoptotic pathways predominantly (Ellis et al., 1998), with contribution from proliferation mechanisms and drug metabolism pathways.

p53 mutation is not usually the initiating genetic event in most breast tumors. It however, seem to be an independent prognostic factor for survival that might prove useful in the clinical management of patients with breast cancer (Eeles et al., 1993). Alteration of the p53 gene in breast cancer is commonly associated with an unfavourable prognosis (Elledge et al., 1993). Accumulation of nuclear p53 or expression of mutant p53 is associated with a number of histological, biological and clinical adverse prognostic factors. The degree of the p53 aberrations and specific mutations seem to vary between different histological types of breast cancers. They are more common in the invasive ductal carcinomas as opposed to lobular carcinomas, and less common in the well differentiated types with more favourable prognosis (Poller et al., 1992). There are exceptions: poorly differentiated medullary carcinomas displaying p53 gene mutations ironically have a more favourable prognosis (Poller et al., 1992). However, p53 abnormalities are generally associated with the higher grade cancers, aneuploid tumors and those with a high S-phase fraction (mitotic rate) (Allred et al., 1992). Therefore the histology of a specific breast tumour should be considered as an adjunct to p53 status when using p53 as a prognostic marker. Abnormal p53 in general is considered to be a marker of more aggressive cancer (Elledge et al., 1993; Elledge and Allred, 1994) both for locally advanced and inflammatory cancers. There are reports of a higher tumor proliferation rate, early disease recurrence, and early death in the node-negative breast cancers (Allred et al., 1993) though there is no clear correlation with tumor size or the axillary nodal involvement. p53 aberrations are associated with low levels of oestrogen and progesterone receptors which are known to be markers of less aggressive tumors with better response rate to systemic hormonal therapies.
(Cattoretti et al., 1988; Thompson et al., 1992; Allred et al., 1993). It has been shown that p53 mutation correlates with negative steroid receptor status and also with overexpression of epidermal growth factor receptor (EGFR). Both of these factors are associated with more aggressive tumors (Horak et al., 1992; Mazars et al., 1992). The independent prognostic power of p53 is weak and is best combined with other cellular and biological parameters. These include oestrogen and progesterone receptor expression as markers for endocrine response to treatment, Bcl-2 proto-oncogene as a predictor for adjuvant hormonal treatment efficacy (Gasparini et al., 1995), c-erb-2 (Her-2/neu) for the proliferative properties of the tumour (Allred et al., 1992), Ki67 antigen as a predictor for survival and relapse, tumour cell characteristics such as microvessel density as a predictor of relapse-free survival (Gasparini et al., 1994) and tumour cell proliferation rate as a marker of malignant potential (Isola et al., 1992). p53 and c-erb-2, protein over-expression are said to be associated with a high malignant potential (Isola et al., 1992), with subsequent prediction of overall survival and metastasis-free interval (MacGrogan et al., 1996) in node-negative tumours. This concurs with earlier studies in node-positive patients, where patients over-expressing c-erb-2 had a worse prognosis (Allred et al., 1992). Other series have excluded c-erb-2 and p53 co-expression as valuable prognostic markers (Rozan et al., 1998).

1.8.4 Strategies for manipulation of the p53 pathway in the treatment of breast cancer

Deregulation of proliferation together with a reduction in apoptosis, creates a platform that is both necessary and can be sufficient for cancer. The secondary traits of diverse neoplasms are a consequence of cell proliferation. Cancer arises from the rare simultaneous acquisition of the two cooperating conditions that permit cell expansion - deregulated cell proliferation and suppressed apoptosis. The greatest problem with the conventional therapeutic approach is that it fails to rectify the tumor specific antiapoptotic lesions and instead employs a crude strategy of simply loading up the proapoptotic side of the equation in the hope of exceeding the apoptotic threshold in the tumor cells before it happens in too many normal ones. If susceptibility to apoptosis is really is the Achilles’ heel of the cancer cell, we need to
know far more about the footwear that protects it or suppresses it (Green and Evan, 2002). Chemotherapy, radiation therapy and immunotherapy, all rely profoundly on apoptosis to kill breast cancer cells. Despite the fact that many tumors initially respond to therapy, cells can subsequently survive and gain resistance to these treatments. This leads to a more phenotypically aggressive cell variant with an inclination to metastasize. Chemosresistance often accompanies the progression of breast cancers from a hormone-dependent, non metastatic, antiestrogen sensitive phenotype to a hormone-independent, invasive, metastatic, antiestrogen-resistant phenotype (Campbell et al., 2001). The activity of the majority of clinically used anticancer agents, including DNA crosslinking agents, antimetabolites and topoisomerase I and II inhibitors are influenced by p53 status. It was found that restoration of wild type p53 function in p53 null HL-60 cells conferred multidrug sensitivity (Ju et al., 1998). However, other studies have produced contradictory results on the relationship between p53 status and chemosensitivity (Weller, 1998). For example inactivation of p53 function in normal human foreskin fibroblast by ectopic expression of the HPV16 E6 resulted in enhanced sensitivity to DNA damaging agents, such as cisplatin, rather than resistance (Hawkins et al., 1996).

1.8.5 Novel strategies involving functional attributes of p53 in breast cancer chemotherapy

Over the last few years, great advances have been made in our understanding of the biology of breast cancer at a molecular level. This research has enabled the identification of novel prognostic and predictive factors, and the emergence of rationally designed targeted therapies into the clinic.
These developments together with an ever-evolving body of clinical trial evidence have revolutionized the management of breast cancer. Hopefully, this will enable further consolidation of improvements in breast cancer mortality already achieved by the use of adjuvant therapies and earlier disease detection over last decade (Peto et al., 2000).

The insights provided by laboratory research done on p53 over two decades are now moving to clinical applications for better diagnosis, prognosis, and treatment of cancer. The detailed strategies for engaging and manipulating the p53 pathway have been comprehensively reviewed in the literature for a wide range of human cancers (Hupp et al., 2000; Lane and Lain, 2002). The future holds promise for specific, individualised targeting of mutant or wild-type p53, or its transcriptional targets, in combination therapies with other cancer-specific drugs, to maximize tumor cell killing while protecting normal cells from toxic side effects. For breast cancers, where not only p53 mutation but also aberrations of the p53 pathway occur commonly, many of these strategies are promising (Table 1). Moreover, therapies against breast cancer with respect to utilization of tumor suppressor p53 at centre stage needs more comprehensive knowledge about the role it plays in the outcome of these treatments. Deciphering these roles by functional inactivation of this tumor suppressor has faced some or other inherent drawbacks. For example, using E6 oncoprotein expression to inhibit p53 functions has a set back as these viral proteins may bind and interfere with the activity of many cellular

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<td>Classical chemotherapy</td>
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<td>Gene replacement</td>
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<td>ONYX-015</td>
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proteins besides p53. On the other hand use of dominant negative mutants of p53 for studying the importance of wild type p53 may lead to confusing conclusions due to unknown gains of function as well as an ineffective reduction of endogenous p53 function. The other modes of attenuation and abrogation of p53 function are either transient or in non-isogenic model systems or are regulated by extra cellular signal. Additionally, all information about relationship between loss and mutated p53 or any genetic and biochemical changes have not been definitely established because these studies were based on tumor biopsies and cell lines already lacking wild type p53 (Xu et al., 1995; Hawkins et al., 1996; Franken et al., 2004). Taken together, the differences in attenuation and abrogation of p53 function has significantly altered conclusions about its role in cell growth and determination of cell fate after chemotherapy. Moreover additional properties of p53 are now emerging including activation of signal transduction pathways and significant involvement of p53. Thus, an appropriate model derived from genetically matched cell systems that only differ in p53 protein status, is a required tool.

Killing of tumor cells by chemotherapy, radiotherapy, immunotherapy, or suicide gene therapy is predominantly mediated by triggering injury in cancer cells which leads to cell death. Cancer chemotherapy suffers major drawback as chemoresistant cells develop within the tumors due to their heterogeneous nature, most importantly in response to therapy under different treatment regimens, and due to inadequate drug delivery methods. Therefore, it is becoming apparent that better understanding of mechanism of drug induced injury to cancer cells will have profound effect on cancer treatment and management due to large therapeutic index that can be achieved. The enforcement of combining anticancer chemotherapeutic agents has been recently underscored by enhanced therapeutic benefit with lessened host toxicity. Combination therapy with multiple drugs or with multiple modalities is common practice in treatment of cancer. When anticancer agents with similar and different modes of action are combined, the outcome can be synergistic, additive or antagonistic. It is also unlikely that a single targeted drug would be successful in majority of cases, because of differences in uptake, metabolism, onset or duration of action, pharmacokinetic behavior, and the mechanism of action of different drugs. Selective synergism against tumor may increase therapeutic efficacy, decrease
toxicity toward the host due to dose reduction, and minimize or delay the development of drug resistance due to less selective pressure at a lower dose required for a given effect. Additionally, role of the p53-induced apoptosis in the modulation of the cytotoxicity of anticancer agents, supported the possibility of p53 being a mediator of broad chemosensitivity in combination chemotherapy.

As a novel strategy to enhance cancer cell killing, it has been demonstrated that alpha particles irradiation also affected population of cells which was not directly hit by alpha particles (Nagasawa and Little, 1992; Hall, 2003), or enhanced cell killing by gene therapy used for prodrug activation underscored the enhanced therapeutic efficacy by propagation of injury or bystander effect (Touraine et al., 1998; Zhou et al., 2000). It has been also well documented that p53 and factors dependent on p53 potentiate cell death in chemo and radiation therapy of various cancers models (Komarova et al., 1998; Nishizaki et al., 1999). Moreover, since chemotherapeutic drugs can induce apoptosis and upregulate death ligands or their receptors that may subsequently play a significant role in death signal amplification via bystander effect in a mixed/heterogeneous population of cells (Petak and Houghton, 2001). Taken together these studies indicate that the bystander phenomenon has great implications in cancer therapies due to the large therapeutic index that can be achieved. Moreover, an improved understanding of the cellular and molecular mechanisms of drug induced cytotoxicity, together with evidence of their occurrence in vivo, will facilitate better outcome of specific breast cancer chemotherapy.

Hence, this thesis documents the delineation of the pathways through which the tumor suppressor p53 act and decipher the basis of its varying actions in specific breast cancer cell types. The research work also comprises the development of new ways to exploit breast cancer chemotherapy regimen for the benefit of cancer patients by increasing its therapeutic efficacy.
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