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Cell Cycle Regulatory Proteins in Breast Cancer: Molecular Determinants of Drug Resistance and Targets for Anticancer Therapies

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1. Introduction

Deregulation of cell-cycle is a distinguishing hallmark of tumor cells (Stewart *et al*, 2003). Regulation of cell cycle is a key mechanism for the maintenance of homeostasis of normal cell growth and viability, and this is a very tightly regulated process. However, deregulation of cell cycle is well known to contribute to tumor development (Maya-Mendoza *et al*, 2009). Normal cells possess an ability to arrest cell-cycle after DNA damage in an attempt to maintain genome integrity whereas tumor-initiating cells are characterized by deregulated cell-cycle whereby the DNA-damaged cells proceed to undergo DNA synthesis and cell division, which leads to the development of tumor mass. Since cancer cells, including breast cancer cells, are known to exhibit uncontrolled cell growth and proliferation, one parameter to judge the efficacy of anti-cancer therapies is through their ability to arrest cell cycle. Therefore, it is not surprising that the acquisition of drug-resistance in cancer cells is often linked with defects in cell cycle regulation. Cell cycle arrest involves down-regulation of cyclins and cyclin-dependent kinases (CDKs), and up-regulation of inhibitory p21 and p27. Several investigations, including those from our own laboratory, have revealed that cell-cycle arrest is an important mechanism responsible for apoptosis-inducing ability of cancer therapeutic agents in breast cancer cells. In particular, functional loss of G1-checkpoint inhibitors, p21 and p27, is believed to be important during the progression of many human malignancies, and most therapeutic agents function via induction of these tumor suppressor proteins. Loss of p21 and p27 has also been implicated in the acquisition of drug-resistance phenotype, and conversely, their up-regulation has the ability to re-sensitize cancer cells to conventional therapeutics. This chapter attempts to update the state-of-our understanding of several key cell cycle proteins that play a crucial role in breast cancer tumorigenesis. We will review modulation of such key players by conventional therapeutics, which eventually results in the induction of apoptotic cell death in the context of cell cycle regulation and drug resistance. In addition to the clinically relevant drugs, we will also introduce readers to the potential utility of natural agents as cell cycle regulators.

2. Cell cycle: An overview

The two main events/phases of the cell cycle are - Interphase and Mitosis (Figure 1). Interphase is the phase of cell cycle in which cell performs the majority of its purposes, including preparation for the division of cell. Mitosis is that phase of the cell cycle when cell prepares for and actually completes cell division. Interphase serves as the checkpoint to ensure that the cell is ready to enter into mitosis. Since the cell cycle is a "cycle", cells are continually entering and exiting the various phases of this dynamic cycle. Cells spend a majority of their time in interphase and this phase has three distinct stages - G1, S and G2.

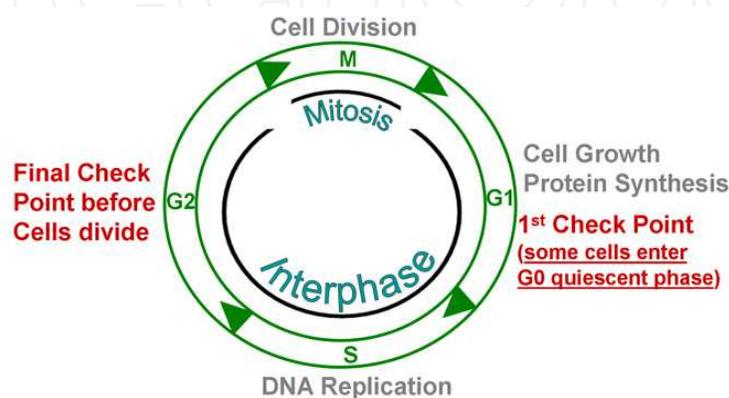


Fig. 1. An overview of cell cycle.

2.1 G1 phase

G1 is the phase of cell cycle immediately following a round of cell division and occupies the time between mitosis and the beginning of DNA replication during S phase. In the G1 phase, cells grow and function normally. Prior to another round of cell division, cell has to make sure that it is completely ready for division. G1 is the phase when this monitoring takes place and if the cell is not ready yet to divide, it will continue to remain in this phase. Cells are even known to enter a phase called G0 if they are not ready to continue in the cell cycle. G0 can last for days, weeks, or even years. However, if cell decides to divide, it grows in size during G1 phase, more cell organelles are synthesized, protein synthesis occurs and cell prepares itself for DNA replication.

2.2 S phase

Immediately following G1 phase is the S (synthesis) phase. It is the phase when DNA replication takes place. This phase represents a particular sensitive point in cell cycle because fidelity of DNA replication is required to ensure that the resulting daughter cells will have exactly the same genetic make-up as the dividing mother cell. Most of the events that occur during S phase are related to DNA replication and this phase is marked by synthesis of proteins/enzymes that are involved in DNA replication machinery. At the end of S phase, cells contain twice the normal number of chromosomes.

2.3 G2 phase

S phase is followed by G2 phase. This phase is marked by further growth of cell in anticipation of mitosis. Since this phase occurs after the duplication of DNA and just before the commencement of cell division in mitosis, it represents another checkpoint in the cell

cycle and a final chance for the cells to make sure that their DNA and other cellular components have been properly duplicated.

3. Regulation of cell cycle

3.1 Cyclins

Cyclins are so named because they undergo a constant 'cycle' of synthesis and degradation during cell division. There are now several recognized classes or types of cyclins, active in different stages of the cell cycle. The D- and E-type cyclins are associated with the G1-S phase transition of the cell cycle (Sherr, 1994). Cyclins are proteins that play important roles in the functioning of CDKs.

3.2 Cyclin-dependent kinases

CDKs, as their names suggest, are kinases that depend on cyclins for their kinase activity i.e. their ability to phosphorylate other molecules. A number of CDKs were discovered independently before their cell cycle regulatory function was recognized, and consequently, the nomenclature of these proteins was not uniform and was often confusing. Based on the outcome of a meeting at Cold Spring Harbor in 1991, the CDK series was born with many of the pre-recognized cell cycle regulators named CDK1, CDK2, CDK3 and so on (Abukhdeir & Park, 2008). CDKs were classified as kinases based on the observation that the total amount of phosphorylated proteins increased following injection of CDKs into the oocytes of a variety of different organisms (Abukhdeir & Park, 2008). A number of CDKs have now been characterized. Cyclins bind to CDKs to form a cyclin-CDK complex (Figure 2). This complex, along with the various phosphorylated targets, acts as a signal for the cell to pass to the next cell cycle phase. Cyclins and CDKs are, therefore, positive modulators of cell cycle. Synthesis of cyclins and CDKs marks the readiness of cells to divide. At the time when cell no longer wants to divide, cyclins are degraded resulting in deactivation of CDKs and arresting the cell cycle.

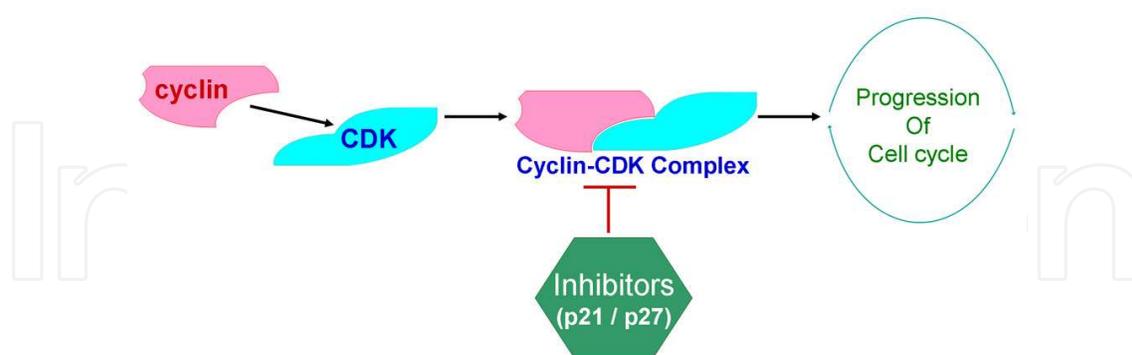


Fig. 2. Role of cyclins, cyclin-dependent kinases (CDKs) and inhibitors in progression of cell cycle.

3.3 Inhibitors of cyclin-dependent kinases

Since CDKs are involved in the progression of cell cycle, molecules that inhibit CDKs are negative regulators of cell cycle and function to induce cell cycle arrest (Figure 2). Cyclin-CDK complexes typically activate their downstream targets by phosphorylation; therefore, inhibitors of cyclin-CDKs modulate cell cycle by preventing or limiting cyclin-CDKs' ability

to phosphorylate their targets. There are two classes of CDK inhibitors. Members of the first class specifically bind to CDK4 and CDK6 and inhibit their association with D-type cyclins. Members of the second class, also known as kinase inhibitor proteins (KIPs) and include p21, p27 and p57, are inhibitors of cyclin A-CDK, cyclin D-CDK and cyclin E-CDK complexes (Abukhdeir & Park, 2008). Similar to existence of many CDKs, many inhibitors of CDKs are also known but we will limit our discussion on two such inhibitors – p21 and p27, to keep our discussion more focused.

3.3.1 p21 (cyclin-dependent kinase inhibitor 1)/WAF1

The p21 is coded by human gene *CDKN1A* and belongs to the *Cip/Kip* family of CDK inhibitor proteins. Two research groups, working independently, published the cloning of its gene in 1993 simultaneously. Using a subtractive hybridization approach, el-Deiry et al. (el-Deiry *et al*, 1993) identified a gene whose expression was directly induced by p53 and it was found to be an important mediator of p53-dependent tumor growth suppression in human brain tumor cells. This gene was named WAF1. Using an improved two-hybrid system, Harper et al. (Harper *et al*, 1993) isolated a 21 kDa protein that regulated CDK2 activity. This protein was found to inhibit phosphorylation of retinoblastoma (Rb) by cyclin A-CDK2, cyclin E-CDK2, cyclin D1-CDK4 and cyclin D2-CDK4 complexes. This gene was named CIP1 (CDK-interacting protein 1). In view of the simultaneous discovery of the same protein by two independent groups, thus p21 is also referred to as p21^{CIP1/WAF1}.

A number of biological effects of p21 are mediated by its binding to and inhibition of cyclin-dependent kinases (CDKs) (Abbas & Dutta, 2009). Since these cyclin-CDK complexes play a role in the progression through G1 phase, p21 regulates the cell cycle progression at G1 phase of the cell cycle. It has been suggested that the ability to mediate p53-dependent gene repression might be a mechanism by which p21 induces cell cycle inhibition (Abbas & Dutta, 2009). This is based on the knowledge that p21 is important for p53-dependent repression of several genes that are involved in cell cycle progression. While p21 might be important for p53 functions in most cases, there is evidence suggesting that p53 can function in a p21-independent manner as well (Xia *et al*, 2011). Also, p21 binds to proliferating cell nuclear antigen (PCNA), and through binding to PCNA, p21 competes for PCNA binding with DNA polymerase- δ and other key factors involved in DNA synthesis leading to the inhibition of DNA synthesis (Moldovan *et al*, 2007; Abbas & Dutta, 2009). Such modulation of PCNA appears to be one of the regulatory roles of p21 in S phase DNA replication and DNA damage repair. Because of its inhibitory action on cell cycle progression, p21 is widely regarded as a tumor suppressor factor. However, there are reports in the literature which suggest its direct role as an oncogene (Roninson, 2002). Mice genetically engineered to lack p21 develop normally and such mice do not exhibit increased susceptibility to cancer. Thus, while the important role of p21 in the regulation of cell cycle is known, its biological functions are far from being clearly understood.

3.3.2 p27 (cyclin-dependent kinase inhibitor 1B) / KIP

The p27 is coded by human gene *CDKN1B* and, like p21, belongs to the *Cip/Kip* family of CDK inhibitor proteins. Similar to the simultaneous identification and characterization of p21 by two independent research groups, p27 was also cloned simultaneously by two independent research groups. Polyak et al. (Polyak *et al*, 1994) cloned p27Kip1 and reported its ability to inhibit cyclin E-Cdk2 complexes leading to obstruction of entry of cells into S phase. It was

found to have a region of sequence similarity to p21Cip1/WAF1 and it inhibited Rb phosphorylation by cyclin E-CDK2, cyclin A-CDK2, and cyclin D2-CDK4 complexes. Using a yeast interaction screen to search for proteins that interacted with cyclin D1-CDK4, Toyoshima et al. (Toyoshima & Hunter, 1994) identified a 27 kDa protein that interacted strongly with D-type cyclins and CDK4. It was identified as a negative regulator of G1 progression.

P27 binds to and prevents the activation of Cyclin A-Cdk2/cyclin E-CDK2/cyclin D-CDK4 complexes. Since these cyclin-CDK complexes also play role in the progression through G1 phase, p27 regulates the cell cycle progression at G1 phase of cell cycle. For the inhibition of cyclin A-CDK2 complex, p27 binds the complex as an extended structure wherein it interacts with both cyclin A and CDK2. In cyclin A, p27 binds within a groove formed by conserved cyclin box residues while to CDK2, it mimics ATP and inserts into the catalytic cleft (Russo *et al*, 1996). Similar to p21, tumor promoting activities have been reported for p27 as well (Besson *et al*, 2007;Blagosklonny, 2002;Lee & Kim, 2009). Despite the dual nature of p27, as a tumor suppressor and tumor promoter, its ability to inhibit CDK complexes is still considered very important. Moreover, in a meta-analysis, reduced p27 has been recognized as an independent prognostic factor for poor overall and disease-free survival breast cancer patients (Guan *et al*, 2010), suggesting that agents that could activate p27 would have clinical utility.

4. Drug resistance

The problem of drug-resistance is a major concern for researchers and clinicians because it is a big hindrance in the successful management of cancer patients. In case of breast cancer, a number of targeted therapies are available for cancer subtypes that are marked by the expression of estrogen receptor (ER), progesterone receptor (PR) and overexpression of Her2/neu. Some cancers do not respond to the therapy at all, right from the beginning, and such phenomenon is called *de novo* drug resistance. However, many cancers actually respond to the targeted therapy initially but with the passage of time and continued administration of therapeutic agent, they eventually develop resistance to that therapeutic agent, and this process is called acquired drug resistance. While *de novo* drug resistance is itself challenging, acquired drug resistance is clinically an even bigger problem. The cancers that have acquired drug resistance are usually far more aggressive and difficult to treat. They are invariably linked to poor prognosis as well as overall poor survival. In the few subsections to follow, we will discuss the problem of drug-resistance as observed in breast cancer patients, and the role that dysregulated cell cycle plays in the development of drug resistant phenotype.

4.1 Cell cycle regulation in tamoxifen resistant breast cancers

For the management of breast cancers that express ER, tamoxifen is the drug of choice for targeted personalized therapy. Tamoxifen can significantly lower the chances of developing recurrent breast cancer and can be very effective in women who initially present with metastatic disease. It remains the primary therapeutic agent for the management of ER- and/or PR-expressing breast cancers, particularly in premenopausal women without or with conventional chemotherapeutic agents. A number of reports have implicated modulation of cell cycle and cell cycle regulatory proteins as prognostic/predictive markers of progression of ER- positive breast cancers during the acquisition of tamoxifen resistance.

In an early attempt (Rostagno *et al*, 1996) to understand the interaction between ER and cell cycle regulators, ER was found to be induced during the G0/G1-phase in MCF-7 cells, the breast cancer cells that are characterized by expression of ER. Increased expression of ER during the G0/G1-phase was followed by a decrease during the S-phase until the late S-phase where a rapid increase was noted. From these observations, it was concluded that estrogens are involved in DNA synthesis since ER was found to be expressed at a maximal level during late G1. A tamoxifen resistant phenotype was developed by long term exposure of MCF-7 xenografts to tamoxifen and this resulted in an altered profile of ER during the G0/G1 phase. This provided an indication for the relevance of cell cycle regulation in tamoxifen resistance of breast cancers *in vivo*. As a molecular signature, it has been shown that tamoxifen-resistant MCF-7 cells express higher levels of cell cycle regulators cyclin E1 and CDK2, compared to parental cells (Louie *et al*, 2010). Further, cell cycle regulatory proteins have also been shown to be differentially expressed in ER-negative vs. ER-positive breast cancer cells. The ER-negative cells have increased expression of cyclin B1, cyclin D1 as well as cyclin E (Skog *et al*, 2004).

In a report suggesting a role for cyclins, specifically cyclin D1, in tamoxifen resistance of ER-expressing breast cancer cells T47D and MCF-7, it was reported that ectopic expression of cyclin D1 was sufficient to reverse the growth inhibitory effect of tamoxifen as well as steroidal anti-estrogens (Wilcken *et al*, 1997). Such ectopic cyclin D1 induction resulted in activation of cyclin D1-CDK4 complexes and entry of cells into S phase of cells indicating a reversal of cell cycle arrest. Later, it was shown that cyclin D1 was not endogenously up-regulated in tamoxifen-resistant MCF-7 cells, compared to parental MCF-7 cells (Kilker *et al*, 2004). It was up-regulated only on exposure to physiological levels of tamoxifen, suggesting a dependence of cyclin D1 expression on ER. The same research group later reported that cyclin D1 expression is necessary for proliferation of tamoxifen-resistant cells and for tamoxifen-induced cell cycle progression (Kilker & Planas-Silva, 2006). The role of cyclin D1 in tamoxifen resistance was further validated by Stendahl *et al*. (Stendahl *et al*, 2004) using tissue samples from 167 postmenopausal breast cancers arranged in a tissue array. It was reported that in 55 ER-over-expressing samples, with moderate or low cyclin D1 levels, patients responded well to tamoxifen treatment. However, in 46 patients with ER – over-expression as well as cyclin D1 over-expression, there was no difference in survival between tamoxifen compared to no tamoxifen group. This clearly suggests that the expression of cyclin D1 is related to the overall response of patients to tamoxifen treatment because the patients with cyclin D1 over-expression were found to be associated with tamoxifen resistant phenotype.

In addition to a positive correlation between cyclin D1 expression and aggressive cancers, as documented above, there is evidence to suggest otherwise as well. For example, a study using microarray analysis of cyclin D1 expression identified high cyclin D1 expression as a low risk factor for local recurrence of breast cancer (Jirstrom *et al*, 2003). Low expression of cyclin D1 expression was, conversely, associated with high risk of cancer recurrence. Thus, overexpression of cyclin D1 was reported to be actually beneficial for breast cancer survival, associated with inverse tumor grade, smaller tumor size, and improved relapse-free and overall survival of breast cancer patients (Ishii *et al*, 2008). As a mechanism for such counter-intuitive function, it has been proposed that cyclin D1 represses the activity of signal transducer and activator of transcription 3 (STAT3) (Ishii *et al*, 2006). Since STAT3 is a potent inducer of cell proliferation and survival, its down-regulation might explain the observed reduced aggressiveness of breast cancer.

Based on a recent report which evaluated p27 expression in 328 primary stage II breast cancers from premenopausal patients, it has been suggested that patients with p27-

overexpressing tumors benefit from tamoxifen treatment, which underscores the role of p27 in predicting response to therapy (Stendahl *et al*, 2010). However no association was found between p27 and recurrence-free survival, suggesting that p27 is not a prognostic marker. There is evidence to suggest the involvement of p21 in tamoxifen resistance as well, and the loss of p21 function has been shown to be associated with a tamoxifen-resistant phenotype (Abukhdeir *et al*, 2008). Studies using immortalized human breast epithelial cells with somatic deletion of the p21 gene showed a growth proliferative response to tamoxifen because absence of p21 enabled cyclin-CDK complexes to aberrantly phosphorylate ER when bound to tamoxifen, resulting in a growth-stimulatory phenotype. On the other hand, p21 wild-type cells demonstrated growth inhibition upon tamoxifen exposure.

4.2 Cell cycle regulation in adriamycin resistant breast cancers

Adriamycin (doxorubicin) is another chemotherapeutic drug that is routinely used in the clinic for the management of breast cancer patients. It is used to treat early-stage or node-positive breast cancers, HER2/neu-positive breast cancers as well as metastatic breast cancers. It primarily interferes with the DNA replication machinery leading to inhibition of cancer cell growth. Since DNA replication is crucial to the progression of cell cycle, its inhibition by adriamycin indicates the involvement of cell cycle regulatory proteins associated with the anticancer action of this drug. Moreover, a number of investigations have actually reported modulation of cell cycle as a mechanism of adriamycin action. Similar to tamoxifen, breast cancers that are managed well by adriamycin initially, eventually develop resistance to adriamycin, which is also a major clinical problem.

While earlier investigations found no correlation between p21 and adriamycin resistant phenotype (Staalesen *et al*, 2004), it was later reported that pre-treatment of adriamycin resistant MCF-7 cells with tumor necrosis factor-alpha (TNF- α), followed by adriamycin treatment, was an efficient way for enhancing the cytotoxic effects of adriamycin (Cao *et al*, 2006). Since TNF- α was earlier shown to down-regulate p21 leading to enhanced killing by adriamycin (Cao *et al*, 2005), these studies provided an evidence for the involvement of inhibitory p21 in resistance of breast cancer cells to adriamycin. A role of p21 in adriamycin-induced, twist-1-mediated induction of epithelial-mesenchymal transition has also been suggested (Li *et al*, 2009). In a study profiling the changes induced by prolonged exposure to adriamycin, it was found that long term culture of MCF-7 cells with adriamycin resulted in inhibition of cyclin D1 and increased expression of p21 (Lukyanova *et al*, 2009). Differential expression of p21 in adriamycin resistant MCF-7 cells has also been reported (Saleh *et al*, 2009) further confirming an involvement of p21 in development of adriamycin resistant phenotype. A role of p27 in adriamycin sensitivity was also suggested when 56 out of a total of 119 breast cancer patients demonstrated p27 overexpression and it was found that the susceptibility of adriamycin in tumors with high expression of p27 was significantly higher than in tumors with low expression (Yang *et al*, 2000). In a later investigation, p27 was found to be a good prognostic marker for disease free and overall survival of breast cancer patients in the context of resistance to preoperative doxorubicin-based chemotherapy in primary breast cancer (Davidovich *et al*, 2008).

4.3 Cell cycle regulation in herceptin resistant breast cancers

The Her2/neu (ErbB2) gene encodes an epidermal growth factor receptor (EGFR) family tyrosine kinase that is overexpressed in about 20-30% of invasive breast cancers. Herceptin

(Trastuzumab) is a humanized monoclonal antibody that targets Her2. It is now utilized in the clinic for the management of breast cancers that over-express Her2/neu. Increased expression of Her2/neu provides a proliferative advantage leading to uncontrolled cell division and growth. Treatment with herceptin effectively blocks the signaling through Her2/neu leading to the inhibition of uncontrolled tumor growth. Herceptin treatment is a very effective way to treat patients with Her2/neu overexpression; however, the phenomenon of resistance to this drug is also seen in a lot of patients who stop responding to the treatment. It is believed that a majority of patients with metastatic breast cancer who initially respond to herceptin/trastuzumab, demonstrate disease progression within 1 year of treatment initiation (Nahta *et al*, 2006) indicating progression to drug resistant phenotype. Similar to the resistance to other drugs documented above, resistance to herceptin also involves modulations in cell cycle regulatory proteins. Cyclin D1 and the inhibitory p27, in particular, have been investigated for their role in herceptin resistance. A number of reports that have detailed the modulation of a specific signaling pathway/ molecule, resulting in re-sensitizing herceptin resistant cells to herceptin treatment, have indicated a role of these cell cycle regulators. Herceptin is known to induce the expression of p27 (Lu *et al*, 2004). Induction of p27 ensures its association with its target CDK leading to the inhibition of cyclin-CDK complex, resulting in cell cycle arrest. It will, therefore, be logical to expect down-regulation of p27 in herceptin-resistant cells which will attenuate the ability of this inhibitory protein to induce cell cycle arrest. Indeed, this was observed in a cell line model when herceptin-resistant variant of Her-2 over-expressing breast cancer cell line, SKBR3, was actually found to harbor significantly reduced expression of p27 (Nahta *et al*, 2004). In addition to induction of p27, herceptin also functions via down-regulation of cyclins (Wu *et al*, 2010). Both of these events - increased p27 and decreased cyclins - ensure an effective arrest of cell cycle progression in response to herceptin treatment. A similar role of CDKs in mechanism of anticancer drugs has also been reported. For example, PD 0332991, a highly selective inhibitor of the CDK4 as well as CDK6, was reported to increase the efficacy of tamoxifen in ER-positive cells and that of herceptin in Her-2 over-expressing cells (Finn *et al*, 2009). In this study, an analysis of 47 human breast cancer cell lines revealed that Rb phosphorylation is blocked only in drug-sensitive cells but not in drug-resistant cells. Since Rb phosphorylation is a measure of CDK activity, an effective inhibition of CDKs by drugs such as tamoxifen and herceptin in sensitive cells results in reduced Rb phosphorylation. The resistant cells become refractory to such CDK inhibitory action implying that CDK inhibition can potentially be beneficial for the management of drug-resistant breast cancers (Sutherland & Musgrove, 2009).

5. Cell signaling crosstalk in drug resistant phenotype: Role of cell cycle regulators

The progression of cancer and the development of drug resistant phenotype is particularly challenging because of the excessive cross-talk between multiple signaling molecules and pathways. Regulation of cell cycle proteins plays a crucial role in most of these processes as well. For instance, tamoxifen treatment is known to result in up-regulation of inhibitory p21 and p27 (Cariou *et al*, 2000). The development of tamoxifen resistance, therefore, involves down-regulation of these cell cycle regulatory proteins (Cariou *et al*, 2000). Since cancer cells

are known to evade processes that may tend to slow down their progress, tamoxifen treatment is often marked by up-regulation of other signaling pathways that may work to overcome the effects caused by tamoxifen treatment. Over-expression of EGFR (Nicholson *et al*, 1990) and Her2/neu (De Placido *et al*, 1998) are often observed in ER-positive cells that have been exposed to tamoxifen. This activation of alternate cell proliferation pathways represents a mechanism by which ER-positive cells respond to anti-estrogen treatment. As a further proof, it has been reported that ectopic expression of Her2/neu results in reduced sensitivity to tamoxifen treatment (Benz *et al*, 1992). Since tamoxifen therapy results in induced expression of EGFR and Her2/neu, a treatment regimen combining tamoxifen with inhibitors of EGFR and Her2/neu might be a better strategy. This was tested by Chu *et al*. (Chu *et al*, 2005) who combined lapatinib (a dual EGFR and Her2/neu inhibitor) with tamoxifen for the treatment of ER-positive breast cancer cell lines. It was found that a combination of these two drugs caused cell cycle arrest more effectively than treatment with either drug alone. This enhanced combinational effect was found to be dependent on increased down-regulation of cyclin D1, cyclin E-CDK2 and marked increase in p27. This study provided clear evidence in support of combinational treatments. When a therapeutic agent suppresses the growth of cancer cells by targeting a specific cell cycle regulatory pathway, the cancer cells look for alternate pathways to overcome this regulation. In the process, alternate pathways are activated to derepress the regulation of cell cycle. In such a scenario, it is necessary to simultaneously target these multiple signaling pathways so as to ensure a sustained blockage of cell cycle progression.

In support of a clinical significance of cell cycle regulatory proteins in cell survival pathways cross-talk, it has been reported that the prognostic importance of Her2/neu is significantly better for breast cancer patients whose tumors overexpress cyclin D1 (Ahnstrom *et al*, 2005). In patients with overexpression of cyclin D1, Her2/neu overexpression strongly correlates with increased risk of recurrence and mortality. Further, in ER-positive patients, tamoxifen treatment was reported to be particularly beneficial in patients with moderate cyclin D1. Another mechanism by which cancer cells can evade tamoxifen is through oncogenic kinase Src. Signaling through EGFR and/or Her2 can lead to increased Src activity (Ishizawar & Parsons, 2004) and overexpression of Src results in repression of p27 (Chu *et al*, 2007). Src represses the inhibitory action of p27 on cyclin-CDK complexes and accelerates p27 proteolysis by phosphorylating it at tyrosine residues 74 and 88. Therefore, it appears that tamoxifen therapy leads to the induction of p27. It also leads to the activation of alternate EGFR and Her2/neu pathways which, in turn, lead to the activation of Src and reverse the induction of p27 via its increased degradation. In this context, Src inhibition increased p27 levels in tamoxifen resistant breast cancer cells (Chu *et al*, 2007) indicating that a combination of Src inhibitors with tamoxifen might also be a good strategy for an effective regulation of cell cycle progression leading to desired inhibitory effects on cancer progression.

There is some evidence to support the role of STAT3 signaling in tamoxifen resistant phenotype. In relation to the role of cyclin D1 in the prognosis of recurrent breast cancers, it was reported that repression of STAT3 by cyclin D1 resulted in reduced cell growth, and treatment with tamoxifen abolished such cyclin D1-mediated repression of STAT3 and resulting effects on cell cycle and growth (Ishii *et al*, 2008). Tamoxifen induced the redistribution of cyclin D1 from STAT3 to the ER, resulting in an efficient activation of STAT3 as well as ER. In another study focusing on another STAT, STAT5b, it was reported

that STAT5b is required for estrogen-induced proliferation of ER-positive breast cancer cells (Fox *et al*, 2008). Inhibition of STAT5b, using specific siRNA, showed reduced proliferation mediated through reduced expression of cyclin D1. This study also identified a role of EGFR and Src kinase in estrogen-induced cyclin D1 expression and cell growth, and thus indicating a cross-talk between ER, Src, EGFR and STAT5b in ER-positive breast cancer cells. Cyclin D1 interacts with a number of different CDKs as well as p27, an inhibitor of cyclin-CDK complexes. Given the detailed investigations on the role of cyclin D1 in drug resistant phenotype, as discussed above, it may make some sense to investigate whether there is a direct role of CDKs, if any, in drug resistant and aggressive phenotype of breast cancer cells. To that end, Johnson *et al*. (Johnson *et al*, 2010) studied the therapeutic potential of inhibiting CDK2 and CDK1 in relation to anti-estrogen resistance. It was observed that CDK2 knock-down results in the accumulation of cells in G1 phase. Knock-down of CDK1, however, resulted in G2-M slowing, and simultaneous knock-down of CDK1 and CDK2 caused further accumulation of cells in G2-M phase transition. This was also accompanied by increased cell death, thus confirming the role of CDK2 and CDK1 as targets for breast cancer therapy.

In yet another demonstration of signaling cross-talk, the role of met receptor in herceptin resistance has been reported (Shattuck *et al*, 2008). This study stemmed from the observation that met receptor is frequently expressed in breast cancer cells that also exhibit Her2 over-expression. Interestingly, Her2 over-expressing cells tend to up-regulate the expression of met in response to herceptin treatment. This might be a way for them to overcome the proliferation inhibition that they are subjected to, post herceptin-treatment. The study (Shattuck *et al*, 2008) observed that the simultaneous inhibition of met might help to overcome resistance to herceptin. This was based on the finding that inhibition of met led to re-sensitization of herceptin resistant cells to herceptin treatment. In this context, the role of cell cycle regulator p27 has been proposed because met-mediated down-regulation of p27 was found to be a significant event that was crucial to the development of herceptin resistant phenotype. Similar to inhibition of met, inhibition of proteasome has also been reported to increase the efficacy of herceptin (Cardoso *et al*, 2006). An effective down-regulation of p27 by the combination treatment, along with down-regulation of NF- κ B, was proposed as the mechanism for the observed results.

A further complex cross-talk between signaling pathways, leading to herceptin resistance, has also been reported. This involves a cross-talk between Her2, Her3 and insulin-like growth factor-I receptor (IGF-IR) pathways (Huang *et al*, 2010). This study reported interactions between the three signaling pathways that were observed exclusively in herceptin resistant breast cancer cells, which suggested a cross-talk that might be important for the progression to as well as sustenance of a herceptin resistant phenotype. Down-regulation of Her3 or IGF-IR was found to result in an efficient induction of p27. Since p27 is an inhibitor of cell cycle, such increased p27 levels were correlated with re-sensitization of previously herceptin resistant cells to herceptin. This study again demonstrated how modulation of cell cycle regulatory protein p27 can play a central role in overcoming drug resistant phenotype.

In summary (as schematically shown in Figure 3), anticancer drugs such as tamoxifen, adriamycin and herceptin act via down-regulation of their cellular targets to induce an efficient cell cycle arrest. This is followed, in many cases, by activation of alternate cell proliferation pathways, which function to restore cell cycle and lead to cancer progression.

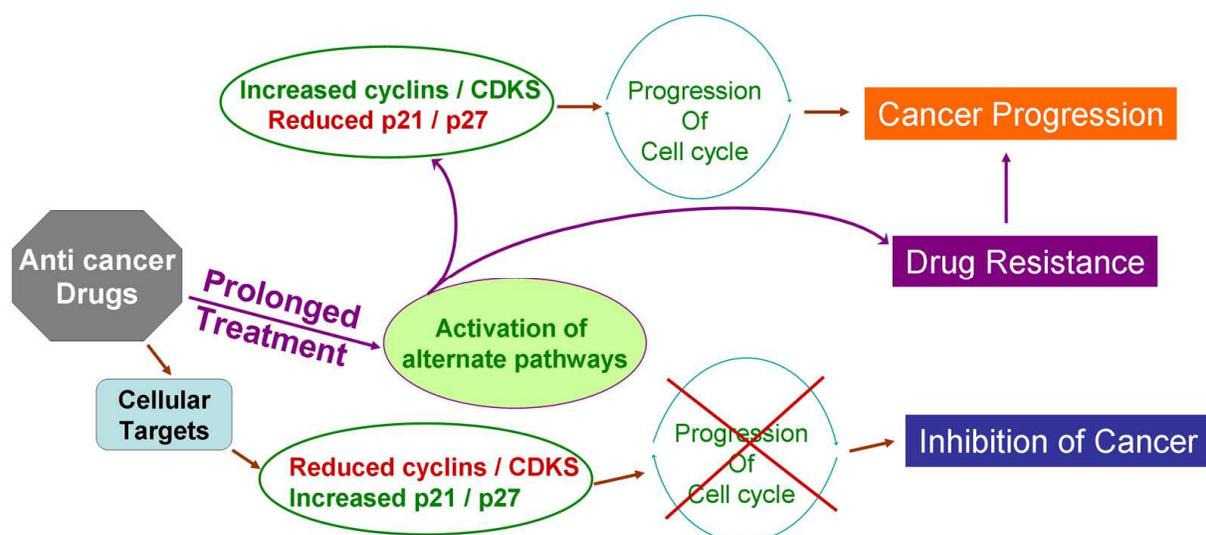


Fig. 3. Complex role of cell cycle regulators in progression of cancer, efficacy of anticancer treatments and development of drug resistant phenotype.

6. Cell cycle regulation by natural compounds: Basis for their apoptosis-inducing and anticancer activity

In addition to various anticancer drugs that are in use for the clinical management of breast cancer patients, a lot of research has been done on plant-derived natural compounds to evaluate their putative role in prevention and progression of cancer. A number of such compounds have shown promise in preclinical studies (Gullett *et al*, 2010). These compounds are potent inducers of apoptosis (Hail, Jr. & Lotan, 2009; Shu *et al*, 2010), and cell cycle arrest is one of the mechanism through which these compounds are able to exert their biological effects (Sarkar & Li, 2004). Cell cycle can be transiently arrested by chemopreventive agents at damage checkpoints which allows for DNA repair, or activation of pathways leading to apoptosis if the damage is irreparable. Our own studies have shown that soy isoflavone genistein can cause G2-M arrest, leading to apoptosis induction, more efficiently in malignant breast cancer cell lines (MCF10CA1a and MDA-MB-231) compared to normal breast epithelial cells (MCF10A and MCF12A) (Upadhyay *et al*, 2001). We found a significant up-regulation of p21 at mRNA as well as protein level in the normal cells. Down-regulation of p21 sensitized normal as well as malignant breast cancer cells to genistein-induced G2-M arrest, suggesting an important role of p21 in determining the sensitivity of normal and malignant breast epithelial cells to genistein. Later, we reported genistein-mediated induction of p21 in ER-positive MCF-7 cells as well (Chinni *et al*, 2003). A similar activity of 3, 3'-Diindolylmethane (DIM; obtained from cruciferous vegetables) was also observed, and DIM was found to inhibit the expression of cyclin E2, survivin and Bcl-2, and induce the expression of p27 leading to the induction of cell cycle arrest and apoptosis in multiple breast cancer cell lines (Rahman *et al*, 2006; Wang *et al*, 2008). Effect of DIM on regulators of cell cycle (cyclin E2 and p21) as well as regulators of apoptotic pathways (survivin and Bcl-2) indicated a close connection and a mechanistic link between these pathways that may define the anticancer activity of natural compounds. Similar to these reports from our laboratory, there are numerous other reports that have documented the anticancer ability of these as well as many other naturally occurring

compounds (Sarkar & Li, 2004;Gullett *et al*, 2010). The interest in research on these compounds is largely based on the observation that natural compounds are part of normal diet and, as such, well tolerated. Further, as discussed above, the development of drug resistance often involves cross-talk between multiple pathways. For example, the drug resistance through cyclin D1 is because of its association with so many cancer progression pathways such as transforming growth factor (TGF)- α , EGFR, Ras, phosphoinositide-3-kinase (PI3K)/Akt and NF- κ B (Liao *et al*, 2007). Natural agents exhibit their inhibitory effects on carcinogenesis and cancer progression through the regulation of multiple cell signaling pathways. Therefore, regulation of multiple cell signaling pathways for controlling the behavior of cancer cells such as inhibition of cell growth, induction of apoptosis, inhibition of invasion/metastasis as well as re-sensitizing drug-resistant cancer cells requires agents that could target multiple pathways, and it is now believed that many of the natural compounds are perfect examples showing that these natural agents could target multiple pathways.

7. Conclusions and perspectives

The role of cell cycle, and the various regulatory factors during the progression of cancer, is now well appreciated. Therefore, one important parameter to judge the efficacy of anticancer therapeutics is via their modulation of cell cycle regulation. Irregularities in cell cycle regulation are hallmarks of aggressive breast cancers as well as those breast cancers that have turned refractory to drug treatment. A number of anticancer drugs used in the clinic, function through modulation of cell cycle. In addition to direct targeting of cell cycle regulatory factors for anticancer therapy, genes such as FoxM1 that modulate cell cycle machinery (Wang *et al*, 2010a), have also been targeted in breast cancer models leading to the inhibition of proliferation and invasion of aggressive cells (Ahmad *et al*, 2010). Furthermore, the anticancer properties of natural non-toxic compounds are increasingly being realized. Although these compounds are well-tolerated and have low toxicity, still they have not yet made their way to clinic. They suffer from inefficient systemic delivery and bioavailability. To that end, nano-chemoprevention, an application of nanotechnology to enhance the efficacy of natural compounds has shown promise (Siddiqui *et al*, 2009). Another approach is to increase the efficacy as well as bioavailability of natural compounds by synthesizing novel analogs of natural compounds, as demonstrated by our recent work on a synthetic analog of curcumin (Padhye *et al*, 2009).

Another emerging area of cancer research involves the regulation of genes by tiny non-coding RNA molecules, microRNAs (miRNAs). It is interesting to note that natural compounds, that have been shown to induce cell cycle arrest, have also been reported to regulate miRNAs (Li *et al*, 2010). Moreover, it is increasingly being realized that the regulation of miRNAs is a good strategy to overcome the problem of drug resistance (Wang *et al*, 2010b). In breast cancer, the role of miRNAs in tamoxifen resistance, through regulation of cell cycle regulatory proteins, has been suggested. In particular, ectopic expression of miR-221/miR-222 was found to render ER-positive MCF-7 cells resistant to tamoxifen. This was mediated through significant down-regulation of their target p27 (Miller *et al*, 2008). Expression of miR-221 and miR-222 was also found to be significantly increased in Her2-positive primary human breast cancer tissues indicating an interrelationship between miR-221/222 expression and Her2 over-expression in primary breast tumors that are generally resistant to tamoxifen therapy. These preliminary reports

are encouraging and a better understanding of the mechanisms, that cause deregulated cell cycle thereby leading to cancer progression, would result in the development of novel anticancer therapeutics.

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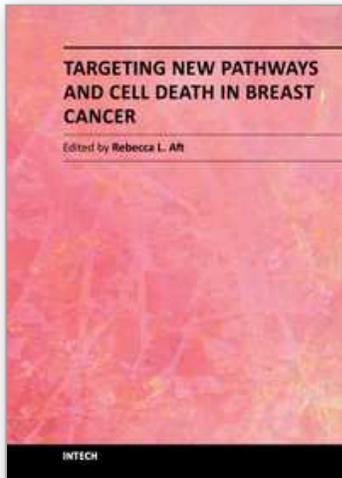
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