Resistance to Chemotherapy in Breast Cancer:
Potential role of *p21B*, *p27* and the p53 apoptotic pathway

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PREFACE

Cancer has been treated with chemotherapeutic drugs for the last fifty years. But the effectiveness of the treatment has not always been as expected. This has been explained by the lack of drug specificity and most importantly drug resistance as the majority of the cancer patients develop resistance against chemotherapy. Therefore, identification of patients with a high probability of responding to specific treatment, and hence, prevent the non-responding patients from ineffective treatment, would improve therapy significantly. Despite this obvious advantage, the number of studies designed specifically to explore the mechanism of drug resistance *in vivo* are surprisingly few compared to the high number of studies done *in vitro* as well as compared to clinical therapy studies in general. Biopsies obtained from primary breast cancers before chemotherapy treatment provides a unique opportunity to investigate *in vivo* mechanisms of chemoresistance. Insight into such mechanisms is expected to lead to improved diagnostication and therapy. Our present project is designed to find possible association between alterations in functional cascades and therapy resistance.

Contents

LIST OF PAPERS

This thesis is based on the following papers, referred to in text by their Roman Numerals:

Paper I:

Ranjan Chrisanthar, Stian Knappskog, Erik Løkkevik, Gun Anker, Bjørn Østenstad, Steinar Lundgren, Elisabet O. Berge, Lars Engebretsen, Louise Mæhle, Terje Risberg, Ingvil Mjaaland, Johan Richard Lillehaug and Per Eystein Lønning (2008). *CHEK2* mutations affecting kinase activity together with mutations in *TP53* indicate a functional pathway associated with resistance to epirubicin in primary breast cancer. PLoS ONE 3(8): e3062 doi:10.1371/journal.pone.0003062

Paper II:

Ranjan Chrisanthar, Stian Knappskog, Erik Løkkevik, Jurgen Geisler, Bjørn Østenstad, Steinar Lundgren, Terje Risberg, Ingvil Mjaaland**,** Johan Richard Lillehaug and Per Eystein Lønning. Neither mutations in the *p27* gene nor its Val109Gly polymorphism is associated with lack of response to epirubicin or paclitaxel in locally advanced breast cancer. *Manuscript Submitted*

Paper III:

Stian Knappskog, **Ranjan Chrisanthar,** Vidar Staalesen, Anne-Lise Børresen-Dale, Inger Torhild Gram, Johan Richard Lillehaug and Per Eystein Lønning (2007). Mutations and polymorphisms of the *p21B* transcript in breast cancer. Int. J. Cancer: 121, 908-910

ABBREVIATIONS

aa	Amino acid
ATM	Ataxia telangiectasia mutated
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CHEK2	Checkpoint kinase 2 gene
Chk2	Checkpoint kinase 2 protein
dsDNA	Double stranded DNA
ER	Estrogen receptor
IHC	Immunohistochemistry
INK	Inhibitor of kinase
KIP	Kinase inhibitor protein
LFL	Li-Fraumeni-like syndrome
LFS	Li-Fraumeni syndrome
LOH	Loss of heterozygosity
Mdm2	Mouse double minute 2 homolog
pRB	Retinoblastoma protein
p53	The protein encoded by the *TP53* gene
SNP	Single nucleotide polymorphism
TP53	Gene encoding p53 protein

DEFINITIONS

Adjuvant

Adjuvant therapy refers to additional treatment, usually given after surgery where all detectable tumor has been removed aiming to eliminate micro-metastases.

Apoptosis

Apoptosis or "programmed cell death" is defined as a mechanism of cellular suicide which occurs after sufficient cellular damage.

Lumpectomy

Lumpectomy is surgery in which only the tumor and some surrounding tissue is removed.

Mastectomy

Mastectomy means surgical removal of the entire diseased breast, in general due to breast cancer.

Neo-adjuvant

Neo-adjuvant therapy refers to chemotherapy or endocrine treatment given to cancer patients prior to surgery. The general reason for neo-adjuvant therapy is to reduce the size of the tumor to facilitate limited surgery (so-called "lumpectomy").

Predictive factor

Predictive factor defines sensitivity of a tumor to a distinct therapeutic agent.

Prognostic factor

Prognostic factor is a term denoting risk of disease relapse or death. Strictly speaking, it should be recorded among patients not undergoing adjuvant systemic therapy to avoid interference from therapy.

Senescence

Cellular senescence is the phenomenon where normal cells lose the ability to divide, a form of permanent cell cycle arrest.

1 INTRODUCTION

1.1 Cancer

Cancer is a class of diseases or disorders characterized by uncontrolled cell growth and/or defects in critical control mechanisms (like growth arrest or ability to undergo apoptosis). Furthermore, cancer cells are characterized by their ability to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis. The unregulated growth that characterizes cancer is caused by mutations in genes that encode proteins controlling cell homeostasis.

Cancer-associated genes are divided into proto-oncogenes/oncogenes and tumor suppressor genes. Proto-oncogenes are genes encoding proteins which promote cell growth and mitosis, a process of cell division, and tumor suppressor genes encodes proteins that discourage cell growth, or temporarily halt cell division in order to allow DNA repair, induce apoptosis and/or senescence. Typically, a series of several mutations to these genes are required before a normal cell transforms into a cancer cell. Most simply defined proto-oncogenes contribute to cancer by over expression or constitutive activation, whereas tumor suppressor genes contribute to cancer by a loss of function. Tumor suppressor genes code for anti-proliferation signals and proteins that suppress mitosis and cell growth. Generally, tumor suppressors are activated by cellular stress or DNA damage. The functions of such genes are to arrest the progression of cell cycle in order to carry out DNA repair, preventing mutations from being passed on to daughter cells. Most tumor suppressor genes are inactivated by mutations leading to absence of wild type proteins.

1.1.1 Breast cancer

Breast cancer is the most commonly occurring cancer among women, causing more deaths in the western world than any other cancer except for cancer of the lung (Greenlee, Murray et al. 2000; Jemal, Thomas et al. 2002). While breast cancer incidence is still increasing, mortality from breast cancer is decreasing in many western societies, probably due to combined effect of early detection and improvements in treatment (Peto, Boreham et al. 2000; Balmain, Gray et al. 2003). The risk of getting breast cancer increases with age. For someone who lives to the age of 90, the chances of getting breast cancer has been estimated to be as high as 14% or one in seven during their lifetime. Men can also develop breast cancer

because the breast is composed of identical tissues in males and females. Male breast cancer incidence is less than 1% of all breast cancer cases (Agrawal, Ayantunde et al. 2007).

The cause of breast cancer is complex and not fully understood. Germline genetic alterations of highly penetrant genes like the tumor suppressor genes *BRCA1*, *BRCA2* and *TP53* are suggested to explain only 5-10% of all breast cancer cases (Ford, Easton et al. 1995; Martin and Weber 2000). Thus, the majority of breast cancer cases is probably caused by life-style factors, environmental (Buell 1973; Kaur 2000), and/or alterations in a variety of low-penetrance breast cancer susceptibility genes (Martin and Weber 2000). The major differences in breast cancer incidence between ethnic groups and geographic areas in general are assumed to be due to life style/environmental factors, probably diet, smoking, high-alcohol consumption and not differences with respect to ethnic background. Evidence in favor of this view was provided when it was revealed that Japanese women immigrating to America over time adapted a breast cancer risk resembling white Americans (Buell 1973).

Low penetrance susceptibility genes are genes with specific sequence variants or polymorphisms that may be associated with an increased breast cancer risk (Weber and Nathanson 2000). Although each variant is only associated with a small increased risk for breast cancer, their common appearance may explain that their attributable risk in the population as a whole may be higher than that of so-called high penetrance genes. In families with several breast cancer cases in the absence of any known germ-line syndrome, interplay between low-penetrance genes and lifestyle factors may contribute to the higher risk for breast cancer (Balmain, Gray et al. 2003).

1.1.2 Breast cancer treatment

Today's treatment of breast cancer includes a multimodal approach consisting mainly of surgery, combined to a variable degree with adjuvant chemotherapy, radiation therapy and hormone therapy depending on tumor and patient characteristics. Once diagnosed, most breast cancers are primarily treated by surgical removal of the tumor (lumpectomy) or the entire diseased breast (mastectomy) following adjuvant systemic therapy to reduce the risk of a relapse. While for most patients surgery is performed upfront to any additional therapy, use of primary, or so-called neo-adjuvant chemo- or endocrine therapy is increasingly used, in particular for larger tumors.

Breast cancer evolves in the epithelial tissue in breast. As these cells proliferate under hormonal control, breast cancer may often be treated by endocrine therapy when the tumor cells have retained the estrogen receptor (ER) (2005). The exception is patients with a

particular low risk of relapse. Tamoxifen, an ER modulator that binds ER and obstructs estrogen from binding, is a general regime often given to premenopausal women. For postmenopausal women, aromatase inhibitors are the first-line therapy, either used alone or sequentially treatment with tamoxifen. Aromatase produces estrogen from androgens in various tissues. This reaction is the main source of estrogen after menopause (Lonning, Dowsett et al. 1990).

Chemotherapy and/or radiation therapy may also be used before or after surgery. While some local relapses may be related to residual cancer tissue deposits, the key problem relates to development of distant micro-metastases. Radiation therapy, which only has a moderate effect on overall survival, is commonly prescribed for women who have undergone lumpectomy or mastectomy surgery with the purpose to reduce the risk that cancer will recur (Overgaard, Hansen et al. 1997). Radiation therapy eliminates the microscopic cancer cells that may remain near the area where the tumor was surgically removed.

Adjuvant therapy has been found to reduce the risk of a relapse, with anthracycline-based treatments most effective (2005).

Combination of cytostatic and endocrine therapy may reduce the death rate by up to 50%, compared to adjuvant therapy (2005). Thus patients with hormone-sensitive tumors are in general treated with endocrine therapy alone.

Chemotherapy, in particular those forms in focus in this study, will be described in more detail in the following section.

1.2 Chemotherapy

Cancer has been treated with chemotherapeutic drugs for the last fifty years. With the exceptions of a few cancer forms, cure rates have not met the initial optimistic expectations. This is explained with lack of specificity and most importantly the development of drug resistance in tumor cells.

Neo-adjuvant chemotherapy is used to reduce the size of a tumor prior to surgery and also to prevent development of micro-metastasis. Neo-adjuvant chemotherapy is being increasingly utilized in patients with locally advanced breast cancer and many centers are favoring treatment with cytotoxic agents and radiotherapy before surgery to "downstage" tumors, opening the possibility of limited surgery such as lumpectomy and thus to avoid mastectomy. Adjuvant chemotherapy is given after surgery to reduce the risk of recurrence and to eliminate micro-metastasis thereby preventing distant metastasis.

There are several different chemotherapy regimens that may be used. The determination of the appropriate regimen depends on many factors including the primary characteristics of the tumor (size and histological grade), lymph node status, and the age and health of the patient. Thus, apart from the old patients, patients harboring lymph node metastases or patients without lymph node metastases but harboring other negative prognostic characteristics are in general treated with a combination of several drugs in general including an anthracycline. The general effect of chemotherapy treatment is believed to be that most anticancer drugs kill cells by inducing apoptosis in the malign cells (Kerr, Winterford et al. 1994; Ellis, Smith et al. 1997). This is achieved through induction of several different intracellular pathways. Even though, it is well documented that anticancer drugs often kill cells by inducing apoptosis, it is still a problem to pinpoint the exact mechanisms as there are indicated that senescence may parallel apoptosis in response to stress (Schmitt, Fridman et al. 2002). However, this is not completely understood. Study of the alterations in the components of the apoptotic pathways may yield information about the molecular pathways associated with chemotherapy response and resistance. There are very few studies that have investigated the biology of the neo-adjuvant chemotherapy *in vivo* and those that exist are limited in the number of time points. Mainly, most of these studies are done in paraffin embedded tissue, due to lack of availability of frozen tissue, at this limited to immunohistochemistry (IHC). However, numerous studies have tried to identify markers or variations in markers that are associated with response or resistance to treatment.

The two classes of cytotoxic drugs that demonstrate most activity towards breast cancers are anthracyclines and taxanes.

1.2.1 Epirubicin

One of the most commonly used anthracycline, doxorubicin, is a natural compound produced by the *Streptomyces* species, whereas epirubicin, which is also widely used in the treatment of breast cancer, is synthetically produced.

Similarity to other anthracyclines, epirubicin acts through binding DNA resulting in inhibition of topoisomerase II and fragmentation of DNA (Smith and Soues 1994), resulting in mechanisms that lead to cell death. Furthermore, anthracyclines can undergo reduction to generate free radicals that cause cell and DNA damage (Cummings, Anderson et al. 1991; Muller, Niethammer et al. 1998). Epirubicin is favored over doxorubicin as it appears to cause fewer side-effects, faster elimination and reduced toxicity (Bhutani, Kumar et al. 2002).

1.2.2 Paclitaxel (Taxol)

Taxol, a brand name of paclitaxel, is one of the most effective anticancer drugs used in the clinic to treat a variety of solid tumors. The first taxane, paclitaxel was initially extracted from the bark of the pacific yew *Taxus brevifolia* in the 1960`s. Since that time its use as an anti-cancer drug has become well established.

During cell division, taxanes interfere with the development of the microtubules needed for the cell duplication thus inhibiting the faster growing tumor cells (Parness and Horwitz 1981). Paclitaxel interferes with normal function of microtubule growth, and stabilizes their structure. This destroys the cell's ability to use its cytoskeleton in a flexible manner. Specifically, paclitaxel binds to the ß-subunit of tubulin. Tubulin is the building block of microtubules, and the binding of paclitaxel locks these building blocks in place. The resulting microtubule-paclitaxel complex does not have the ability to disassemble (Jordan, Wendell et al. 1996), a requisite for microtubule dynamic function. This adversely affects cell function because the shortening and lengthening of microtubules is necessary for their function as a mechanism to transport other cellular components. Especially in the M-phase of the cell cycle, when chromosomes are separated into daughter cells by dissembling of the microtubules they are attached to.

1.2.3 Resistance to chemotherapy

The main obstacle to chemotherapy treatment is resistance to cytostatic regimes. Resistance to chemotherapy remains a major challenge in the treatment of breast cancer. Apart from applying effective treatment, to avoid patients having ineffective toxic treatment is a main goal in cancer therapy.

As mentioned above, defective apoptosis is considered to be a central event in malignant tumors. The general effects of most chemotherapeutic drugs are in generally believed to require functioning apoptotic pathways to induce cell death, this could be one explanation of why also drug-naive tumors can be resistant. We know that alterations leading to disturbances in the apoptotic pathway results in drug resistance (Evan and Vousden 2001). However, at the present time there is no reliable means of predicting chemotherapy responsiveness. Biopsies obtained from breast cancers during neo-adjuvant chemotherapy provide a valuable opportunity to observe the pathways involved in cell death. The molecular characterization may provide an improved understanding of the mechanisms of chemoresistance and lead to new, targeted treatment strategies.

1.3 The p53 signaling pathway network

Cells respond to DNA damage by activating a DNA damage-response pathway that includes cell cycle arrest, transcriptional and posttranscriptional regulation of genes associated with repair and programmed cell death (Vogelstein, Lane et al. 2000). The pathway provides a mechanism for transmitting a signal from a sensor that recognizes the damage, through a transduction cascade, to a series of downstream effectors, which implement the appropriate response.

The p53 signaling pathway is in "standby" mode under normal cellular conditions. Activation occurs in response to cellular stresses or damage, and several independent pathways of p53 activation have been identified (Vogelstein, Lane et al. 2000). Recent research has confirmed the existence of at least three independent pathways by which p53 network can be activated. One pathway is indeed triggered by DNA damage, caused by drugs like anthracyclines and ionizing radiation. This activation of the network is dependent on two protein kinases, ATM (*Ataxia Telangiectasia Mutated)* and Chk2 (Checkpoint kinase 2) (described in detail below). The second pathway may be induced by ultraviolet light and protein kinase inhibitors. This pathway is distinguished from others because it is not dependent on intact ATM or Chk2. Instead, this pathway involves kinases called ATR (ataxia telangiectasia related kinases). The third pathway for activating p53 is triggered by aberrant growth signals, such as those resulting from the expression of the oncogenes Ras or Myc. These oncogenes stimulate the transcription and stabilization of the p14 protein, which binds to Mdm2 and inhibits its activity (Sherr and Weber 2000), thereby preventing p53 binding and degradation.

As a result of activation, numbers of genes have been identified as transcriptional targets of p53. These can be placed into a number of classes according to their functions. Protein products of these genes control processes such as cell cycle arrest, DNA repair and in many cases they even cause the programmed death (apoptosis) in response to genotoxic damage as well as oncogene activity to protect the organism (Vousden and Lu 2002; Lee and Schmitt 2003). The p53 protein therefore provides a critical brake on tumor development, explaining why it is so often mutated in cancers.

1.3.1 The p53 apoptotic pathway

In normal cells DNA damage triggered by anthracyclines and ionizing radiation is detected by the protein product of the ATM, transducted towards cell cycle arrest and/or apoptosis through the p53 pathway (Figure 1).

A single dsDNA break is sufficient for ATM activation. ATM activates Chk2 by phosphorylating amino acid Thr68 (Matsuoka, Rotman et al. 2000). Phosphorylation of Thr68 leads to autophosphorylation of Thr383 and Thr387 in the Chk2 kinase domain (Lee and Chung 2001). ATM can also phosphorylate p53 directly at Ser15 (Delia, Mizutani et al. 2000) a site close to the interaction region of Mdm2. Phosphorylation of Ser15 will obstruct binding of Mdm2 and increase the stability of p53 (Delia, Mizutani et al. 2000). ATM can also phosphorylate Mdm2 directly on Ser395 to decrease its affinity to p53 (Khosravi, Maya et al. 1999).

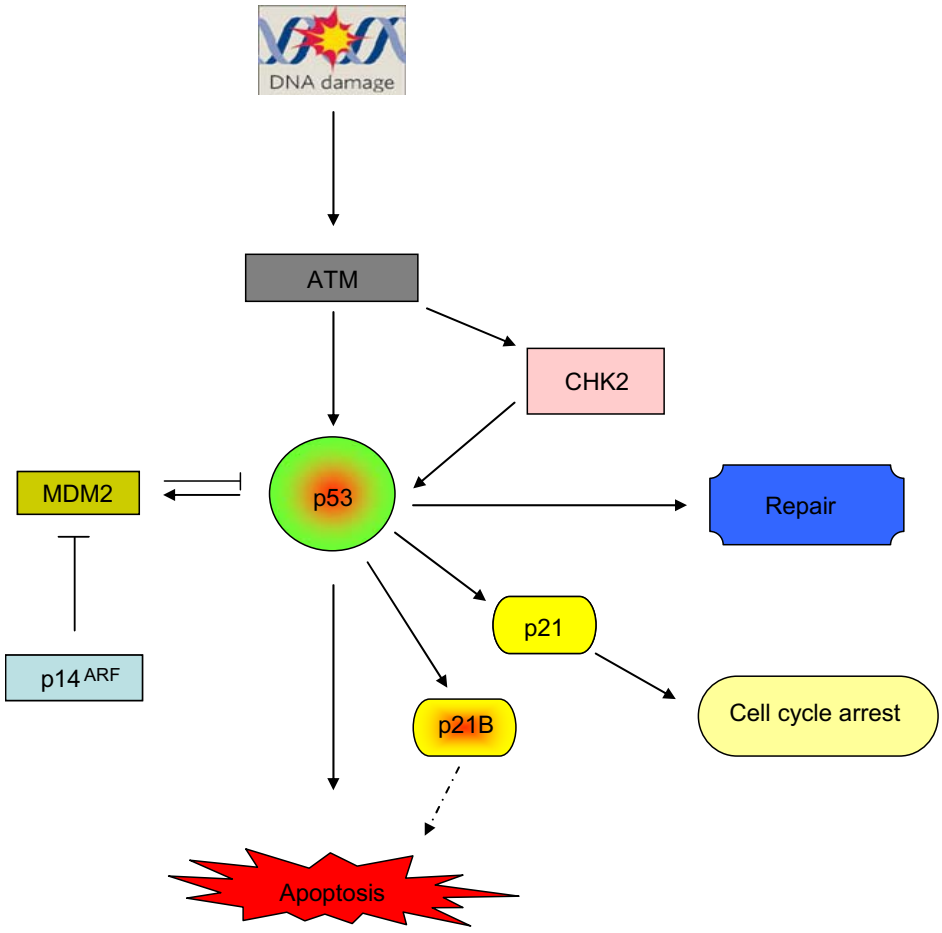

Figure 1: Schematic illustration of p53 apoptotic pathway following DNA damage. ATM activates Chk2 by phosphorylation which in turn phosphorylates p53. Arrows indicate activation/induction while butt ends indicate inhibition.

The *CHEK2* gene is located on chromosome 22q12.1. The gene contains 14 exons, and encodes a protein of 543 amino acids. The protein Chk2, a serine/threonine kinase, plays an important role in double strand break responses leading to cell cycle checkpoint arrest, apoptosis and DNA repair, as mentioned above. The Chk2 protein (Figure 2) consists of three distinct functional domains. The most amino terminal of these are the SQ/TQ cluster domain (aa 19 – 69), which contains Thr68, the site phosphorylated in response to DNA damage by the upstream kinase, ATM, as mentioned above. Downstream of the SQ/TQ domain the forkhead associated domain (aa 115 – 165) is located, which function in trans to modulate protein interactions and in cis to affect other functional domains within the protein itself. The kinase domain (aa 225 – 490) is located almost on the entire C-terminal half of Chk2 (Ahn, Urist et al. 2004). On DNA damage signal, oligomerization of Chk2 is activated by phosphorylation of Thr68, which serves as a ligand for the forkhead-associated domain of another Chk2 molecule (Ahn, Li et al. 2002). This allows autophosphorylation of Thr383 and Thr387 resulting in the release of active Chk2 monomers, which function in DNA damage induced cellular responses by activating p53 by phosphorylating p53 at Ser20 (Khanna, Lavin et al. 2001). These phosphorylation events disrupt the binding interface between Mdm2 and p53, increasing the p53 population.

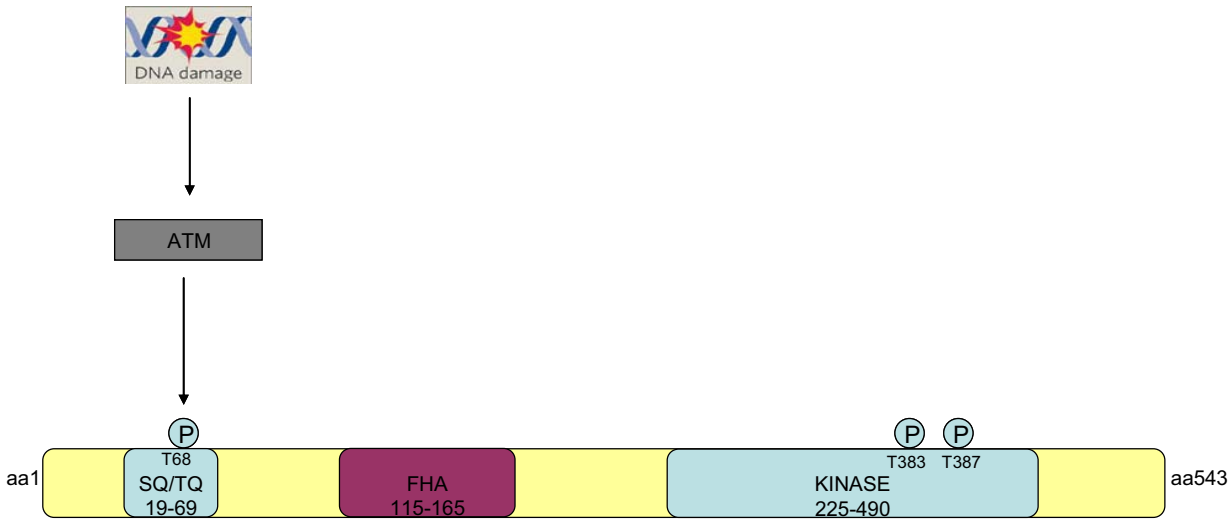

Figure 2: Chk2 structure is shown schematically with the functional domains. Following DNA damage ATM activates Chk2 by phosphorylating Thr68.

The *TP53* gene, located on chromosome 17q13.1, contains 11 exons and encodes a protein of 393 amino acids with the central region harboring the DNA binding domain (Figure 3). Activation of p53 by Chk2 phosphorylating Ser20 in response to DNA damage result in an increase in the levels of p53 protein due to reduced Mdm2 dependent proteolytic degradation, and increased affinity of p53 for sequence specific DNA binding activity.

As mentioned above, an increasing number of genes are being identified as transcriptional targets of p53 (Vogelstein, Lane et al. 2000; Kannan, Amariglio et al. 2001). Stabilized p53 regulates transcription of several downstream factors, including members of KIP family (p21/p27/p57) (Bates and Vousden 1999) (described below), GADD45, 14-3-3-Sigma genes leading to cell cycle arrest and repair (el-Deiry 1998). There are several potential mediators of p53 induced apoptosis, mediators like Bax, p53AIP1, Noxa proteins are located in mitochondria and when activated/over-expressed induce apoptosis (el-Deiry 1998). Other potential mediators of p53 induced apoptosis include TNF (tumor necrosis factor) receptor and FAS (Lin, Ma et al. 2000). p53 can also cause cell death by directly stimulating mitochondria to produce an excess of highly toxic reactive oxygen species (Vogelstein, Lane et al. 2000). Although most of effects of p53 are ascribed to its function as transcription factor, several reports have suggested that p53 can also induce apoptosis in transcription independent mechanisms (reviewed by (Chipuk and Green 2003).

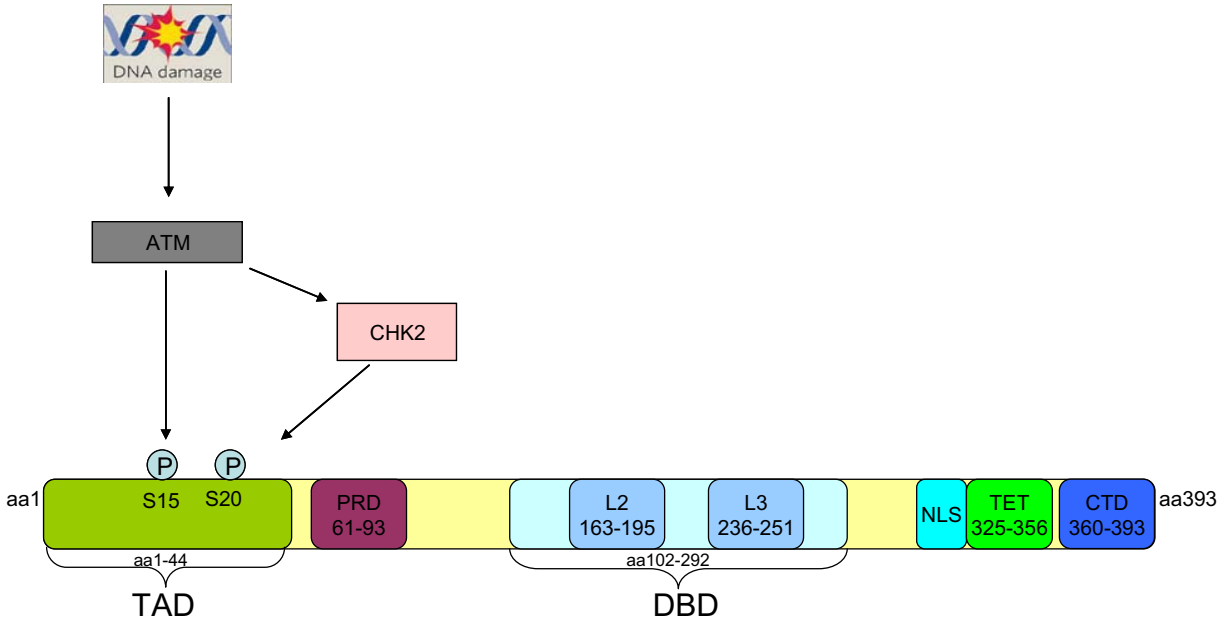

Figure 3: p53 structure is shown schematically with the functional domains. The p53 contains the TAD (trans activation domain) domain, which is responsible for activating target genes, a PRD (Proline rich domain) domain, and the DBD (DNA-binding domain) which consists of a variety of structural motifs including L2/L3.

1.4 Kinase Inhibitor Protein (KIP Family)

Progression through the cell cycle is governed by cyclin-dependent kinases (CDKs). Activation and deactivation of these kinases leads to proper phosphorylation of their downstream targets, pRB (retinoblastoma protein) which inhibits cell cycle progression through interacting transcription factors such as E2F1. When pRB becomes phosphorylated by CDKs, E2F1 is released and stimulates proliferation. CDK inhibitors have been suggested to be indirectly involved in apoptosis through regulation of CDKs. Improper regulation of CDK can send erroneous signals for cell division and cell cycle arrest. Two families of CDK inhibitors, INK4 (inhibitor of CDK4) and KIP (kinase inhibitor family) negatively regulate CDK activities and mediate cell cycle arrest following growth inhibitory signals (Tsihlias, Kapusta et al. 1999).

The INK4 family of proteins, composed of p15 (INK4B), p16 (INK4A), p18 (INK4C) and p19 (INK4D) inhibits specifically cyclin D-associated kinases (Parry, Bates et al. 1995). The KIP family of proteins, composed of p21 (WAF1, CIP1), p27 (KIP1) and p57 (KIP2), binds and inhibits cyclin E/CDK2, cyclin A/CDK2, and cyclin B/CDK1 complexes (Figure 4) (Sherr and Roberts 1999). All three members of the KIP family contain characteristic motifs in their N-terminal domains, enabling them to bind both the cyclin and the CDK subunits of the active cyclin/CDK complexes (Warbrick, Lane et al. 1995; Russo, Jeffrey et al. 1996).

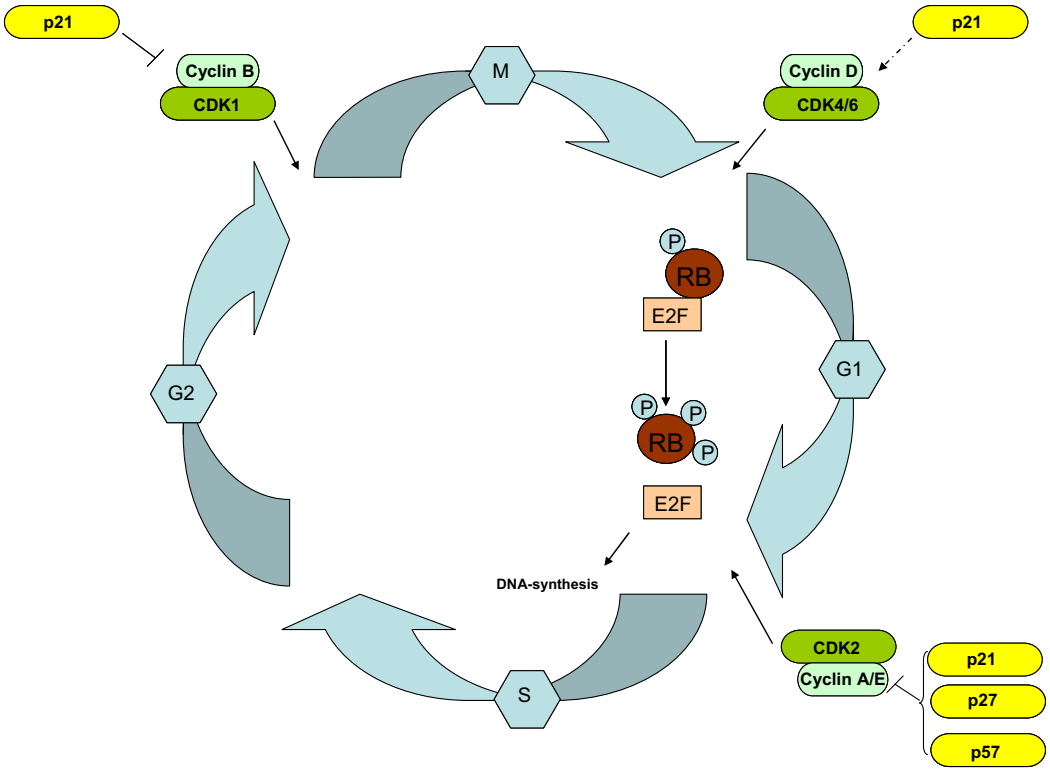

Figure 4: Schematic illustration of KIP family influence on cell cycle at several stages. Arrow indicates activation/induction while butt ends indicate inhibition.

Several factors affect p21 levels and its activity in the cell. p21 is synthesized during the p53 dependent G_1 cell cycle arrest, where its primary function is cell cycle arrest (Waldman, Kinzler et al. 1995). However, p21 has been reported to express both anti- or pro-apoptotic functions (el-Deiry, Harper et al. 1994), depending on the experimental conditions, p21 is capable of binding and inhibit the kinase activity of the complexes of cyclin A or E and CDK2 during G1. Its repression of G1/S transition is considered to be its main role in cell cycle control. This leads to activation of the E2F family of transcription factors by phosphorylation of the pRB, promoting release of E2F1 from the pocket domain (Harbour, Luo et al. 1999). Further downstream effects include RB-mediated apoptosis. p21 can also bind and inhibit cyclin B/CDK1 during G2 cell cycle arrest (Caldon, Daly et al. 2006).

p21B is a recently discovered protein translated from an alternative transcript of the *CDKN1A* gene. Expression of p21B is transcribed by an independent promoter, and the processed transcript consists of a short first exon (Exon IB) spliced to a longer exon (Exon IIB) that comprises half of intron II, exon II, intron III and exon III harboring the p21B coding region (Figure 5). p21 and p21B do not share any amino acid sequence (Nozell and Chen 2002). As p21B was recently described, and the only study which have addressed the functional aspect of p21B indicated that over-expression of p21B leads to apoptosis through the mitochondrial pathway (Figure 1) (Nozell and Chen 2002). The results from the same work also indicated that p21B expression is induced by p53 through p53 binding. However, so far, these data are not confirmed by other groups.

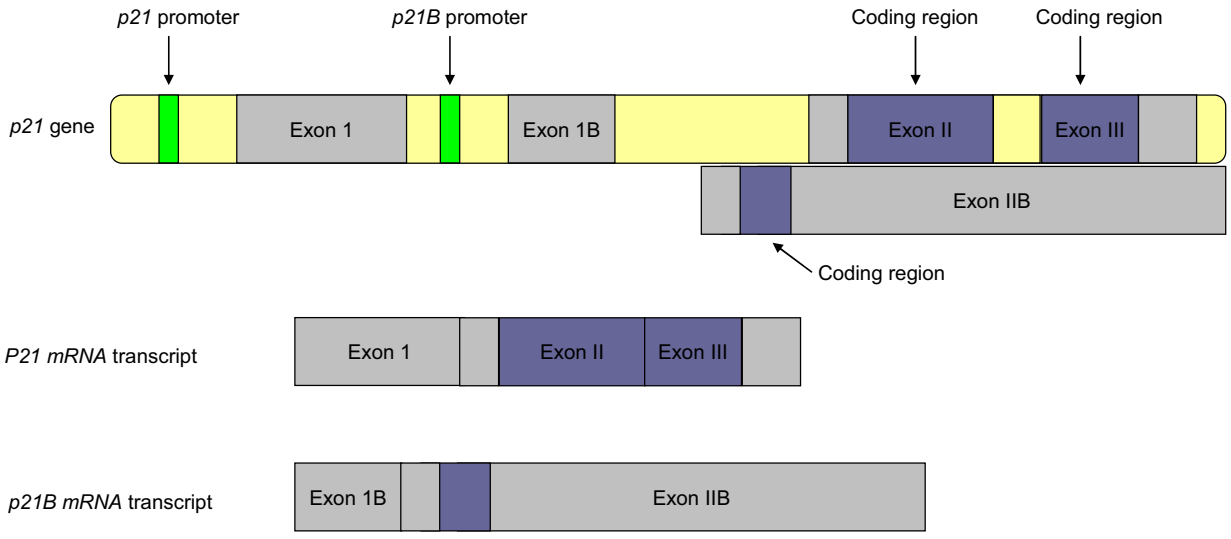

Figure 5: Schematic representation of the *CDKN1A* gene with *p21* and *p21B* transcripts.

p27 is found to execute its function by inhibiting the cyclin E/CDK2 complex (Figure 4) (Tsihlias, Kapusta et al. 1999). The importance of p27 in regulating the cell cycle is further supported by the fact that mice lacking the encoding p27 have rapid growth arrest (Kiyokawa, Kineman et al. 1996). Therefore it appears more and more likely that p27 is involved as a tumor suppressor gene and triggers apoptosis (Katayose, Kim et al. 1997). Moreover p27 inhibits angiogenesis and migration (Goukassian, Diez-Juan et al. 2001) and therefore it may also inhibit distant metastasis.

p57, another member of the KIP family, contains three structural domains: an N-terminal region bearing homology with p21 and p27, a central proline-rich domain, and a C-terminal region (QT) domain shared with p27 (Lee, Reynisdottir et al. 1995). The tumor suppressor p57 functions as a negative regulator of cellular proliferation (Lee, Reynisdottir et al. 1995). It is reported that ablation of the p57 results in increased apoptosis and plays a major role in embryonic development where its loss leads to developmental disorder (Yan, Frisen et al. 1997).

It seems clear that the main function of the KIP family is to negatively regulate the activity of CDKs. However, it seems like that p21 have some additional roles which makes the overall picture more complex. Some data indicate that p21's interaction with the cyclin D/CDK4 complex (Figure 4) was not of an inhibitory kind, but rather an interaction required for proper assembly of the functional active kinase complex, which will promote the cell progression through G1 (LaBaer, Garrett et al. 1997; Cheng, Olivier et al. 1999). This raises the question of why p21 inhibits cyclin E/CDK2 but stabilizes cyclin D/CDK4 when downstream target for both these kinases is pRB.

1.5 Prognostic and predictive value of gene mutations in breast cancer

Several studies have investigated the prognostic (survival or disease free survival) and predictive (factors associated with sensitivity or resistance to a specific treatment) value of different genetic alterations in patients with tumor response to treatment in various cancers. However, different clinical and methodological settings have been used and the results have often been heterogeneous and conflicting (Lonning 2007; Lonning, Knappskog et al. 2007).

1.5.1 Prognostic and predictive value of *TP53* mutations in breast cancer

A large number of studies have evaluated the prognostic and predictive role of the *TP53* alterations in cancer yielding conflicting results. Mainly two different methodologies have been used to assess *TP53* alterations: DNA sequencing and IHC. As the majority of *TP53* point mutations leads to the synthesis of a stable, non-functional and non-degradable protein that accumulates in tumor cells, increased protein expression has been used as a surrogate parameter for *TP53* mutations. The correlation between p53 protein accumulation measured by IHC and *TP53* mutations detected by sequencing is, however, less than 75% in breast cancer (Sjogren, Inganas et al. 1996; Geisler, Lonning et al. 2001). One of the reasons for this is that not all mutations yield a stable protein and some mutations result in protein truncation and are thus not detected by IHC. Non-mutated p53 protein may also accumulate in some cells as a result of response to DNA damage, giving a positive IHC result. Studies that have used sequencing to detect mutations all showed a strong association to prediction whereas most studies using IHC failed to detect such an strong association (Soussi and Beroud 2001).

It has been reported that patients with mutations effecting or disrupting the zink binding domain L2 (codon 163-195) and L3 (236 – 251) have worse prognosis than patients with mutations outside L2/L3 domains (Borresen, Andersen et al. 1995). Mutations affecting amino acids directly involved in DNA binding (Figure 3), many of these residing in the L2/L3 domain, were related with the worst prognosis (Berns, Foekens et al. 2000) and displayed aggressive phenotype with a short survival (Alsner, Yilmaz et al. 2000). Mutations causing loss of the native protein structure have also been associated with poor prognosis (Powell, Soong et al. 2000).

There are strong biological reasons for investigating whether mutations in the *TP53* gene are predictors of response to chemotherapy, since *TP53* is involved in control of the cell cycle, in repair after DNA damage, and in apoptosis. Several studies have reported that for locally advanced breast cancers treated with different anthracyclins in a neo-adjuvant setting, there was strong evidence that *TP53* mutations in general and especially mutations disrupting the zink binding domains (L2/L3) correlated with primary resistance (Aas, Borresen et al. 1996; Kandioler-Eckersberger, Ludwig et al. 2000; Geisler, Lonning et al. 2001; Geisler, Borresen-Dale et al. 2003). Berns and collaborators (Berns, Foekens et al. 2000) studied whether *TP53* mutations can predict response to tamoxifen chemotherapy in patients with advanced disease. A total of 243 patients were included in the study. Patients with *TP53* mutations in codons that directly affected DNA binding or mutations within the zink binding domain L3 showed the lowest response to tamoxifen. Another study found that adjuvant therapy, with tamoxifen along with radiotherapy was of less value for patients with *TP53* mutation (Bergh, Norberg et al. 1995). In another small study of advanced breast cancer patients treated with anthracyclines (35 patients) or paclitaxel (32 patients) in a neo-adjuvant setting, a strong correlation between lack of response and presence of a *TP53* mutation was observed in the patients treated with anthracyclines but not in the paclitaxel treated group (Kandioler-Eckersberger, Ludwig et al. 2000).

At least 14 different single nucleotide polymorphisms (SNP) have been reported in the *TP53* gene, with Arg72Pro as the most common variant. Ethnic differences are observed for this SNP, the Arg72Arg variant being more prevalent in Caucasians, whereas the Arg72Pro variant is more prevalent in Chinese and African-Americans (Beckman, Birgander et al. 1994). Few studies have analyzed the impact of the Arg72Pro mutation on cancer risk, the results are conflicting and no clear consensus has been reached. For the impact on cancer outcome, one study on breast cancer patients showed that those carrying a Pro/Pro variant may be less sensitive to anthracyclines-based treatment than those with Pro/Arg or Arg/Arg and suggests that analysis of p53 codon 72 polymorphisms may provide a simple predictive marker for selecting the right breast cancer patients to anthracycline-based neo-adjuvant chemotherapy (Xu, Yao et al. 2005). This result, however, needs confirmation.

All the data reported point to a significant clinical implication of *TP53*. However, there are no clear consensuses on the specific type of mutations carrying a worse prognosis or prediction, as different classifications of mutations have been used and the comparison of individual mutations was limited by a lack of statistical power. In addition, data clearly show

that the type of the mutation and its biological function should be considered in the analysis of the prognostic and predictive factor.

1.5.2 Prognostic and predictive value of *CHEK2* mutations in breast cancer

A recurrent mutation in the *CHEK2* gene, 1100delC, was first to be associated with family cancer (Meijers-Heijboer, van den Ouweland et al. 2002). Since then, numerous studies have documented the prevalence of this single founder mutation in various populations. In terms of pathology, 1100delC tumors did not seem to be appreciably different from the mutation-negative tumors in 1479 women from the Netherlands treated for invasive breast cancer before the age of 50 years, the mutation 1100delC was identified in 3.7% of the patients (Schmidt, Tollenaar et al. 2007). However the women in the *CHEK2* subgroup harbored a two-fold increased risk of developing breast cancer in that study. A prospective study of 9231 individuals from the Danish general population, observed for 34 years, suggested that 1100delC heterozygosity is associated with a three-fold risk of breast, prostate and colorectal cancer (Weischer, Bojesen et al. 2007). Other reports have also suggested that breast cancer patients with a 1100delC mutation have an increased risk of second breast cancer and have a worse long-term relapse-free survival rate (Meyer, Dork et al. 2007; Schmidt, Tollenaar et al. 2007). Several studies have reported germline *CHEK2* mutations Arg117Gly or Ile157Thr not to be associated with hereditary breast cancer (Allinen, Huusko et al. 2001; Schutte, Seal et al. 2003). However, *CHEK2* mutations are found at low incidence in human cancer in general, and the combination of lack of functional studies evaluating different mutations makes evaluation of *CHEK2* mutations difficult.

1.5.3. Prognostic and predictive value of *KIP* family mutations in breast cancer

Because of its central role in cell cycle control, p21 has been frequently studied. Several groups have addressed the potential role of *p21* mutations and polymorphisms as risk factors for cancer development (Sjalander, Birgander et al. 1996; Facher, Becich et al. 1997; Chen, Wu et al. 2002). However, until now the only study exploring *p21* mutation or polymorphism status in relation to response to chemotherapy treatment in breast or other type of cancers *in vivo* was conducted by us (Chrisanthar, Knappskog et al. 2007). In our study, totaling 521 breast cancer patients, no *p21* mutations were observed. We identified three polymorphisms in addition to the earlier reported $p21^{G251A}$ polymorphism, which was found to be associated with locally advanced breast cancer (Staalesen, Knappskog et al. 2006). However, none of these polymorphisms predicted response to chemotherapy treatment. Galmarini et al. (Galmarini, Bouchet et al. 2006) found a T insertion in codon 104, resulting in truncation of the p21 protein, to be associated with a poor response to paclitaxel in noncancerous breast epithelial cells *in vitro*. However, this was linked to a single mutation only (the same mutation appearing in several drug resistant populations of a mammary cell line).

As only limited experimental information is available about the p21B, it is not clear whether or not this protein plays a role as a prognostic or predictive factor. Mutation screening in breast tumor samples has so far not revealed any high frequency of mutation in the coding region or promoter hypermethylation, indicating that p21B does not play an important role in breast cancer development (Staalesen, Leirvaag et al. 2004). Both studies revealed none of the polymorphisms in *p21B* so far identified to be associated with resistance to chemotherapy (Paper III).

Reduced p27 expression has been shown to predict a poor prognosis in breast cancer (Catzavelos, Bhattacharya et al. 1997; Porter, Malone et al. 1997; Tan, Cady et al. 1997). However, until now there are no studies exploring *p27* mutation or polymorphism status in relation to response to chemotherapy treatment or as a predictive factor in human cancers. Recently, we discovered the $p27^{Val109Gly}$ polymorphism not to be a factor predicting epirubicin or paclitaxel resistance in breast cancer (Paper II).

Deletions within the proline-alanine-rich region of the p57 are relatively common in human populations (Kondo, Matsuoka et al. 1996; Matsuoka, Thompson et al. 1996). *p57* germline deletions within the proline-alanine-rich region has been suggested to be associated with increased risk of a variety of cancers, including breast cancer (Tokino, Urano et al.

1996). In the same study the investigators found that a silent single-base pair substitution polymorphism in codon 185 (GCT→GCC) was not associated with increased cancer risk.

1.5.4 Prognostic and predictive value of functional pathways

Finding that alterations in individual genes are associated with but not fully predictive to response to chemotherapy treatment has lead to increased focus on the probability of testing not only alterations in individual genes but rather, multiple genes in concert using methods such as microarrays.

Microarrays have been used to explore the biology pattern of most tumor types. Several studies (Perou, Sorlie et al. 2000; Sorlie, Perou et al. 2001) showed that breast cancer may be classified on the basis of hierarchal analysis of gene expression patterns identified by microarray. The problem of using such patterns to select patients for therapy lies in the fact that we here, as for individual genes, lack useful clinical information associated with predictive value, even though several studies have reported gene expression patterns associated with sensitivity to different chemotherapy drugs (Chang, Wooten et al. 2003; Ayers, Symmans et al. 2004; Ma, Wang et al. 2004; Rouzier, Perou et al. 2005; Sorlie, Perou et al. 2006). However, none of these microarray patterns have come to clinical use for a couple of reasons. These patterns have been developed in studies enrolling a limited number of patients only or they consistently reported sensitivity as well as specificity predicting therapy outcome of ~70-80%, which is below what should be required if a parameter is to be used to select patients for different therapies. In addition, microarray classification cannot be used prospectively to classify new tumors, as the dendograms of hierarchical clustering analysis are re-organized when a new sample is added (Chang, Hilsenbeck et al. 2005).

Each individual factor found to be associated with chemotherapy so far has been involved in specific biological processes, either processes involved in targets of the drug or associated with processes such as cell cycle arrest/DNA repair or apoptosis. Most of these gene products carry out their function in complex with other factors. They are activated by certain upstream factors and then activate downstream factors in functional pathways. Therefore, it is likely to postulate that inactivation of specific factors up- or downstream in the same pathways in a functional cascade, may lead to similar effects.

A key problem applying to such a model is that for instance in the p53 apoptotic pathway, we lack exact knowledge about which specific factors are involved downstream of p53. Data from *in vitro* experiments, show that p53 activates several hundred genes (Kannan,

Amariglio et al. 2001; Yeoh, Ross et al. 2002). However, this represents a huge reduction in the number of candidate genes to explore compared with the use of general microarray. Thus, gene arrays including genes involved in certain biological pathways may be a way to advance. However, as each tumor will express different gene patterns (Lonning, Knappskog et al. 2007) in respect to the genes involved in different pathways, we believe that microarrays gene expression data must be used in combination with other methods for gene alterations.

1.6 Family Cancer Syndromes

A family cancer syndrome is a phenotypical condition characterized by an abnormal high incidence and (in general) early diagnosis of one or several specific forms of cancer. The underlying genetic factors characterized so far, the hallmark of families with inherited cancer syndromes are germline mutations of central cell cycle, DNA repair and apoptosis regulating genes. Approximately 5-10% of all breast cancers are assumed to be inherited. The majority of these cancers are inherited in an autosomal dominant manner. There are many cancer predisposing genes and each one of these genes, has a specific variant of cancer associated with it (Nagy, Sweet et al. 2004). Normally, the term hereditary or familial cancer syndrome is used only for families with the following characteristics: More than two relatives with the same type of cancer on the same side of the family, diagnosis at early ages than what is typically seen for that cancer type and individuals with multiple primary cancers. However, these criteria can be different for different types of cancer. Because of differences in phenotypic variability, gender specific cancer risks and age related penetrance, many families with cancer predisposing genetic alterations may not fulfill these criteria. Also, with respect to the fact that cancer is relatively common in the western population, for some families with a high incidence of cancer cases which look like an inherited cancer syndrome, cancers may still occur by chance. In the following, the most important cancer predisposing genes are briefly described.

The *RB* gene, located at chromosome 13, was the first gene identified in which a germline mutation was found to be associated with a particular cancer. A novel germline mutation in *RB* leads to development of familial and sporadic retinoblastoma tumors (Friend, Bernards et al. 1986). As mentioned above, the pRB acts as an inhibitor of the cell cycle progression stimulator E2F1. E2F1 is a key transcription factor in late G1 of cell cycle, regulating expression of genes needed to enter S-phase. Mutations in *RB* cause a high risk for early onset retinoblastoma.

As mentioned above, *BRCA1* and *BRCA2* gene mutations are associated with an increased risk for breast and ovarian cancer. These genes have been shown to play a role in a multitude of cellular processes, including but not limited to DNA transcription, cell cycle regulation, DNA damage repair, and apoptosis (Jasin 2002; Somasundaram 2003).

The *CDKN2A* gene encodes two cell cycle regulatory proteins, p16(INK4a) and p14(ARF). p16, which is a tumor suppressor, executes its function, as a specific inhibitor of the catalytic activity of cyclinD/CDK4 complexes (Serrano, Hannon et al. 1993). While

CDKN2A are the gene for which germline mutations frequently have been linked to hereditary cases of malignant melanoma, there are continued discussions about how the inactivation of p14 leads to malignant melanoma. Mutations in the locus, CDK4, are much rarer and have been linked to the melanoma in only three families worldwide. (Molven, Grimstvedt et al. 2005; Helsing, Nymoen et al. 2008). Even though p16 and pRB are functionally linked, germline genetic alterations of these genes encoding these two proteins predispose to different malignancies, malignant melanoma and retinoblastoma, respectively.

Common for the above mentioned genes are that when inactivated, a major increase in the risk of one major, or limited number of cancer forms, is observed. However, there are genes that when mutated, cause an increased risk for multiple cancer forms. Mutations in genes which cause syndromes like Li-Fraumeni (described below) severely increase the risk of breast cancer in addition to other forms of cancer.

1.6.1 Li-Fraumeni Syndrome (LFS)

Li-Fraumeni syndrome (LFS) is a rare autosomal dominant syndrome and was first described by Fredric Li and Joseph Fraumeni in 1969. LFS is characterized by a wide variety of cancer types seen in affected individuals. Li-fraumeni syndrome is diagnosed if the following three criteria are met: 1. The patient has been diagnosed with any bone or tissue sarcoma when younger than 45 years of age; 2. A first degree relative with any cancer under 45 years of age; 3. Another first or second degree relative in the same linkage has been diagnosed with any cancer at an age below 45 or with a sarcoma diagnosed at any age. Typically, breast, brain, adrenocortical cancers, sarcomas and acute leukemia are diagnosed when younger than 45 years, but also other tumors (Li, Fraumeni et al. 1988). Having discovered families with a less severe cancer phenotype presented in the criteria for LFS, a somewhat milder definition of LFS, named the Li-Fraumeni like (LFL) syndrome has been defined with the following criteria: A proband with any childhood tumor or sarcoma, brain tumor or adrenocortical tumor under 45 years plus a first or second-degree relative with a typical LFS cancer at any age and another first or second-degree relative with any cancer under the age of 60 (Birch, Hartley et al. 1994).

The underlying genetic defect in many Li-fraumeni families is believed to be a germline mutation in the *TP53* gene as first described by Malkin et al. (Malkin, Li et al. 1990). *TP53*, a tumor suppressor gene, has proven to be one of the most important genes

involved in the development of cancer. In addition, to the gene's role in non-inherited mutations in human tumors, it is also found as germline mutation. Germline mutations in the *TP53* gene have been observed in approximately 63% of the (LFS) and 27% of LFS like families, respectively (Eng, Schneider et al. 1997) and with mutations affecting the DNA-binding domain of the *TP53* gene have generally more cancers and at younger ages than families with mutation not affecting DNA-binding domain or with no germline *TP53* mutation (Birch, Blair et al. 1998).

CHEK2 is a tumor suppressor gene that, when mutated, produces effects similar to those of p53 mutations and are involved in the development of several human cancers. *CHEK2* germline mutations have been found to confer an increased risk of some cancer and are identified in Li-Fraumeni syndrome families lacking mutations in *TP53* (Bell, Varley et al. 1999). Bell and coworkers found three distinct *CHEK2* alterations (1100delC, Ile157Thr and 1422delT) in three of the families in total out of four LFS and 18 LFL families. However, there is increased discussion and evidence about the fact that germline mutation in *CHEK2* does not cause Li-Fraumeni Syndrome. Nayanta et al. (Sodha, Bullock et al. 2002) showed that one of the mutations 1422delT, is a variant in a homologous fragment of the gene present in Chromosome 15. The second mutation Ile157Thr, has been reported to be a polymorphism in the Finnish population (Allinen, Huusko et al. 2001) while the mutation 1100delC, leads to truncated protein and is therefore likely to be pathogenic. This raise the intriguing question about *CHEK2* mutations related to LFS.

On the basis of that 1100delC is a predominant founder mutation in *CHEK2* which is carried by 0.7% of the Northern European population. In contrast, the LFS is believed to affect only one in 20 000 births or approximately 100 times less (Lalloo, Varley et al. 2006). Further, a detailed analysis of 67 *CHEK2* families (Thompson, Seal et al. 2006) failed to identify an increased risk of cancer for any site other than breast cancer, in a resent paper Evans and colleagues (Evans, Birch et al. 2008) have concluded that *CHEK2* mutations are not associated with the cancer types seen in the LFS or LFL.

2 PREVIOUS WORK AND AIMS OF THE PRESENT STUDY

In breast cancer, only a minority of the patients fully benefit from the different chemotherapy regimens in use, considering that many patients will fail to respond and that chemotherapy has toxic side effects. Identification of markers that could predict response to certain chemotherapy schedules would be of great value. Despite this, the number of studies designed specifically to explore the mechanism of drug resistance *in vivo* is low compared to the high number of studies done *in vitro* and clinical trials assessing responses in general.

Even though microarray data provide large amounts of information revealing gene expression in single analysis, it is clear that such arrays may not identify every potential mechanism of gene inactivation. Alternative splicing, as well as single base mutation or even a deletion or a splice defect located downstream of the probing area would be missed. For single base substitutions within the area covered by the probe, the result is less predictable, as the transcript may not bind the probe at all, or in other cases it may still bind partly or completed and thus the substitutions may be overlooked. A resent paper (Michiels, Koscielny et al. 2005) have intriguing questions about the validity of several extensively published expressing profiling studies. Using a simple but insightful approach, these authors showed that in five of these seven studies on array expression the signatures perform no better than what could be expected for random variation or in more popular terms “tossing the coin”.

Because not all mutations yield a stable protein and some mutations result in truncated protein and are thus not detected by IHC as well as the fact that wild type proteins may accumulate in some cells, the research activity of our team has focused on exploring mechanisms of chemoresistance in breast cancer by conventional sequencing of the coding region of candidate genes of functional pathways and especially exploring mutational and epigenetic alterations of genes involved in apoptosis and cell cycle arrest. Mutations in many of these genes are associated with hereditary disposition for cancer development, and findings by our group and others have shown that mutations in *TP53* are associated with chemoresistance in breast (Kandioler-Eckersberger, Ludwig et al. 2000; Geisler, Lonning et al. 2001; Geisler, Borresen-Dale et al. 2003) but also hematological malignancies (Diccianni, Yu et al. 1994; Wattel, Preudhomme et al. 1994; Preudhomme, Dervite et al. 1995). Also, the pathways in which the products of these genes execute their function are important for the response to chemotherapy (Lonning 2004).

Previously, our group reported mutations in the zink-binding domains L2 (codons 163-195) and L3 (codons 236-251) of p53 critical to DNA binding (Cho, Gorina et al. 1994) to be associated with, but not fully predictive for resistance to anthracycline (Geisler, Lonning et al. 2001) or a mitomycin plus 5-fluorouracil (Geisler, Borresen-Dale et al. 2003) regimen. In that study, the finding that some tumors harboring wild type *TP53* and be resistant to anthracycline therapy made us postulate that other genes involved in the p53 pathway could be mutated in these tumors. In addition, the finding that some patients are sensitive to therapy despite their *TP53* mutation made us postulate that redundant mechanisms may be involved in initiating apoptosis in response to anthracycline therapy.

The primary goal of the present study was to investigate and possibly identify the cause of therapy failure in patients with non-responding tumors. The patients analyzed in this work are from two separate treatment regimes, where patients were treated with either epirubicin (n = 109) or paclitaxel (n = 114) monotherapy. The reason for randomizing patients according to protocol was not to compare response between the two therapeutic options but to achieve balance between the two arms with respect to patient selection in as much as the protocol endpoint was to explore potential mechanisms of drug resistance to the regimens. Biopsies of the patients were collected neo-adjuvant (before treatment) to explore molecular parameters predicting response to epirubicin and paclitaxel monotherapy by analyzing for mutations in *TP53, CHEK2* and other factors up/downstream of p53 along the entire coding region of these genes and not only in the "hotspot" areas. In addition, analyzing for mutations in genes involved in the cell cycle arrest as the alternative factors involved in resistance to chemotherapy.

In case *CHEK2* mutations are found to be germline, explore cancer distribution among relatives harboring the mutation in view of the continued debate about the cancer spectrum (LFS/LFL) associated with mutation in this gene in an unselected breast cancer cohort (unselected for family history).

Furthermore, to evaluate the biochemical functions of the different mutant variants corresponding to previously mutations in the *CHEK2* gene associated with resistance to chemotherapy.

3 SUMMARY OF PAPERS

The results presented in this thesis have provided new insight with respect to functional pathways associated with chemoresistance in breast cancer *in vivo* and insight in family cancer incidence in relation to *CHEK2* germline mutations.

Paper I

Previously, our group reported mutations affecting the L2/L3 domains of *TP53* to predict resistance to a doxorubicin low dose (14 mg/m^2) weekly regimen and to mitomycin combined with 5-fluorouracil in breast cancer. However, while mutations affecting the L2/L3 domains of *TP53* were associated with drug resistance, some tumors were found to be therapy resistant despite harboring wild type *TP53*.

In this study we explored alterations in the *TP53* gene with respect to resistance to a regular dose epirubicin regimen in patients with primary, locally advanced breast cancer. Further to identify critical mechanisms activating p53 in response to DNA damage in breast cancer. To do so, we analyzed for inactivating mutations in the p53 activation genes *CHEK2* and *p14(ARF)* as potential causes of drug resistance in tumor harboring wild type *TP53*. When mutations are detected evaluate the *in vitro* function of potential Chk2 and p14 protein mutant variants corresponding to identified mutations in the *CHEK2* and *p14(ARF)* genes. In addition, when *CHEK2* or *p14(ARF)* mutations were found to be germline, explore the incidence of different cancer distribution among relatives.

Snap-frozen biopsies were collected from 109 patients with locally advanced breast cancer prior to treatment with epirubicin (90 mg/m^2) administered at 3 weekly intervals as preoperative therapy. Each sample was investigated for *TP53, CHEK2* and *p14(ARF)* mutations with complete sequencing of the coding region. In addition, *p14(ARF)* was analyzed for promoter methylation.

Mutations in the *TP5*3 gene were associated with lack of response to epirubicin for all mutations and especially for those affecting loop domains L2/L3 of the p53 protein. No statistically significant correlation between *TP53* LOH and response to therapy was found. *CHEK2* mutations were for the first time associated with lack of response (p = 0.0026). Notably, *CHEK2* mutations associated with therapy resistance were found to abrogate Chk2 protein dimerization and kinase activity *in vitro*. The occurrence of mutations affecting *CHEK2* and/or *TP53* strongly predicted therapy resistance, in particular when *TP53* mutations

were restricted to those affecting the L2/L3 domains of p53. The phenotypes of the two families harboring the *CHEK2* mutation Arg95Ter expressed increased incidence of different cancers, but in a manner not typical for the Li-Fraumeni or the Li-Fraumeni-like syndromes. No mutation or promoter methylation in *p14(ARF)* were detected.

This study, (one of the largest studies performed to date) revealed a significant association between lack of response to epirubicin *in vivo* and mutations in the *TP53* gene, particularly those affecting the L2/L3 domains of the p53 protein for the patients treated with epirubicin at regular doses. Most importantly, our finding that mutations in the *CHEK2* gene abrogating dimerization and kinase activity were associated with drug resistance, confirms activation of p53 through phosphorylation by Chk2 to be a key event executing breast cancer response to anthracycline treatment *in vivo*.

To our knowledge, this is the first study that directly relates response to epirubicin chemotherapy to *CHEK2* alterations in human cancers. Moreover, it is the first study documenting mutations in two genes acting direct up/down-stream to each other to cause therapy failure, documenting not only the importance of a single gene but the role of a functional cascade. The tumor suppressor gene *CHEK2*, when mutated, produces effects similar to those of *TP53* mutations. The evidence suggests that it is part of the same functional pathway for controlling cell growth and replication.

Paper II

The finding of patients treated with epirubicin contains wild type *TP53* and *CHEK2* and not responding to treatment (Paper I) and mutated tumors responding to treatment suggested that additional pathway may be involved in initiating apoptosis in response to chemotherapy in breast cancer.

Thus, to this end we have searched for alterations in other genes, involved in growth arrest/cell cycle control to explain this observation.

p27 expression detected by immunostaining has previously been associated with prognosis in breast cancer. In contrast to data on prognostication, we are not aware of any studies evaluating a potential role of p27 with respect to drug resistance. Here, we report on the p27 status with respect to mutations effecting the coding domains and distribution of a well defined polymorphism with respect to drug resistance. Also, we analyzed for a loss of heterozygosity in relevant samples. A total of 223 patients were equally divided (according to randomization) between paclitaxel and epirubicin monotherapy for primary/locally advanced breast cancers.

Our findings are negative in the way that we discovered no mutations affecting the p27 gene and we did not find any statistically significant association between presence of the polymorphism Val109Gly and drug sensitivity.

Paper III

In Paper III, we reported the identification of a novel *p21B*^{G128T} polymorphism in our tumor samples, in addition to the previously reported *p21B*$^{T35C.}$ Both polymorphism variants were found to be equally distributed not only among breast cancer and non-breast cancer individuals but also between different subgroups of breast cancer. Finding that mutations in *p21B* coding sequence are rare, as we detected only one mutation among 521 tumor samples analyzed, indicates that the protein product of this transcript does not play a key role in carcinogenesis of breast cancer or in relation to chemotherapy. However, based on its proposed function in apoptosis, it may well be that inactivation of other genes involved in the same pathway may be a mechanism causing resistance to chemotherapy in human breast cancer. Our studies on breast cancer do not exclude that p21B may be of importance for malignancies other than breast cancer.

4 DISCUSSION

During the last few years a number of possible mechanisms of resistance against chemotherapy have been proposed. While these have been evaluated under *in vitro* conditions, data on their clinical relevance are lacking. Even though there are some *in vivo* studies (Hamilton and Piccart 2000; Bertheau, Plassa et al. 2002) focusing on resistance to chemotherapy, these are not directly comparable, making a direct evaluation of possible mechanisms of resistance to individual drugs difficult. In addition, previously research *in vivo* has often been investigated single genes that can be used as predictive markers, while its important to identify these individual genes, a broader understanding of the biological process has been missing. This study has taken a step further to gaining insight into biologically pathways involved in resistance to chemotherapy in breast cancer.

Our first paper (Paper I) is to our knowledge one of the largest studies thus far directly relating response to chemotherapy to *TP53* mutations in breast cancer patients. The study verifies our previous findings (Geisler, Lonning et al. 2001; Geisler, Borresen-Dale et al. 2003) regarding the predictive value of *TP53* mutations as a whole and mutations affecting or disrupting the L2 and/or L3 domains of the p53 protein with respect to primary resistance to anthracycline (epirubicin) therapy "normal dose" (90 mg/m^2) administered at 3 weekly intervals in breast cancer, contrasting to primary treatment consisted of weekly doxorubicin "low dose" (14 mg/m^2) (Geisler, Lonning et al. 2001) and receiving combination chemotherapy with 5-fluorouracil (1000 mg/m^2 on days 1 and 2) and mitomycin (6 mg/m^2 on day 2), administered every 3 weeks (Geisler, Borresen-Dale et al. 2003).

As experimental evidence suggests a key role for p53 is apoptosis in response to genotoxic agents (Lowe, Ruley et al. 1993; Lowe, Bodis et al. 1994). We defined the p53 apoptotic pathway as follows: Epirubicin will lead to DNA damage in the cell, DNA damage activates ATM, ATM activates Chk2, Chk2 activates p53 by phosphorylation and activation of p53 leads to transcription of downstream genes which will lead to that cell can induce apoptosis. A key part of this model is that there are no alternative mechanisms. Thus all proteins are targets for mutations that lead to therapy failure. No single protein may substitute for a single inactivated protein in the p53 apoptotic pathway. There is however the finding that some patients treated with epirubicin contain *TP53* mutations affecting the L2/L3 domains and respond to treatment suggests that redundant pathway may be involved to compensate for the entire p53 apoptotic pathway (Lonning 2003; Lonning 2004). Tumors

being sensitive to therapy despite their *TP53* mutation in the L2/L3 domains may have the redundant pathway intact, while tumors being *TP53* mutated and resistant have this redundant pathway inactivated. Tumors that are resistant to therapy despite expressing wild type *TP53* may have a gene upstream or downstream of *TP53* inactivated, in addition to inactivation of the redundant pathway. Postulating that these tumors may harbor genetic disturbances in genes playing a key role in the p53 pathway, we here sequenced *TP53* along with *CHEK2* and *p14(ARF)*, the latter two known to play a critical role as p53 activators.

Our findings have two major implications. First, we confirm that mutations in genes encoding proteins located within the same functional pathway may substitute for each other with respect to drug sensitivity, revealing for the first time a functional pathway critical to chemotherapy response *in vivo*. Second, this study is also the first reporting an association between *CHEK2* mutations and therapy resistance in human cancers. Further, the identification of mutations in the *CHEK2* but not in the *p14(ARF)* gene in resistant tumors suggests that Chk2 mediated phosphorylation of p53 is a critical event in executing anti-tumor effect as a response to DNA damaging agents in breast cancer. This adds to our understanding not only the function of p53 but Chk2 as well. The tumor suppressor gene *CHEK2*, when mutated, produces effects similar to those of *TP53* mutations. The evidence suggests that it is part of the same pathway for controlling cell growth and replication. Further, it shows that p53 status alone cannot determine if the p53 pathway is intact and verify our hypothesis that tumors can have alterations in other components of the p53 apoptotic pathway even though p53 itself is normal.

Even though mutations often are associated with loss of function, mutations in the *TP53* gene have different biological effects (Di Como and Prives 1998; Flaman, Robert et al. 1998; Monti, Campomenosi et al. 2002). Some mutated p53 proteins still bind to DNA with variable efficiency (Rolley, Butcher et al. 1995), while other mutant proteins may perform a dominant-negative effects on normal p53 or other upstream or downstream regulators (Chene 1998). Even though these biological effects have been observed *in vitro*, their importance of the mechanisms to chemoresistance *in vivo* is incompletely understood. Our *in vivo* data indicate that mutations in functional domains may be of particular importance predicting resistance to epirubicin therapy. Grouping the mutants based on functional status will further improve the predictive value of certain *TP53* and *CHEK2* mutations.

The results of Varley et al. (Varley, McGown et al. 1995) and our study demonstrate the importance of looking for mutations along the whole gene as our studies have shown that

point mutations in other domains than hot-spot locations of p53 or Chk2 can be functionally disruptive.

Taken together, our *in vivo*, *in vitro* and family history data strongly suggest that the novel *CHEK2* mutations identified in the present study (Arg95Ter) will affect Chk2 function *in vivo*, contribute to cancer development and chemoresistance to anthracyclines in the tumors carrying this variant. *In vivo*, *in vitro*, and family pedigree of variant Ile364Thr behaved almost like the wild type Chk2, and this alteration alone may not be a disease-causing or resistance causing mutation, although the alteration may affect associations with other proteins.

The role of Chk2 with respect to cancer family syndromes has been controversial. Following a report of a Li-Fraumeni family harboring a germline *CHEK2* mutation (Bell, Varley et al. 1999), current opinion is that the most frequently observed mutation del1100C as well as the more infrequent I157T and the rare R117G point mutation may cause syndromes of moderately increased risk of breast as well as colorectal cancers (Meijers-Heijboer, Wijnen et al. 2003; Kilpivaara, Vahteristo et al. 2004; Kilpivaara, Alhopuro et al. 2006; Sodha, Mantoni et al. 2006). Observing that *CHEK2* germline mutations revealed different types of cancers in an unselected breast cancer cohort (unselected for family history) in their family, may indicate variable phenotypic expression in the families with *CHEK2* mutations or yet other genes may account for the Li-Fraumeni and Li-Fraumeni like families. A resent report by Evans and colleague (Evans, Birch et al. 2008) verify our data and conclude that *CHEK2* mutations are not associated with the cancer types seen in the LFS or LFL and it is no longer reasonable to consider *CHEK2* mutations to be a cause of LFS.

As we suggested that mutations in *CHEK2* might provide the same effect as mutations in *TP53.* However, one critical point questioning this hypothesis is the fact that *CHEK2* mutations do not account for LFS or LFL, like p53 mutations do. One plausible explanation for this is that cancer susceptibility and resistance to therapy are two different processes that might be caused by slightly different patterns of mutations. Even though mutation in *CHEK2* and *TP53* cause resistance to anthracylines, they may have different aspect on cancer development. This view is supported by similar phenomenon in other functional cascades, for example, the RB-pathway. Even though, pRB, cdk4 and p16 are functionally linked and mutations are reported to have the same effect on cell cycle progression, however, germline genetic alterations of these genes predispose to very different malignancies, retinoblastoma and malignant melanoma, respectively. However, our family history data are consistent with

the hypothesis that *CHEK2* is a multi organ cancer susceptibility gene that acts in synergy with other genes or factors to cause cancer.

In Paper II and paper III we investigated for mutations in genes involved in cell cycle arrest as cause and effects for chemoresistance since it is known that p53 has two separate roles in the responsiveness of cells to anticancer agents, apoptosis and cell cycle arrest (Bunz, Hwang et al. 1999). Further, a critical role of the p53 response to DNA damage is the CDK inhibitor p21 (Harper, Adami et al. 1993). Since *p21* and *p21B* are downstream genes transcriptionally regulated by p53, and linked to the key cellular events growth arrest and apoptosis, respectively, alterations affecting the function of these transcripts may potentially influence the function of the p53 pathway and as our previous study showed that disturbances in p21 function are unlikely to play a major role in chemotherapy resistance in breast cancer *in vivo* (Chrisanthar, Knappskog et al. 2007), to this end we have searched for alterations in other members of KIP family. The very low prevalence of p*21B* and *p27* mutations together with the observation that none of the identified polymorphisms correlated to breast cancer therapy indicates that these genes are unlikely to play a major role in chemotherapy resistance.

However, it may well be that these genes are disturbed by other mechanisms, or disturbance of other genes involved in the same pathway may be a mechanism causing chemoresistance in human breast cancer.

That both polymorphisms in *p21B* (T35C and G128T) (Paper III) were equally distributed among breast cancer and non-breast cancer individuals and also between different subgroups of breast cancer, indicates that either p21B does not play an important role in breast cancer development.

These findings should have significant impact on future studies to identify potential factors involved in *TP53*-dependent drug resistance *in vivo* aiming at improved diagnostification and therapy.

5 FUTURE PERSPECTIVES

The data described above provide an insight into the biology of the chemoresistance *in vivo*, but reveal the need for further studies of this type to provide conclusive valuable information for future use in the clinic.

The finding that some patients treated with epirubicin carry *TP53* mutations affecting the L2/L3 domains and *CHEK2* mutations respond to treatment suggests that either the mutants are not functionally inactivated or redundant mechanisms may be involved in initiating apoptosis in response to chemotherapy in breast cancer. Finding that other pathways must be able to compensate for the entire apoptotic p53 pathway, either in response to DNA damage, or through other effects the chemotherapy may have on cells leads to search for genetic and epigenetic alterations in upstream and downstream genes and also other pathways should be investigated. The question is; what may be the alternative pathway in patients harboring *TP53* and/or *CHEK2* mutations that respond to epirubicin treatment? Pathways to be examined on the basis of biological processes either they represent the target for drug or involved in processes such as growth arrest, apoptosis, or DNA repair should be a target.

Grouping the mutants based on functional status will further improve the predictive value of certain *TP53* mutations.

In addition, it would be important to look at genomic deletions and insertions (one or more entire exons) which are frequent causes of cancer by using high resolution methods like MLPA (Multiplex ligation-dependent probe amplification).

While we found that mutations of both *TP53* and *CHEK2* can occur in the same breast tumor, it will be of interest to determine whether such mutations are more likely to occur in the same or alternative subsets of tumors and also what may be the order of such events during the progress of tumor development. Clarifying these issues should not only help to better understand the biology of the p53 apoptotic pathway, but also help develop better treatment for cancer patients. Further work is required to determine the role of the identified mutations in breast cancer as predictive factors. Experimental systems have to be developed to evaluate the biochemical functions of the different mutations associated with and resistance to chemotherapy and poor prognosis.

Paclitaxel is one of the most effective anticancer drugs used in the clinic to treat a variety of solid tumors. Despite its increasing use, especially as chemotherapy, few *in vivo* data are currently available to shed light on the mechanism of drug resistance. Despite this,

analyzing potential factors involved in paclitaxel resistance would without question be of value. Biopsies obtained during neo-adjuvant paclitaxel treatment provide a valuable opportunity for the investigation of factors that may be used as predictors.

Understanding chemoresistance will represent a great improvement in our attempts to cure not only breast cancer but also cancer in general.

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