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# *In vivo* model for research of breast cancer biomarkers

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**ABSTRACT**

*The preoperative (neoadjuvant) setting of breast cancer treatment is an optimal in vivo model by which to allow the characterization of biomarker expression pattern with the tumor remaining in situ throughout treatment as an in vivo measure of response to particular therapy. Elucidating surrogate molecular or cellular markers of tumor response to therapy, may provide biological insight into both, the mechanism of tumor growth dynamics and drug sensitivity/resistance. Owing to the knowledge that many drugs are effective on actively proliferating cells and more intriguingly, that many anticancer agents with differing modes of action achieve cytotoxic effects by inducing apoptosis, has lead to a reappraisal of traditional views of tumor response/resistance to cytotoxic drugs in vivo. Accordingly, this review article will focus on discussing apoptosis phenomena and the p53 and bcl-2 protein as its regulators of principal importance; a cell proliferation determined by the Ki-67 expression, as the major counterbalancing process to apoptosis is also considered. This paper reviews the rationale for the use of these proteins as indices of tumor response to therapy, as well as the published literature regarding their clinical relevance. So far, no firm conclusions can be made concerning their predictive utility.*

**KEY WORDS**: *Breast Neoplasms; Tumor Markers, Biological; Apoptosis; Tumor Suppressor Protein p53; Proto-Oncogene Proteins c-bcl2; Ki-67 Antigen; Cell Proliferation*

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## INTRODUCTION

The scenario of presurgical therapy of breast cancer within neoadjuvant setting has been proposed as an ideal *in vivo* model for studying the tumor biological features that might be reliable markers for the assessment of tumor response to therapy and/or valuable indices for long-term disease outcome. After exposure to a cytotoxic agent *in vitro*, a single cell will survive (with or without a temporary growth arrest) or die. Thus, sensitivity and resistance should be defined as mutually exclusive states. However, the *in vivo* situation differs because cell populations are heterogeneous (1). Therefore, the use of primary (neoadjuvant) chemotherapy offers the opportunity to test clinical relevance of the pattern of modifications in the cell phenotype induced by therapy with the tumor remaining *in situ* throughout treatment as an *in vivo* measure of response. In addition, testing could be done on intact human breast tissue, with vasculature, stroma, and other components, conditions that cannot be replicated in laboratory experiments. This treatment modality allows for accurate tumor measurements and assessment of objective tumor response by use of well-defined response criteria; furthermore, the response of macroscopic tumors could be assessed at the cellular level. In addition, serial determinations of intratumoral characteristics, due to available samples throughout treatment, can be obtained, allowing for comparison of biological features before and after therapy. Accordingly, the neoadjuvant clinical setting has been increasingly evaluated as an optimal study designed for the determination of breast cancer biological features, and the significance of their expression pattern as well as possible variations induced by therapy, to address the reliability of these biomarkers as prognostic/predictive factors. In an advance, elucidating surrogate molecular or cellular markers of tumor response to therapy, may provide, earlier biological insight into both, the mechanism of tumor growth and drug sensitivity/resistance (2).

## RATIONALES FOR THE HYPOTHESIS OF PREDICTIVE VALUES OF APOPTOSIS PHENOMENA, p53, bcl-2 AND Ki-67 IN BREAST CANCER

Tumor growth is the net result of deregulated cell proliferation in relation to cell death, in the form of apoptosis, as the key determinants of cancer growth dynamics and kinetic cell activity (3). These observations were highlighted the new insight into the tumor growth control. Furthermore, the proliferation kinetics and apoptosis pathway are so far considered as the most relevant biological phenomena that are associated with cellular effects induced *in vivo* by chemotherapy. Owing to the knowledge that many drugs are effective on actively proliferating cells (4) and more intriguingly, laboratory evidence which have shown that many anticancer agents with differing modes of action achieve cytotoxic effects, at least partly, by inducing apoptosis (5), has led to a reappraisal of traditional views of tumor response and resistance to cytotoxic drugs. Since the mechanism that disturbed apoptosis may be relevant in development of intrinsic or acquired resistance to cytotoxic drugs (6), of the factors controlling and regulating this complex phenomenon, the bcl-2 and p53 as prototype apoptotic markers, are considered of principal importance, in common with the Ki-67 as indicator of proliferation, which is the main counterbalancing process to apoptosis in tumor growth dynamics.

### *Apoptosis*

Apoptosis is a physiological mechanism of an actively regulated cellular process that leads to destruction of individual cells, and is characterized by distinct morphological features (7). Of the factors controlling and regulating this complex phenomenon, the p53 and bcl-2 as the prototype protein of the bcl-2 gene family, are considered of principal relevance.

Biochemically, the end result of this complex phenomenon is DNA fragmentation by endonucleases and nuclear destruction, which are mediated by a family of proteases, caspases, which participate in the induction and execution of the apoptotic process (8).

Despite the multitude of intracellular targets for the different triggers of apoptosis, many of antineoplastic agents induce apoptosis in susceptible cells through the activation of a few common pathways (9). Two separable pathways, leading to caspase activation, have been characterized. The extrinsic, receptor-mediated killing involves members of TNF superfamily, with the best known participant in apoptosis – Fas(Apo-1/CD95), TNF, and TRAIL (Apo-2) receptors that activate activator caspase -8, and -10 which in turn activate effectors caspases-3 and -7. The second, well characterized intrinsic pathway (mitochondria-mediated killing) requires disruption of the mitochondrial membrane and release of cytochrome-c, (and other proteins) which is involved in the formation of the apoptosome complex (cyt c, Apaf-1, pro-caspase-9) leading to activation of caspase-9 that subsequently activates caspase-3.

### *Bcl-2*

The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins (10), which governs mitochondrial membrane permeabilisation by the opposing actions of pro- and antiapoptotic bcl-2 family members. These proteins may interact physically with each other trough a network of homo- and heterodimers, the relative proportions of which ultimately control the sensitivity or resistance of cells to apoptotic stimuli. Family members are classified on the basis of structural similarity to the bcl-2 homology (BH) domains, with main BH3 domain that is essential for heterodimerisation among members, and constitutes the minimum domain required for the proapoptotic function. The bcl-2 family is devisable into three classes: prosurvival-antiapoptotic, whose members are most structurally similar to bcl-2 ( such as bcl-xl); pro-apoptotic proteins (Bax, Bak), that antagonize prosurvival functions of bcl-2: and the pro-apoptotic " BH3-only" proteins. Proapoptotic bcl-2 proteins (Bax and Bak) can be activated directly following interaction with Bid (BH3-only protein). Alternatively, binding of other BH3-only proteins (Noxa, Puma, Bad, Bim) to antiapoptotic proteins (notably bcl-2 and bcl-xl) results in activation of Bax and Bak., thus promoting apoptosis (11). An important regulatory factor in many cells is the balance between concentrations of Bax and bcl-2 proteins. Since bcl-2 may heterodimerize with Bax , its increased expression leads to formation of their heterodimers, thereby shift a balance which favor abrogation of apoptosis. In line with their antiapoptotic function, bcl-2 inhibit the release of cytochrome c or Apaf-1 from mitochondria, keeping cells alive by preventing caspase activation. It is ascertained that there is considerable cross-talk between the extrinsic and intrinsic pathway.

*In vitro* studies suggest that bcl-2 may contribute to resistance to a series of cytotoxic drugs in breast cancer and that some combination of agents resulted in reduced bcl-2 expression; therefore the degree of bcl-2 expression may somewhat contribute to the level of resistance to chemotherapy.

### *p53*

A sensor of cellular stress, p53 is a critical initiator of the intrinsic apoptotic pathway, though it appears that p53 can function as a master regulator of the apoptotic program, capable of coordinating the process at multiple levels via several mechanisms (12).

The role of p53 protein as a multifunctional transcriptional regulator is to maintain genomic stability, through its participation in the cell cycle regulation (at G1 and/or G2 phase) (13). Cells can undergo a p53-dependent G0/G1 phase cell cycle arrest (mediated at least in part by p21), followed by DNA repair (gadd-45, PCNA). In cells where p53 activation predominantly stimulates this effect (rather than apoptosis) p53 would function as a survival gene, and its loss would be expected to sensitize the cell to genotoxic stress (14). However, being a key player in apoptosis induction, which is a desirable goal of different treatment approaches, p53 activity is thought to be a crucial predictor of their effectiveness (15). This assumption is largely based on some laboratory evidence that observed that alteration in TP53 gene function (p53 is its encoded protein) predict for resistance to chemotherapy, both *in vitro* (16) and animal *in vivo* (17). This finding has lead to the merging investigation of the p53 function in relation to the different treatment -induced effects (18).

Intriguingly, a key subset of the bcl-2 family genes are p53 targets (9,12). P53 can initiate apoptosis by transcriptionally activating proapoptotic bcl-2 gene family (Bax, Bak, Noxa, and PUMA) and repressing antiapoptotic bcl-2 proteins (bcl-2, bcl-xl) and IAPs proteins which inhibit caspase activity. One pathway of apoptosis induction by p53 is through its transcriptional activation of the enigmatic *bax* gene in the context of PUMA that is also directly induced by p53 in response to DNA damage. PUMA expression promotes mitochondrial translocation and dimerization of Bax, thus culminating in apoptosis induction. Bax is absolutely required for PUMA-mediated apoptosis, and participates in death response as an indirect target of p53 through PUMA. Thus, it appears that, in response to DNA damage, p53 activates the intrinsic mitochondrial apoptotic pathway (12,19). P53, also, promotes cytochrome c release through induction of target genes encoding BH-3 only proteins, and Apaf-1 expression, thus mediating the apoptosome complex activation. The extrinsic apoptotic pathway, drug-induced suicide mediated by Fas/CD95 expression and TRIAL-receptor gene DR5/KILLER, are also p53 induced in response to DNA damage, which is mainly cell-type specific. It is also obvious that certain therapies may also induce apoptosis via a p53-independent pathway (9,14,19).

So, p53 modulates treatment responsiveness, but the criteria that influence p53 to stimulate cell cycle arrest or apoptosis are only partially understood. The summation of the type of stress signals, p53 expression levels, and the cell type in particular cellular context, are general factors that influence the net result of altering p53 status. These alternative scenario of protective versus sensitizing roles of p53 status, also raises the important notion that p53 may do so in opposite directions for different drugs or treatments, that are applied on the different cellular context, that is, cell cycle phase, state of differentiation, and others, associated molecular aberrations being most relevant. Presumably, the key to predicting the consequence of p53 loss lies in understanding which p53-dependent action is the dominant output of a specific treatment in a given cell (9,14).

### *Ki-67*

Another major area in a breast cancer research is the process of cell proliferation that takes place through a defined process in which several phases can be recognized rendering the concept of cell cycle (20). Many studies evaluating the role of individual genes and factors regulating these processes, has been attempted, but the functional end result of this process -a cell dividing– has remained the most important factor so far. An optimal assessment of proliferation rate of a tumor includes measurements of the growth fraction, in addition to cell cycle time (21). The different methods to assess proliferation have been available, and it is proposed that the growth fraction of tumor cells could be easily measured by means of immunohistochemistry (22). The expression of Ki-67 protein throughout the cell cycle in proliferating cells and their absence in quiescent cells created interest on its potential role as a marker of cell proliferation (23). The cellular appearance and location of the Ki-67 protein throughout the cell cycle is not homogenous, with overall evidence that the levels of this protein are low during G1- and early S-phase and progressively increased to reach a maximum during mitosis. There is little known about essential function of Ki-67 in active cycling cells. Apoptosis provides the major counterbalancing determinant of tumor growth to proliferation, and index based on the Ki-67 – apoptosis ratio might be crude indices of cell turnover or growth index (24).

## *The contribution of molecular markers to the prediction of response to chemotherapy in neoadjuvant setting: A review of the literature on apoptosis, Ki-67, bcl-2 and p53*

It is beyond a scope of this article to consider an extensive literature about numerous biological markers that have been of interest of basic scientist and clinicians in the elucidation of the cancer cell response to anticancer agents, as well as the published clinical correlative study addressing the use of each one to predict response to particular therapy in breast cancer patients. The determination of relevant predictive factors in breast cancer is an important issue to address, since knowledge of the expression patterns of these factors

is necessary in selecting the best option for further therapy. If the chemotherapy elicit biomarker phenotype changes their reliability might be reduced and their predictive value might be uncertain, and one has to raise the question whether these factors should be determined in the tumor samples prior to chemotherapy or in excised tumor after therapy.

So far there have been a paucity of studies examining the issue of chemotherapy induced apoptotic phenomena in a human solid tumor, notably in patients with breast cancer. Rasbridge et al. (25) first noted morphological changes including apoptosis, which were induced by neoadjuvant therapy and found variable response pattern. Chemotherapy-induced histologic changes in neoplastic tissue ranged from nuclear and cytoplasmic alteration to stromal degeneration and fibrosis, which are not uniformly present throughout the tumor, and correlate with pathologic tumor response (26). It is a more difficult to recognize and quantify apoptosis in solid tissues for several reasons. Apoptosis itself is relatively rapid process and most of apoptotic cells are efficiently removed from a tissue by phagocytic elements, thus escaping detection. Cancer tissue undergo additional changes, such as mutation and/or abnormal expression of critical death regulating genes which may greatly reduced apoptosis, as well as altered interactions with the surrounding extracellular matrix. Other biological properties, such as proliferation rate and the growth fraction may also influence the rate of cell death in tumors. All these caveats may explain why there appears to be so few apoptotic cells in tumor tissue compared to physiologically active tissue or *in vitro* tumor cell systems. As central biochemical event of apoptosis is endonucleolysis, which results in DNA cleavage into oligonucleosomal-sized fragments – DNA fragmentation, this, form the basis of TUNEL assay on histological sections, that has been a reproducible method to assess apoptotic cells in clinical samples (27).

It might be important and useful to compare apoptotic values of cancer specimens at different time-points during chemotherapy, because in this way we could distinguish the endogenous apoptotic activity in patient's tumors from their apoptotic levels induced by the chemotherapy. In a pilot study by Ellis et al (28) it was shown a significant, more then 50 % increase in the apoptotic index (AI) in breast cancer biopsy following 24 hours after commencing treatment. The later published reports corroborated this findings of early induction of apoptosis within 24-48 hours after drug administration (29,30), or even later on 7 days (31). Moreover, in a several studies an obvious increase in apoptosis elicited by chemotherapy was observed in post-therapy specimens at the time of surgery (32,33,34). These corresponding findings related to the chemotherapy-induced increase in apoptotic activity of cancer samples following the various chemotherapy regimens, suggest that apoptosis is not restricted to a specific drugs but rather is valid for many drugs with different mechanism of action; and they might be conclusive evidence of an *in vivo* response and pattern of modifications in cell phenotype induced by different agents (6,9). However, taken all together, these studies also revealed that the apoptotic index may have a different relevance in the estimation of objective response of breast cancer to chemotherapy; the increase in apoptosis levels accompanied by pronounced changes in its values during chemotherapy were significantly associated with greater pathological (30) or clinical (31,34) tumor response, or no correlation was seen (29). Besides, there was no found correlation either between pathological (30) or clinical response (34) to chemotherapy and pretreatment, baseline measurements of apoptosis levels; while post-chemotherapy AI correlated with clinical response and increased relapse and overall survival (32,33).Therefore, measurement of apoptosis warranted to confirm its clinical usefulness as a predictive biomarker in the larger collaboration studies.

In a line with the proposed hallmark of tumor growth dynamics that cell proliferation is the main counterbalancing determinant to apoptosis (24) a changes in tumor cell proliferation that are induced by chemotherapy have been evaluated in substantial number of correlative clinical studies. Proliferative activity in tumor cells has been suggested as a prognostic marker, but conflicting data have been presented on its predictive value. Several groups have found that Ki-67 index decreases after primary chemotherapy compared to its baseline pre-treatment values (34-37) but with various clinical relevance; tumors displaying more than 75% reduction were more likely to achieve a pathological response (36) or high proliferation of tumor cells could be indicator of obtaining complete pathological response (35). Reduced proliferation in post-therapy tumor samples may reflect a direct effect of therapy on the cycling cells, with reduced proliferation in residual tumor cell population surviving therapy (28). Whereas most studies reported that high proliferation, the baseline level in pre-treatment samples, were related to objective tumor response, in some papers the finding of reduced Ki-67 expression in tumors were not related to response to therapy, furthermore, a low proliferative activity might be associated with improved pathological response (35). Another reports failed to observe a significant change in Ki-67 expression in paired samples before and after primary chemotherapy, though high Ki-67 expression was associated with pathological response (38). Some studies have focused on the evaluation of early changes in cell proliferation during treatment, by analyzing Ki-67 index in repeat tumor samples taken at variable time-points during chemotherapy (29,31,39,40). The clinical relevance of the modulation in Ki-67 expression are inconsistent; a significant fall in Ki-67 was seen 24-48 h following chemotherapy, but these changes was not related to clinical response (29,31) and disease outcome (29). Further, decrease in proliferation fraction of more than 25% gave the best predictive value after the first course of chemotherapy (39), and absolute and percentage change in Ki-67 in first 21 days from baseline level, were only significant in responders but not in non-responder patients (40). A more recent study reported that neither pre- nor post-treatment median Ki-67 index nor median AI at different time points differed significantly between clinical or pathological responders or non-responders, and also found that the initial reduction in Ki-67 index was often followed by a rebound increase in cellular proliferation by the time of surgery; besides, there was no consistent pattern of changes in apoptosis throughout treatment (41). Taking into account all these inconclusive results, especially related to the clinical relevance of the expression pattern of both apoptosis and proliferation in tumor cells, and their modulation which might be elicited by chemotherapy, an index based on the Ki-67 – apoptosis ratio could be of more importance. Though it is clear that this cannot accurately reflect the tumor growth dynamics, this may have utility as an surrogate marker of response to treatment (24).

Over-expression of bcl-2, an antiapoptotic protein, has been reported in up to 70% of all cases of breast cancer and was usually related with relatively indolent tumor cells due to association with markers of favorable prognosis in breast cancer patients. As far as the bcl-2 expression concerned in relation to the effect of primary chemotherapy, in some reports it was not observed a change in bcl-2 expression levels in paired tumor samples before and after chemotherapy (28,34,42); it was also noted the inverse relationship to apoptotic index but that was not retained after chemotherapy (34) and, with no relation to objective response. However, absence of bcl-2 expression in pre-chemotherapy specimens was associated with more frequent complete pathological response (42), as documented by others (30) who also observed an early decrease in bcl-2 expression on 14 days after commencing the treatment. Other studies only examined pre-chemotherapy bcl-2 levels and subsequent response to chemotherapy. Key pilot study by Ellis et al (28) showed that bcl-2 levels were lower in patients with clinical complete response, and others have shown that the absence of detectable bcl-2 predicted a better pathological response (43). On the contrary, it was reported that bcl-2 expression did not predict the ultimate clinical and pathological responses in either responding or non-responding tumors, but high rates of apoptosis and proliferation at baseline were associated with improved pathological response (44). So far, no firm conclusions can be made based on the articles studied.

A suggestion that the loss of p53 function is related to lower chemosensitivity has been extensively investigated. p53 mutation rates in breast cancer have been estimated between 20 %- 40%, with some studies reaching up to 60%, depending on the methods used (14). Controversial data have been published as regard as the role of p53 in conferring increased resistance to chemotherapeutic agents. Some data from the literature revealed that the expression of p53 increases after chemotherapy (38,45) although it was not predicted to pathological response to treatment A change in p53 protein expression in terms of reduction

in its expression after chemotherapy was observed in patients who has a clinically objective response but no changes in p53 expression was in non-responders (34). Some other studies reported the opposite results; p53-negative expression in tumors correlated with a significant advantage towards better clinical and complete pathological response (46), and changes in p53 expression after treatment were mainly confined to p53-positive tumors at baseline, and it was a significant predictor for poor clinical response (47). In another study it was shown that p53 positivity did not correlate to complete clinical response, but it was associated with higher risk of progression of disease and death (48). In addition, there were not observed significant changes in p53 expression pattern during or after chemotherapy, and no relevance to treatment response (43,44). Current clinical correlative studies on p53 and chemosensitivity are mostly based in that they screen most TP53 gene alterations, point mutations, leading to the synthesis of a stable, and non-degradable protein that accumulates in tumor cells, and thus can be detected by means of immunohistochemistry (p53 protein overexpression). The use of different antibodies, staining standards, scores for positivity with different threshold values might be reason, at least in part, for less than optimal technique for determination of the p53 status. Additionally, with this method there is a risk of false negative cases related to the type of TP53 gene mutations; specific gene mutations encode unstable proteins, so they do not cause p53 overexpression detectable by IHC. Therefore, the method of DNA sequencing might be more sensitive to get reliable information of p53 status (49) but with this molecular biology methods complete gene should be sequenced because different p53 mutation sites may be associated with different functional implications.

Thus the integration of laboratory and clinical approaches into the search for reliability of mentioned biomarkers yield to no firm conclusions. Different chemotherapy regimens used, and definition of objective response, clinical versus pathological, might also contribute to the lack of concordance among reported results (1). Moreover, there is no standards method of assessing pathological response to primary chemotherapy in breast cancer patients (50) although patients who achieve pathologic complete response (7%-32 %) have a significantly better disease outcome than do those with residual disease.

Selection bias related to different patients` selection criteria, clearly may influence the final results. Larger studies on population-based cohorts evaluating a panel of biomarkers are warranted, and ideally the studies should have prospective design.

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