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PRINCIPAL INVESTIGATOR: Wiliam N. Hait, M.D., Ph.D.

CONTRACTING ORGANIZATION: Robert Wood Johnson Medical School
New Brunswick, New Jersey 08901

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p53 mutations are found in 15%-50% of breast cancers. The microtubule associated protein 4 (MAP4) is repressed by p53 in cells expressing temperature sensitive p53 and by physiological induction of p53 by DNA-damage. We found increased paclitaxel and decreased vinca alkaloid sensitivity in cells expressing non-functional p53 and higher MAP4 protein levels. Therefore, the purpose of these studies was to determine whether there is a correlation between p53 status and MAP4 expression in human breast cancer which could predict responsiveness to taxane and vinca alkaloid chemotherapy. In a panel of breast cancer cell lines of known p53 status, we found that whereas transcriptionally-activated targets of p53, p21/WAF1 and MDM2 were expressed at high levels in wild-type p53 containing cells, there was no significant difference in MAP4 levels between wild-type or mutant p53 containing cell lines. Treatment of MCF-7 cells (wtp53) with the DNA-damaging drug, doxorubicin, led to an increase in p53, MDM2 and p21 and a decrease in MAP4 protein levels. These findings suggest that DNA-damage of wild-type p53 containing breast cancer cells is necessary to increase steady-state p53 levels high enough to repress MAP4, and that breast cancer patients with wild-type p53 may benefit from combination doxorubicin/vinca alkaloid treatment.

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FOREWORD

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William [signature] 7/28/99

PI - Signature Date

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INTRODUCTION:

The treatment of breast cancer patients with chemotherapy is empirical. Even the most active drugs produce meaningful responses in <50% of patients. As a result, too many patients are needlessly exposed to highly toxic drugs and suffer the side effects without reaping the benefits. p53 is commonly mutated in human breast cancer (1). Recent studies demonstrate that p53 mutations also affect the sensitivity to cancer chemotherapeutic drugs (2-5). In studying the mechanism(s) underlying this observation, we found that increased expression of microtubule associated protein 4 (MAP4), which occurs when p53 is transcriptionally silent, is associated with increased sensitivity to paclitaxel and decreased sensitivity to vinca alkaloids (6). Using murine fibroblasts transfected with MAP4, we directly demonstrated that these changes in drug sensitivity were a consequence of overexpression of MAP4. Immunofluorescent staining of the microtubule network revealed that cells with increased MAP4 expression contained greater polymerized microtubules and bound more fluoresceinated paclitaxel than controls (6). Since MAP4 stabilizes polymerized microtubules (7, 8), overexpression of this gene provides a plausible mechanism to explain the sensitivity to microtubule-active drugs in the presence of mutant p53. The overall purpose of our present studies is to test the idea that overexpression of MAP4, as a consequence of functional loss of p53, can predict responsiveness to taxane and vinca alkaloid chemotherapy in human breast cancer. For these studies, we begin by analyzing a panel of breast cancer cell lines whose p53 status is known to determine whether or not there is a correlation between p53 transcriptional activity and expression of MAP4.

BODY:

Task #1 of our approved Statement of Work proposes to determine whether the loss of p53 function leads to increased expression of MAP4 in human breast cancer. Using model rodent cell lines, we and others have found that p53 mutations that inactivate the transcriptional activity of p53 lead to overexpression of MAP4 (6, 9). Furthermore, the physiological induction of wild-type p53 by UV irradiation in the murine mammary tumor cell line C127 led to the repression of MAP4 mRNA and protein levels (9, 10). However, this relationship has never been reported for human breast cancer. Our initial examination as to whether there is a correlation between loss of p53 function and overexpression of MAP4 in breast cancer begins by verifying the transcriptional activity of p53 in several breast cancer cell lines whose p53 status is known by measuring the expression of transcriptional activation targets for p53, p21/WAF1 and MDM2 (11, 12), by immunoblotting.

Briefly, extracts were made from exponentially growing breast cell lines with wild-type p53 (ZR-75-1, MCF-7, MCF-10A) and mutant p53 (MDA-MB-231, MDA-MB-468, BT-20, BT-474, BT-549 and T47D). Extracts were normalized for protein concentration using the BIO-RAD protein assay (BIO-RAD) and p21/WAF-1 and MDM2 immunoblotting was done as previously described (13, 14) using WAF1(Ab-1) or MDM2(Ab-1) antibodies (Oncogene Research Products) and developed with peroxidase-conjugated anti-mouse antibodies using a direct chemiluminescence system (Amersham). Coomassie Brilliant Blue R250 staining of post-transfer gels verified complete transfer of proteins (data not shown). As shown in Figure 1 (see Appendix), as expected, all three wild-type p53 expressing cells had higher levels of MDM2 and p21 proteins (lanes 1-3) compared to mutant p53 containing cell lines (lanes 4-9). Interestingly, relatively high levels of MDM2 were seen in T47D cells despite the presence of mutant p53. This is in agreement with findings by others (15) and suggests that other cellular factors besides p53 account for the high levels of MDM2 in this cell line. A control blot for p53 verified that high levels of p53

protein was detectable in all six mutant p53 containing cell lines whereas low levels were found in wild-type p53 containing cells. This agrees with the short half-life of wild-type p53 and the increased stability of mutant p53 proteins (16, reviewed in 17).

We next analyzed the expression of MAP4 in these cell lines by immunoblotting using a rabbit polyclonal antibody against the C-terminus of human MAP4 (generously provided by Gary Borisy, 18) followed by a peroxidase-conjugated anti-rabbit antibody using a direct chemiluminescence system (Amersham). As shown in Figure 1, no significant correlation ($p > 0.05$) was seen between p53 status and MAP4 expression. For instance, while two out of six mutant p53 containing cell lines showed high levels of MAP4, the other four cell lines showed similar MAP4 levels as those found in the three cell lines with wild-type p53. As addressed in our original grant discussion, it is likely that the basal expression of wild type p53 in these cells is too low to suppress MAP4. For this reason we have begun to carry out studies with stimuli that will induce DNA damage and therefore p53 transcriptional activity. As shown in Figure 2 (see Appendix), preliminary data indicates that treatment of MCF-7 cells with 24 nM ($<IC_{15}$) of a topoisomerase II inhibitor, doxorubicin, not only led to the induction of p53, MDM2 and p21 proteins but also led to the repression (~ 2 fold) of MAP4. Further studies are warranted to confirm this preliminary observation in other p53 wild-type containing breast cancer cell lines, with other DNA-damaging drugs and stimuli such as bleomycin, UV-treatment and γ-irradiation. Furthermore, we will determine whether this pre-treatment of MCF-7 cells with doxorubicin will lead to changes in microtubule polymerization and increased sensitivity to vinblastine. This would be a very important observation since DNA damaging agents (e.g., topoisomerase inhibitors such as doxorubicin) are commonly used in combination with vinca alkaloids in the treatment of breast cancer (19). In support of these observations, preliminary work suggests that treatment of metastatic breast cancer patients with doxorubicin leads to the induction of p53, p21 and MDM2 and a repression of MAP4 in peripheral blood mononuclear cells (unpublished observations).

We found that the p53 wild-type containing MCF-7 and ZR-75-1 breast cancer cell lines also express high levels of the bcl-2 anti-apoptotic protein (Figure 3, Appendix). It is likely that the majority of breast cancers that have wild-type p53 have been selected to contain other genetic alterations to enhance their tumorigenic phenotype, such as amplified bcl-2 (20). It has previously been shown that bcl-2 is capable of blocking the repression of MAP4 by p53 (9). Thus, the high levels of bcl-2 in these cells may reduce the ability of p53 induced by DNA damage to repress MAP4 (Figure 2). For this reason, we are analyzing breast cancer cell lines with wild-type p53 and low bcl-2 levels for our DNA-damage studies. For instance, we will carry out these experiments using an MCF-7 breast cancer cell variant that has been shown to have low levels of bcl-2 yet proliferates with a doubling time similar to MCF-7 cells with high levels of bcl-2 (21). Additionally, treatment of MCF-7 cells with low concentrations of 13-cis-retinoic acid (22) may reduce bcl-2 levels enough to allow DNA-damage induced p53 to repress MAP4. Alternatively, as it has been shown that estrogen can stimulate the expression of bcl-2 (23) and that depletion of estrogen from the medium of estrogen-receptor positive cells such as MCF-7 leads to a downregulation of bcl-2, we are currently investigating the growth characteristics and bcl-2 expression of MCF-7 cells grown in the presence or absence of estrogen-containing medium. Additionally, antisense bcl-2 transcripts or the antiestrogen compound 164,384 can render MCF-7 cells grown in the presence of estrogen more sensitive to doxorubicin (23) through the repression of bcl-2 and may therefore prove useful in our studies.

KEY RESEARCH ACCOMPLISHMENTS:

• We have found no significant correlation between p53 status and MAP4 expression in breast cancer cell lines uninduced by DNA damage.

• Treatment of the wild-type p53 containing MCF-7 breast cancer cell line with the DNA-damaging drug, doxorubicin, increased p53, MDM2, and p21 protein levels and repressed MAP4.

• These findings confirm our hypotheses and encourage us to next investigate the microtubule polymerization state of doxorubicin treated wild-type p53 containing breast cancer cells and whether they exhibit enhanced sensitivity to vinca alkaloids.

REPORTABLE OUTCOMES:

- A poster based on this work has been submitted to the Molecular Targets and Cancer Therapeutics meeting in Washington, DC from November 16-19, 1999.

- This award is supporting the research activities of a second year graduate student, Elizabeth Alli.

CONCLUSIONS:

These preliminary studies indicate that basal levels of wild type p53 are not sufficient to repress MAP4. These studies also demonstrate high levels of MAP4 in human breast cancer cells with mutant p53. Our idea based on studies done in model rodent cell lines (6, 9) was that the loss of p53 function in human breast cancer would lead to an increase in MAP4 protein levels and this could identify patients who would respond to taxane therapy and fail to respond to vinca alkaloids. Interestingly, we found that although there was significant levels of p53 transcriptionally-activated genes, p21/WAF1 and MDM2 in wild-type p53 containing breast cell lines, there was no significant repression of MAP4 protein levels in these cells. It is likely that there is not enough p53 in these cells to lower transcriptionally-repressed genes and that there needs to be a DNA-damaging stimulus in order to raise the levels of p53 high enough to repress MAP4. In fact, we found that doxorubicin, a DNA-damaging drug, led to an induction of p53 and a repression of MAP4 protein levels in treated MCF-7 cells. Others have found that DNA-damage is necessary to result in repression of MAP4, which agrees with our observations (M. Murphy, personal communication). We also have plans to manipulate the levels of bcl-2, which blocks the repression of MAP4 by p53, in breast cancer cells with wild-type p53 to investigate whether we can further repress MAP4 by reducing the levels of bcl-2.

In summary, these studies suggest that sequential chemotherapy of breast cancer may prove effective in treatment. A DNA-damaging drug, such as doxorubicin, combined with a suppressor of bcl-2 (antisense or cis-retinoic acid) followed by vinca alkaloid treatment may lead to enhanced survival of breast cancer patients. We look forward to testing these combinations in cell culture with the hopes of their use in the therapy of patients someday in the future.

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Appendix

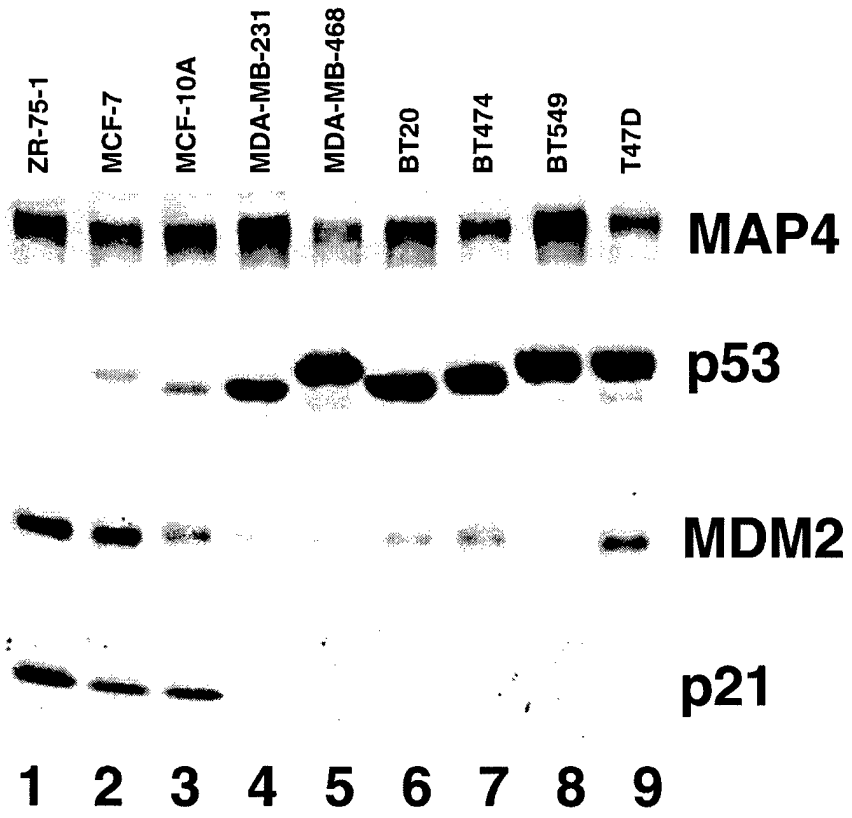

Figure 1: Western blot analysis of MAP4, p53, MDM2 and p21 expression in breast cancer cell lines. Cell extracts (30 µg) from exponentially growing ZR-75-1, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, BT20, BT474, BT549 and T47D breast cancer cells were analyzed by enhanced chemiluminescence-immunoblotting with antibodies specific for MAP4, p53, MDM2 and p21.

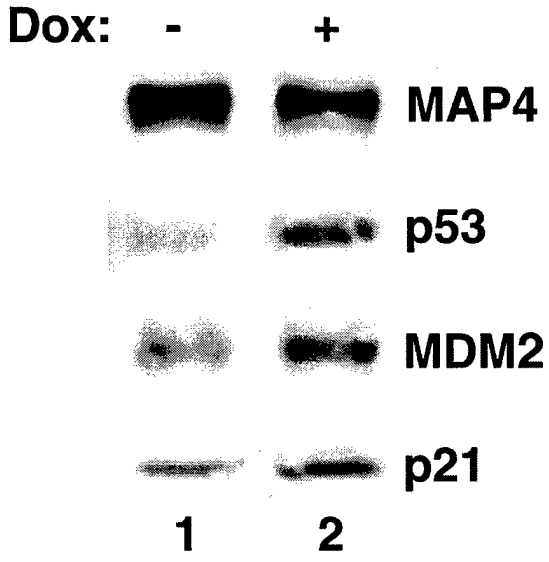

Figure 2: Western blot analysis of MAP4, p53, MDM2 and p21 expression in doxorubicin treated MCF-7 cells. Cell extracts (30 μg) from MCF-7 cells grown in the absence (lane 1) or presence of 24 nM of doxorubicin (lane 2) were analyzed by enhanced chemiluminescence-immunoblotting with antibodies specific for MAP4, p53, MDM2 and p21.

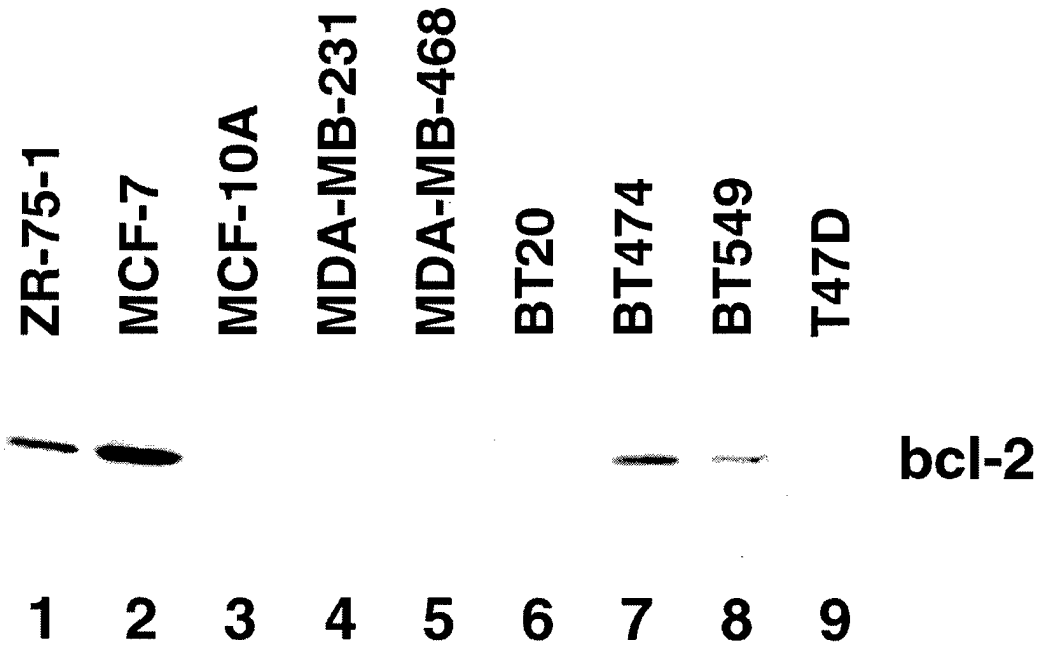

Figure 3: Western blot analysis of bcl-2 expression in breast cancer cell lines. Cell extracts (30 μg) from exponentially growing ZR-75-1, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, BT20, BT474, BT549 and T47D breast cancer cells were analyzed by enhanced chemiluminescence-immunoblotting with antibodies specific for bcl-2.