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The Impact of a Common Mdm2 SNP on the Sensitivity of Breast Cancer To Treatment

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14. ABSTRACT

The discovery of a single nucleotide polymorphism (SNP) in the mdm2 promoter uncovered a previously unknown role of this SNP in predicting early onset of breast and the possibility that this germ line variation could decrease the effectiveness of treatment. These outcomes are likely due to the increased expression of mdm2 protein in SNP309 individuals, which blunts the p53-mediated apoptotic response to DNA damage. The objective of this proposal is to test the hypothesis that SNP309 decreases the effectiveness of radiation and chemotherapy in breast cancer and that this negative impact can be overcome by targeted down-regulation of mdm2. There appears to be a trend toward excess contralateral events with the variant and enrichment of the variant in ER+ breast cancer recurrences. We observed that anti-estrogen agent, fulvestrant, causes a decrease in mdm2 protein half-life, leading to a reduction in mdm2 following treatment with this agent. We demonstrate that combined use of fulvestrant with chemotherapeutic drugs doxorubicin, etoposide and paclitaxel can enhance the sensitivity of breast cancer cells to these cytotoxic agents. We observed that mdm2 expression is differentially modulated by estrogen, the anti-estrogen tamoxifen, and genistein in a genotype-specific manner. The largest effects on reduction in mdm2 expression at the protein level occur in the mdm2 SNP309 cell line. We will continue to explore mechanistic studies in vitro while evaluating the clinical outcome associations of SNP309 to chemotherapy, hormonal therapy and radiation therapy.

15. SUBJECT TERMS
mdm2, breast cancer, polymorphisms

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INTRODUCTION

The recent discovery of a single nucleotide polymorphism (SNP) in the mdm2 promoter uncovered a previously unknown role of this SNP in predicting early onset of breast and the possibility that this germ line variation could decrease the effectiveness of treatment. These outcomes are likely due to the increased expression of mdm2 protein in SNP309 individuals, which blunts the p53-mediated apoptotic response to DNA damage. The objective of this proposal is to test the hypothesis that SNP309 decreases the effectiveness of radiation and chemotherapy in breast cancer patients and that this negative impact can be overcome by targeted down-regulation of mdm2. The rationale in support of these objectives are molecular epidemiological data showing that individuals harboring SNP309 are at increased risk for early onset breast cancer, and laboratory studies showing that SNP309 decreases the activity of DNA damaging agents. If we are to achieve better results of treatment for patients with breast cancer, the choice of treatment must eventually benefit from a more precise understanding of the genetic abnormalities that are present in each individual's tumor. Using the same dose of drug or amount of radiation for each breast cancer patient cannot possibly be consistent with our understanding of modern molecular medicine. For example, subtle variations in our genetic code (called single nucleotide polymorphisms, [SNPs "snips"]) exist in the human population and make us susceptible to certain diseases and resistant to others. Similarly, these polymorphisms can make us more or less sensitive to treatment. Since these polymorphisms exist both in breast cancer and in normal tissues, understanding their impact on both the patient and the tumor will eventually guide the choice and dose of drug and amount of irradiation. Therefore, our objective is to improve the ways in which patients with breast cancer are evaluated and treated through an understanding of subtle variations in the human genome. The proposal brings together a team of molecular biologists/epidemiologists, pharmacologists, radiation and medical oncologists, and statisticians to focus on this novel approach to breast cancer treatment. We anticipate that our results may be applicable to patients by the end of the three-year grant period.

BODY

Task 1. Determine the impact of mdm2 SNP309 on the results of breast irradiation

Updating and assuring complete clinical data has been ongoing. Paperwork for IRB in accordance with recommendations from the IRB at CINJ and the human investigations committees of the DOD was completed and IRB-approval obtained. Patient accrual was initiated through the Radiation Oncology Clinics.

We have completed analysis of mdm2 on the cohort of patients whom we have long term follow-up. We confirmed an association of SNP309 with young patient age in the population of over 250 patients previously treated with long-term follow-up. While all patients in the previously treated database were in the younger age group, a larger percentage of patients of the GG genotype were under age 40 compared to the TT/TG

genotypes (65% vs 35%, $p < .01$). We also found a correlation with race, with few African American patients having the GG homozygous genotype at SNP309. There were no other strong correlations between the SNP309 status and clinical-pathologic variables such as histology, ER status, Her2 status, nodal status, T-stage, family history. There did not appear to be strong correlations with local-regional outcome in this dataset. There appears to be a trend toward excess contralateral events with a 10-year event rate of 9% in the TT/TG subset compared to over 20% in the GG. This will be analyzed in the larger prospective data set in the future. In addition, in this data set there was a difference in distant metastasis in the GG subtypes, with the 10 year rate of distant metastasis-free survival 89% in the TT/TG subset compared to 76% in the GG subtype ($p = .04$). This will be further explored in multivariate analysis. Although there were no clear differences in local control, further exploratory subset analysis will be performed to determine if there are subsets within this cohort with higher local relapse rates.

In the prospective cohort, we continue to recruit patients in the radiation therapy clinic as well as in CINJ breast clinic. In the radiation therapy clinic we continue to actively accrue patients and continue accrual in the CINJ clinics such that we will have reached our accrual goals of patients treated with breast conserving surgery and radiation by years end. We will then analyze this larger cohort for SNP309 and evaluate outcomes and clinical-pathologic correlations over the next year.

Task 2 Determine the impact of mdm2 SNP309 on the results of adjuvant chemotherapy.

A total of 1629 women have been consented for participation in the parent study protocol as of May 12, 2009 (CINJ Protocol #040406, IRB# 0220044862). Of these, genomic DNA has been isolated from 1,100 patients. The information contained in this table reflects data available from chart review for study participants (this chart review was completed as of November 15, 2008). Annual chart re-review in currently ongoing for update of data on recurrence in patients with breast cancer.

We will be using this cohort to determine the genotype-specific recurrence free survival for the following: 1) hormone receptor positive and hormone receptor negative breast cancers; 2) hormone receptor positive breast cancer patients receiving hormonal therapy alone; 2) breast cancer patients receiving chemotherapy only (hormone receptor positive and negative disease); 3) breast cancer patients receiving chemotherapy followed by hormonal therapy (hormone receptor positive only).

Table 1- Demographics of Study Cohort at The Cancer Institute of New Jersey.

Race	Number of Patients	% of Patients
African American	48	5.5
Asian	37	4.2
Caucasian	680	77.9
Hispanic	54	6.2
Indian	22	2.5
Other	32	3.7
Not reviewed	103	

Tumor Type	Number of Patients	% of Patients
Colloid/Mucinous	12	1.4
DCIS	71	8.4
Invasive Ductal	644	75.8
Invasive Lobular	88	10.3
LCIS/Atypical hyperplasia	5	0.6
Medullary	4	0.5
Metaplastic	4	0.5
Other	22	2.6
Unknown	131	

ER Status	Number of Patients	% of Patients
Positive	584	74.3
Negative	202	25.7

PR Status	Number of Patients	% of Patients
Positive	486	63.1
Negative	284	36.9

Her2/Neu Status	Number of Patients	% of Patients
Not amplified or 0-2+ IHC	471	80.7
Amplified or 3+ IHC	113	19.3
(all 2+ by IHC were reflexed for FISH) 6 ambiguous		

Stage	Number of Patients	% of patients
Control/LCIS/Atypical hyperplasia	5	0.7
0	66	8.7
1	274	36.1
IIA	172	22.3
IIB	118	15.5
IIIA	68	8.9
IIIB	8	1.1
IIIC	11	1.4
IV	37	4.9

Tumor	% of Patients
T0	8.3
T1	53.6
T2	27.9
T3	5.8
T4	4.4

Node status	
N0	59.1
N1	33.1
N2	5.9
N3	1.7

Metastatic Status	
M0	95.4
M1	4.6

Recurrence Status	% of patients
Yes	19.3
No	80.7
(excludes stage IV at diagnosis)	

Patients Receiving Each Treatment	No (%)	Yes (%)
Radiation	25	75
Chemotherapy	40	60
Hormonal therapy	25	75
trastuzumab	90	10

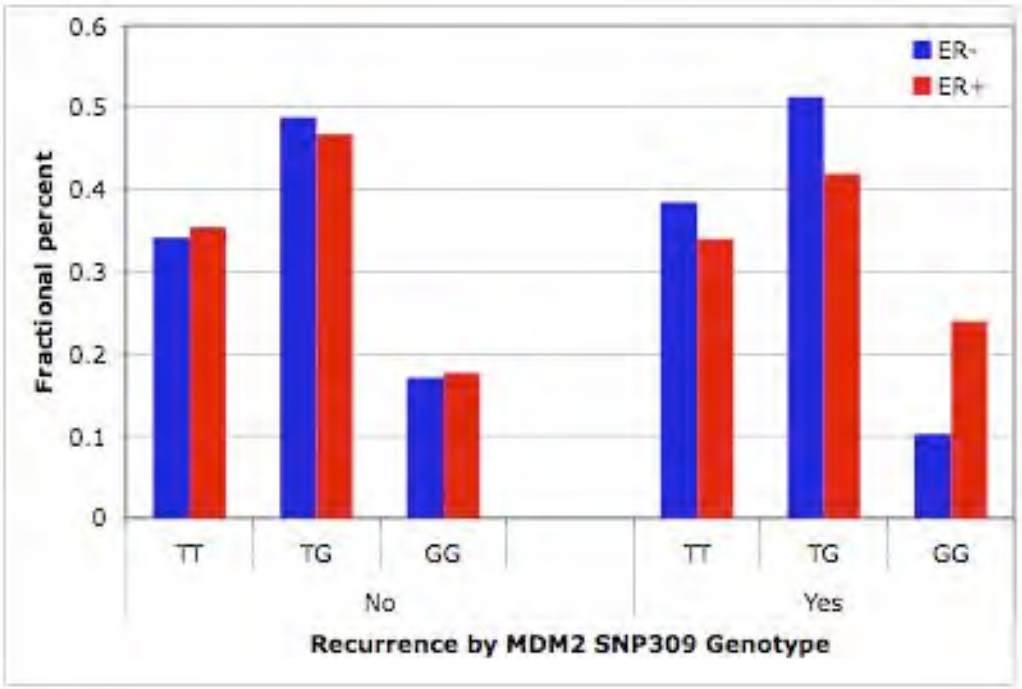


Figure 1. Recurrence of breast cancer by estrogen receptor status and MDM2 SNP309 genotype.

For n=135 patients with recurrence, 68% of recurrence occur by 5 years. Of those with recurrences after 5 years, those are mostly ER+ breast cancers. When comparing recurrences in ER- and ER+ breast cancers by genotype for MDM2 SNP309, there is a trend toward enrichment in the GG genotype in ER+ recurrences as compared to ER- recurrences (Figure 1). Numerically, the GG genotype in ER- and ER+ recurrences was 10.3% and 24%, respectively. In those patients with recurrences, the genotype distribution for ER- and ER+ for TT was 38.5% vs 34% and for TG 51.3% vs 42%, respectively. For patients without recurrences, there was no difference in the distribution

of MDM2 genotypes for ER- and ER+ breast cancers (TT 34.2% vs. 35.5%, TG 48.8% vs 46.8%, GG 17.1% vs 17.7%). There is no difference in the risk of recurrence in GG carriers regardless of ER status. In general, ER negative breast cancers are known to have a poorer prognosis with higher risk of relapse as compared to ER+ breast cancer. This suggests that ER+ breast cancers in women carrying the GG genotype may have worse prognosis. Due to the low number of recurrences within each group, stratification of data further by type of recurrence (local vs regional vs distant), stage at diagnosis, chemotherapy+/- hormonal therapy +/- trastuzumab results in too few numbers for adequate power in the analyses. However, through *in silico* analysis from SNP array data publicly available on the NCI-60 cell lines, those cell lines with the TT genotype are more likely to have higher MDM2 copy numbers than those cell lines with the GG genotype and this correlates with protein expression. This implies that in those lines with the GG genotype, the GG mechanism drives higher expression of MDM2. However, cell lines with the TT genotype may undergo selective pressure to acquire an alternative mechanism of increasing mdm2 protein expression.

Task 3 Determine the ability of anti-estrogens to restore drug and irradiation sensitivity by decreasing mdm2 expression

In this grant period, we have investigated and confirmed the effects of anti-estrogen agent, fulvestrant, on mdm2 expression and sensitivity of human breast cancer cells to chemotherapeutic drugs. We found that in both MCF7 (T/G) and T47D (G/G) human breast cancer cell lines, fulvestrant decreases mdm2 expression to similar extents (Figure 2). As has been previously established, fulvestrant decreases the level of estrogen receptor protein. Further, fulvestrant not only abolished the effect of estradiol (E_2), but also was also able to suppress mdm2 protein levels below the control (no E_2) level (Figure 3). At lower concentrations of fulvestrant, the ability to block effects of estradiol was reduced as compared to the higher concentration of fulvestrant. Mdm2 depletion by fulvestrant did not correlate with an increase in p53 activation (slight decrease) and no change in p21 levels was observed (Figure 4). Estrogen receptor is known to bind and stabilize p53 and this stabilization may be reduced in the presence of fulvestrant where ER levels are reduced. Fulvestrant did not cause a reduction in mdm2 mRNA in MCF7, but reduces mdm2 protein half-life (Figure 5). Of note, there was both a reduction of mRNA and protein levels in T47D. The combination of fulvestrant and chemotherapeutic drugs doxorubicin, etoposide or paclitaxel showed synergism in MCF7 and T47D cells (Figure 6). The combination of Fulvestrant (an FDA-approved compound) and three traditional chemotherapeutic agents (Doxorubicin, Paclitaxel and Etoposide) showed different degrees of synergism in both, MCF7 and T47D estrogen receptor positive breast cancer cell lines, thus enhancing the sensitivity of these cells to chemotherapy.

Epidemiologic evidence suggests that genistein intake is inversely related to the risk of several tumors including breast cancer but its mechanism of action is not completely understood. However, conflicting data exists on the effect of genistein on the expression of the estrogen-dependent mdm2 gene. We hypothesized that if genistein acted like an anti-estrogen, it could bind estrogen receptor (ER), preventing binding to the ERE at the mdm2 promoter and lead to down-regulation of mdm2 expression. For those cells in which SNP309 is present, we anticipated even stronger effects. To explore this, we grew

breast cancer cells under conditions of no estrogen (PF), normal media (N), with estradiol (E2), with Tamoxifen (T), and with genistein (G). We selected three ER+ breast cancer cell lines representing the three mdm2 SNP309 genotypes: ZR75-1 (TT), MCF-7 (TG), and T47D (GG). Protein was isolated from the cells grown in the various conditions and Western blot analysis was performed (Figure 7).

In MCF-7 cells (TG), mdm2 protein is reduced when cells are grown in the absence of estrogen media as compared with normal media or with estradiol. With Tamoxifen or genistein, relative to estradiol, mdm2 was reduced, but remained at levels higher than that in the absence of estrogen. In T47D (GG genotype), the response in the absence of estrogen, normal media, and with estradiol treatment is similar to that of MCF-7 cells (TG genotype). However, by comparison, mdm2 levels are reduced to levels nearly equivalent to those in the absence of estrogen when treated with Tamoxifen and genistein. Of interest, the ~50kDa isoform of mdm2 is reduced further with genistein as compared with Tamoxifen, suggesting an effect on alternative splicing. In ZR75-1 cells (TT), no 50kDa isoform is expressed. In contrast to the MCF7 and T47D cells, genistein and Tamoxifen treatment resulted in ***increased*** mdm2. Increased expression may be the result of increased transcription or posttranslational changes leading to reduced degradation and longer half-life. These results suggest a genotype-specific effect of genistein and may explain contradictory effects observed in studies.

The P2 promoter of mdm2 has an ERE and we previously demonstrated that mdm2 levels are estradiol dose-dependent and genotype dependent (preliminary data for proposal). Therefore, we had hypothesized that Tamoxifen, an anti-estrogen that binds ER, would result in decreased mdm2 as well as decreased binding at the promoter as determined by chromatin immunoprecipitation (figure 8). While this was true in ZR75-1 cells and to a much lesser degree in MCF7 cells, binding occurred in the presence of Tamoxifen in T47D. As genistein is thought of as an anti-estrogen, we hypothesized that genistein treatment would result in decreased binding to the ERE. With genistein treatment, ER still bound the P2 promoter region but transcription was reduced in MCF7 and T47D. Interestingly, binding appeared to be reduced in ZR75-1 for treatment with estradiol, Tamoxifen, and genistein. Since protein levels were increased in ZR75-1 with Tamoxifen and genistein, this suggests that post-translational modification leading to longer half-life may play a role in increased mdm2 levels with these treatments. It is not clear if this is truly a genotype-specific effect or if this is related to this particular cell line.

KEY RESEARCH ACCOMPLISHMENTS

- We observed that anti-estrogen agent, fulvestrant, causes a decrease in mdm2 protein half-life but not transcript, leading to a reduction in mdm2 following treatment with this agent. This occurs in the absence of changes in p53 and p21 protein or gene expression levels.

- We demonstrate that combined use of fulvestrant with chemotherapeutic drugs doxorubicin, etoposide and paclitaxel can enhance the sensitivity of breast cancer

cells to these cytotoxic agents. The combination of paclitaxel and fulvestrant demonstrated the most consistent and strongest synergistic effects.

- We observed that mdm2 expression is differentially modulated by estrogen, the anti-estrogen tamoxifen, and genistein in a genotype-specific manner. The largest effects on reduction in mdm2 expression at the protein level occur in the mdm2 SNP309 cell line.

- We observed that binding of estrogen receptor alpha to the mdm2 promoter is less efficient in the wildtype mdm2 breast cell line in the presence of estrogen, tamoxifen, and genistein as compared with cell lines carrying at least one variant allele.

- We have accrued the patients needed to evaluate the role of SNP309 in mdm2 on outcomes associated with chemotherapy and hormonal therapy. Preliminary data demonstrate a trend toward enrichment of recurrent ER+ breast cancer compared to ER- breast cancer with no difference in rate of recurrence.

REPORTABLE OUTCOMES

Manuscript

In preparation

Abstracts

Annual AACR poster presentation- April 2009
Annual New Jersey Cancer Retreat- May 2009

Degree obtained that are supported by this award

None

CONCLUSIONS

1. Selective estrogen receptor down-regulator, fulvestrant, decreases MDM2 expression and enhances sensitivity of human breast carcinoma cells to chemotherapeutic drugs (such as doxorubicin, etoposide and paclitaxel).
2. The anti-estrogen tamoxifen decreases MDM2 expression in a genotype-specific manner.
3. Recurrence of ER+ breast cancer in GG patients is not significantly different from those individuals with ER- breast cancer.
4. There is a trend toward enrichment of the GG genotype frequency in ER+ recurrences as compared with ER- recurrences and ER+ patients without recurrence.

REFERENCES: none

APPENDICES: none

SUPPORTING DATA

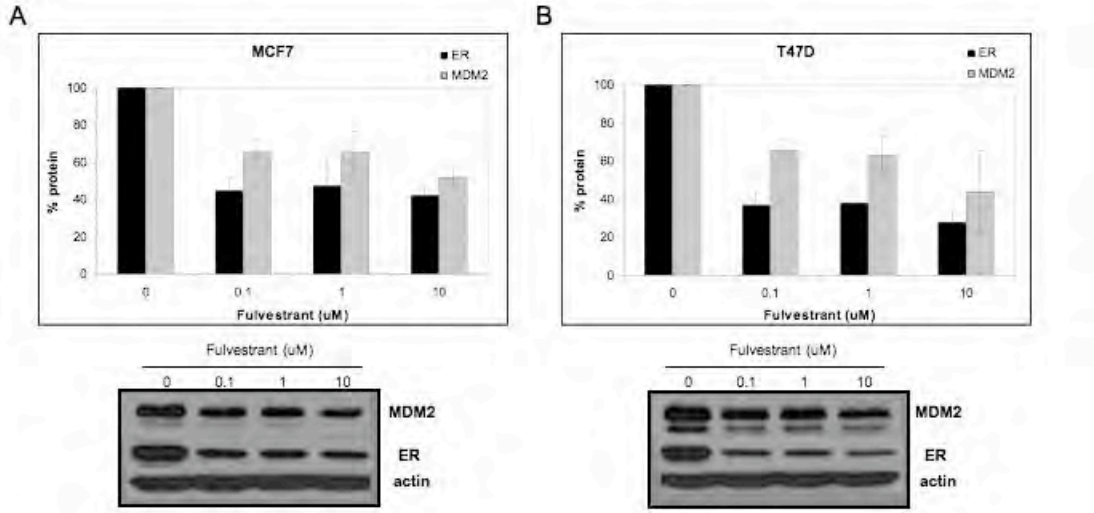


Figure 2. Effect of the antiestrogen fulvestrant on expression of estrogen receptor and mdm2 protein. Two breast cancer cell lines MCF7 and T47D were grown at various concentrations (0-10 micromolar) of fulvestrant for 66 hours. Protein was then harvested and levels of estrogen receptor and mdm2 were assayed by Western blot. The upper plots demonstrate the dose-dependent reduction of both proteins in each cell line.

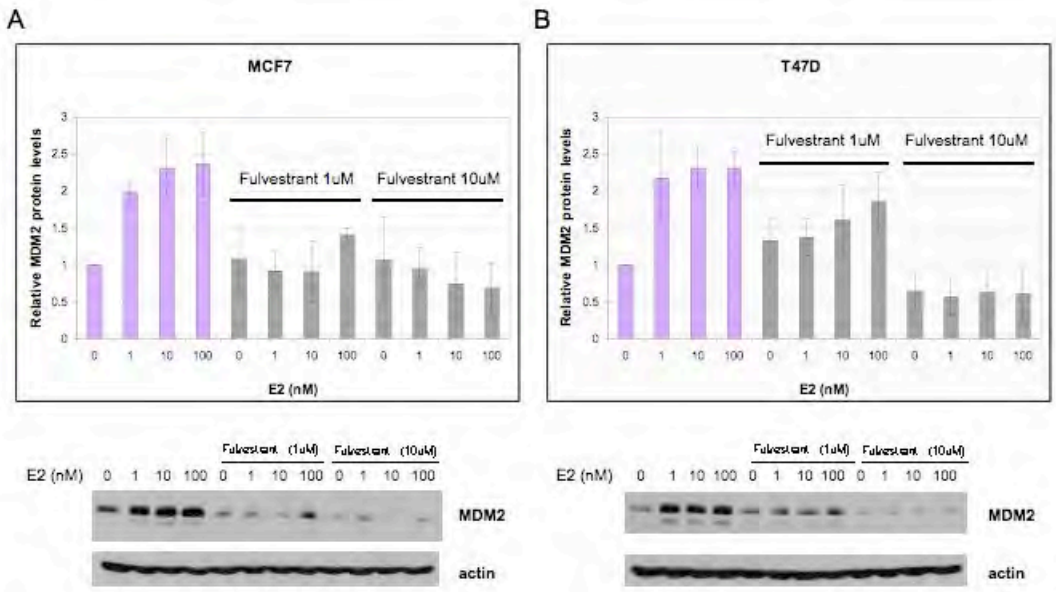


Figure 3. Effect of the antiestrogen fulvestrant on mdm2 levels in breast cancer cells grown in the presence of estradiol. Two breast cancer cell lines MCF7 and T47D were grown in the presence of estradiol, and estradiol with one of two concentrations of fulvestrant. Thee lower plots represent the Western blot analysis corresponding to the quantification in the upper graphs.

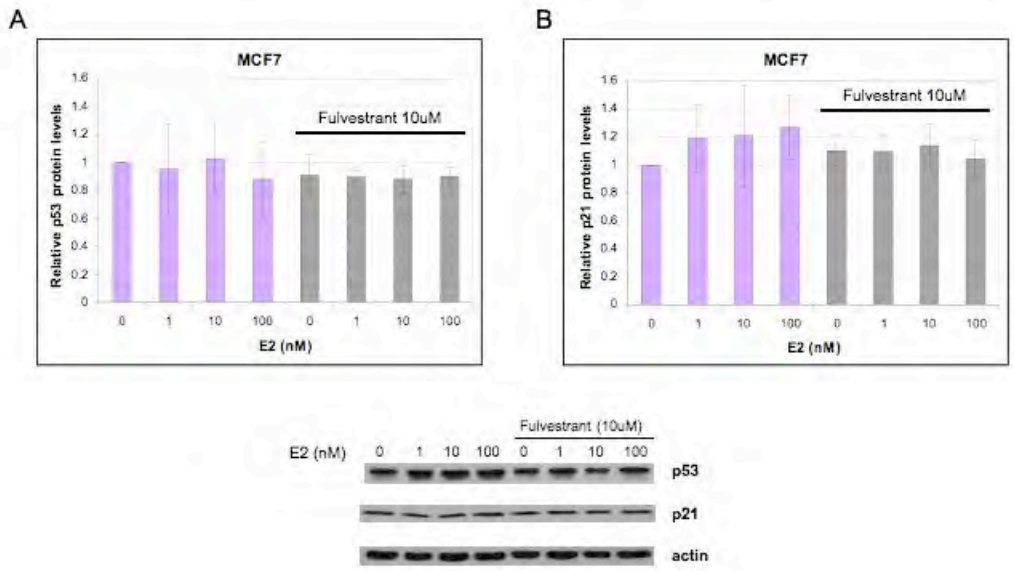


Figure 4. Effect of estradiol and the antiestrogen fulvestrant on p53 and p21 in breast cancer cell lines. The breast cancer cell lines MCF7 was grown in estradiol alone or with the presence of 10micromolar fulvestrant. Protein was harvested and Western blot analysis performed to detect p53 and p21. The lower plot depicts the Western blot for each protein using actin as a loading control. This plot was used to quantitate protein levels expressed in the upper curves.

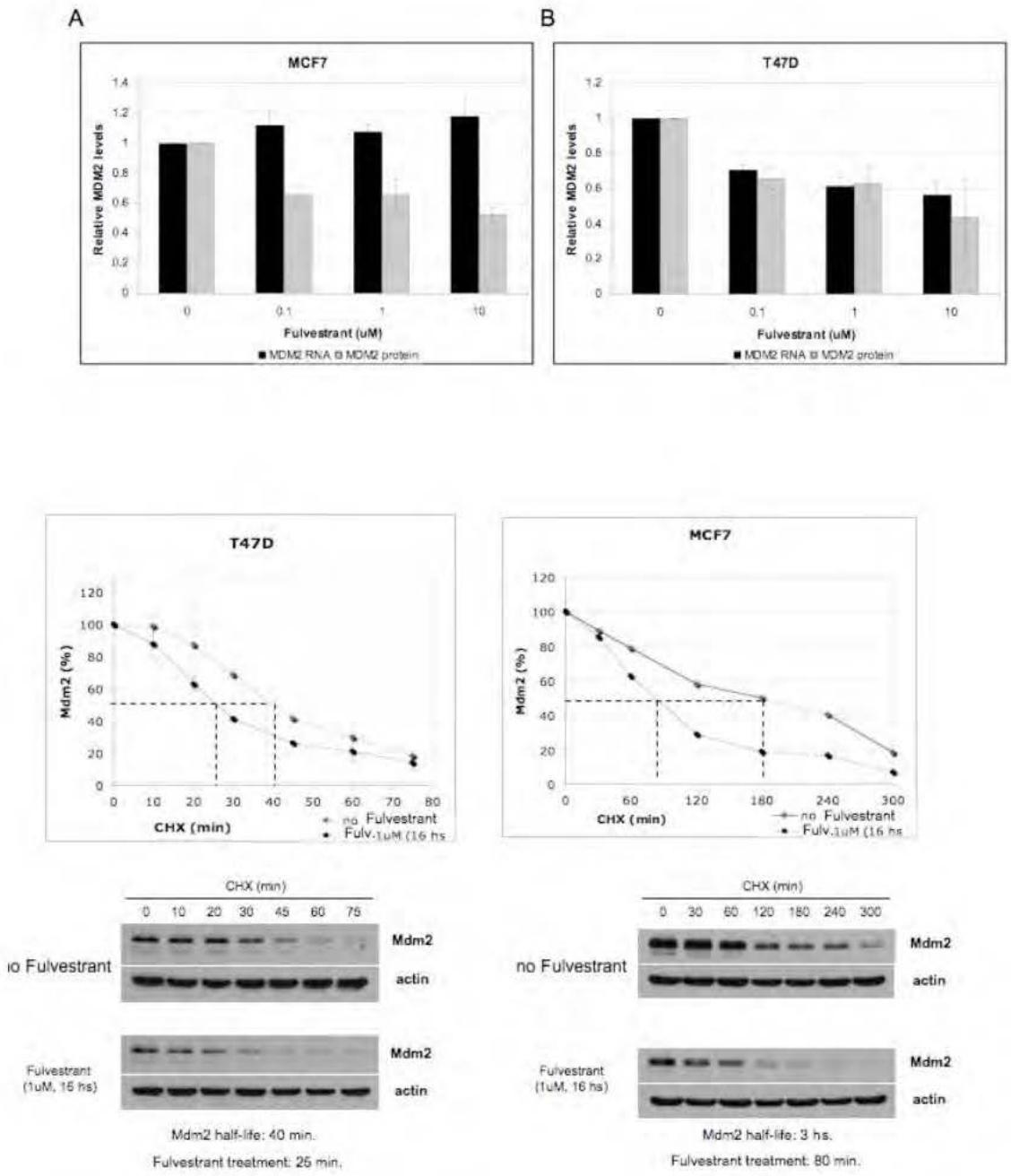


Figure 5. Effect of fulvestrant on the half-life of mdm2 protein and MDM2 transcript. Two breast cancer cell lines T47D and MCF7 were grown in the absence and the presence of the antiestrogen fulvestrant. The upper curves demonstrate the effect of fulvestrant on MDM2 transcript levels in comparison to protein levels. For the half-life of protein (lower curves), cells were treated with cycloheximide (CHX) and mdm2 protein expression was determined at various time points. The lower curves show Western Blot analyses from each cell type using actin as a loading control and were used to quantitate mdm2 levels given in the corresponding curves above.

Figure 6

A

MCF7

DOXORUBICIN - FULVESTRANT

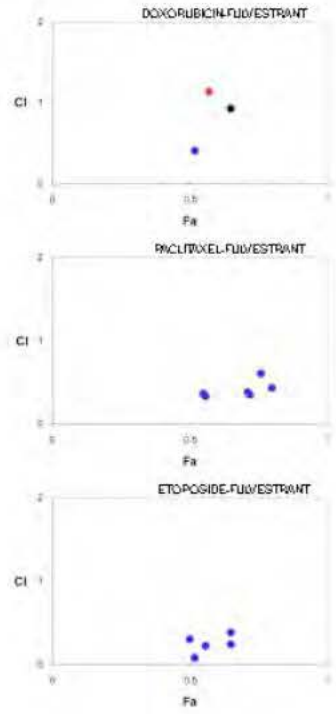
Fulv (uM)	DOX (uM)	Fa	CI	Assigned Symbol
5	0.3	0.65	0.93	±
1	0.06	0.52	0.41	+++
1	0.3	0.57	1.14	-

PACLITAXEL - FULVESTRANT

Fulv (uM)	TAX (uM)	Fa	CI	Assigned Symbol
1.25	0.005	0.55	0.36	+++
5	0.02	0.72	0.35	+++
12.5	0.05	0.80	0.43	+++
1	0.005	0.56	0.33	+++
1	0.02	0.71	0.38	+++
1	0.05	0.76	0.61	+++

ETOPOSIDE - FULVESTRANT

Fulv (uM)	VP16 (uM)	Fa	CI	Assigned Symbol
0.3	1	0.52	0.08	+++++
1.7	5	0.56	0.22	++++
5	15	0.65	0.24	++++
1	1	0.50	0.3	++++
1	15	0.65	0.38	+++



B

T47D

DOXORUBICIN - FULVESTRANT

Fulv (uM)	DOX (uM)	Fa	CI	Assigned Symbol
50	0.05	0.48	0.82	++
100	0.1	0.50	1.07	±
1	0.05	0.41	0.84	++
1	0.1	0.53	0.99	±

PACLITAXEL - FULVESTRANT

Fulv (uM)	TAX (uM)	Fa	CI	Assigned Symbol
100	0.008	0.67	0.69	+++
1	0.004	0.38	0.69	+++
1	0.008	0.54	0.74	++

ETOPOSIDE - FULVESTRANT

Fulv (uM)	VP16 (uM)	Fa	CI	Assigned Symbol
10	0.25	0.53	0.21	++++
20	0.5	0.46	0.6	+++
1	0.5	0.43	0.55	+++

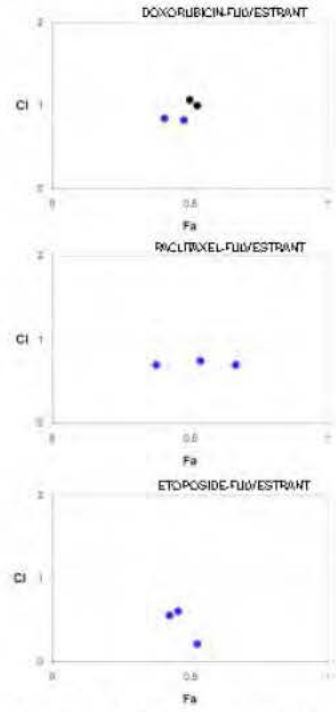


Figure 6. Effect of combining the antiestrogen fulvestrant with doxorubicin, paclitaxel, or etoposide in two breast cancer cell lines. Analysis of cell response was determined using the CompuSyn program. Each combination was observed at two concentrations of

chemotherapeutic agent while keeping the concentration of fulvestrant constant. The last column indicates the type observed effect of the combination for each drug and dose.

CompuSyn analysis. Effect value (Fa): fraction of the population affected by the treatment at the specified dose. Combination Index (CI): quantitative measure of the degree of drug interaction in terms of synergism and antagonism.

Assigned Symbols:

Synergism (CI ‹ 1):
+++++(very strong), ++++(strong), +++(synergism), ++(moderate), +(slight)

Additive effect (CI =1): ±

Antagonism (CI › 1):
- - - - - (very strong), - - - - (strong), - - - (antagonism), - - (moderate), - (slight)

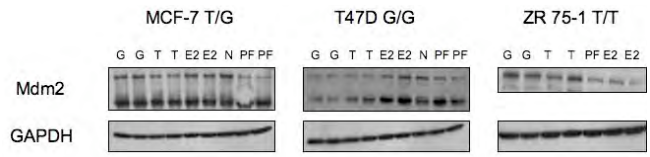


Figure7. Western blot demonstrates mdm2 protein expression in three ER+ breast cancer cells lines representing the three SNP309 genotypes: ZR75-1 (TT), T47D (GG), MCF7 (TG). Cells were grown under different conditions: phenol-free, charcoal stripped media (PF), normal media (N), estradiol (E2), Tamoxifen (T), or genistein (G).

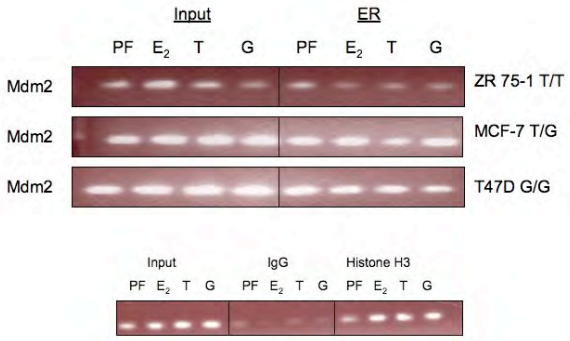


Figure 8. Chromatin immunoprecipitation using anti-ERalpha antibody with PCR of the mdm2 P2 promoter region was performed in the three ER+ breast cancer cell lines representing each of the three mdm2 genotypes.