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TITLE: 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. Mdm2 expression negatively correlates with breast cancer survival. 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 chemotherapeutics 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. There was no association between the mdm2 SNP309 and clinical outcome of breast cancer with chemotherapy, hormonal therapy and radiation therapy.					
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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.

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 a 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%, $p < 0.01$). 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 GG carriers. 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 ($p=0.04$). This will be further explored in multivariate analysis. There were no clear differences in local control. Although further exploratory subset analyses were performed to determine if there are subsets within this cohort with higher local relapse rates, inadequate sample size resulted in no other significant associations on subset analysis (defined as $p<0.05$).

Immunohistochemical Analysis of MDM2

Cells suffering DNA damage ultimately progress through apoptotic pathways, in which p53 plays a central role. Murine double minute 2 (MDM2) and its related ortholog MDM4, are involved in the negative regulation of p53 including ubiquitin-mediated targeting of p53 for proteolytic degradation and transcriptional activity. Moreover, MDM2 is induced by p53 thus creating a negative feedback loop (Wu, 1993; Barak, 1993; Kussie, 1996; Lin, 1994; Freedman, 1999). Apart from its p53 ubiquitination function, MDM2 has other functions including nuclear-cytoplasmic shuttling of p53 and prominent interactions with various ribosomal proteins (Roth, 1998; Marechal, 1994). MDM4 has similar structure and function to MDM2; however, it can be degraded by MDM2 (Shvarts, 1997; Shvarts, 1996; Okamoto, 2005). Appropriate expression of p53 propels cells down apoptotic pathways, but this progression may be counteracted by overexpression of MDM2/MDM4 and subsequent degradation of p53. It follows that dysregulation of p53 by MDM2/MDM4 could potentially cause radiation resistance through inability of the cell to undergo p53-mediated apoptosis.

Several studies have reported the significance of MDM2 in cancers of the prostate, breast and ovary (Marchetti, 1995; Marchetti, 1995; McCann, 1995; Khor, 2005). In breast cancer, MDM2 has been extensively studied as prognostic marker for overall and disease specific survival. Over expression of MDM2 has found to be associated with worse breast cancer specific survival and has been demonstrated to have a role in enhancing estrogen receptor alpha ($ER\alpha$) mediated gene expression and altering $ER\alpha$ stability (Kim, 2011; Turbin, 2006; Marchetti, 1995; Duong, 2007). However, the significance of MDM2 on local recurrence of breast cancer has not been adequately explored with most reports focusing on either recurrence-free survival or overall survival (Khor, 2005). Specifically, there have been no reports assessing the significance of MDM2 on local recurrence in early stage breast cancer treated with BCS + RT. Additionally, relatively little is known regarding the effects of over/under expression of MDM4 and p53 on local recurrence.

Because MDM2 SNP309 correlates with expression of *mdm2* (Bond, 2005), we also explored whether *mdm2* expression correlated with in-breast, regional (lymph node), and distant recurrence of breast cancer in women treated for early stage breast cancer. MDM4, another negative regulator of p53, and p53 itself were also evaluated for prognostic value in this cohort. This analysis was limited as germline DNA from peripheral blood was not available genotyping. Genotyping was not performed on tumor tissue as patient tissue did not have consent for such analysis. Breast cancer tissue cores were compiled into a tissue microarray (TMA), $n=514$, that was evaluated for MDM2, MDM4, p53. All patients had histological evidence of invasive breast carcinoma with early stage (I/II) disease and were treated with breast conserving surgery and radiation. The size of the primary tumor was considered to be the largest tumor diameter reported

by the pathologist after surgical excision. Margin status was defined as positive if tumor cells were present on the most peripheral slide of the tumor. Following surgery, patients received standard whole breast irradiation to a total median dose to the breast of 48 Gy and a total tumor bed dose of 64 Gy; regional nodes were treated to a median dose of 46 Gy, as clinically indicated. Adjuvant systemic chemotherapy and/or adjuvant hormone therapy was administered as clinically indicated in accordance with standard practices during this time interval. Local recurrence was defined as clinically and biopsy-proven relapse in the ipsilateral breast. Ipsilateral breast recurrence free time was defined from the time of initial diagnosis to ipsilateral breast tumor relapse; nodal relapse free time was defined as the time from initial diagnosis to the time of biopsy proven nodal relapse; locoregional recurrence free time was defined as time from initial diagnosis to either ipsilateral breast recurrence or nodal recurrence. Descriptive statistics comparing MDM2 expression with conventional markers of tumor aggressiveness were analyzed by standard chi-squared tests, or, when appropriate, Fisher's exact test. Estimates of disease-free survival were calculated by the Kaplan–Meier product-limit method and the differences were assessed by the log-rank test. Probabilities of survival were calculated from the date of breast cancer diagnosis to either the date at which relapse was clinically identified or the date of last contact. The staining profile of each tumor was correlated with ipsilateral breast recurrence survival (IBRFS), nodal recurrence free survival (NRFS) and loco-regional recurrence free survival (LRFS). The associations between staining and outcomes were assessed in a multivariate model. Univariate and multivariate survival analysis was carried out using Cox's proportional hazard regression model. Multivariate analysis was used to assess the independent contribution of each variable to survival. A computer program package SAS (Version 9.2, SAS Institute, Cary, NC) was used for all statistical testing and management of the database.

Results: Results are depicted in the appendix: Neboori *et al.*, submitted to Breast Journal, under review. Note that this manuscript was originally submitted to the journal Cancer but despite the favorable initial review (attached with our response to reviewer) was then rejected. The median follow up of the cohort was 7.23 years. Positive scoring of MDM2 was correlated with worse 10 year IBRFS (75.0% v. 90.0%; $p=0.032$) by Kaplan-Meier analysis. Neither MDM4 nor p53 correlated with any endpoints. Combinations of markers did not enhance prognostic value. On multivariate analysis incorporating MDM2, tumor size, margin status and nodal status, MDM2 was significant for IBRFS ($p=0.0009$). As MDM2 is an estrogen-responsive gene, its prognostic value was assessed on ER+ and ER- subsets. MDM2 was found to be predictive of local recurrence only in the ER+ subset on multivariate analysis for IBFRS ($p=0.0037$). Our data indicate that MDM2 is an independent predictor for local recurrence in conservatively treated, early stage breast cancer, suggesting its possible use as a prognostic marker.

In this study, we showed that MDM2 overexpression is associated with significantly worse local recurrence in stage I and stage II invasive breast cancer treated with BCS+RT as defined by ipsilateral breast recurrence free survival (IBRFS) and loco-regional recurrence free survival (LRFS). When examining MDM4 and p53 expression however, we were not able to appreciate a similar prognostic value. Lastly, as MDM2 expression has been linked to active ER α signaling, (Kim, 2011; Marchetti, 1995) we sought to determine if the prognostic significance of MDM2 was associated with this subset. The fact that MDM2 was only found to have predictive value in the ER+ subset may be explained by higher biologic activity of MDM2 in estrogen responsive tumors. Numerous studies have identified associations between MDM2 and ER α expression in

breast tissue and breast cancer cell lines [Hori, 2002; Sheikh, 1993; Marchetti, 1995]. In vitro data have demonstrated that MDM2 is an estrogen-responsive gene through action of activated ER α on the estrogen response element in the first intron of MDM2 (Hu, 2007; Brekman, 2011; Okumura, 2002; Phelps, 2003). Furthermore, data support two separate interactions between MDM2 and estrogen receptor signaling. Duong *et al.* (Duong, 2007) demonstrate that MDM2 plays a role in ER α turnover through its ubiquitin-ligase activity and targeted ER α degradation and down-regulation. In contrast to these findings, Kim *et al.* (Kim, 2011) demonstrated MDM2-enhanced ER α -mediated transactivation in the presence of wildtype p53. Both studies however emphasize protein-protein interactions between MDM2 and ER α leading to these functional responses.

These findings suggest that while both MDM4 and MDM2 are involved in the negative regulation of p53 and subsequent arrest of apoptosis, only MDM2 protein expression may have prognostic value in determining local outcomes in early stage breast cancer treated with BCS+RT. These results add to a growing body of evidence demonstrating that increased expression of MDM2 has negative prognostic value for various endpoints in multiple tumor types (Bueso-Ramos, 1996; Khor, 2005; Kim, 2011; Marchetti, 1995; Marchetti, 1995; McCann, 1995; Turbin, 2006; Lukas, 2001). The prognostic value of MDM2 found to be independent of MDM4 and p53 status of the tumor cores. Additionally, it should be noted that MDM2 was found to be an independent predictor for local outcomes in early stage breast cancer regardless of patients having received chemotherapy or hormone therapy.

Interestingly, there is a correlation between MDM2 expression and Her2 phenotype, *i.e.* higher expression of MDM2 was more common in Her2 overexpressors (Table 1). Nearly an equal number of tumors stained positive for Her2 as were positive for p53. P53 expression, normally low in the absence of cell stress, is thought to increase in the presence of p53 mutation due to resultant stabilization of the dysfunctional protein. P53 mutations are more common in Her2 overexpressing breast tumors. At least one study has identified a relationship between Her2 expression with MDM2 expression (Casalini, 2001). However, in that study, MDM2 is downregulated in the presence of wild type p53. Therefore, the association observed in this dataset may, in part, reflect the p53-Her2 pathway interaction. This dataset though, does not have sufficient information to validate this hypothesis.

To our knowledge this is the first study assessing the significance of protein expression of MDM2, MDM4 and p53 for local recurrence in conservatively treated, early stage breast cancer. This cohort demonstrated that increased expression of MDM2 correlated with reduced ipsilateral breast recurrence free survival, and worse locoregional relapse free survival in early stage breast cancer treated with breast conserving surgery and radiotherapy. Moreover, on subset analysis, it was found that MDM2 was only found to have prognostic value in the ER + subset alluding to the importance of this protein in ER+ breast cancer. These results add to the growing body of evidence assessing the prognostic value of MDM2 expression, and its potential as a therapeutic target in combination with radiation therapy. If confirmed in larger studies, these results can have significant clinical implications. However, further studies are needed to assess its importance in regional recurrence, and of MDM4 and combinations of other markers in prognosis.

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

A total of 3462 women have been consented for participation in the parent study protocol as of May 12, 2012 (CINJ Protocol #040406, IRB# 0220044862). Of these, genomic DNA has been isolated from 2,400 patients and 1,319 with completed chart reviews and study follow-up. The information contained in Table 1 reflects data available from chart review and patient-completed questionnaires for study participants (this chart review was completed as of December 15, 2011).

The timing of recurrence is an important variable in this dataset since the median follow-up time is 7.66 years. Of 192 recurrences, however, 79.9% occur by the end of 5 years (Table 2). The majority of recurrences beyond five years reflect estrogen receptor positive disease.

The nature of recurrence reflects the initial stage, molecular features, and type of adjuvant therapy. Table 3 depicts the distribution of adjuvant therapies delivered in this cohort of breast cancer patients. The majority of patients received radiation, chemotherapy, and/or hormonal therapy. Only about 12% of patients received trastuzumab.

We used 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).

Breast Cancer Recurrence as a Function of Receptor Status, MDM2 SNP309 Genotype, and Adjuvant Therapy. Of 192 recurrences with known genotypes, more than 50% were in estrogen receptor negative (ER-) breast cancers, as expected. In estrogen receptor negative breast cancer, the recurrence rate was 29.8% as compared to 16.8% in estrogen receptor positive (ER+) disease. There is no significant difference in risk of recurrence by genotype for either estrogen receptor positive or estrogen receptor negative breast cancers (Table 4). For ER- disease risk of GG vs. TT genotype, OR 1.11 CI [0.619-1.993], p=0.445. For ER+ disease, OR for recurrence for GG as compared with TT was 1.18 CI [0.701-1.98], p=0.323. The frequency of recurrence for GG ER- is 19% and for ER+ is 18% are similar.

Because of the lack of targeted therapy for hormone receptor negative disease, its more aggressive behavior and propensity to recur, the majority of patients with hormone receptor negative disease received chemotherapy and we could not perform analysis due to inadequate numbers for patients with ER- tumors *not* receiving chemotherapy. However, in ER+ patients receiving chemotherapy, there was no association between carrying the G allele or GG genotype and risk of recurrence with receipt of chemotherapy (data not shown).

Association of MDM2 SNP309 with Recurrence of Early Stage Breast Cancer

Because stage III disease has the highest risk of recurrence due to its advanced nature, early stage disease was then analyzed separately. This included stage 0 through stage IIB disease. Again, there is an insignificant enrichment of the G allele in recurrent ER+ disease with hormone therapy. Overall, recurrence rates were similar between ER- disease and ER+ disease by genotype (Table 5). This finding is significant because hormone receptor positive disease has a better prognosis than hormone receptor negative disease in general.

Site-Specific Recurrence as a Function of MDM2 SNP309

We analyzed the site of recurrence for stages 0-III breast cancer as a function of MDM2 genotype. There were few cases where recurrences were multiple sites including local, regional, and distant loci (n=11). Therefore, most recurrences were either local/regional (n=70) or distant only (n=90). G allele carriers were more likely to have a pattern of distant recurrence as compared to local/regional recurrence: OR 2.06 CI [1.06-4.01], p=0.043. This suggests that MDM2 may play a role in biologic behavior of breast cancer, making metastasis more likely, but does not reflect response to specific therapies.

Risk of New Malignancy in Breast Cancer Patients as a Function of MDM2 SNP309

A significant number of patients with breast cancer are diagnosed with either a second breast cancer or cancer at another site. Therefore, we evaluated MDM2 SNP309 for its association with risk of developing second malignancies. Analysis was performed comparing TT vs. G allele. Patients carrying the G allele were more likely to develop a second breast cancer OR 1.75 [0.893-3.4]. However, this only represented a trend (p=0.09). There was no statistically significant risk for any other site of malignancy and MDM2 SNP309 genotype.

Combinatorial Analysis of MDM2 SNP309 with MDM4 Genotypes

Because we had previously shown that the variant G allele of MDM2 SNP309 associates with earlier age of diagnosis of ductal breast cancers (Bond and Hirshfield *et al.*, 2006) and more recently demonstrated in the same population that the variant T allele of MDM4 also results in earlier age of diagnosis of ductal breast cancers (appendix: Kulkarni *et al.*, 2009), we asked whether the combination of each risk allele would further modify the age at diagnosis of ductal breast cancers. The combination of the risk genotypes of MDM4 with MDM2 results in the earliest onset of estrogen receptor negative breast cancer. The mean age of diagnosis for MDM4/MDM2 combinations were 41.9 and 50.8 for TT/TG and CC/TG, respectively ($\Delta=8.9$ years; p=0.0099). There were insufficient numbers to compare homozygous variants for both MDM4 and MDM2 with the combination wildtype. There was only one TT/GG combination, diagnosed at age 42. In contrast, in estrogen receptor positive breast cancer, the MDM4 risk allele appears to negate the previously-observed earlier onset of the MDM2 SNP309 G allele. For example, when MDM4 was homozygous wildtype, there was a 1.8 year difference in age of onset where the GG combination was diagnosed earlier. When the MDM4 homozygous variant TT was combined with MDM2 SNP309, the age of diagnosis was 54.2 years and 51.9 years for the TT/TT and TT/TG combined genotypes. Although the combined TT/GG variants showed an age of diagnosis of 64 years, there were only 3 cases, underpowering this comparison.

Mdm2 is a protein that is highly regulated and has function that is highly coordinated with other proteins participating in the p53 stress response pathway. The importance of this regulation is that dysregulation of any member, e.g. as may occur due to presence of SNPs through any number of mechanisms, can weaken the tumor surveillance system. As such, we evaluated SNPs in other genes within the p53 pathway. We have already alluded to mdm2. However, our analysis extended to the following co-regulated genes: mdm4, p53, tp53bp1, ppp2r2b, tsc1, tsc2, perp.

PERP (appendix, manuscript submitted to JCO, Kulkarni *et al.*):

Purpose: A single nucleotide polymorphism (SNP) in PERP (rs2484067, G>A) has been associated with apoptotic efficiency of lymphoblastoid cell lines (LCLs) after gamma irradiation. Therefore, this SNP may be important in clinical outcomes of cancer. In this study, the association of this SNP with breast cancer recurrence was evaluated in conjunction with other prognostic factors.

Patients and Methods: Genotyping was performed on patient blood DNA and genotypes were linked with annotated clinical information. Recurrence analysis was limited to Caucasian cases (n=790) from a cohort of 1020 patients.

Results: Homozygous perp SNP genotypes had a similar distribution among Caucasian cases (AA: 25%; GG: 26%). The AA genotype was found to be an independent predictor of recurrence-free survival (RFS, hazard ratio [HR]: 1.9, 95% CI: 1.4-2.7, p=0.001) in Cox proportional hazards survival analysis, with AG and GG carriers having similar and more favorable RFS. The effect of AA genotype on RFS was more pronounced in patients receiving breast-conserving surgery followed by radiation (HR for AA vs. AG+GG: 2.23, 95% CI 1.29-3.83, p=0.0037), patients diagnosed at or above 51 years of age (10 year RFS rates AA 55%, AG+GG 79%; p<0.0001) or patients with stage III disease (HR: 2.5, 95% CI 1.26-4.95, p=0.009). AA genotype decreased the RFS in subgroups of Caucasian cases stratified by hormone receptor status, HER2 status, and whether they received chemotherapy.

Conclusion: PERP SNP rs2484067 may be a novel independent predictor of breast cancer prognosis and might be used as a tool to optimize treatment strategies.

P53 (abstract Rahim *et al.*, 2008):

Purpose: The tumor suppressor p53 regulates a variety of cell responses to stress signals. Alteration of p53 function is associated with tumorigenesis and may be related to mutation, LOH, or polymorphisms. The two variant alleles of the p53 codon 72 arginine/proline single nucleotide polymorphism (SNP) possess differential apoptotic and DNA repair abilities. The clinical relevance of these differences was evaluated through a genetic association study to determine its relevance in breast cancer.

Patients/Methods: DNA isolated from whole blood samples of breast cancer patients via a spin column method, were evaluated with a 5' nucleotidase-based assay on the ABI Prism Sequence Detection System. Genotypes for p53 codon 72 were then linked to clinical information. All genotypes were in Hardy Weinberg equilibrium. To reduce population heterogeneity, we focused on Caucasians with invasive ductal carcinoma. Sub-group analysis was performed by stratifying the Caucasians based on clinical phenotypes and molecular characteristics.

Results: A significant mean earlier age of onset was found for individuals homozygous for the arginine allele (GG) when compared to other genotypes (GC, CC) in estrogen receptor (ER) negative invasive ductal carcinomas (p<0.02). The mean age of diagnosis was 45.5 years versus 51.0 years of age respectively. The frequency of the arginine homozygous genotype was enriched in premenopausal women with breast cancer (72% vs. 49%). No difference was detected in estrogen receptor positive individuals.

Conclusion: P53 codon 72 could have particular relevance for assessing the risk of premenopausal women developing ER negative invasive ductal carcinoma. While arginine homozygotes have better apoptotic efficiency, a reduced DNA repair may result in genomic instability and somatic mutations as compared with proline homozygotes.

MDM4 (appendix, Kulkarni *et al.*, 2009):

Purpose: Murine double minute 4 (MDM4) shares significant structural homology with murine double minute 2 (MDM2) and interacts and regulates transcriptional activity of the tumor suppressor p53. In tumors with wild-type p53, there is often overexpression of MDM2 or MDM4 leading to functional inactivation of p53. A single-nucleotide polymorphism (SNP) in the promoter of human MDM2 (SNP309) was shown to associate with increased MDM2 expression and increased risk of cancer. This study evaluated the association of a SNP in human MDM4 (C>T) with age of onset of breast cancer in two independent cohorts.

Materials/Results: In cohort 1 of 675 patients, the average age of diagnosis for women with estrogen receptor (ER)-positive and ER-negative breast cancers was 53.2 and 48 years, respectively. In this cohort, homozygous variant (TT) carriers developed ER-negative carcinomas at an earlier age than homozygous wild-type (CC) or heterozygous (TC) such that the age at diagnosis was accelerated by 5.0 years ($P = 0.018$). This association was validated in a second cohort of breast cancer patients ($n = 148$), where TT carriers with ER-negative cancer developed the disease 3.8 years earlier than CC carriers ($P = 0.006$). The effect was more pronounced in Caucasians with ER-negative ductal carcinomas with TT homozygotes developing disease 7.5 years ($P = 0.031$) and 6.2 years ($P = 7 \times 10^{-5}$) earlier than CC carriers in cohorts 1 and 2, respectively. No association was seen in ER-positive ductal cancers.

Conclusion: The data support that the SNP in MDM4 only has a functional association in ER-negative breast cancer.

PPP2R2B (appendix, Vazquez *et al.*, 2011):

Purpose: A recent candidate gene association study identified a single nucleotide polymorphism (SNP) in the PPP2R2B gene (rs319217, A/G) that manifests allelic differences in the cellular responses to treatment with chemotherapeutic agents (Vazquez *et al.*, 2008). This gene encodes a regulatory subunit of protein phosphatase 2A (PP2A), one of the major Ser/Thr phosphatases implicated in the negative control of cell growth and division. Given the tumor suppressor activities of PP2A, here we evaluate whether this genetic variant associates with the age of diagnosis and recurrence of breast cancer in women.

Materials: To investigate the linkage disequilibrium in the vicinity of this SNP, PPP2R2B haplotypes were analyzed using HapMap data for 90 Caucasians. It is found that the A variant of rs319217 tags a haplotype that appears to be under positive selection in the Caucasian population, implying that this SNP is functional. Subsequently, associations with cellular responses were investigated using data reported by the NCI anticancer drug screen and associations with breast cancer clinical variables were analyzed in a cohort of 819 Caucasian women.

Results: The A allele associates with a better response of tumor derived cell lines, lower risk of breast cancer recurrence, later time to recurrence, and later age of diagnosis of breast cancer in Caucasian women.

Conclusion: Taken together these results indicate that the A variant of the rs319217 SNP is a marker of better prognosis in breast cancer.

TP53BP1 (appendix, Haffty *et al.*, 2011):

Purpose: TP53BP1 is a key component of radiation-induced deoxyribonucleic acid damage repair. The purpose of this study was to evaluate the significance of a known common single nucleotide polymorphism in this gene (rs560191) in patients treated with breast-conserving surgery and whole-breast irradiation (BCS + RT). **Methods and Materials:** The population

consisted of 176 premenopausal women treated with BCS + RT (median follow-up, 12 years). Genomic deoxyribonucleic acid was processed by use of TaqMan assays. Each allele for rs560191 was either C or G, so each patient was therefore classified as CC, CG, or GG. Patients were grouped as GG if they were homozygous for the variant G allele or CC–CG if they carried at least one copy of the common C allele (CC or CG).

Results: Of the 176 women, 124 (71%) were CC–CG and 52 (29%) were GG. The mean age was 44 years for GG vs. 38 years for CC–CG ($p < 0.001$). GG was more common in African-American women than white women (69% vs. 13%, $p < 0.001$) and more commonly estrogen receptor negative (70% vs. 49%, $p = 0.02$). There were no significant correlations of rs560191 with other critical variables. Despite the fact that GG patients were older, the 10-year rate of local relapses was higher (22% for GG vs. 12% for CC–CG, $p = 0.04$).

Conclusions: This novel avenue of investigation of polymorphisms in radiation repair/response genes in patients treated with BCS + RT suggests a correlation to local relapse. Additional evaluation is needed to assess the biological and functional significance of these single nucleotide polymorphisms, and larger confirmatory validation studies will be required to determine the clinical implications.

TSC1/TSC2 (appendix, Mehta *et al.*, 2010):

Background: TSC1 acts coordinately with TSC2 in a complex to inhibit mTOR, an emerging therapeutic target and known promoter of cell growth and cell cycle progression. Perturbation of the mTOR pathway, through abnormal expression or function of pathway genes, could lead to tumorigenesis. TSC1 and TSC2, expressed in normal mammary epithelial cells, have reduced expression in invasive breast cancer. Mutations in either TSC1 or TSC2 cause tuberous sclerosis, a disorder characterized by hamartomas affecting multiple organ systems. Single nucleotide polymorphisms (SNPs) have been implicated in risk and age at diagnosis of breast cancers. Our laboratory has previously reported on polymorphisms in p53 pathway genes that associate with age at diagnosis of breast cancer and on how these associations depend on breast cancer phenotypes. Systematic SNP association studies have yet to be performed on TSC1. We evaluated SNPs in TSC1 for their associations with clinical features of breast cancer.

Experimental Procedure: Eighteen TSC1 loci were genotyped in DNA from healthy volunteers and a haplotype constructed. SNPs were selected for further study using a bioinformatics approach based on SNP associations with drug response in NCI-60 cell lines and evidence of selection bias for haplotype frequencies. TaqMan allelic discrimination assays were performed on genomic DNA isolated from whole blood from over 900 women, recruited through CINJ clinics for four SNPs.

Results: We evaluated four TSC1 loci as potential genetic biomarkers of breast cancer risk and outcomes: TSC1+134802822 (SNP1), TSC1-134765855 (SNP2), TSC1+134760121 (SNP3) and TSC1-134794556 (SNP4). We found that postmenopausal women who were TT carriers of SNP1, had a 7 year later age at diagnosis of ER+, but not ER-, ductal carcinomas ($p=0.027$). We also found that SNP1 may modify the effect of BMI on age at diagnosis. Although high BMI is thought to increase risk for developing breast cancer; in our cohort, it is significantly associated with a 4 year later age at diagnosis of breast carcinoma. This effect was exaggerated in TT carriers of SNP1 whereby TT carriers had a 10.9 year later age at diagnosis as compared with CC carriers ($p=0.007$). None of the four TSC1 SNPs showed association with recurrence or other breast cancer phenotypes. SNPs 2-4 showed no association with age at diagnosis.

Conclusions: We have shown that TSC1 SNP1 may have a functional role in age at diagnosis of ER+, but not ER-, breast cancer and may be modified by BMI. We postulated that the presence of the T allele, but not the C allele, creates a site for estrogen receptor (ER) to bind an ERE in TSC1, resulting in activation of TSC1 transcription and increased inhibition of mTOR. Modulation of age of diagnosis by BMI may be further enhanced due to correlations between BMI with altered hormone levels.

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

In the grant period, we investigated the effects of anti-estrogen agent, fulvestrant, on mdm2 expression and sensitivity of human breast cancer cells to chemotherapeutic drugs. The results of this work are depicted in Jager *et al.* (manuscript in preparation, appendix). However, the summary of results is described below.

Purpose: MDM2 is overexpressed in several human malignancies and contributes to the development of cancer mainly through the inhibition of p53 tumor suppressor activity. MDM2 is an E3 ubiquitin ligase that catalyzes the polyubiquitylation of p53, marking p53 for proteasomal degradation. MDM2 overexpression is strongly related to the presence of estrogen receptor (ER) as the MDM2 promoter contains an estrogen response element. We tested the hypothesis that by blocking expression of MDM2 with antiestrogens, sensitivity to chemotherapeutic drugs could be restored in ER+ breast cancer cell lines. We focused on the antiestrogen fulvestrant since it is known to downregulate ER expression.

Experimental Design: We investigated the effects of fulvestrant on MDM2 expression and sensitivity of the ER+ human breast cancer cell lines T47D and MCF7 to chemotherapeutic drugs. MDM2 expression was measured at protein and mRNA levels by Western and qPCR. Cells were treated with either fulvestrant alone, chemotherapy alone, or in combination. Chemotherapeutic drugs included doxorubicin, etoposide or paclitaxel. Drug sensitivity assays (MTT assays) were performed. The CompuSyn computer program for quantitation of synergism and antagonism was used to determine if there was any change in the sensitivity of the breast cancer cells to these cytotoxic agents with fulvestrant.

Results: Fulvestrant down-regulated Mdm2 expression through increasing the turnover rate of this oncoprotein in the ER positive human breast cancer cell lines MCF7 and T47D. Fulvestrant not only blocked the up-regulation Mdm2 caused by estradiol, but also decreased Mdm2 protein to the level below that seen in the breast cancer cells cultured in the absence of estradiol. Fulvestrant had no effects on activity of p53 and level of MDM2 mRNA, but enhanced the turnover rate of MDM2 protein. Combination of fulvestrant with doxorubicin, etoposide or paclitaxel showed a synergistic effect on these chemotherapeutic drugs.

Conclusion: This study demonstrates that fulvestrant possesses a suppressive effect on Mdm2 expression and a synergistic effect with chemotherapeutic drugs in estrogen receptor positive human breast cancer cells. These results provide a rationale and support for testing the combination of fulvestrant with chemotherapy as a new therapeutic strategy for patients with advanced breast cancers.

MDM2 SNP309 allele-specific effects of genistein

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 the anti-estrogen 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 1).

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 2). 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 cell line-specific.

KEY RESEARCH ACCOMPLISHMENTS

- 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 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 breast cancer patients for evaluation of the role of MDM2 SNP309 on outcomes associated with chemotherapy and hormonal therapy.
- We analyzed associations between MDM4, PERP, PPP2R2B, p53, TP53BP1, and TSC1/TSC2 SNPs and breast cancer phenotypes.
- We analyzed associations between MDM2 SNP309 and breast cancer phenotypes.
- We observed that mdm2 tissue expression in primary breast tumors correlates with local and locoregional recurrence of breast cancer in women with stage I or stage II tumors undergoing breast conserving surgery and radiation. Our data indicate that MDM2 is an independent predictor for local recurrence in conservatively treated, early stage breast cancer, suggesting its possible use as a prognostic marker.
- We have analyzed associations between SNPs in genes that co-regulate the p53 pathway in conjunction with MDM2 and breast cancer phenotypes. These genes include PPP2R2B, MDM4, PERP, TSC1, TSC2, p53.

REPORTABLE OUTCOMES

Manuscripts

Neboori H, Wu H, Kulkarni D, Goyal S, Schiff D, Moran MS, Yang JM, Hirshfield KM, Haffty BG. The Prognostic Value of MDM2 Expression in Early Stage Breast Cancer Treated with Breast Conserving Surgery and Radiotherapy (BCS+RT), *Breast Journal*, submitted for review.

Jäger AV, Dolfi SC, Hait WN, Toppmeyer D, Haffty B, Yang JM, Kim M, Hirshfield. Effects of Fulvestrant on Mdm2 Expression and Sensitivity of Human Breast Carcinoma Cells to Chemotherapeutic Drugs. Manuscript in preparation.

Kulkarni D, Dolfi, S, Lu SE, Chen C, Moore, D, Vazquez A, Harris S, Toppmeyer DL, Levine AJ, Demissie K, Hirshfield KM. A Polymorphic Variant in Perp Associates with Increased Risk of Breast Cancer Recurrence, *Journal of Clinical Oncology*, *submitted for review*.

Kulkarni D, Vazquez A, Haffty B, Bandera E, Hu W, Sun Y, Toppmeyer D, Levine A, Hirshfield KM. A polymorphic variant in MDM4 associates with accelerated age of onset of estrogen receptor negative breast cancer. *Carcinogenesis* 2009, 30:1910-5.

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Haffty BG, Goyal S, Green C, Schiff D, Yang Q, Moran MS, Kulkarni D, Ganesan S, Hirshfield KM. Evaluation Of Single Nucleotide Polymorphisms (SNP) In The P53 Binding Protein Gene (TP53BP1), In Breast Cancer Patients Treated With Breast Conserving Surgery And Whole Breast Irradiation (BCS+RT). *International Journal of Radiation Oncology, Biology, Biophysics*, 2011, 80: 385-391.

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Hirshfield KM, Rebbeck TR, Levine A. Germline mutations and polymorphisms in the origins of cancers in women. *J Oncol.* 2010, 2010:297671. (Invited review)

Abstracts

Nayak M, Hait WN, Hirshfield KM, Haffty B, Yang, JM. A Single Nucleotide Polymorphism in the MDM2 Promoter (SNP 309) Alters the Sensitivity to Topoisomerase II-Targeting Drugs. Presented (poster presentation) at the Era of Hope Meeting 2008, Washington, D.C.

Kulkarni DA, Vazquez A, Levine AJ, Hirshfield KM. A polymorphic variant in PP2A strongly associates with risk and recurrence of breast cancer. Presented (poster presentation) at the American Association for Cancer Research Special Conference in- Candidate Pathways, Whole Genome Scans: Reconciling Results, Looking into the Future, AACR Molecular Epidemiology Working Group 2008, Carefree, A.Z.

Kulkarni DA, Levine AJ, Haffty B, Hirshfield, KM. Polymorphic variants in *Perp* show genotype-specific risk of recurrence and development of second hormone receptor-positive breast cancers. Presented (poster presentation) at the American Association for Cancer Research 2008, San Diego, C.A.

Rahim H, Levine AJ, Hirshfield, KM. P53 codon 72 Genotype is Linked to an Accelerated Age of Diagnosis in Caucasian Women with Estrogen Receptor Negative Invasive Ductal Carcinoma. Presented (poster presentation) at the Annual Retreat on Cancer Research 2008, Piscataway, N.J.

Kulkarni, DK, Vazquez A, Levine AJ, Hirshfield, KM. A polymorphic variant in PP2A strongly associates with risk and recurrence of breast cancer. Presented (oral presentation) at the Annual Retreat on Cancer Research 2008, Piscataway, N.J.

Jager A, Hirshfield K, Hait WN, Haffty, B, Yang, JM. The selective estrogen receptor down-regulator, fulvestrant, decreases MDM2 expression and enhances sensitivity of human breast carcinoma cells to chemotherapeutic drugs. Presented (poster presentation) at the AACR 100th Annual Meeting 2009, Abstracts manual p. 280.

Jäger A, Hait WN, Toppmeyer D, Haffty B, Hirshfield K, Yang JM. Fulvestrant Decreases MDM2 Expression and Enhances Sensitivity of Human Breast Carcinoma Cells to Chemotherapeutic Drugs. Presented (poster presentation) at the Annual Retreat on Cancer Research 2009, Piscataway, N.J.

Rahim H, Levine A, Hirshfield KM. P53 Codon 72 Genotype is Linked to an Accelerated Age of Diagnosis in Caucasian Women with Estrogen Receptor Negative Invasive Ductal Carcinoma. Presented (poster presentation) at Aresty 2009, Rutgers University, New Brunswick, N.J.

Sreenath M, Vazquez A, Hirshfield KM. Tuberous sclerosis genes TSC1 and TSC2, and breast cancer risk. Presented (poster presentation) at the Annual Retreat on Cancer Research 2009, Piscataway, N.J.

Kulkarni D, Vazquez A, Hu W, Sun Y, Toppmeyer D, Levine A, Hirshfield KM. A Polymorphic Variant in MDM4 Associates with Accelerated Age of Onset of ER Negative Breast Cancer. Presented (oral presentation) at the Annual Retreat on Cancer Research 2009, Piscataway, N.J.

Haffty BG, Goyal S, Green C, Schiff D, Yang Q, Moran MS, Kulkarni D, Ganesan S, Hirshfield KM. Evaluation Of Single Nucleotide Polymorphisms (SNP) In The P53 Binding Protein Gene (TP53BP1), In Breast Cancer Patients Treated With Breast Conserving Surgery And Whole Breast Irradiation (BCS+RT). Presented (oral presentation) at ASTRO 2009.

Sreenath M, Vazquez A, Hirshfield KM. Polymorphic variants in TSC1 associate with breast cancer phenotypes. Presented (poster presentation) at the AACR Annual Meeting 2010, Chicago, I.L.

Neboori H, Wu H, Schiff D, Goyal S, Moran M, Yang JM, Hirshfield KM, Haffty BG. The prognostic value of MDM2, MDM4 (MDMX), and p53 for local recurrence in early stage breast cancer treated with breast conserving surgery and radiotherapy. Presented (oral presentation) at the Annual Retreat on Cancer Research 2011, Piscataway, N.J.

Neboori H, Wu H, Schiff D, Goyal S, Moran M, Yang JM, Hirshfield KM, Haffty BG. The prognostic value of MDM2, MDM4 (MDMX), and p53 for local recurrence in early stage breast cancer treated with breast conserving surgery and radiotherapy. Presented (poster presentation) at the Era of Hope Meeting 2011, Orlando, F.L.

Dolfi SC, Kulkarni D, Lu S, Chen C, Moore D, Vazquez A, Harris S, Toppmeyer D, Levine AJ, Hirshfield KM. Prognostic Role of Perp in Breast Cancer. Presented (poster presentation) at the AACR Annual Meeting 2012, Chicago, I.L.

Degree obtained that are supported by this award:

None

Development of DNA Repository:

- Collection of peripheral blood from breast cancer patients and healthy controls (n=3,426)
- Purification of DNA from peripheral blood
- Collection of self-reported information from study participants
- Development of clinical data warehouse for follow-up on study participants
- Collection of clinical information on breast cancer patients

List of personnel receiving pay from the research effort:

Kim M. Hirshfield, M.D., Ph.D.

Adriana Jager, Ph.D.

Sonia Dolfi, Ph.D. (nee Picinich)

JinMing Yang, Ph.D.

Bruce Haffty, M.D.

Funding Based on Work Supported by DOD Grant:

Current-

Breast Cancer Research Foundation

“Single Nucleotide Polymorphisms in the p53, p63, and p73 Pathways”

October 1, 2011- September 30, 2012

Role: Co-PI

The goals of this project are the identify SNPs in genes in the p53, p63, and p73 pathways that impact on the risk of development of breast cancer in offspring of parents that carry these genetic variations.

New Jersey Commission on Cancer Research

“Epithelial Integrity and Breast Cancer Recurrence”

July 1, 2008- June 30, 2010 in no-cost extension

Role: PI

This project is to elucidate the molecular mechanism of genes associated with epithelial integrity on breast cancer cell behavior while characterizing SNPs in those genes for associations with recurrence.

Completed-

Ruth Estrin Goldberg Memorial in Cancer Research

“mTOR Pathway Dysregulation in Breast Cancer”

July 1, 2009- June 30, 2010

Role: PI

This funded sample collection from breast cancer patients.

Breast Cancer Research Foundation

“Single Nucleotide Polymorphisms in the p53 and Mdm2 Genes Lower the Age of Onset of Estrogen Receptor Positive Breast Cancers in Women”

October 1, 2009- September 30, 2010

Role: Co-PI

The goals of this project are to identify SNPs in genes in the p53 pathway that impact on age of onset of breast cancer, investigate the role of interactions with SNPs in the steroid hormone metabolism pathway, and to construct haplotypes in genes in the p53 pathway.

Breast Cancer Research Foundation, “Single Nucleotide Polymorphisms in the p53, p63, and p73 Pathways”

October 1, 2010- September 30, 2011

Role: Co-PI

The goals of this project are to identify SNPs in genes in the p53, p63, and p73 pathways that impact on the risk of development of breast cancer in offspring of parents that carry these genetic variations.

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. MDM2 SNP309 G allele associates with increased risk of distant recurrence of breast cancer.
4. MDM2 SNP309 G allele may associate with increased risk of contralateral breast cancer events.
5. Mdm2 tissue expression in primary breast tumors is prognostic for both local and locoregional recurrence of breast cancer in women with stage I or stage II tumors undergoing breast conserving surgery and radiation.
6. SNPs in p53, PERP, MDM4, TSC1/TSC2, PPP2R2B, TP53BP1 demonstrate significant associations with breast cancer phenotypes, e.g. risk and/or treatment response, in a manner that reflects breast cancer molecular features.

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APPENDICES

Publications/Manuscripts

- a) Neboori H, Wu H, Kulkarni D, Goyal S, Schiff D, Moran MS, Yang JM, Hirshfield KM, Haffty BG. The Prognostic Value of MDM2 Expression in Early Stage Breast Cancer Treated with Breast Conserving Surgery and Radiotherapy (BCS+RT), *Breast Journal* 2012, *under review*.
- b) Neboori H, Wu H, Kulkarni D, Goyal S, Schiff D, Moran MS, Yang JM, Hirshfield KM, Haffty BG. The Prognostic Value of MDM2 Expression in Early Stage Breast Cancer Treated with Breast Conserving Surgery and Radiotherapy (BCS+RT), *review and author reply provided from submission to the journal Cancer*.
- c) Kulkarni D, Dolfi SC, Lu SE, Chen C, Moore D, Vazquez A, Harris S, Toppmeyer DL, Levine AJ, Demissie K, Hirshfield KM. A Polymorphic Variant in Perp Associates with Increased Risk of Breast Cancer Recurrence, *Journal of Clinical Oncology*, *submitted for review*.
- d) Kulkarni D, Vazquez A, Haffty B, Bandera E, Hu W, Sun Y, Toppmeyer D, Levine A, Hirshfield KM. A polymorphic variant in MDM4 associates with accelerated age of onset of estrogen receptor negative breast cancer. *Carcinogenesis* 2009, 30:1910-5.
- e) Vazquez A, Kulkarni D, Grochola LF, Bond GL, Barnard N, Toppmeyer DL, Levine AJ, Hirshfield KM. A genetic variant in a PP2A regulatory subunit encoded by the PPP2R2B gene associates with altered breast cancer risk and recurrence. *International J Cancer* 2011, 128: 2335-43.
- f) Haffty BG, Goyal S, Green C, Schiff D, Yang Q, Moran MS, Kulkarni D, Ganesan S, Hirshfield KM. Evaluation Of Single Nucleotide Polymorphisms (SNP) In The P53 Binding Protein Gene (TP53BP1), In Breast Cancer Patients Treated With Breast Conserving Surgery And Whole Breast Irradiation (BCS+RT). *International Journal of Radiation Oncology, Biology, Biophysics*, 2011, 80: 385-391.
- g) Mehta MS, Vazquez A, Kulkarni DA, Kerrigan JE, Atwal G, Metsugi S, Toppmeyer DL, Levine AJ, Hirshfield KM. Polymorphic variants in TSC1 and TSC2 and their association with breast cancer phenotypes. *Breast Cancer Research and Treatment* 2010, 125:861-8.
- h) Hirshfield KM, Rebbeck TR, Levine A. Germline mutations and polymorphisms in the origins of cancers in women. *J Oncol*. 2010, 2010:297671. (Invited review)
- i) Jäger AV, Dolfi SC, Hait WN, Toppmeyer D, Haffty B, Yang JM, Kim M, Hirshfield. Effects of Fulvestrant on Mdm2 Expression and Sensitivity of Human Breast Carcinoma Cells to Chemotherapeutic Drugs. *Manuscript in preparation*.

The Prognostic Value of MDM2 Expression in Early Stage Breast Cancer Treated with Breast
Conserving Surgery and Radiotherapy (BCS+RT)

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Running Head: MDM2 expression in breast cancer

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Abstract:

Purpose: MDM2 and MDM4 are involved in negative regulation of p53 protein and apoptosis.

As such, we hypothesized aberrant MDM2/MDM4/p53 protein expression to have prognostic value in determining local outcome in early-stage breast cancer treated with BCS + RT.

Methods: Breast tumors from 514 women with early-stage invasive breast cancer treated with BCS+RT were constructed into a tissue microarray and stained for MDM2, MDM4 and p53. The staining profile of each tumor was correlated with ipsilateral breast recurrence survival (IBRFS), nodal recurrence free survival (NRFS) and loco-regional recurrence free survival (LRFS). The associations between staining and outcomes were assessed in a multivariate model.

Results: The median follow up of the cohort was 7.23 years. Positive scoring of MDM2 was correlated with worse 10 year IBRFS (75.0% v. 90.0%; $p=0.032$) by Kaplan-Meier analysis. Neither MDM4 nor p53 correlated with any endpoints. Combinations of markers did not enhance prognostic value. On multivariate analysis incorporating MDM2, tumor size, margin status and nodal status, MDM2 was significant for IBRFS ($p=0.0009$). As MDM2 is an estrogen-responsive gene, its prognostic value was assessed on ER+ and ER- subsets. MDM2 was found to be predictive of local recurrence only in the ER+ subset on multivariate analysis for IBFRS ($p=0.0037$).

Conclusion: Our data indicate that MDM2 is an independent predictor for local recurrence in conservatively treated, early stage breast cancer, suggesting its possible use as a prognostic marker.

Introduction:

Murine double minute 2 (MDM2) and its related ortholog MDM4, are involved in the negative regulation of p53 including ubiquitin-mediated targeting of p53 for proteolytic degradation. Moreover, MDM2 is induced by p53 thus creating a negative feedback loop¹⁻⁵. Apart from its p53 ubiquitination function, MDM2 has other functions including nuclear-cytoplasmic shuttling of p53 and prominent interactions with various ribosomal proteins^{6,7}. MDM4 has similar structure and function to MDM2; however, it can be degraded by MDM2⁸⁻¹⁰. Appropriate expression of p53 propels cells down apoptotic pathways, but this progression may be abated by overexpression of MDM2/MDM4 and subsequent degradation of p53. It follows that dysregulation of p53 by MDM2/MDM4 could potentially cause resistance to treatment through inability of the cell to undergo p53-mediated apoptosis.

Several studies have reported the significance of MDM2 in cancers of the prostate, breast and ovary¹¹⁻¹⁴. In breast cancer, MDM2 has been extensively studied as prognostic marker for overall and disease specific survival. Over expression of MDM2 has been found to associate with worse breast cancer specific survival and to have a role in enhancing estrogen receptor alpha (ER α) mediated gene expression^{11, 15-17}. However, the significance of MDM2 on local recurrence of breast cancer has not been adequately explored¹⁴. Specifically, there have been no reports assessing the significance of MDM2 on local recurrence in early stage breast cancer treated with BCS + RT. Additionally, relatively little is known regarding the effects of over/under expression of MDM4 and p53 on local recurrence.

The purpose of this study was to ascertain if MDM2/MDM4/p53 expression in a cohort of women with Stage I and II invasive breast cancers treated with BCS + RT, will have prognostic value in predicting locoregional relapse.

Materials & Methods

Tissue microarray and patient characteristics

The protocol was reviewed and approved by the Human Investigations Committee at Yale University, School of Medicine. Patients selected for the study were treated at the Yale University Department of Therapeutic Radiology, New Haven, CT between 1975 and 2003 and had their breast cancer tissue cores compiled into a tissue microarray (TMA). Only patients that had paraffin embedded tissues in the pathology archives of the hospital were included in the array. The TMA comprised of tumor cores from 514 patients was used for this study. Patients had tumor cores evaluated for MDM2, MDM4, p53 and hormone receptors. Information about the patients' clinical history was obtained from a clinical database as previously described¹⁸. All patients had histological evidence of invasive early stage breast carcinoma and were treated with BCS+RT. The size of the primary tumor was considered to be the largest tumor diameter reported by the pathologist after surgical excision. Margin status was defined as positive if tumor cells were present on the most peripheral slide of the tumor specimen. Following surgery, patients received standard whole breast irradiation to a total median dose to the breast of 48 Gy and a total tumor bed dose of 64 Gy; regional nodes were treated to a median dose of 46 Gy, as clinically indicated^{19,20}. Adjuvant systemic chemotherapy and/or adjuvant hormone therapy was administered as clinically indicated in accordance with standard practices during the time interval.

Ipsilateral breast tumor recurrence was defined as clinically and biopsy-proven relapse in the ipsilateral breast. IBRFS was defined from the time of initial diagnosis to ipsilateral breast tumor relapse; NRFS was defined as the time from initial diagnosis to the time of biopsy proven nodal

relapse; LRFS was defined as time from initial diagnosis to either ipsilateral breast recurrence or nodal recurrence. The vast majority of local recurrences occurred prior to distant recurrences or were diagnosed concurrently. There were 3 patients in which local failure occurred 2 months or more after the diagnosis of distant failure.

Immunohistochemical study

Immunohistochemical analysis was performed on 5 μ m thick tissue sections prepared from formalin-fixed, paraffin-embedded tissue from the constructed tissue microarray block. Tissue sections were de-paraffinized and then quenched in 2% hydrogen peroxide–methanol solution. Samples were then pretreated to promote antigen retrieval with the DAKO Target Retrieval Solution (DAKO, Carpinteria, CA, USA). Slides were then incubated with MDM2 antibody (1:200; Neomarker, CA, USA, Rb 9218), MDM4 antibody (1:200; Bethyl laboratories, Montgomery, TX, USA) and p53 antibody (1:4000; DAKO, Carpinteria, California, M7001). After incubation, the slides were washed in phosphate buffered saline, and a biotinylated secondary antibody was applied. Samples were then applied with DAKO streptavidin-horseradish peroxidase using LSAB + Kit. DAKO DAB (3,3-diaminobenzidine tetrahydrochloride dehydrate) was then applied as a chromogenic substrate. A known positive case was included as positive control. For the negative control, the primary antibody was replaced with non-immune rabbit serum. Slides were read by a pathologist blinded to clinical outcome. For MDM2 staining, a tumor core was only considered positive if the pathologist reported staining in the cytoplasm of more than 70% of tumor cells. Others have used a similar cutoff¹⁶. MDM4 staining was considered positive if more than 10% of cells were stained. This was similar to the cutoff chosen by others.²¹ For p53, a tumor core was considered positive if more than 10% of cells were stained. As per NCCN guidelines, ER/PR were considered positive

when more than 1% of cells stained were stained positive and Her2 neu was considered positive if Her2/ neu staining had an intensity of 2+ or more, and negative if staining intensity was 0 or 1 (I think this is the correct criteria).

Statistical analysis

Descriptive statistics comparing MDM2 expression with conventional markers of tumor aggressiveness were analyzed by standard chi-squared tests, or, when appropriate, Fisher's exact test. Estimates of disease-free survival were calculated by the Kaplan–Meier product-limit method, and the differences were assessed by the log-rank test. Probabilities of survival were calculated from the date of breast cancer diagnosis to either the date at which relapse was clinically identified or the date of last contact. **Date of death from breast cancer, date of death from another cause, and date of last contact were all defined as 'date of last contact.'** The occurrence of contra lateral breast cancer was treated as an occurrence of a new primary and did not impact our analysis. Univariate and multivariate survival analysis was carried out using Cox's proportional hazard regression model. Multivariate analysis was used to assess the independent contribution of each variable to survival. All P values were two-tailed, and < 0.05 level was considered statistically significant. A computer program package SAS (Version 9.2, SAS Institute, Cary, NC) was used for all statistical testing and management of the database.

Results

Descriptive statistics and correlations with clinicopathologic markers

The description of the entire patient cohort is shown in Table 1. The median age at diagnosis of the entire patient cohort was 55 years (range 25-88 years) with 40% of patients being younger than 50 at the time of diagnosis. 63% ($n= 183$), 29% ($n= 86$) and 10% ($n= 40$) of the patient population were ER, PR and HER2/*neu* positive respectively. 25% ($n= 72$) of the patient population was negative for all three markers. 46% ($n= 140$) of patient population received adjuvant hormonal therapy and 36% ($n= 108$) of the patient population received adjuvant chemotherapy. 14% ($n= 72$) received both adjuvant hormone and chemo therapy.

As of September 2009, median follow-up on this cohort was 7.23 years during which 17.5% ($n=90$) of patients died. 9.7% ($n=50$) of patients experienced ipsilateral breast recurrence, 2.3% ($n=12$) experienced nodal relapse and 11.3% ($n=58$) experienced locoregional recurrence.

Immunohistochemical staining results

The predominant intracellular staining of MDM2 was cytoplasmic. Immunoreactivity was completely absent in some tumor cores while in others, the number of immunoreactive cells ranged from very few to the majority of cells. Samples of both positive and negative cores and slides are shown in Figure 1. 8.6% ($n=26$) of the cores were scored as positive for MDM2 staining while 91.4% were scored as negative.

Staining for MDM4 was less specific with 56.7% ($n=174$) of tumor cores being scored as positive (data not shown). Immunoreactivity for MDM4 encompassed a range from very few

cells stained positive to almost all cells being stained positive. Staining for MDM4 was predominantly nuclear. 31.0% ($n=140$) of patients stained positive of p53 with the staining being predominantly nuclear (data not shown).

Association between MDM2 and patient outcomes

10 year survival analysis was performed for ipsilateral breast recurrence free survival (IBRFS), nodal recurrence free survival (NRFS), and locoregional recurrence free (LRFS) as a function of MDM2, MDM4 and p53 expression. Only MDM2 was found to be a significant predictor of IBRFS (75.0% v. 90.0%; $p=0.032$) and LRFS (65.6% v. 88.5%; $p=0.017$) by log rank test (Fig. 2).

Combinations of the three markers (*i.e.* MDM2 positive and p53 positive) were assessed to see if any combination could add to the prognostic value that MDM2 expression alone offered. MDM2 positivity predicted for worse IBRFS (89.16% v. 61.0%; $p= 0.0063$) and LRFS (87.3% v. 40.6%; $p= 0.0015$) in the p53 negative group; but was unable to predict for worse local or regional outcomes in the p53 positive group. If any combination of three markers was used, the sample size and number of events for each combination became too small. Expression of these markers was also analyzed for other endpoints including overall survival and distant metastatic free survival; all p -values were non-significant (data not shown).

Univariate analysis was performed using MDM2, age, race, ER status, PR status, HER2/*neu*, systemic therapy, triple negative status, nodal status, tumor size and margin status. Relevant results are displayed in Table 2. When assessing for IBRFS, only tumor size, margin status, and

MDM2 positivity were found to be significant ($p=0.0002$; $p=0.0137$; $p=0.0416$, respectively). When assessing for LRFS, tumor size, margin status and MDM2 were again found to be significant ($p=0.0001$; $p=0.0367$; $p=0.0229$, respectively). It should be noted that while nodal status approached significance for NRFS ($p=0.0686$), no variable was significant for NRFS possibly due to the small number of events. Considering that MDM2 was the only marker that was significant on univariate and log-rank tests, it was the only IHC marker used for multivariate analysis.

A multivariate analysis was done for IBRFS and LRFS using the three variables that were significant in univariate analysis: margin status, tumor size and MDM2 expression. Nodal status was also included as it approached significance for nodal recurrence on univariate analysis; which in turn may have an impact on LRFS. MDM2 was again found to be a significant predictor of IBRFS and LRFS ($p=0.0009$ and 0.0003 respectively). Additionally tumor size was once again found to be independently predictive of IBRFS and LRFS ($p=0.0007$ and $p=0.0010$). The results are shown in table 3. A separate analysis was performed excluding margin positive patients and MDM2 expression again was significant for local IBRFS and LRFS.

As MDM2 is an estrogen responsive, prosurvival gene, a subset analysis of ER+ and ER- tumors was done. MDM2 was found to be predictive of IBRFS and LRFS on univariate analysis only in the ER+ subset ($p=0.0003$ and $p=0.0011$ respectively, data not shown). The results were similarly validated in multivariate studies ($p=0.0037$ and $p=0.0037$ respectively). The prognostic value of MDM2 was however not similarly observed in the ER- subset (table 4).

Discussion:

Through ubiquitination, MDM2 marks p53 for degradation and hence diminishes its cellular capacity to carry out p53-mediated apoptosis. *In vivo* and *in vitro* studies have shown MDM2 to be a key negative regulator of p53 and its apoptotic pathways¹⁻⁴. While many studies have explored the significance of MDM2 in prostate and breast cancers with a focus on overall recurrence, there is a lack of data reporting the significance of MDM2 protein expression on local and regional outcomes following breast radiation^{11, 13, 14}. In addition, much evidence has recently been shed on the structurally similar protein MDM4, but few have explored its significance in breast cancer and no studies have explored its significance in relation to local and regional outcomes in early stage breast cancer treated with BCS+RT. Lastly, although p53 has been well studied *in vivo* and *in vitro*, associations between expression and outcomes have not yielded the predicted results. As such we hoped to study the prognostic potential of these protein markers for assessing local response to radiation in early stage breast cancer.

In this study, we showed that MDM2 overexpression is associated with significantly worse local recurrence in stage I and stage II invasive breast cancer treated with BCS+RT as defined by ipsilateral breast recurrence free survival (IBRFS). When examining MDM4 and p53 expression however, we were unable to appreciate a similar prognostic value. We acknowledge that 11.3% local regional recurrence rate is somewhat high by modern standards, however it should be noted that many of these patients were treated in an earlier era and a much larger proportion of this cohort has triple negative breast cancer. Lastly, as MDM2 expression has been linked to active ER α signaling,^{11, 15} we sought to determine if the prognostic significance of MDM2 was associated with this subset. The fact that MDM2 was only found to have predictive value in the

ER+ subset may be explained by higher biologic activity of MDM2 in estrogen responsive tumors. Numerous studies have identified associations between MDM2 and ER α expression in breast tissue and breast cancer cell lines^{11, 22, 23}. In vitro data have demonstrated that MDM2 is an estrogen-responsive gene through action of activated ER α on the estrogen response element in the first intron of MDM2²⁴⁻²⁷. Kim et al.¹⁵ demonstrated MDM2-enhanced ER α -mediated transactivation in the presence of wildtype p53 suggesting a protein-protein interactions between MDM2 and ER α leading to these functional responses. Additionally, the finding of the significance of MDM2 only in the p53 negative subset may allude to greater importance of MDM2 expression in p53 wild type tumors. P53 expression, normally low in the absence of cell stress, is thought to increase with the presence of p53 mutation due to resultant stabilization of a dysfunctional protein. In p53 mutant tumors, the mutation alone may play a dominant role in the dysregulation of the p53 pathway, negating need for alterations in other p53 pathway genes to provide a survival advantage.

These findings suggest that while both MDM2 and MDM4 are involved in the negative regulation of p53 and subsequent arrest of apoptosis; only MDM2 protein expression may have prognostic value in determining local outcomes in early stage breast cancer treated with BCS+RT. These results add to a growing body of evidence demonstrating that increased expression of MDM2 has negative prognostic value for various endpoints in multiple tumor types^{11-16, 28, 29}. Additionally, it should be noted that MDM2 was found to be an independent predictor for local outcomes in early stage breast cancer regardless of patients having received chemotherapy or hormone therapy.

Interestingly, there is a correlation between MDM2 expression and Her2 phenotype, *i.e.* higher expression of MDM2 was more common in Her2 overexpressors (Table 1). Nearly an equal number of tumors stained positive for Her2, as were positive for p53. A hypothesis has been put forth that patients having p53 mutations are more likely to develop Her2 overexpressing breast tumors³⁰. At least one study has identified a relationship between Her2 expression with MDM2 expression³¹. However, in that study, MDM2 is downregulated in the presence of wild type p53. Therefore, the association observed in this dataset may, in part, reflect the p53-Her2 pathway interaction. This dataset though, does not have sufficient information to validate this hypothesis.

The limitations of this study include: that is retrospective; not all patients were treated in the same time period and hence received varying chemotherapy and hormone therapy regimens contributing to higher rates of locoregional relapse; and lack of direct p53 gene sequencing to assess if what mutations are present in each individual tumor.

To our knowledge this is the first study assessing the significance of protein expression of MDM2, MDM4 and p53 for local recurrence in conservatively treated, early stage breast cancer. This cohort demonstrated that increased expression of MDM2 correlated with worse local outcomes in early stage breast cancer treated with breast conserving surgery and radiotherapy. Moreover, on subset analysis, it was found that MDM2 was only found to have prognostic value in the ER + subset alluding to the importance of this protein in ER+ breast cancer. These results add to the growing body of evidence assessing the prognostic value of MDM2 expression, and its potential as a therapeutic target in combination with radiation therapy. If confirmed in larger studies, these results can have significant clinical implications. However, further studies are

needed to assess its importance in regional recurrence, and of MDM4 and combinations of other markers in prognosis.

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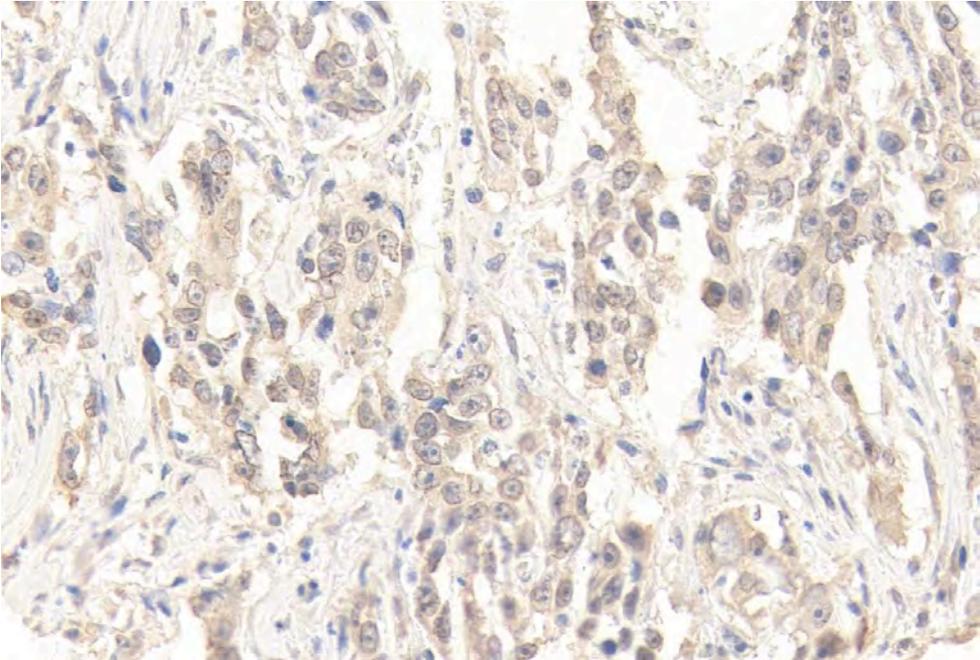
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Figure1: Representative immunohistochemical staining of MDM2 in breast tumors (40x): a.

MDM2 positive; b. MDM2 negative

a.



b.

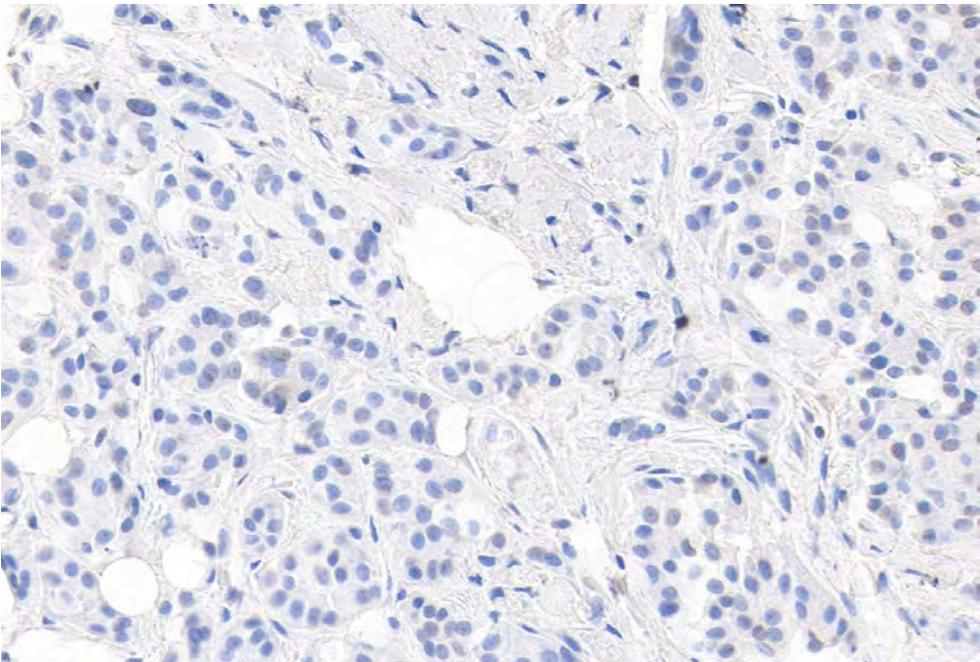
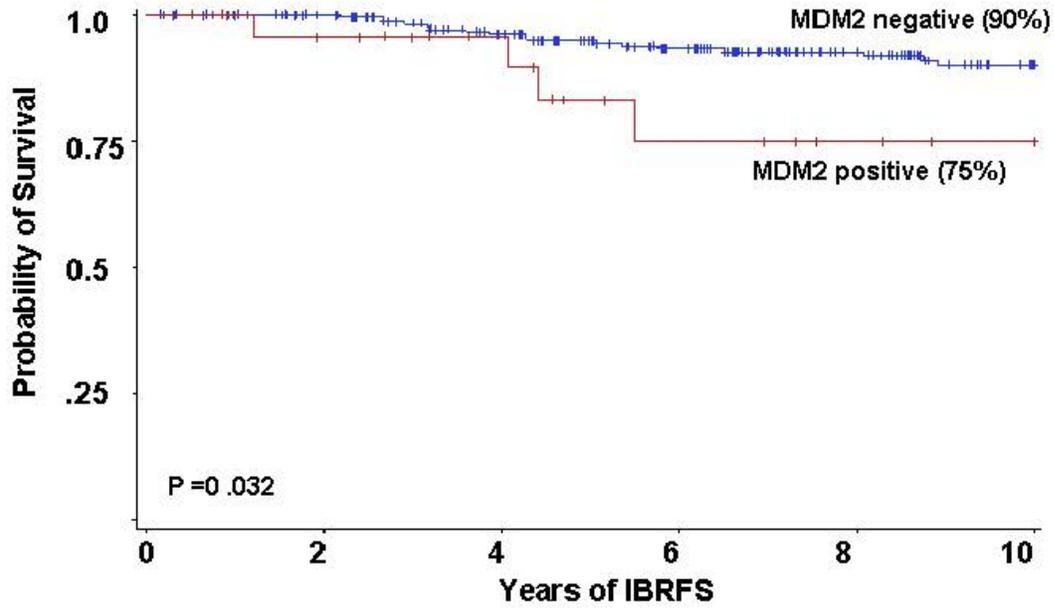
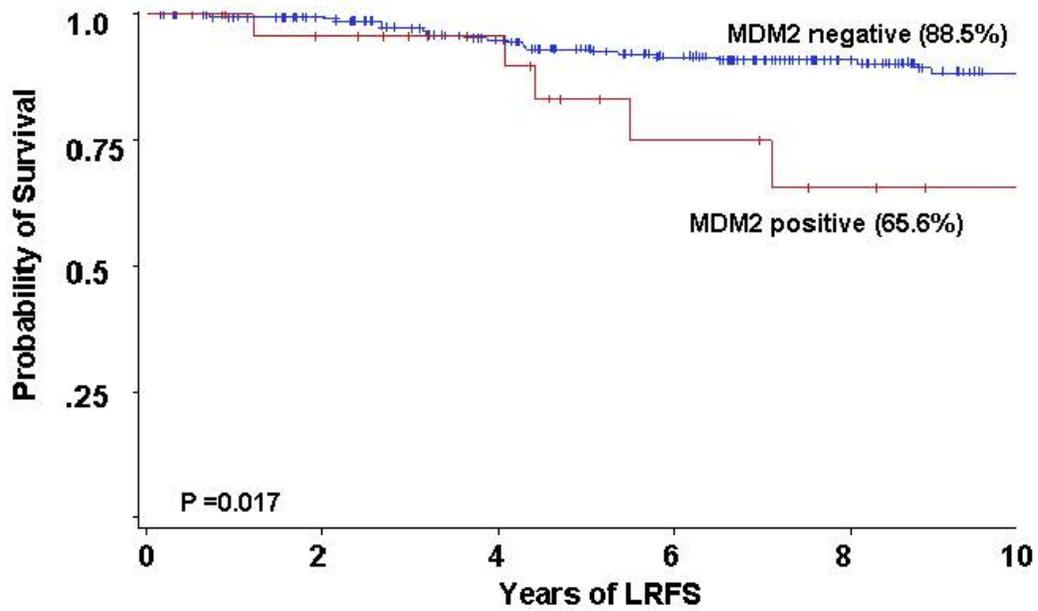


Figure2: Kaplan Meier survival curves of MDM2+ (red) v. MDM2- (blue) for: a. Ipsilateral breast recurrence b. Locoregional recurrence

a.



b.



Dear Dr. Pollock,

Thank you for the reviewer of our paper entitled “The Prognostic Value of MDM2 Expression in Early Stage Breast Cancer Treated with Breast Conserving Surgery and Radiotherapy (BCS+RT)”. The reviewers’ comments were most helpful and we believe the attached revised manuscript is much improved as it addresses all comments and concerns. We appreciate the constructive comments. We have revised the manuscript in accordance with the reviewer comments as follows:

Reviewers: 1

Comments to the Author

The authors provide an interesting analysis showing a relationship between MDM2 expression and local-regional recurrence after breast conservation therapy. These data offer original findings of a new and potentially important biomarker for local-regional outcome. The paper is well written and analyzed.

- We appreciate this reviewer’s favorable comments regarding the novelty and quality of the paper.

General

1. Please provide relationship of these biomarkers to DM and OS

- A summary of the relationship of the markers with distant metastasis and overall survival are now included in the results. As shown below, MDM2 was not seen to be a predictor for worse 10 year distant and overall survival outcomes.

Outcome	P-value
Overall survival	0.1622
Distant Metastatic Free Survival	0.8333

2. What was the relationship of MDM2 positivity and LRR in the p53 normal cases and did this differ compared to the p53 mutated cases?

- The differential results of MDM2 for the p53 mutated and wildtype cases are given in the results. We apologize for having omitted these results earlier. MDM2 positivity was able to predict for worse outcome in the p53 negative group but was unable to predict for worse outcome in the p53 positive group.

Specific

page 4 - first paragraph - DNA damage doesn't "ultimately progress through apoptotic pathways" - what about reproductive cell death and necrosis - is this not felt to be the major mechanism by which radiation causes cellular death?

- We apologize for overstating this and have modified the statement regarding apoptotic cell death.

page 4 - line 13 - I don't understand "and transcriptional activity" - the sentence structure is worded such that this happens after proteolytic degradation.

- The sentence has been modified as requested. We apologize for the confusing sentence structure.

page 7 - suggest, "read by a pathologist blinded to the clinical outcome"

- The statement regarding the blinded pathologist has been modified as suggested.

page 9 - in interpretation of the results would you consider that 11.3% LRR rate with a median f/u of 7 yrs somewhat high by modern standards?

- The LRR of 11.3% is somewhat high by modern standards, but many of these patients were treated in an earlier era, and this is consistent with results reported. We have added a statement in the discussion regarding this.

page 14 - suggest a short paragraph concerning the limitations

- retrospective

- higher rate of LRR

- lower rate of hormone therapy (only 2/3 of ER+ received hormonal therapy)

- lack of p53 sequencing for mutations

- We have added a paragraph in the discussion regarding the limitations and appreciate this comment.

Reviewers: 2

Comments to the Author

In this manuscript, Naboori et al. present a biomarker study of MDM2 and its prognostic value in local outcomes in breast cancer. While the result of the study is interesting, there are some statistical and methodological concerns with the study:

How were patients selected for inclusion into the tissue microarray? There are relatively few patients for a long time interval (1975 to 2003).

- We apologize for not stating selection criteria more clearly. All patients treated at the facility with BCS+RT who had available paraffin embedded tissues in the pathology archives of the hospital were included in the tissue microarray. This is now more clearly stated in the methods.

The study mentions 514 patients - however, in Table 1, approximately 300 patients are included for statistical analysis. Due to technical reasons, it is expected that not all patients will have complete biomarker data, but the authors need to explain why a large proportion of their patient cohort was not included in statistical analysis. Similarly, does

the number of locoregional events (n = 58) refer to the entire cohort of 514 patients or the 300 patients that were analyzed?

- Again we apologize for not clearly stating the patients who were in the final analysis. Not all portions of all cores were present in every section of the array. Only the cores which were completely evaluable were included in the study ... i.e. if a large portion of the core was missing then it was not analyzed and not included for subsequent analysis. This decision was left to the pathologist. The number of loco-regional events refers to the entire cohort of 514 patients.

For outcomes analysis, how were competing events analyzed (i.e. distant metastasis, death from breast cancer, death from other causes, contralateral breast cancer, etc.)?

- All local recurrences were included in the analysis. The vast majority of local relapses occurred prior to distant metastasis. Death from breast cancer, death from another cause, and loss to follow up were defined as censoring events. The occurrence of contra-lateral breast cancer was treated as an occurrence of a new primary and did not impact our analysis. This is now stated in the methods.

10-year outcomes are reported, however, the median follow-up of this cohort is 7 years. Would 5-year outcomes be more appropriate for this study?

- With a median follow up of 7 years we feel that both 5 and 10 year results are relevant. However, as the 10 year results provide a better indication of patient outcomes and may be of better clinical value to the readers of your journal, we have chosen to include the 10 year results for the results section of the manuscript.

The authors analyze 3 outcomes, however, no variables were found to be significant for nodal recurrence free survival (NRFS). There are very few nodal recurrences, so I would question the need to present outcomes for both local recurrence and locoregional recurrence (the difference in survival curves in Figure 2a and 2b are minimal).

Consequently, the authors should emphasize that this study is primarily examining the effect of MDM2 on ipsilateral breast recurrence.

- We agree with the comments of the reviewer regarding the small number of nodal events and have shifted the emphasis from loco-regional to local recurrence.

The statement, "MDM2 expressors were found to be 9.1 times more likely to experience locoregional recurrence free survival (Page 11, Line 32) is not the correct interpretation of the statistical test.

- The statement has been modified as per the request of the reviewer. Thank you for notifying us of this inconsistency.

Patients with positive surgical margins were included in the survival analysis. Surgical margins are a treatment related variable that has a significant effect on local recurrence. Because this manuscript focuses on the molecular biology of MDM2 and its effect on local recurrence, it may be preferable to exclude patients with positive surgical margins.

- We concur that margin status is a critical factor in local relapse and both our univariate and multivariate analysis has confirmed that. Given that both MDM2 and margin status were significant in the multivariate analysis, we felt that all patients with and without margin status should be included so as not to add additional selection bias into the study. However, as the reviewer suggests we did perform a separate analysis with the positive surgical margin patients included and MDM2 remained a significant prognostic factor for local relapse. This information is included in the revised paper.

The authors use a cut-off of 70% for MDM2 status. In the study referenced by the authors (Turbin DA et al), the cut-off used is any staining of breast cancer cells. The referenced study also applied a cut-off of 80% for “strong positivity,” but these cases were combined with the “weak positive” cases for statistical analysis. The authors need to justify why they decide to use a different cut-off for MDM2.

- We used the 70% cutoff as we believed it most closely replicated the criteria used by Turbin et al. While the 80% cutoff by Turbin et al. is more specific than our 70% criteria, they combined their “strong positives” with weak positives, hence including more patients and decreasing specificity of their staining. To account for the lowered specificity, we similarly decreased our threshold.

In the discussion, the authors refer to additional studies on MDM2, but it is not clear how these additional studies support the authors’ findings in this manuscript. For example, Page 13, Paragraph 2 (Line 11) does not appear to support the finding that the prognostic effect of MDM2 is isolated to the ER positive subgroup. As well, Page 13, Paragraph 4 (Line 49) does not provide an hypothesis for the association between MDM2 and Her2.

- Thank you for bringing these to our attention, the statements no longer appear in the revised version of the manuscript.

Page 13, Line 40 – MDM2 cannot be said to have prognostic significance that is independent of another variable if that variable was not included in the multivariable analysis.

- We have deleted this statement from the manuscript.

We would like to thank both reviewers for their thoughtful and constructive criticism; we believe the paper is stronger because of it.

Sincerely,
Hanmanth Neboori, Bruce Haffty.

A Polymorphic Variant in Perp Associates with Increased Risk of Breast Cancer
Recurrence

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Abbreviations

PERP p53 effector of apoptosis related to PMP22

LCL lymphoblastoid cell line

SNP single nucleotide polymorphism

ER estrogen receptor

PR progesterone receptor

BCS breast conserving surgery

XRT radiation therapy

RFS recurrence free survival

LOH loss of heterozygosity

IHC immunohistochemistry

FISH fluorescence in situ hybridization

HR hormone receptor

PV pemphigus vulgaris

Abstract:

Purpose:

A single nucleotide polymorphism (SNP) in PERP (rs2484067, G>A) has been associated with apoptotic efficiency of lymphoblastoid cell lines (LCLs) after gamma irradiation. Therefore, this SNP may be important in clinical outcomes of cancer. In this study, the association of this SNP with breast cancer recurrence was evaluated in conjunction with other prognostic factors.

Patients and Methods:

Genotyping was performed on patient blood DNA and genotypes were linked with annotated clinical information. Recurrence analysis was limited to Caucasian cases (n=790) from a cohort of 1020 patients.

Results:

Homozygous perp SNP genotypes had a similar distribution among Caucasian cases (AA: 25%; GG: 26%). The AA genotype was found to be an independent predictor of recurrence-free survival (RFS, hazard ratio [HR]: 1.9, 95% CI: 1.4-2.7, p=0.001) in Cox proportional hazards survival analysis, with AG and GG carriers having similar and more favorable RFS. The effect of AA genotype on RFS was more pronounced in patients

receiving breast-conserving surgery followed by radiation (HR for AA vs. AG+GG: 2.23, 95% CI 1.29-3.83, $p=0.0037$), patients diagnosed at or above 51 years of age (10 year RFS rates AA 55% , AG+GG 79%; $p<0.0001$) or patients with stage III disease (HR: 2.5, 95% CI 1.26-4.95, $p=0.009$). AA genotype decreased the RFS in subgroups of Caucasian cases stratified by hormone receptor status, HER2 status, and whether they received chemotherapy.

Conclusion:

PERP SNP rs2484067 may be a novel independent predictor of breast cancer prognosis and might be used as a tool to optimize treatment strategies.

Introduction:

PERP (p53 apoptosis effector related to PMP-22), a tetraspan plasma membrane protein, is a novel p53-inducible effector of apoptosis and is a direct target of p63, a p53 family member¹⁻³. PERP is known to be downregulated in melanomas and carcinomas including breast, more so in metastatic lesions as compared to primary tumors^{2,3}. PERP is selectively induced by p53 in cells undergoing apoptosis but not G1 cell cycle arrest^{1,4,5}. Moreover, overexpression of PERP can induce apoptosis even in the absence of p53 activation⁵. PERP also plays an important role in p63-regulated development of stratified epithelium through localization in desmosomes⁶. *Perp*-null mice develop severe blistering of the skin and die postnatally, pointing towards a critical role in desmosome assembly and maintenance of epithelial integrity^{6,7}. PERP is also implicated in the pathogenesis of pemphigus vulgaris, an autoimmune disease with desmosomal defects of the skin and oral mucosa,⁸ and in ankyloblepharon ectodermal dysplasia, a disorder with multiple defects in organs of ectodermal origin⁹.

A functional SNP in the second intron of human *PERP* gene (rs2484067, IVS2-76 G>A), identified through a cell culture assay system using lymphoblastoid cell lines (LCLs), showed that the SNP was associated with varying efficiency of p53-induced apoptosis in LCLs subjected to gamma-radiation^{10,11}. In a recent evaluation, the same SNP in PERP did not appear to impact the risk of breast cancer development in BRCA1 or BRCA2 mutation carriers of Ashkenazi Jewish descent¹².

Age at diagnosis, stage at diagnosis, hormone receptor status, and altered gene expression due to mutation or amplification are several factors implicated in the alteration of therapeutic response and prognosis of breast cancer¹³⁻¹⁷. A growing number of genetic polymorphisms, many within genes of the p53 stress response pathway, have been implicated in the overall risk of developing breast cancer,¹² as well as in determining the clinical phenotypes such as age of onset¹⁸⁻²⁴, lymph node metastasis, aggressiveness of breast cancer, and relapse-free and overall survival²⁵⁻²⁷. Because *PERP* has been implicated in both apoptotic response and cell adhesion, the association of the previously identified intronic SNP (rs2484067) in *PERP* with breast cancer recurrence was examined in this study.

Methods:

Patient Population and Study Design:

Patients diagnosed with stage 0-III breast cancer and evaluated at The Cancer Institute of New Jersey (CINJ) were sequentially enrolled from 2004-2009 in an Institutional Review Board-approved protocol. Diagnosis for all subjects was biopsy-confirmed by a breast pathologist. Clinical data abstraction from medical records was performed for date of and age at diagnosis, race, histologic subtype and stage of breast cancer (determined by AJCC Tumor Node Metastasis classification), molecular features, treatment and date of breast cancer recurrence. Date of diagnosis was defined as the first date of biopsy-confirmed breast cancer. Breast cancers were considered estrogen (ER) and progesterone receptor

(PR) positive if immunohistochemistry (IHC) was > 10%. HER2 IHC score of 3+ or fluorescent-in situ-hybridization (FISH) score >2.0 were considered positive. Subjects were considered to have breast cancer recurrence when the disease was detected and confirmed by biopsy (in the majority of cases) at local, regional and distant sites at a time greater than one month from initiation of adjuvant therapy.

DNA Isolation and Genotyping:

After obtaining informed consent, venipuncture was performed to procure five mL of whole blood from which genomic DNA was isolated using a spin column based DNA extraction kit according to manufacturer's protocol (QIA-Amp midi-kit, Qiagen, Valencia, CA). The rs2484067 locus was analyzed using a TaqMan allelic discrimination assay. Briefly, the 20 μ L reaction volume was comprised of 5-10ng of genomic DNA, allele-specific fluorescent probes (50nM each VIC and FAM), 225nM each forward and reverse primers, and 1X Genotyping Master Mix (Applied Biosystems, Foster City, CA). Amplification steps were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Amplicons were measured with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Alleles at this SNP locus were A or G.

Statistical Methods:

Deviation from Hardy-Weinberg equilibrium for this locus by race was performed using web-based software (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>).

Recurrence-free survival (RFS) was defined as time from date of initial diagnosis until date of recurrence. Patients who were alive with no recurrent disease at the time of analysis were considered censored. Survival functions for RFS were computed using the Kaplan-Meier method²⁸ and correlated with PERP genotype using log-rank tests²⁸.

Adjustment for additional covariates was performed using Cox proportional hazards regression analysis, e.g. stage at diagnosis, adjuvant medical treatment. Stratified analyses were performed by cancer treatment (breast conserving surgery [BCS] and mastectomy), age of diagnosis, and stage to examine the association between PERP genotype and recurrence by various levels of these variables. Results were presented as a hazard ratio (HR) and 95% confidence interval. Computations were carried out using SAS Version 9.1 (SAS Institute, www.sas.com) and R Version 2.11.1 (R Foundation for Statistical Computing, Vienna, Austria, www.R-project.org).

Results:

PERP SNP rs2484067 has population-specific frequencies:

Genotyping of 1020 cases revealed that the PERP locus (rs2484067) demonstrated race-specific genotype frequencies. The A allele was prevalent in 69% of African Americans (n=59), 79% of Indians (n=26), and 92% of the Asian population (n=41). However, A and G alleles were equally represented among Caucasians (n=790) with frequencies of 49% and 51%, respectively, and were not a function of Ashkenazi Jewish descent (data not shown). Hispanics (n=63) had similar allelic distributions as Caucasians (A: 53%; G:

47%). Forty cases either did not have available information for race (n=4) or because of multi-national heritage, did not match a homogeneous group (n=36). Overall, genotype frequencies were consistent with those in the HapMap consortium ²⁹. Despite these inter-racial variations, this SNP was in Hardy-Weinberg equilibrium in all subgroups (data not shown).

Patient Characteristics:

The primary objective of this study was to determine associations between PERP genotypes at SNP locus rs2484067 and risk of breast cancer recurrence. Although the recurrence analysis was performed on breast cancer cases of all ethnicities, only the analysis of Caucasian patients (77% of cases) is presented to reduce heterogeneity in the study population. Sample size was not sufficient to perform analysis on other racial subgroups. Table 1 depicts the characteristics of Caucasian patients in the breast cancer cohort. Median age at diagnosis was 51 years, with a range of 19-95 years. The majority of breast cancers were of ductal origin, stage 0-II at diagnosis, hormone-receptor positive, and HER2 negative. Most cases received chemotherapy or hormone therapy after initial surgery (65% and 73%, respectively). The frequency of PERP genotypes among Caucasians cases was 25% AA, 49% AG, and 26% GG.

Distribution of PERP SNP by Tumor Characteristics and Treatment:

Table 1 presents the distribution of PERP genotype by tumor characteristics and treatment status. The frequency of PERP genotypes was similar by ER, PR and HER2 status. On the other hand, while AG/GG carriers were more frequent in stage I breast cancer the frequency of AA carriers was higher in stage II and III breast cancers. There were no marked differences in the distribution of PERP genotypes by age, histologic subtype, choice of definitive surgery, or use of chemotherapy and hormonal therapy (Table 1).

Impact of SNP rs2484067 on Breast Cancer Prognosis:

Association between PERP genotypes and breast cancer recurrence was assessed before and after adjusting for clinicopathologic variables. Among AA carriers, 28% developed breast cancer recurrences as opposed to 16% and 14% cases of AG and GG carriers. Caucasian breast cancer patients carrying the AA genotype were twice as likely to develop a recurrence as compared with those carrying the GG genotype (HR 2.4; 95% CI 1.5-3.8, $p=0.0007$). Subjects with the AA genotype were more likely (HR 1.9; 95% CI: 1.4-2.7, $p=0.001$) to exhibit recurrence than those carrying the G allele (AG+GG). Because of similarity of heterozygotes to GG homozygotes, AA carriers were compared to AG+GG carriers in subsequent analyses. Kaplan-Meier survival analysis among Caucasian cases demonstrated significant differences in recurrence-free survival (RFS)

for AA versus AG+GG carriers (Fig 1A, $p=0.0001$) with a 10-year RFS of 56% and 77% and a median time to recurrence of 11.9 years and 24.6 years, respectively.

To assess the effect of AA genotype on outcomes of initial treatment, Caucasian cases were stratified by definitive breast surgery: breast-conserving surgery plus radiation (BCS+XRT; $n=368$) or mastectomy alone (mastectomy-XRT; $n=117$). The stage distribution among patients who received BCS plus XRT was not statistically different from patients receiving mastectomy alone ($p=0.40$). As displayed in Table 2, the AA genotype was associated with a 2.2-fold higher risk of breast cancer recurrence in patients who underwent BCS plus XRT (HR 2.2; 95% CI: 1.3-3.8, $p=0.0037$). This association persisted after adjusting for stage at diagnosis (HR 1.9; 95% CI 1.12-3.35, $p=0.018$).

There was no significant association between PERP genotype and breast cancer recurrence among patients who underwent mastectomy alone (crude HR 1.71; 95% CI: 0.88-3.30, $p=0.11$; adjusted HR 1.8; 95% CI 0.88-3.51, $p=0.11$). Kaplan-Meier survival analysis showed worse RFS for AA carriers compared to AG+GG carriers in patients receiving BCS plus XRT (Fig 1B, 10-year survival rate for AA: 55% vs. AG+GG: 82%; $p=0.0029$) but no such genotype-specific effect on RFS in the mastectomy alone group (Fig 1C, 10-year RFS rate for AA: 79% vs. AG+GG: 84%; $p=0.2957$).

In order to test the interaction of AA genotype with age at diagnosis, Caucasian cases were stratified by age at diagnosis: younger than 51 years ($n=384$); 51 years and older ($n=347$). Kaplan-Meier survival analysis indicated that PERP AA genotype did not further diminish the RFS as compared to AG or GG carriers in patients younger than 51

years of age (Fig 2A, $p=0.22$). The 10 year RFS and median time to recurrence for AA were 55% and 10.2 years, while AG+GG were 75% and 24.6 years, respectively. On the other hand, the deleterious effects of AA genotype on RFS became more apparent in patients diagnosed at 51 years of age and older (Fig 2B, 10 year RFS rate for AA vs. AG+GG at 55% and 79%, respectively; $p<0.0001$).

To assess whether there was any stage-specific enrichment of a PERP genotype that could in turn influence the rate of recurrence, Caucasian breast cancer cases were stratified by PERP genotype and stage at diagnosis. Kaplan Meier survival analysis in patients stratified by stages I ($n=286$), II ($n=254$), and III ($n=85$) and PERP genotype demonstrated that AA carriers had lower RFS at all stages (Fig 3), but only a statistically significant association with worse RFS was evident among stage III (Fig 3C, 10 year RFS for AA: 24%, for AG+GG: 55%, and median time to recurrence for AA: 4.1 years, for AG+GG: 12.3 years; $p=0.0058$). After accounting for the effect of chemotherapy using the Cox-Proportional hazard model, the AA genotype was associated with 2.5 times higher risk of breast cancer recurrence in patients with stage III disease at diagnosis (Table 3; adjusted HR 2.5; 95% CI 1.26-4.95, $p=0.009$).

PERP AA Genotype Interacts with Other Prognostic Indicators in Predicting RFS in Multivariate Analysis:

To assess the interaction of PERP genotype with the other known prognostic indicators of breast cancer, Caucasian breast cancer cases were stratified by hormone receptor status, HER2 status, stage, and use of adjuvant chemotherapy (Table 3). After accounting for the effects of stage at diagnosis and use of chemotherapy, the AA genotype was associated with 80% higher risk of recurrence (HR 1.8; 95% CI: 1.10-2.94, $p=0.019$) in ER-positive cases. Among ER-negative cases, the AA genotype was associated with a 73% higher risk of recurrence (HR 1.73; 95% CI 0.91-3.29), but this estimate did not achieve statistical significance ($p=0.095$). Similarly, the AA genotype was associated with a two-fold (HR 2.2; 95% CI: 0.74-6.54) and a 56% (HR 1.56; 95% CI: 0.96-2.52) elevated risk of recurrence in HER2 positive and HER2 negative subjects, respectively. There was an appreciable difference in the association between AA genotype and breast cancer recurrence in PR-negative cases (HR 1.81; 95% CI: 1.06-3.09, $p=0.03$), but no significant association in PR-positive cases (HR 1.64; 95% CI: 0.93-2.89).

Discussion:

This is the first association study that analyzes the impact of the SNP rs2484067 in PERP on breast cancer prognosis. While a case-control study performed using the same SNP did not yield any positive associations between the SNP and the risk of developing breast cancer in BRCA1/2 mutation carriers of Ashkenazi Jewish descent¹², the current study demonstrates an association of this SNP with increased risk of breast cancer recurrence. In particular, Caucasian breast cancer patients carrying the AA genotype at the PERP

SNP locus exhibit an increased propensity to develop recurrent disease compared to those carrying AG or GG genotypes.

The standard of definitive surgical care for locally-confined breast cancer is either BCS+XRT or mastectomy where both treatments have equivalent clinical outcomes³⁰. In this study, PERP AA genotype was associated with worse clinical outcomes in patients receiving BCS plus XRT, but not in those receiving mastectomy alone. This suggests that cases with AA genotype at this PERP locus may benefit from more radical surgical treatment. PERP AA genotype also has deleterious effect on RFS in an age- and stage-specific manner, *i.e.* those diagnosed ≥ 51 years age and stage 3 cancers. Breast cancers diagnosed at a later age are more likely hormone receptor positive and tend to have better prognosis than cancers diagnosed at a younger age¹³. However, p53-dependent apoptotic efficiency of cells decreases with age, leading to increased susceptibility for tumor development³¹. PERP has well-documented roles in p53-dependent or independent apoptotic response^{1,5} as well as cell-cell adhesion⁶: both mechanisms implicated in response to radio- and chemotherapy, cell motility and in turn, cancer metastasis. It is possible that PERP AA genotype downregulates PERP expression or function, further reducing the apoptotic potential of tumors in patients diagnosed at a later age (≥ 51), leading to residual disease and recurrence. Similarly, PERP AA genotype may increase the invasive potential of the tumors by diminishing desmosome function, leading to decreased cell adhesion and hence advanced stage disease at diagnosis and enhanced propensity to travel to distant sites. The observed interaction of PERP AA genotype was also seen with other known prognostic indicators to modify the risk of recurrence.

PERP expression is diminished in cancer cell lines and metastatic lesions of a variety of tissues^{2,3} as well as being downregulated at protein and mRNA levels in the more aggressive and highly metastatic subtypes of uveal melanoma³². Esophageal cancers show an association between diminished expression of PERP and less than complete pathological response to neoadjuvant chemoradiation suggesting a role of PERP induced apoptosis in response to therapy³³. Furthermore, another SNP in PERP (rs648802) has been shown to significantly enhance the risk of GERD patients to develop adenocarcinoma of the esophagus by two-fold³⁴. Nonetheless, *Perp* knock-out mice are resistant to papilloma development suggesting that lack of PERP may not be required for tumor initiation, but, required for tumor maintenance and progression³⁵. The attenuation of PERP expression in tumors has been attributed to mechanisms such as loss of heterozygosity (LOH) at the PERP locus^{3,36} on chromosome 6q, a frequent site for LOH in many cancers including breast cancer³⁷.

The SNP rs2484067 is located in the second intron of human *PERP*, 76 nucleotides away from the 5' boundary of the second exon. Haplotype analysis of *PERP* accounting for about 30-35 SNPs spanning the gene (Fig. 4) suggests that no other SNPs in *PERP* have similar frequencies as this locus in the Caucasian population. In other words, the SNP does not tag a haplotype that may be under positive selection pressure and it is not linked to any other functional SNP in *PERP*. Bioinformatic analysis suggests that it does not alter any transcription factor binding sites, splice site or any predicted micro-RNA

binding site (A. Vazquez, personal communication). The SNP may still affect PERP expression and/or function in ways that are yet to be determined.

One strength of this analysis is that extensive pathologic information was available for examining associations between PERP genotypes and known prognostic factors.

Nonetheless, the length of follow up on this cohort could limit the associations because time to recurrence is related to molecular features of breast cancer, *i.e.* known long duration between hormone receptor positive primaries and recurrence. Furthermore, because of heterogeneity in breast cancer and its treatment, the number of patients for evaluating the interaction of PERP AA genotype with multiple known prognostic indicators combined was too small. For example, to assess the combined effect of PERP genotype, definitive treatment, age and stage at diagnosis on RFS, Caucasian cases receiving either BCS+XRT (n=368) or mastectomy alone (n=117) were divided into multiple subgroups. The subgroup of Caucasian AA carriers, diagnosed \geq age 51 years with stage 2 disease and receiving BCS+XRT had worse 10 year RFS compared to matched cases undergoing mastectomy alone (49% and 91%, respectively; $p=0.078$). The finding suggests a trend but power is limited by small numbers in each subgroup. If true, the result would suggest that a subgroup of patients with PERP AA genotype that are older than 51 years and are stage 2 or higher may benefit from mastectomy over breast conserving surgery to improve their RFS. A larger sample sizes is required to confirm these findings. Similarly, effects associated with specific hormonal therapies, specific site of recurrence or use of trastuzumab was limited by number of cases. Finally, it is not clear whether the data could be generalized to other populations based on the unique

genotype distribution in Caucasians as compared with other ethnic groups and differing genetic background in other populations.

Acknowledgments

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Figure 1: Kaplan-Meier recurrence-free survival analyses of A) all Caucasian cases, B) Caucasian cases undergoing BCS+XRT, and C) Caucasian cases undergoing mastectomy-XRT, stratified according to the genotype at PERP SNP locus rs2484067 as AA and GG/AG, are represented. The p-values are by log rank test.

Figure 2: Kaplan-Meier recurrence-free survival analysis of Caucasian cases A) below 51 and B) at or above 51 years at the time of diagnosis divided based on the genotype at PERP SNP locus rs2484067 as AA and GG/AG is represented. The p-values are by log rank test.

Figure 3: Kaplan-Meier recurrence-free survival analysis of Caucasian cases with A) stage 1, B) stage 2, and C) stage 3 breast cancer at diagnosis, stratified according to the genotype at PERP SNP locus rs2484067 as AA and GG/AG is represented. The p-values are by log rank test.

Figure 4: Haplotype structure of PERP. The PERP locus rs2484067 was evaluated in this study. Haplotype frequencies for the major haplotypes are represented on the right for each allele of rs2484067.

Table 1. Subject and tumor characteristics by PERP genotype^a

	AA (n= 187)	AG/GG (n= 563)	All subjects (n= 750)	p-value ^b
Age groups, %				0.127
≤ 40	16.6	17.4	17.2	
40-50	33.2	29.5	30.4	
50-60	32.1	26.1	27.6	
60-70	11.2	18.1	16.4	
>70	7.0	8.9	8.4	
Histological subtype, %				0.788
Ductal	75.0	73.0	73.5	
Lobular	10.7	10.7	10.7	
Other	14.3	16.3	15.8	
Stage at diagnosis, %				0.006
0	10.1	11.8	11.4	
I	30.7	43.9	40.6	
II	43.6	33.5	36.0	
III	15.6	10.8	12.0	
Definitive surgery, %				0.361
BCS plus XRT	58.4	61.1	60.4	
BCS alone	2.0	4.6	3.9	
Mastectomy plus XRT	18.8	15.6	16.4	
Mastectomy alone	20.8	18.7	19.2	
ER-positive, %	75.1	76.5	76.1	0.716
PR-positive, %	62.4	63.9	63.5	0.710
HER2-positive, %	19.0	20.1	19.7	0.785
Chemotherapy				0.107
Yes	70.1	63.2	64.9	
No	29.9	36.8	35.1	
Hormonal therapy				0.905
Yes	73.7	73.3	73.4	
No	26.3	26.8	26.6	

^a This cohort was derived from a total number of 1020 cases. Numbers represent cases for which the variable of interest was known. Only cases with all known variables were included in analyses.

^b p-value based on chi-square test comparing the variable distribution between PERP genotype AA vs. AG/GG.

Table 2: Recurrence-free survival (RFS) by PERP genotype as a function of definitive surgery, radiation, hormone therapy, and chemotherapy.

Factor	Type	HR (Unadjusted)	95%CI lower	95%CI upper	p- value	HR ^a (Adjusted)	95%CI lower	95%CI upper	p- value
Definitive surgery	BCS+xrt	2.23	1.29	3.83	0.004	1.94	1.12	3.35	0.018
	BCS-xrt	3.84	0.34	43.38	0.277	-	-	-	-
	Mastectomy+xrt	1.71	0.88	3.30	0.111	1.76	0.88	3.51	0.108
	Mastectomy-xrt	1.67	0.63	4.39	0.301	1.36	0.46	4.03	0.576
Hormone Therapy ^b	Yes	1.94	1.09	3.45	0.025	1.57	0.86	2.87	0.145
	No	2.42	1.07	5.46	0.033	2.40	1.04	5.52	0.041
Chemotherapy ^b	Yes	2.02	1.22	3.37	0.007	1.80	1.05	3.07	0.032
	No	1.11	0.24	5.21	0.898	1.31	0.28	6.17	0.737

a: HR adjusted for stage at diagnosis.

b: Analysis includes only those patients undergoing either BCS+XRT and Mastectomy-XRT.

Table 3: Effect of PERP AA genotype on recurrence-free-survival (RFS) of various subgroups among Caucasian cases

Factor	Type	HR	95%CI	95%CI	p-value	HR	95%CI	95%CI	p-value
		(Crude)	lower	upper		(Adjusted ^b)	lower	upper	
Stage at diagnosis	0	2.39	0.39	14.35	0.341	0	0	0.99	-
	1	2.13	0.92	4.93	0.079	1.99	0.82	4.79	0.127
	2	1.45	0.70	2.99	0.316	1.45	0.70	2.99	0.318
	3	3.10	0.79	12.23	0.106	3.02	0.77	11.91	0.114
ER Status	Positive	2.44	1.32	4.52	0.005	1.85	0.95	3.62	0.071
	Negative	1.79	0.77	4.15	0.175	1.64	0.68	3.97	0.272
PR Status	Positive	1.89	0.89	4.04	0.099	1.69	0.77	3.69	0.192
	Negative	2.17	1.13	4.15	0.020	1.69	0.85	3.39	0.134
Her2 Status	Positive	1.12	0.23	5.39	0.888	1.37	0.24	7.81	0.723
	Negative	2.03	1.12	3.68	0.020	1.77	0.95	3.31	0.072

a: Analysis includes only those patients undergoing either BCS+XRT and Mastectomy-XRT.

b: HR adjusted for chemotherapy and stage at diagnosis, except for stage at diagnosis which only chemotherapy is adjusted.

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Figure 1.

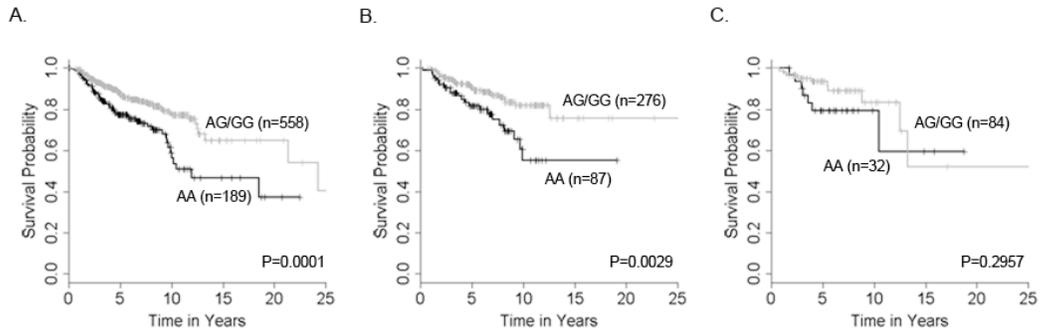


Figure 2.

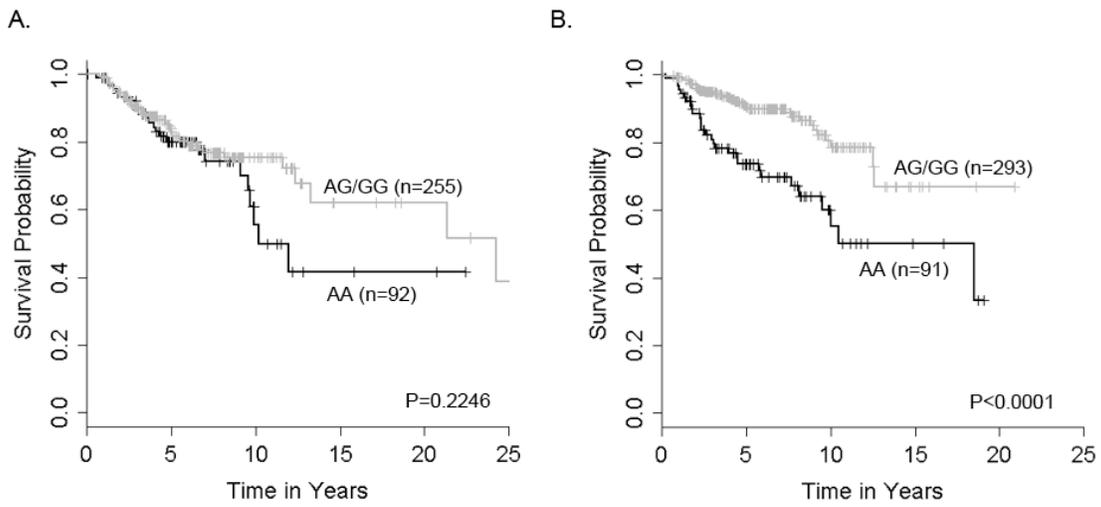


Figure 3.

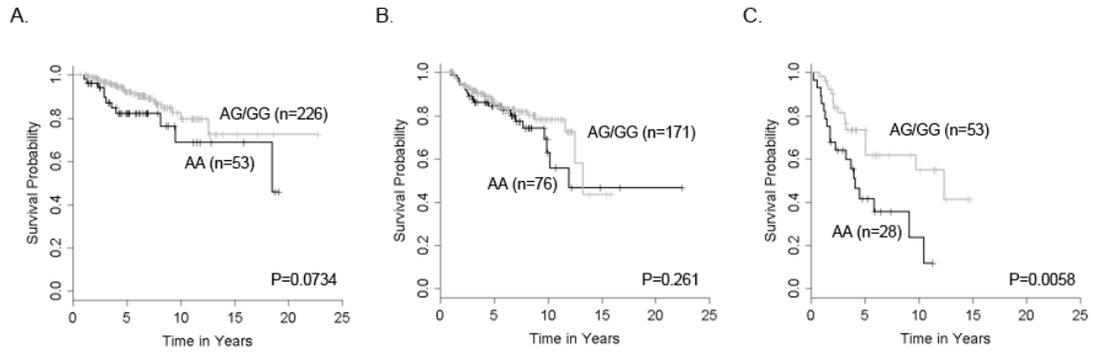
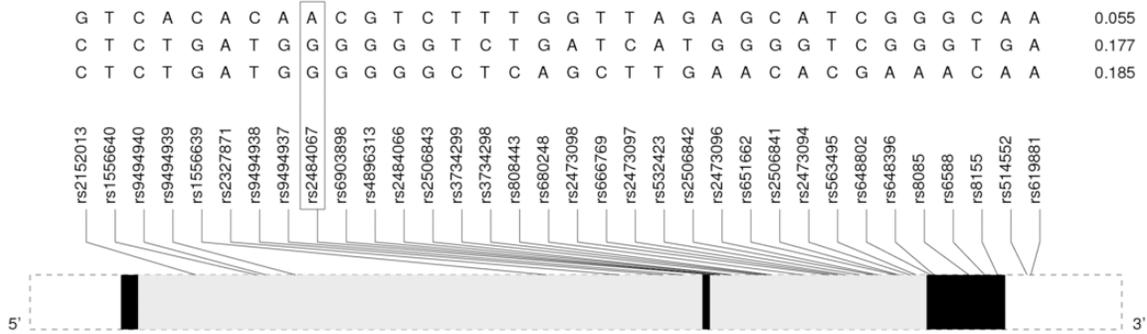


Figure 4.



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A polymorphic variant in human MDM4 associates with accelerated age of onset of estrogen receptor negative breast cancer

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Murine double minute 4 (MDM4) shares significant structural homology with murine double minute 2 (MDM2) and interacts and regulates transcriptional activity of the tumor suppressor p53. In tumors with wild-type p53, there is often overexpression of MDM2 or MDM4 leading to functional inactivation of p53. A single-nucleotide polymorphism (SNP) in the promoter of human MDM2 (SNP309) was shown to associate with increased MDM2 expression and increased risk of cancer. This study evaluated the association of a SNP in human MDM4 (C>T) with age of onset of breast cancer in two independent cohorts. In cohort 1 of 675 patients, the average age of diagnosis for women with estrogen receptor (ER)-positive and ER-negative breast cancers was 53.2 and 48 years, respectively. In this cohort, homozygous variant (TT) carriers developed ER-negative carcinomas at an earlier age than homozygous wild-type (CC) or heterozygous (TC) such that the age at diagnosis was accelerated by 5.0 years ($P = 0.018$). This association was validated in a second cohort of breast cancer patients ($n = 148$), where TT carriers with ER-negative cancer developed the disease 3.8 years earlier than CC carriers ($P = 0.006$). The effect was more pronounced in Caucasians with ER-negative ductal carcinomas with TT homozygotes developing disease 7.5 years ($P = 0.031$) and 6.2 years ($P = 7 \times 10^{-5}$) earlier than CC carriers in cohorts 1 and 2, respectively. No association was seen in ER-positive ductal cancers suggesting that the SNP in MDM4 only has a functional association in ER-negative breast cancer.

Introduction

The ubiquitous role of p53 in multiple signaling pathways and response to cell stress underlines its role as a tumor suppressor (1). Complex protein interactions regulate the activity and expression of the p53 protein (2). Murine double minute 2 (MDM2), the key regulator of p53, not only binds and inhibits the p53 transcriptional domain (3,4) but also catalyzes p53 ubiquitination by virtue of its RING (really interesting new gene) dependent E3 ligase activity and marks p53 for proteasomal degradation (5,6). The structurally homologous protein murine double minute 4 (MDM4) also binds to the transactivation domain of p53, inhibiting its activity (7). However, there are conflicting reports about the role of MDM4 in regulating p53 protein stability. While it has been reported that overexpression of MDM4 inhibits MDM2 mediated p53 degradation thus stabilizing p53 (8-11), there is also evidence that MDM4 stimulates MDM2 mediated p53 ubiquitination, MDM2 auto ubiquitination (12) and contributes to MDM2 E3 ubiquitin ligase activity (13,14).

Abbreviations: ER, estrogen receptor; MDM2, murine double minute 2; MDM4, murine double minute 4; SNP, single nucleotide polymorphism.

Mouse knockout studies have shed significant light on the dynamics of the p53-MDM2-MDM4 interaction and have highlighted the biological role of these molecules during embryonic development as well as oncogenesis. Whereas the *p53* null mouse is viable and prone to oncogenesis (15), the *Mdm2* single knockout exhibits a p53 dependent early embryonic lethal phenotype as evidenced by excessive apoptosis, which in turn is rescued with *p53* knockout (16,17). Similarly, the *Mdm4* knockout in mice results in p53 dependent embryonic lethality with defects in proliferation and not apoptosis but also becomes viable in a *p53* null background (18-20). The distinct phenotypes seen in *Mdm2* and *Mdm4* null mice suggest that MDM2 and MDM4 have non overlapping roles in p53 regulation (20). This is further supported by the fact that deletion of *Bax*, a proapoptotic gene, marginally delays the embryonic lethality of *Mdm2* null mice (21,22), whereas the lethality in *Mdm4* null mice is partially rescued by deletion of *p21* (23). However, recent evidence suggests that the MDM4 deficiency can be completely rescued by *Mdm2* transgene revealing functional overlap of MDM2 and MDM4 during development (24). Lastly, mice lacking *p53*, *Mdm2* and *Mdm4* are viable but are susceptible to tumorigenesis similar to that of *p53* Δ mice (25).

It is a well established fact that the human *MDM2* gene is amplified to high copy numbers or overexpressed in a wide variety of human cancers (26-28). The majority of these malignancies possess wild type p53, with functional inactivation of p53 due to overexpression of MDM2. The human *MDM4* gene is also amplified or overexpressed in a subset of malignant gliomas, soft tissue sarcomas and retinoblastomas (29-31). Tumors showing absence of *p53* mutations or *MDM2* amplification often display MDM4 overexpression as an alternative and independent molecular mechanism for tumorigenesis. The overall data support the hypothesis that deregulated expression of MDM2 or MDM4 leads to functional inactivation of wild type p53 and carcinogenesis.

Earlier age onset breast cancer is more probably to have genetic associations, yet most women with breast cancer do not have mutations in known breast cancer susceptibility genes (32,33). Our laboratory has previously reported a single nucleotide polymorphism (SNP) located in the promoter region of human *MDM2*, known as SNP309, associating with higher protein expression, but only in the presence of active estrogen signaling (34-36). Furthermore, the presence of the variant associates with early onset of estrogen receptor (ER) positive, but not ER negative breast cancers (34). A recent report described the haplotype structure and correlation between SNPs in *MDM4* with risk of breast and ovarian cancers (37). In this study, the association of a SNP in *MDM4* with clinical phenotypes of breast cancer, in particular age of onset of breast cancer based on tumor subtype, was evaluated.

Materials and methods

Subjects

Cases consisted of two cohorts. Cohort 1 was derived from consecutive patients evaluated at The Cancer Institute of New Jersey who were invited to participate in this prospective study from 2004 to 2009. Greater than 95% of eligible individuals gave consent for participation. Eligibility included a history of biopsy proven breast cancer verified by pathology records and confirmed on review by our institutional breast pathologist. In <5% of cases, slides were not available for review and pathological features were based on available pathology reports from other institutions. Clinical information was abstracted through chart review. ER staining <10% was considered negative. BRCA1/2 testing was performed where clinically indicated. Patients with known BRCA1/2 mutations were excluded from age association analyses due to potential confounding bias. Investigations were performed with approval by the University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School Institutional Review Board.

Cohort 2, an independent group of cases, consisted of individuals evaluated and enrolled consecutively through an institutional review board approved protocol at Yale Medical School from 1996 to 2007. This cohort included 148 patients treated with conservative surgery and radiation therapy to the intact breast who were ≤ 52 years at the time of diagnosis. All patients in this cohort had lumpectomy followed by radiation therapy to the intact breast for early stage I or II breast cancer. Clinical information was obtained from clinical records, de identified and stored in a clinical database.

Genotyping

Genomic DNA was extracted from 1 ml of peripheral blood, obtained through venipuncture, using a spin column based method according to manufacturer's protocol (QIAGEN, Valencia, CA). Genotyping for the human *MDM4* SNP (rs1563828) was performed using an Applied Biosystems Taq Man assay on the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, reactions were performed with 5–10 ng genomic DNA in a 20 μ l vol. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The assay failed in <1% of cases. The alleles at the locus are C and T where T is defined as the ancestral allele.

Statistical analysis

A permutation test (10^8 permutations) was performed to determine the statistical significance of differences in mean age at diagnosis between different genotype groups (e.g. CC or CT versus TT homozygous). This test was chosen because it is non parametric, making no assumptions about the age of diagnosis distribution. The statistical significance in categorical values was determined using Fischer's exact test. The associated odds ratio and 95% confidence interval were computed using a Bayesian estimate for the odds ratio posterior distribution.

Results

The polymorphic locus in human *MDM4* tags a haplotype under recent natural selection

A recent report indicates that a major haplotype of the human *MDM4* gene has undergone recent selection in the Caucasian population (37). This haplotype is tagged by several SNPs across the *MDM4* gene that are in high linkage disequilibrium (37), including the polymorphic locus rs1563828 located within intron 10. To confirm this, the haplotype structure of the human *MDM4* gene in the Caucasian population was analyzed. To this end, genotypes from the HapMap project [http://www.hapmap.org] were used, specifically for the HapMap CEU samples (Centre d'Etude du Polymorphisme Humain) representing 90 Utah residents with ancestry from northern and western Europe. Fifteen SNPs were found to be in linkage disequilibrium with rs1563828 (normalized mutual information above 0.7). These linked SNPs include 13 within the *MDM4* gene and two downstream (Figure 1) with two

major haplotypes accounting for 95.5% of the genotypes observed in the CEU samples. More importantly, these two haplotypes are tagged by the genotype at rs1563828: the C allele and T allele containing haplotypes being 70 and 25.5%, respectively. In practice, this means that, with 95.5% confidence, whenever the C or T alleles are observed, the associated haplotype is actually being observed. Furthermore, any association found between the genotype at rs1563828 and clinical phenotypes is in fact an association between the haplotype and the clinical variable. Based on these findings, the SNP locus was selected for genotyping in the two breast cancer cohorts.

Patient characteristics and human *MDM4* genotypes in the two study cohorts

The demographics of both cohorts are depicted in Table I. The data demonstrate that the majority of women were Caucasian and the majority of cases were of ductal origin in both groups. Overall in cohort 1, the average age at diagnosis was 51.9 years where half of women were diagnosed <51 of age; the average age of menopause for women in the USA (38). In cohort 2 that predominantly consisted of women diagnosed with breast cancer at a younger age, the average age at diagnosis was 38.8 years. While nearly 75% of breast cancers in cohort 1 were positive for ER, cohort 2 had nearly equal representation of ER positive and negative breast cancers. The latter group reflects the higher propensity of early onset breast cancer to be ER negative.

As distribution of genotype frequencies may be population specific, *MDM4* genotype frequencies were analyzed by race (Table II). Consistent with the ancestral allele information [dbSNP: (http://www.ncbi.nlm.nih.gov/sites/entrez)], the T allele was more prevalent in African American women in both cohorts. The major allele in Caucasians was the C allele. Although *MDM4* genotypes had population specific frequencies, these frequencies did not deviate from Hardy Weinberg Equilibrium except in African Americans in both the cohorts.

Because of the age association with hormone receptor status (39), the frequency of *MDM4* genotypes in ER negative and ER positive breast cancers was evaluated. The distribution of C and T allele frequencies for ER positive and ER negative breast cancers was not statistically significant in both the cohorts (data not shown). Because breast cancer represents a very heterogeneous disease, the distribution of genotype frequencies among the different cancer subtypes, such as ductal carcinoma *in situ*, invasive ductal carcinomas, invasive lobular carcinomas and other subtypes, was examined as well. There was no significant allele enrichment by breast cancer subtype (data not shown).

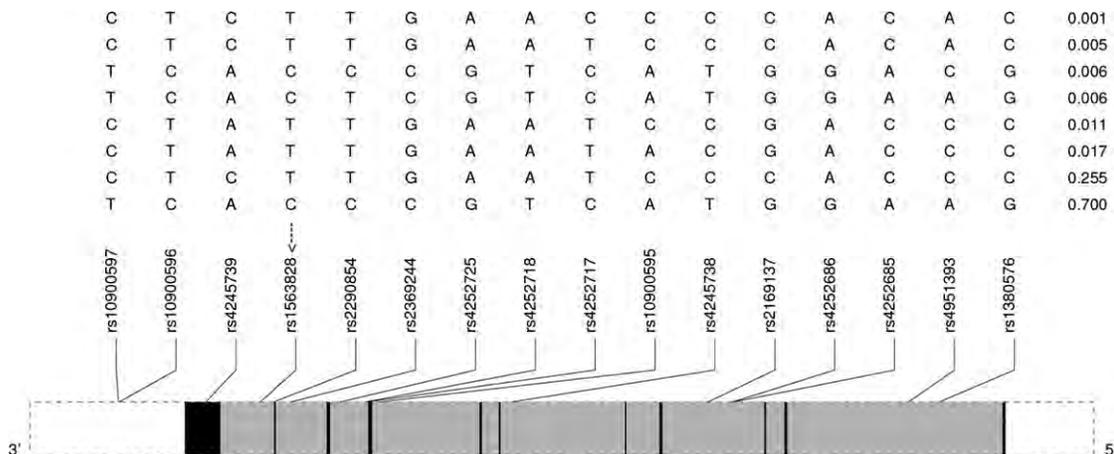


Fig. 1. The haplotype structure of human *MDM4* gene. The introns and exons in human *MDM4* are denoted in gray and black, respectively. The SNP rs1563828 present in intron 10 is marked by an arrow and is in linkage disequilibrium with 15 other SNP loci, 13 within the *MDM4* gene and two downstream. The possible haplotypes and their frequencies in HapMap CEU population are shown. Two major haplotypes (70 and 25.5%) are observed in Caucasians, each tagged by the two alleles (C or T) of this SNP.

Table I. Demographics of breast cancer patients enrolled in the CINJ cohort (Cohort 1) and Yale cohort (Cohort 2)

	Cohort 1		Cohort 2	
	Cases (n)	Percentage (%)	Cases (n)	Percentage (%)
Ethnicity				
African American	40	5.9	45	30.4
Asian	26	3.9	0	0.0
Caucasian	564	83.6	98	66.2
Hispanic	45	6.7	0	0.0
Other	0	0.0	5	3.4
Total	675	100.0	148	100.0
Type of breast cancer				
DCIS	35	5.2	12	8.1
IDC	538	79.7	121	81.8
ILC	72	10.7	4	2.7
Others: colloid, medullary, metaplastic	30	4.4	11	7.4
Age at diagnosis (in years)				
Mean	51.9		38.8	
Median	50.5		40.0	
Range	19-89		25-52	
ER status				
Negative	170	25.2	78	52.7
Positive	505	74.8	70	47.3

DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.

Table II. Distribution of genotype and allelic frequencies for MDM4 SNP by race

	Genotype frequency (%)			Allele frequency (%)	
	CC	TC	TT	C	T
Cohort 1					
Race					
Caucasian	0.40	0.49	0.11	0.64	0.36
African American	0.20	0.30	0.50	0.35	0.65
Asian	0.46	0.42	0.12	0.67	0.33
Hispanic	0.27	0.53	0.20	0.67	0.40
Cohort 2					
Race					
Caucasian	0.39	0.42	0.19	0.60	0.40
African American	0.13	0.22	0.65	0.24	0.76
Other	1.00	0.00	0.00	1.00	0.00

The TT genotype associates with an earlier age at diagnosis of ER negative breast cancers

Age of onset analyses in cohort 1 revealed that African American and Hispanic women were diagnosed at an earlier age than Caucasian and Asian women (Table III; $P = 0.0045$). In contrast, Caucasian women were diagnosed earlier than African American women in the second cohort (Table III; $P = 3.59 \times 10^{-6}$). Overall and as expected, ER negative breast cancers were diagnosed at an earlier age as compared with ER positive breast cancers regardless of MDM4 genotype in cohort 1. Of note, African Americans were diagnosed with ER positive breast cancers at an age earlier than those with ER negative disease in this cohort. This difference was not significant and probably reflects one outlier diagnosed with an ER negative breast cancer in her 70s. The average at diagnosis was similar in cohort 2 irrespective of hormone receptor status (Table III). The vast majority of ER negative breast cancers were of ductal origin and invasive in both the cohorts (data not shown).

Table III. Distributions of mean age at diagnosis grouped by race, MDM4 genotype and ER status in Cohorts 1 and 2

	Cohort 1			Cohort 2		
	Age in years by ER status			Age in years by ER status		
	All ER	ER	ER+	All ER	ER	ER+
Race						
Caucasian	52.4	48	53.8	37.2	37.4	37.0
African American	48.4	50.6	47.6	42.3	42.3	42.3
Asian	51.9	51.2	52.1			
Hispanic	48.9	45.3	50.9			
Other				40.2	40.2	
Genotype						
CC	51.8	48.9	52.7	39.7	41.0	38.7
TC	52.5	48.7	53.8	38.8	41.3	36.6
TT	50.1	43.8	52.6	38.0	37.2	39.6

The mean age at diagnosis by genotype without grouping by ER status demonstrated no significant difference in both the cohorts (Table III). However, subgroup analysis of age at diagnosis by genotype and ER status revealed distinct differences with women homozygous for the T allele demonstrating an earlier mean age at diagnosis of ER negative breast cancer compared with women homozygous for the C allele (Table III). The mean age of onset for TC genotype carriers was not significantly different from CC carriers in both the cohorts. The most significant increase occurred between the TT and CC+TC groups, with a difference in mean age at diagnosis of 5.0 years ($P = 0.018$) in cohort 1 and a difference of 3.8 years ($P = 0.006$) in cohort 2. Finally, when comparing alleles, it was observed that the T allele associates with a mean age at diagnosis 2.0 years earlier than C allele ($P = 0.053$) in cohort 1 and 2.8 years ($P = 0.002$) earlier in cohort 2. In contrast, no genotype specific difference in age at diagnosis was observed for ER positive breast cancer in either cohort (Table III; $P > 0.05$).

As a consequence of population specific genotype frequencies, potential differences due to genetic background and breast cancer characteristics, any analysis made pooling together all samples may be misleading. One would conclude that the TT genotype associates with an earlier age at diagnosis in ER negative breast cancers independent of ethnicity. However, this may be a consequence of the fact that the majority of cases are of Caucasian origin. To reduce the heterogeneity in the study population and any age specific differences due to breast cancer subtypes and/or ethnicity, samples were stratified into groups with the same cancer subtype and ethnic background. However, only the group of ductal carcinomas (patients with ductal carcinoma *in situ* and invasive ductal carcinoma) in Caucasian women was represented by a significant number of samples in both the cohorts ($n = 476$ in cohort 1 and $n = 90$ in cohort 2). Thus, the association between the genotype and the age at diagnosis of ductal carcinomas was evaluated in Caucasian women. For ER positive ductal carcinomas in Caucasian females, there was no statistically significant association between any of the MDM4 genotypes and the age of diagnosis (Figure 2A and B). For the homozygous wild type (CC), the heterozygote (CT) and the homozygous variant (TT), the mean age at diagnosis were 52.7, 53.5 and 53.8 years, respectively, in cohort 1 ($P > 0.3$) and 38.1, 36.0 and 36.2 years, respectively, in cohort 2 ($P = 0.17$). However, there was a left shift in the cumulative incidence curve for ER negative ductal cancers corresponding to a 7.5 year earlier onset in women homozygous for TT (41.7 years) as compared with the CC homozygote (49.2 years; $P = 0.031$) in cohort 1 (Figure 2C). Similarly, there was a 6.2 year earlier onset in TT carriers (33.2 years) as compared with CC carriers (39.4 years; $P = 7 \times 10^{-5}$) in the second cohort (Figure 2D). Finally, when comparing alleles, the T allele associates with a mean age of diagnosis 2.9 years earlier than C allele ($P = 0.017$) in cohort 1 and 4.0 years earlier than C allele ($P = 1 \times 10^{-4}$) in cohort 2.

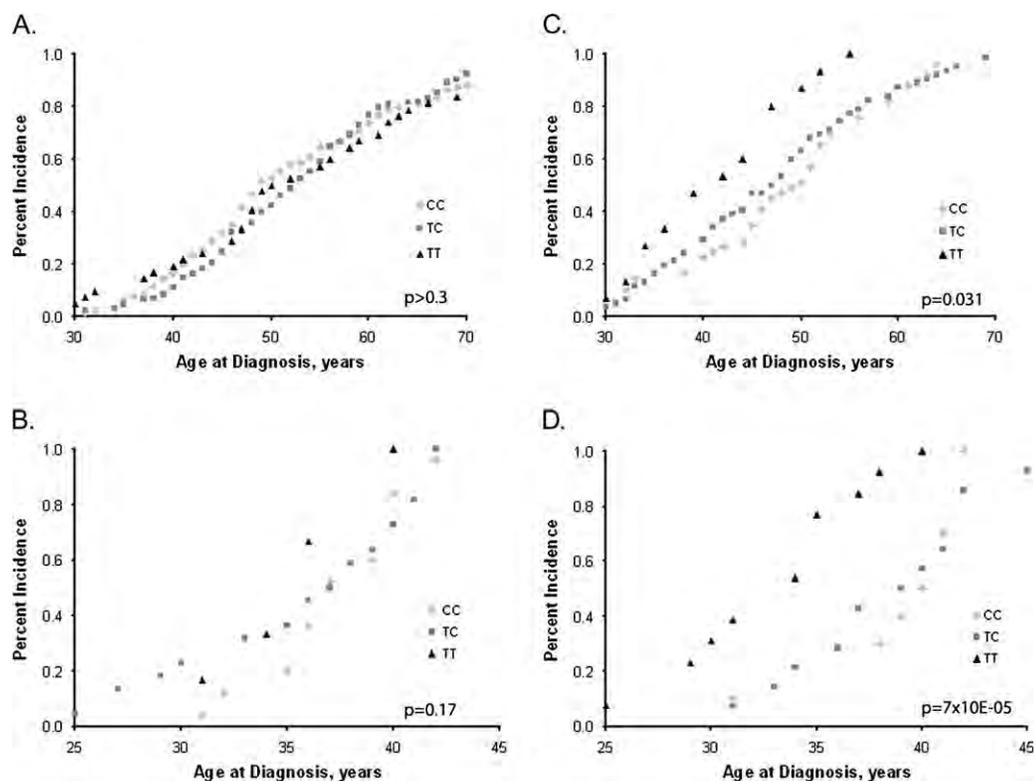


Fig. 2. The TT genotype of human *MDM4* associates with an accelerated age of onset of ER negative but not ER positive ductal breast cancers in Caucasian women. The cumulative incidence of cancer for individuals with TT genotype (black triangles), TC (dark gray squares) or CC genotype (light gray diamonds) is plotted as a function of age at diagnosis. The analysis was limited to a sample size of 476 patients (cohort 1) and 90 patients (cohort 2) consisting of Caucasian women with ductal breast cancers. Panels (A and B) depict ER positive and panels (C and D) represent ER negative ductal breast cancers from cohorts 1 and 2, respectively.

The MDM4 TT genotype shows enrichment in ER negative breast cancers developing at a younger age

Because the risk for breast cancer is related to age at menopause, the data from cohort 1 were also analyzed to evaluate the genotype specific risk of developing ER negative breast cancer before 51 years, the average age of menopause in USA (38). Thus, women diagnosed with cancer at ≥ 51 years were used as a comparison group for the cases diagnosed at an earlier age (Figure 3). The genotype frequencies for the < 51 year old cohort were 29% CC, 49% TC and 21% TT. For those ≥ 51 years at diagnosis, that distribution was 44, 48 and 8%, respectively. This indicates that there was enrichment of the TT genotype in the younger age group as compared with the older age group ($P = 0.021$, one tailed Fischer's exact test). The odds ratio of 4.6 and 95% confidence interval [0.94 10.42] demonstrates an increased risk for developing ER negative breast cancer under age 51 years for TT over CC genotypes. Furthermore, while 51 and 63% of Caucasian women carrying the CC or TC genotypes, respectively, were diagnosed with ER negative ductal breast cancers by the age of 51, 87% carrying the TT genotype were diagnosed by age 51 years ($P = 0.026$, one tailed Fischer's exact test). This corresponded to an odds ratio = 12.5, 95% confidence interval (1.14 28.6) where the TT genotype associated with a higher risk of developing ER negative ductal breast cancers in Caucasian women before age 51.

The second cohort, which is particularly enriched in women diagnosed with breast cancer before the age of 51 years, also demonstrated a similar trend in age at diagnosis when this cohort was stratified into women diagnosed with ER negative breast cancer before the age of 40 years or ≥ 40 years. The cut off of 40 years was chosen since that was the median age at diagnosis in cohort 2 by when $\sim 50\%$ women were diagnosed with breast cancer. The genotype frequencies for the < 40 year old cohort were 17% CC, 25% TC and 58% TT and those for ≥ 40 years at diagnosis were 38% CC, 36% TC

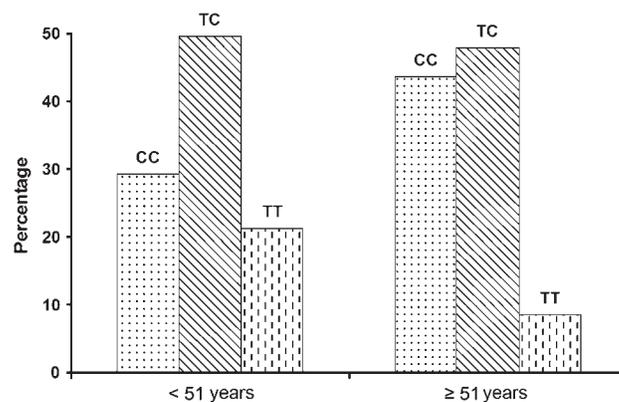


Fig. 3. The TT genotype of human *MDM4* is enriched in ER negative breast cancers developing at a younger age. Genotype frequencies were calculated for women developing ER negative breast cancers before age 51 and at age 51 and older including all ethnicities and cancer subtypes from cohort 1. CC represents the wild type and TT the homozygous variant.

and 26% TT, respectively, confirming the enrichment of TT genotype in younger patients ($P = 0.0039$). Similarly, 40 and 50% Caucasian women carrying CC or TC genotypes were diagnosed with ER negative ductal cancers by the age of 40 years, by which time 92% of the TT carriers had the disease ($P = 0.0056$). Taken together, the data suggest that women carrying the T allele or homozygous for this variant are at increased risk of developing ER negative breast cancer at a younger age. There were no differences noted in the development of ER positive breast cancer, even when looking at ductal and lobular breast cancers separately (data not shown).

Discussion

In this descriptive, case only study design of breast cancer patients, a SNP located in an intronic region of *MDM4* (rs1563828), was found to be associated with earlier age at diagnosis of ER negative breast cancers, but not ER positive breast cancers. To our knowledge, this is the first study describing such an association. Although these studies identify an association with a single SNP, by virtue of being in possible linkage disequilibrium with a different locus, an alternative locus may represent the true functional polymorphism. The haplotype structure including other SNP loci in *MDM4* that are linked to this polymorphism supports this hypothesis (37). *MDM4* represents an attractive candidate for study of potentially functional polymorphisms due to its role in the p53 pathway. Our laboratory had similarly evaluated a SNP in the human *MDM2* gene known as SNP309 that associated with increased levels of MDM2 and increased risk of tumorigenesis in familial as well as sporadic breast cancers (34,35). It was further demonstrated that this polymorphism also associated with earlier age of onset of ER positive but not ER negative breast cancers as well as other tumors, e.g. diffuse large B cell lymphoma and soft tissue sarcoma, in a gender specific manner (34). The presence of this polymorphic locus within an Sp1 transcription factor binding site and existence of an estrogen response element within 10 base pairs of this locus led to the hypothesis that both ER and Sp1 increase MDM2 levels through synergistic activation of the *MDM2* promoter (34,35,40). This was further substantiated when estrogen was shown to selectively increase MDM2 levels in cells containing the G allele of SNP309 (36).

Unlike SNP309 in *MDM2*, the *MDM4* SNP appears to associate with earlier age at diagnosis of ER negative breast cancer. However, the SNP is not associated with an increased risk of developing this subtype of breast cancer over ER positive disease. A growing body of literature supports the hypothesis that ER positive and ER negative breast cancers derive from different progenitor or breast stem cells (41,42). Altered expression of MDM4 may more effectively disrupt normal homeostasis in a cell type dependent manner, e.g. mammary stem cell/progenitor or ER negative duct epithelial cell, or in a developmental time point specific manner.

In general, ER negative breast cancers are thought to have higher genomic instability (43). DNA repair occurs when cells are in cell cycle arrest mediated by p53. If MDM4 directly binds p21 and disrupts p21 (44) in addition to p53, this may help explain the propensity of this *MDM4* genotype to display a phenotype in ER negative breast cancers. In presence of DNA damage, MDM4 interacts with isoforms of 14 3 3, which is achieved through phosphorylation of MDM4 at residue S367 by CHK2 kinase (45). The binding with 14 3 3 in turn promotes nuclear import of MDM4 and its subsequent MDM2 dependent ubiquitination and degradation, resulting in p53 stabilization.

MDM4 is also known to have shorter isoforms, one arising from a short internal deletion of 68 base pairs and giving rise to a truncated protein known as MDM4 S, and is better at binding and inhibiting p53 induced transactivation than full length MDM4 (46). There is evidence for human *MDM4* gene amplification or overexpression of human MDM4 S messenger RNA splice variant in soft tissue sarcomas, malignant gliomas and retinoblastomas with the expression of the splice variant being associated with worse prognosis (29 31). Another isoform of MDM4 (MDMX), the MDMX211 splice variant stabilizes MDM2 (47). It is possible that the SNP in *MDM4* associates with expression of one of the shorter isoforms of MDM4. The effect on splicing could be a direct effect of this SNP or an indirect effect mediated through one of the linked SNPs within the haplotype. If this SNP indeed promotes the expression of a shorter MDM4 isoform with more efficient binding to p53 or MDM2 and less efficient binding to 14 3 3, this would result in less degradation of MDM4 and enhanced p53 inactivation even in the presence of DNA damage.

Both MDM2 and MDM4 play distinct but coordinated roles in p53 regulation. Unlike *MDM2*, *MDM4* is not known to be hormonally regulated (Entrez Gene) despite some computational predictions for an estrogen response element in the promoter and first intron of

MDM4 (A.Vazquez, personal communication). We hypothesize that while SNP309 of MDM2 is functionally active in the presence of active estrogen signaling, the negative effects of MDM4 become more dominant in the absence of that active hormone signaling and the ratio of MDM4 to MDM2 increases. In an *in vitro* model system of point mutations in the C terminus of MDM2, MDM4 was shown to contribute to the E3 function of MDM2 (14), evidence pointing toward a cooperative action between MDM2 and MDM4. Furthermore, there is also functional overlap between MDM2 and MDM4 as evidenced by complete rescue of *Mdm4* null phenotype in mice by *Mdm2* transgene (24). Thus, it can be hypothesized that in ER negative breast cancers where MDM2 expression is lower, MDM4 acts both cooperatively with MDM2 and independently from MDM2 in exerting its effects.

Strengths of this study include the availability of information for all study participants from pathology reports and that the association analysis incorporated disease subtype. The latter is especially important given the heterogeneity of breast cancer as a disease. Although the effect of carrying the TT genotype in *MDM4* on ER negative breast cancers may represent a true biologic effect, the shift to earlier age at diagnosis may represent a bias in detecting more rapidly growing breast cancers that are detected earlier. This is particularly important as breast cancers are thought to be present for several years before they reach a threshold for detection. That being said, the distribution in stage at diagnosis using the TNM (Tumor Node Metastasis) classification was not significantly different between *MDM4* genotypes. The association in this study was strongest for ductal carcinomas but may not be representative of all breast cancer types, e.g. lobular, metaplastic, colloid, tubular breast cancers. In fact, lobular and tubular breast cancers would be predicted to have no association given their strongly positive ER status. Lastly, one would also expect that younger women might suffer from delay in diagnosis due to dense breast tissue relative to the sensitivity of mammography and lower suspicion for diagnosis of breast cancer in a young woman. This effect would tend to decrease the differences in age at diagnosis.

The specific ER dependent associations observed in this descriptive study are hypothesis generating for the role of human *MDM4* in hormone receptor negative disease that will subsequently need to be tested in additional patient populations. Genetic association studies are often criticized for lack of reproducibility (48). However, in this study and in spite of its small size, cohort 2 provides reproducibility in the observed association with the *MDM4* SNP locus. Differences in the genotype frequencies among ethnicities between the two cohorts can be attributed to factors such as smaller sample size in cohort 2 and admixture in different regions of the country. Due to the small size of cohort 2 with limited statistical power, a larger comprehensive study should be performed for confirmation of these associations. This would be particularly true since the smaller groups resulting from subgroup analysis are more probably to lead to Type I errors. Analyses using larger patient populations would help refute this type of error. As this study is a case only design, it does not examine the overall risk of developing breast cancer. However, Atwal *et al.* (37) demonstrated risk associated with this SNP locus in human *MDM4* in both breast and ovarian cancers. That being said, defining the molecular mechanism of SNP functionality, or that of a closely linked SNP, would even further support the observed positive associations. Because of the known role of MDM2 in breast cancer, the role of MDM4 in regulating the tumor suppressor p53, and known interactions between MDM2 and MDM4, there is biological plausibility for this association in breast cancer. Future studies may also benefit from controlling for potential confounders such as gynecological factors, e.g. age at menarche, age at first live birth, number of pregnancies.

In summary, we found that SNP rs1563828 located in intron 10 of human *MDM4* associated with earlier age at diagnosis of ER negative breast cancer but not ER positive breast cancer. These findings were confirmed in a second cohort of breast cancer cases. However, further studies are needed to confirm our findings and molecular studies to identify functionality of this polymorphism are underway.

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A genetic variant in a PP2A regulatory subunit encoded by the *PPP2R2B* gene associates with altered breast cancer risk and recurrence

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A recent candidate gene association study identified a single nucleotide polymorphism (SNP) in the *PPP2R2B* gene (rs319217, A/G) that manifests allelic differences in the cellular responses to treatment with chemotherapeutic agents (Vazquez et al., *Nat Rev Drug Discov* 2008;7:979-87). This gene encodes a regulatory subunit of protein phosphatase 2A (PP2A), one of the major Ser/Thr phosphatases implicated in the negative control of cell growth and division. Given the tumor suppressor activities of PP2A, here we evaluate whether this genetic variant associates with the age of diagnosis and recurrence of breast cancer in women. To investigate the linkage disequilibrium in the vicinity of this SNP, *PPP2R2B* haplotypes were analyzed using HapMap data for 90 Caucasians. It is found that the A variant of rs319217 tags a haplotype that appears to be under positive selection in the Caucasian population, implying that this SNP is functional. Subsequently, associations with cellular responses were investigated using data reported by the NCI anticancer drug screen and associations with breast cancer clinical variables were analyzed in a cohort of 819 Caucasian women. The A allele associates with a better response of tumor derived cell lines, lower risk of breast cancer recurrence, later time to recurrence, and later age of diagnosis of breast cancer in Caucasian women. Taken together these results indicate that the A variant of the rs319217 SNP is a marker of better prognosis in breast cancer.

Several studies support the hypothesis that single nucleotide polymorphisms (SNPs) can affect the predisposition to cancer development and the response to cancer therapy¹⁻¹³ (cancer

Key words: PP2A, SNP, natural selection, cellular response, breast cancer

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functional SNPs or functional SNPs to abbreviate). However, the identification of all or most functional SNPs is a challenging problem given the large number of genetic variations in the human population. Genome wide association studies, designed to screen for potential genetic associations are also limited given the implicated assumptions for such analyses and that loci may not be functional loci but rather linked to the functional locus.

Recently we performed a candidate gene association study to uncover functional SNPs using data generated by the NCI anticancer drug screen (NCI60 screen).¹⁴ Specifically, statistical computing methods were developed to analyze correlations between the response of tumor derived cell lines to standard chemotherapeutic agents and the genotype of SNPs within candidate genes in the p53 pathway.¹⁵ The analysis identified six SNPs with significant genotype drug response associations. Genetic variants of two of these SNPs, residing in the *YWHAQ* and *CD44* genes, have been recently shown to associate with cancer risk and response to chemotherapy in patients with soft tissue sarcomas,¹⁶ thus validating the NCI60 candidate gene study.

The analysis of the NCI60 data also predicted a functional SNP in the *PPP2R2B* gene, encoding for a regulatory subunit of protein phosphatase 2A (PP2A). PP2A is a ubiquitously expressed heterotrimeric protein that accounts for a large fraction of phosphatase activity in eukaryotic cells¹⁷ and it has been implicated in breast tumorigenesis and progression. Phosphorylation of PP2A is associated with progression of breast tumors.¹⁸ The inactivating glycine 90 to aspartate somatic mutation in the structural subunit encoded by *PPP2R1B*, observed at higher frequency in breast cancer patients, results in reduced protein function.¹⁹ Both tumorigenicity and functional haploinsufficiency have been attributed to mutation in the A subunit of PP2A where mutation in this scaffold subunit promotes degradation of the regulatory subunit.^{20,21} Furthermore, a case control study using haplotype analysis of tagged SNPs in *PPP2R1A* and *PPP2R2A* demonstrated associations protective for breast cancer and modified risk for women with proliferative breast lesions.²² However, this study did not include evaluation of *PPP2R2B*. Therefore, we further investigate the hypothesis that SNPs in *PPP2R2B* will associate with breast cancer phenotypes, focusing on a putative functional genetic variant in the *PPP2R2B* gene.

Material and Methods

Haplotype analysis

The haplotype analysis was based on HapMap genotypes for 90 Caucasians of Northern and Western European ancestry (HapMap CEU).²³ Genotypes for 500 SNPs within *PPP2R2B* were available. Among them, 43 SNPs with genotype calls having relative mutual information of 0.7 or higher with the rs319217 genotypes were selected. The ancestral allele information was available from dbSNP.²⁴ The relative mutual information ($M_{ij} = I_{ij}/I_{ii}$) is a measure of linkage disequilibrium, defined as the ratio between mutual information ($I_{ij} = \sum_{ab} m_{ij,ab} \ln[nm_{ij,ab}/g_{i,a}g_{j,b}]$) of a probe SNP (j) and a reference SNP (i) (here rs319217) and the self mutual information (I_{ii}) of the reference SNP (i), where n is the number of samples, $g_{i,a}$ is the number of samples with genotype a at locus i , and $m_{ij,ab}$ is the number of samples with genotype a at locus i and genotype b at locus j . The relative mutual information takes the values between 0 (independent SNPs) and 1 (perfectly linked SNPs). Based on the genotypes for the selected 43 SNPs, the associated haplotypes were estimated using the SNP HAP program <http://www.gene.cimr.cam.ac.uk/clayton/software/>.

Cellular drug responses

The mutational status of p53, the genotypes of 109,687 SNPs (Affymetrix 125K chip) and the GI50 data for the NCI60 cell panel were obtained from the NCI/NIH Developmental Therapeutics Program web site, <http://www.dtp.nci.nih.gov>. The genotypes of the rs319217 SNP in the NCI60 samples have been determined using accurate allelic discrimination assays (Applied Biosystems).¹⁶ A univariate test was undertaken for

132 drugs to evaluate allelic differences in the GI50s. Specifically, the average log GI50 [$X = -\log_{10}(\text{GI50})$] for cells for each of the three genotypes of a given locus (AA, Aa and aa) were calculated for cells either wild type or mutant for p53. Subsequently, the probability (p value) was computed that just by chance the difference for the following groupings either was equal to or larger than the actual measurement: (a) $X_a X_{AA}$ or (b) $X_{aa} X_A$, or (c) $X_{AA} X_a$, or (d) $X_A X_{aa}$, or (e) [$X_{aa} X_{aA}$ and $X_{aA} X_{AA}$] and (f) [$X_{AA} X_{aA}$ and $X_{aA} X_{aa}$]. These probabilities were estimated using a permutation test (10^6 permutations) that preserved the allele or genotype group sizes but permuted the samples among the groups. Results $p < 0.05$ were considered significant and $p < 0.1$ marginally significant. A multiple hypothesis test was performed for allelic differences in the GI50s across the entire panel of drugs. This test took advantage of the fact that 132 well characterized compounds were tested against the NCI60 cell panel, which provided a set of independent measurements. A Fisher's exact test to compute the statistical significance of observing h univariate hits for a SNP on a total of $D = 132$ drugs, given that overall H significant hits are observed after testing S reference SNPs on the D drugs. All 109,687 Affymetrix genotyped SNPs were chosen as a reference set.

Breast cancer cohort

The case cohort consisted of 819 Caucasian women diagnosed with breast cancer. It was derived from patients evaluated at The Cancer Institute of New Jersey who were invited to participate in this study from 2004 to 2009. Greater than 95% of eligible individuals gave consent for participation. Eligibility included being at least 18 years of age and a history of biopsy proven breast cancer verified by pathology records and confirmed on review by our institutional breast pathologist. Samples were collected retrospectively for cases diagnosed before 2004. Occurrence of asynchronous primary breast tumors and recurrent tumors were not considered in age at diagnosis analyses. For those women with more than one primary, age of diagnosis analysis was performed based on first primary. In 5% of cases, slides were not available for review and pathological features were based on available pathology reports from other institutions. Clinical information was abstracted through chart review. Estrogen receptor alpha and progesterone receptor were measured by immunohistochemistry for over 99% of tumors and were negative if staining was less than 10%. For those measured by protein, tumors with less than 5 fmol/mg was considered negative. BRCA1/2 testing was performed where clinically indicated through Myriad Genetic Laboratories using standard assays including full sequencing and rearrangement tests unless otherwise indicated. Patients with known BRCA1/2 mutations were excluded from age and recurrence association analyses due to potential confounding bias. Recurrence was defined as the time between the date of biopsy proven diagnosis to date of biopsy proven recurrent disease. Local, regional and distant recurrences were defined as biopsy proved recurrence

Table 1. Demographics of breast cancer cohort¹

Caucasian women, <i>n</i>	819
Age at diagnosis, years	
Median age at diagnosis	53
Range	19–95
Stage at diagnosis, <i>n</i> (%)	
0	78 (11%)
I, II	503 (73%)
III, IV	107 (16%)
Type of breast cancer, <i>n</i> (%)	
Invasive ductal	532 (77%)
Invasive lobular	82 (12%)
DCIS	77 (11%)
Estrogen receptor status, <i>n</i> (%)	
Positive	535 (76%)
Negative	166 (24%)
Her2 status, <i>n</i> (%)	
Positive	102 (19%)
Negative	429 (81%)
Progesterone receptor status, <i>n</i> (%)	
Positive	430 (63%)
Negative	252 (27%)
Chemotherapy, <i>n</i> (%)	
Yes	234 (37%)
No	393 (63%)
Radiation, <i>n</i> (%)	
Yes	452 (75%)
No	151 (25%)
Adjuvant hormonal therapy, <i>n</i> (%)	
Yes	457 (72%)
No	178 (28%)
Observation time ²	
Mean (months)	77.9
Range (months)	1–415
Recurrence, <i>n</i> (%)	137 (21%)
Received adjuvant chemotherapy, <i>n</i>	393

¹Percent figures are reported relative to the number of patients for which annotations were available. ²Time on observation until recurrence or total time observed without a recurrence.

in breast, in lymph node basins, or in other organs beyond the breast or lymph nodes, respectively. Patients with stage IV disease at diagnosis were excluded from the recurrence analysis. DCIS was not included in recurrence analyses as a function of chemotherapy as this treatment is not standard of care for DCIS. Because there were no associations between genotype and risk of noninvasive vs. invasive ductal carcinomas, DCIS was included in analysis of risk of recurrence, particularly as several individuals experienced distant recur-

rence in the absence of evidence local or regional recurrence. Investigations were performed with approval by the University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School Institutional Review Board. Demographics of the breast cancer cohort are depicted in Table 1.

Genotyping

Genomic DNA was extracted from 1 ml of peripheral blood, obtained through venipuncture, using a spin column based method according to manufacturer's protocol (QIAGEN, Valencia, CA). Genotyping for the human *PPP2R2B* SNP (rs319217) was performed using an Applied Biosystems TaqMan assay on the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, reactions were performed with 5–10 ng genomic DNA in a 20 µl vol. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The assay failed in <1% of cases. The alleles at the locus are A and G where G is defined as the ancestral allele.

Statistical analysis of the clinical data

A permutation test was done to determine the statistical significance of the noted increase of the average age of tumor diagnosis between patients carrying the A allele and patients carrying the GG homozygous. The statistical significance for the enrichment of GG carriers among patients manifesting recurrence events was computed using the Fischer's exact test. The odds ratio (OR) estimates were computed using, first, a Bayesian estimate of the probability density function of the fraction of GG homozygous in patients manifesting recurrence events, and second, a binomial test to compute the probability to observe (*n*) GG genotypes in (*N*) patients manifesting recurrence events. The analyses of recurrence free survival was performed using the Kaplan Meier analysis and the Cox's multivariate proportional hazards regression model with the SPSS 17.0 software (SPSS, Chicago, IL).

Results

Genetic variant and haplotype analysis

The *PPP2R2B* gene is a relatively long gene of about 500 kb. It is composed of 10 exons with long first and second introns (Fig. 1). The SNP of interest (rs319217) is located in the first intron and it is linked to several other SNPs expanding over a region around the second exon (Fig. 1 and Supporting Information Fig. 1). There are two major haplotypes associated with the genotype calls of those SNPs in strong linkage disequilibrium with rs319217, overall accounting for 95% of the predicted haplotypes in the HapMap Caucasian samples. The rs319217 SNP locus displays two alleles A or G, each tagging one of the two major haplotypes. G is the ancestral allele and it is at lower frequency in the Caucasian population (about 0.3), compared to that for the derived allele A (about 0.7). Together, the fact that the derived allele is at high frequency and tags a very long haplotype appears to be inconsistent with a neutral model of evolution. Under

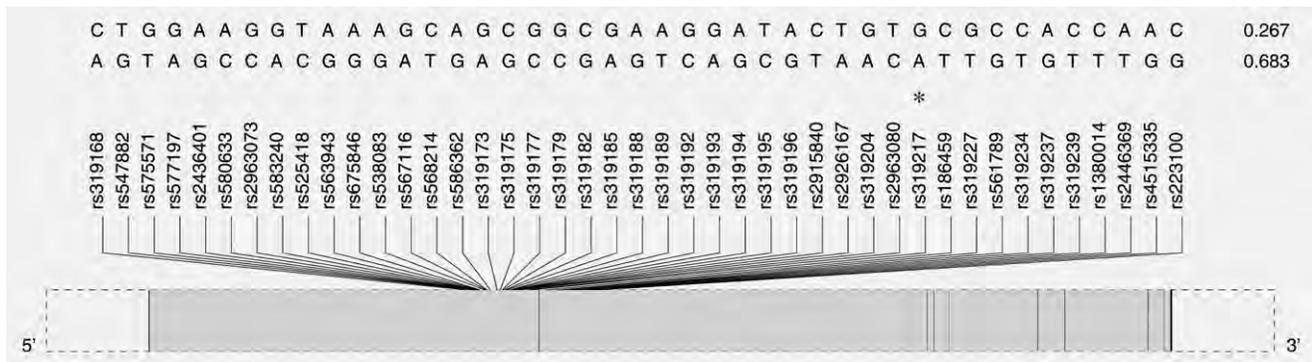


Figure 1. Haplotypes associated with the rs319217 SNP in the PPP2R2B gene. The figure shows the PPP2R2B gene, with black vertical bars representing exons and gray rectangles representing introns in their relative sizes. The solid lines from the gene map to the rs numbers indicate the positions of 43 SNPs in PPP2R2B with strong linkage disequilibrium with rs319217. On the top, the two major haplotypes associated with these SNPs is depicted.

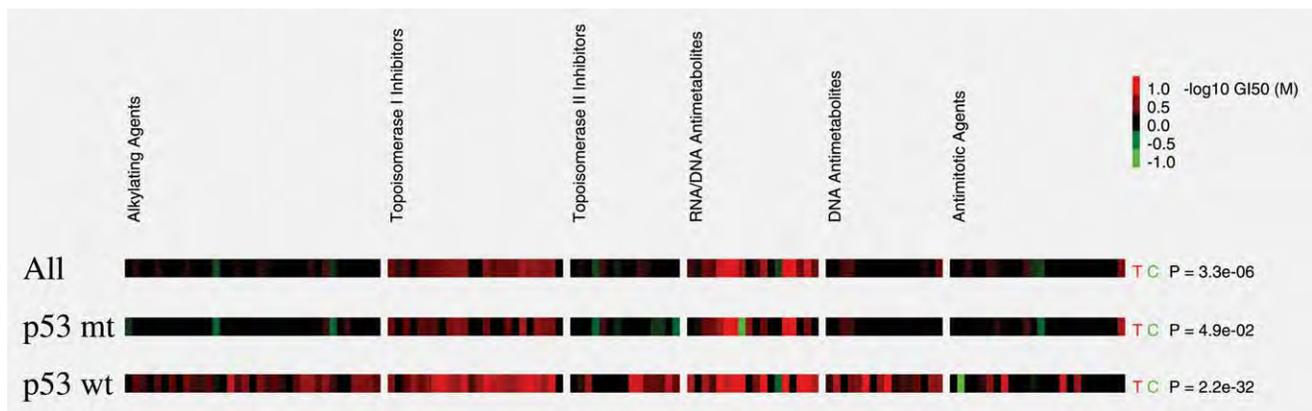


Figure 2. Heat map summarizing the response of tumor derived cell lines to 132 chemotherapeutic agents, grouped according to their mechanism of action. The red and green colors indicate that the A and the G allele, respectively, correlate with a better response (*i.e.*, lower GI50), while the black color denotes that absence of a significant association. The color intensity is proportional to the average GI50 fold change between the homozygous as indicated by the scale. The top, middle and bottom panels correspond to the analysis made using all, p53 mutant and p53 wild type cell lines, respectively.

neutrality, high frequencies can only be achieved after a long period of evolution and after such long period the linkage disequilibrium with nearby SNPs should have significantly decreased due to recombination events. The observation of both a high frequency and a long haplotype is thus indicative of a selective sweep acting on the haplotype tagged by the A variant. This hypothesis is supported by the statistical analysis of Haplotter,²⁵ reporting that the *PPP2R2B* gene is under selection in the Caucasian population with a statistical significance of 0.045. Note that, based on the same Haplotter analysis, this gene is not under selection in Africans and, therefore, this is a population specific effect.

The evidence for selection indicates that the rs319217 is a functional SNP or it is linked to some functional genetic variation. Indeed, for a genetic variant to be selected for it must lead to some phenotype affecting reproduction. The fact that PP2A is one of the major Ser/Thr phosphatases opens the window for several possibilities. In particular, PP2A has been

implicated in the negative regulation of cell growth and division, which are essential processes during development. PP2A activity and regulation is necessary during developmental different stages.^{26–29}

Allelic differences in drug responses in cancer cell lines

The first evidence of the association of the rs319217 alleles and cancer related phenotypes comes from the response of tumor derived cell lines to standard chemotherapeutic agents.¹ This evidence is recapitulated in Figure 2, showing the average response of tumor derived cell lines carrying the A or G alleles to 132 standard chemotherapeutic agents, where the red color indicates that the A allele associates with a higher sensitivity to the drug (lower GI50), the green color indicates the same but for the G allele, and black represents the absence of an association. This heat map clearly shows that in general the A allele associates with a better response (predominance of the red color). The probability that this

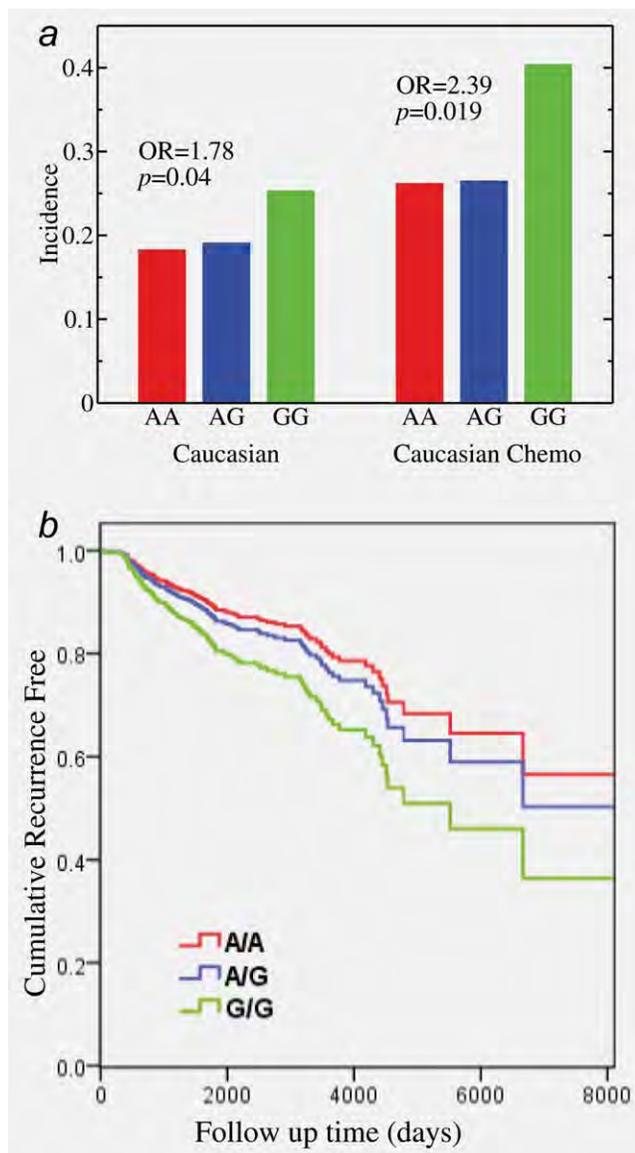


Figure 3. (a) Fraction of patients with breast cancer recurrence events stratified according to their genotype at the rs319217 SNP, for all Caucasian patients (Caucasian, left) and all Caucasian patients receiving chemotherapy (Caucasian Chemo, right). The *p* values indicate the statistical significance for an enrichment of patients with the homozygous GG genotype among patients manifesting recurrence events. (b) The Cox multivariate regression survival analysis of *PPP2R2B* SNP rs319217 shows an association of the G allele with an increased risk for and earlier recurrence. The graphs display the survival curves of breast cancer patients for each genotype and are plotted against the survival time in days. Patients with a GG genotype (green line) showed a 1.81 fold increased risk for tumor recurrence when compared to patients homozygous for the A allele (red line), while heterozygous individuals showed an intermediate course (blue line). The Cox multivariate regression survival analysis was adjusted to tumor stage and histopathological subtype of the tumor.

would happen for a randomly chosen SNP is of 3.3×10^{-6} , 2.2×10^{-32} and 4.9×10^{-2} when considering all cell lines, cell lines with a wild type p53, and cell lines carrying a mutant p53, respectively, indicating that this association is independent of the p53 status.

Allelic differences in breast cancer recurrence

The NCI60 cell response analysis indicates that patients carrying the A variant of rs319217 should manifest a better response to cancer treatment, and specifically to chemotherapy. To test this prediction, recurrence events following breast cancer treatment were analyzed in cohort of 667 Caucasian women diagnosed of breast cancer (Fig. 3). Among these patients, there were 247, 324 and 96 individuals with the AA, AG and GG genotypes. In carriers of the GG genotype at rs319217, patients manifest a higher frequency of recurrence events compared to patients carrying the A variant, with an average odds ratio 1.78 CI (0.97 2.69) and statistical significance of $p = 0.043$ (Fig. 3a). Thus, as predicted by the analysis of cell response to chemotherapeutic agents, patients carrying the A variant are predicted to have a better response to the chemotherapeutic treatment.

Stratified analysis was also performed according to ER, PR, Her2 status, the type of therapy received (chemotherapy, radiation, adjuvant hormonal therapy), the stage at diagnosis and the cancer subtype. No association was observed when stratifying by patients that received or not radiation therapy. The association became more significant in the group of patients receiving adjuvant hormonal therapy ($p = 0.024$), while no association was observed in the group of patients that did not received hormonal therapy ($p = 0.43$). Regarding the status of the different receptors, no association was observed except for the group of progesterone negative patients ($p = 0.019$).

The noted association became more significant ($p = 0.016$) when the analysis was restricted to patients that received chemotherapy as part of their treatment ($n = 348$), resulting in an average odds ratio 2.39 CI (1.01 3.71) (Fig. 3b), while no significant association was observed in the group of patients that did not received chemotherapy ($p = 0.41$). The majority of the patients receiving chemotherapy were treated with a combination of a topoisomerase II inhibitor (doxorubicin or epirubicin) and an alkylating agent (mainly cyclophosphamide). In some cases the latter received an additional agent, which was either an antimetabolic agent (Docetaxel or Paclitaxel) or a DNA antimetabolite (5 Fluorouracil). On the other hand, some patients received a combination of an alkylating agent (cyclophosphamide), a DNA antimetabolite (5 Fluorouracil) and a RNA/DNA antimetabolite (Methotrexate). All these class of agents are represented in the 132 standard agents used in the NCI60 analysis (Fig. 2), allowing the comparison between associations in the tumor derived cell line responses and the patient responses to chemotherapy. There is a precise concordance with the cell lines study, both indicating that the A variant is

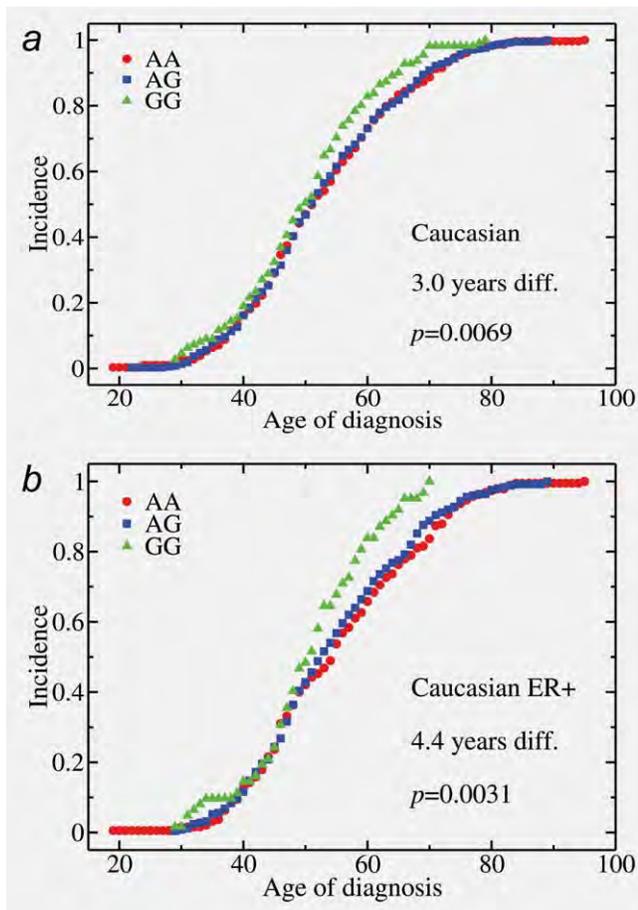


Figure 4. Fraction of individuals diagnosed with breast cancer as a function of age, in a cohort of Caucasian women diagnosed with (a) breast cancer and (b) ER+ breast cancer. The inset figures represent the average difference in age of diagnosis between the homozygous groups (AA/GG) and the corresponding statistical significance.

associated with a better response to standard chemotherapeutic treatments.

To further assess the impact of the *PPP2R2B* SNP on breast cancer outcome, Kaplan Meier analysis of regression free survival was performed. The analysis revealed that GG carriers had the worst recurrence free survival with a mean survival time of 7,059 days (232.1 months), followed by patients heterozygous for the G allele [mean survival time of 6,406 days (210.6 months)] and subsequently patients with an AA genotype [mean survival time of 5,682 days (186.8 months); $p = 0.050$, log rank test]. To exclude potential biases from other independent prognostic factors, a multivariate Cox's regression survival analysis was performed, adjusted to tumor stage and histological subtype including receptor status. This analysis further confirmed the initial results, showing a relative risk (RR) for recurrence of 1.81 for patients with the GG genotype when compared with individuals homozygous for the A allele ($p = 0.028$; Fig. 3b). In line

with the previous observations, this trend was also more pronounced when the analysis was restricted only to those patients who had received chemotherapy (RR = 1.85, $p = 0.042$), while no significant association was observed in the group of patients not receiving chemotherapy (RR = 1.63, $p = 0.52$).

Allelic differences in age of diagnosis of breast cancer

Previous studies suggest that, genetic variants implicated in the variable response to cancer treatment, can alter the rate and age at which individuals manifest cancer as well. Thus, the association between the rs319217 genotypes and the age of diagnosis of breast cancer in Caucasian women was evaluated. Genotypes and age of diagnosis was obtained for 760 patients, stratified into 284, 365 and 111 patients with the AA, AG and GG genotypes. On average, women carrying the GG genotype at rs319217 are diagnosed of breast cancer 3.0 years earlier than those carrying the A variant, with a statistical significance $p = 0.0069$. This association is a consequence of the increase in the incident rate in patients with the GG genotype (Fig. 4a), particularly after the age of 50 years, where the average age of menopause is 51 for women in the U.S.

Data were further stratified according to ER, PR and Her2 status, the stage at diagnosis and the cancer subtype for additional analysis. The noted association became stronger when restricting the analysis to women diagnosed with ER positive breast cancer (Fig. 4b). In this case patients carrying the GG genotype are diagnosed 4.4 years earlier ($p = 0.0031$). The genotype specific associations were preserved for ductal carcinomas. The same trend was noted for invasive lobular carcinomas, but due to small group size ($n = 82$), statistical significance was not reached ($p = 0.09$). No genotype specific associations were observed by Her2 status or stage at diagnosis.

Discussion

Taken together our results indicate that the A variant of the rs319217 SNP associates with a better response to chemotherapy. In both, tumor derived cell lines and breast cancer patients, carriers of the A variant manifest a better response to standard chemotherapy. In addition, we also observed that breast cancer patients carrying the GG genotype were diagnosed 3.0 years earlier than those carrying the A variant, with a 4.4 years difference for ER positive breast cancers.

The rs319217 SNP resides within the *PPP2R2B* gene, encoding a regulatory subunit of PP2A. PP2A is a ubiquitously expressed heterotrimeric protein that accounts for a large fraction of phosphatase activity in eukaryotic cells.¹⁷ PP2A phosphatase activity has been shown to interact directly with the p53 pathway, causing the dephosphorylation of key residues of p53 and MDM2, resulting in the regulation of p53 activity levels in cells.^{30–34} ADP ribosylation factor like 2 (Arl2) modifies chemosensitivity to conventional drugs, e.g., taxanes and doxorubicin, used to treat breast cancer in

the adjuvant setting. The mechanism implicated is through PP2A effects on p53 phosphorylation.³⁵ PP2A can antagonize Ras signaling by dephosphorylating c Myc and RalA and by negatively regulating the PI3 kinase/Akt signaling pathway.¹⁷ Further evidence of the importance of PP2A activity in suppressing cellular transformation lies in the wide range of mechanisms that transformed cells have evolved to inhibit its activity. For example, inhibition of PP2A activity has been shown to be mediated by the small tumor antigen of DNA tumor viruses³⁶ by up regulation of the c Myc specific inhibitor CIP2A,³⁷ by BCR/ABL via SET up regulation,³⁸ through biallelic mutational inactivation of the A β subunit,³⁹ or by decreased expression of the A α subunit.²⁰ PP2A has also been implicated in inhibition of nuclear telomerase in human breast cancer cells.⁴⁰

The various forms of PP2A contain an active core dimer, made up of the catalytic (C) subunit and a structural scaffold A subunit. The scaffold subunit mediates interaction of the core dimer with a great variety of regulatory (B) subunits in a tissue specific manner. The rs319217 resides within the gene *PPP2R2B*, encoding for a regulatory subunit. Interestingly, a recent report implicates a genetic variant of *PPP2R5E*, encoding for another regulatory subunit of PP2A, has been also shown to alter the age of diagnosis and survival in patients with soft tissue sarcomas.⁴¹ Furthermore, both *PPP2R2B* (here) and *PPP2R5E*⁴¹ appear to be under natural selection in the Caucasian population, as indicated by the analysis of their haplotypes. Haplotype analysis of tagged SNPs in *PPP2R1A* and *PPP2R2A* demonstrated that certain genotypes were protective for breast cancer, while others modified the risk for women with proliferative breast lesions.²² No previous reports have described associations for SNPs in *PPP2R2B* with either risk of breast cancer or its recurrence. Data from this cohort are consistent with associations between *PPP2R2B* alleles and breast cancer outcomes. However, longer follow up is necessary to determine the relationship between survival curves while accounting for late relapses.

The role of PP2A in breast cancer suggests how functional SNPs may play a role in breast cancer biology. For instance, reduced stability of ER α mRNA has been linked with

reduced PP2A activity.⁴² Direct interactions between ER α and PP2A through the catalytic subunit of PP2A result in ER dependent gene expression, even in the absence of estrogen.⁴³ In addition, Loss of PP2A expression in human breast cancer results in endocytosis of e cadherin, a key player in the beta catenin pathway.⁴⁴ Reduced or absent e cadherin expression is observed in breast tumors. As a cell cell adhesion molecule, reduced e cadherin membrane expression may contribute to the metastatic potential of breast cancer cells. PP2A also has demonstrated effects on chemosensitivity. Reduced PP2A expression and activity have been observed in adriamycin resistant MCF 7 human breast cancer cells, while adenoviral E1A mediated sensitization of a human breast cancer cell line to paclitaxel appears to occur through PP2A upregulation.^{45,46}

The association reported here has not been detected in a recent genome wide association study (GWAS) searching for common genetic variants with an association with breast cancer prognosis.^{4,5,47} The latter can be attributed to several factors, including the multiple hypothesis testing complexity of GWAS studies and the lack of appropriate stratification of the patient population. Our preliminary NCI60 analysis offered the advantage of providing a candidate SNP (rs319217), the allele associated with better prognosis (A allele), and the specific context (response to chemotherapy).

The associations reported here remain to be validated in other patient cohorts, and importantly, the regulatory changes associated with *PPP2R2B* rs319217 or linked SNPs are yet to be determined. The latter is a challenging task given that the variant and ancestral alleles of rs319217 tag two long haplotypes expanding a 60 kb region. Furthermore, more exhaustive searches for other genetic variants closely linked to these SNPs, but not included in the HapMap project, will be necessary to develop a complete list of candidate functional SNPs that merit further experimental investigation into the molecular and cellular mechanisms behind the significant allelic differences in haplotype structure, cancer risk, and response to cancer treatment noted in this report. However, these data strongly suggest that *PPP2R2B* harbors genetic variants that can affect human cancer and may be under evolutionary selection pressure.

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CLINICAL INVESTIGATION

Breast

EVALUATION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN THE p53 BINDING PROTEIN 1 (*TP53BP1*) GENE IN BREAST CANCER PATIENTS TREATED WITH BREAST-CONSERVING SURGERY AND WHOLE-BREAST IRRADIATION (BCS + RT)

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Purpose: *TP53BP1* is a key component of radiation-induced deoxyribonucleic acid damage repair. The purpose of this study was to evaluate the significance of a known common single nucleotide polymorphism in this gene (rs560191) in patients treated with breast-conserving surgery and whole-breast irradiation (BCS + RT).

Methods and Materials: The population consisted of 176 premenopausal women treated with BCS + RT (median follow-up, 12 years). Genomic deoxyribonucleic acid was processed by use of TaqMan assays. Each allele for rs560191 was either C or G, so each patient was therefore classified as CC, CG, or GG. Patients were grouped as GG if they were homozygous for the variant G allele or CC CG if they carried at least one copy of the common C allele (CC or CG).

Results: Of the 176 women, 124 (71%) were CC CG and 52 (29%) were GG. The mean age was 44 years for GG vs. 38 years for CC CG ($p < 0.001$). GG was more common in African-American women than white women (69% vs. 13%, $p < 0.001$) and more commonly estrogen receptor negative (70% vs. 49%, $p = 0.02$). There were no significant correlations of rs560191 with other critical variables. Despite the fact that GG patients were older, the 10-year rate of local relapses was higher (22% for GG vs. 12% for CC CG, $p = 0.04$).

Conclusions: This novel avenue of investigation of polymorphisms in radiation repair/response genes in patients treated with BCS + RT suggests a correlation to local relapse. Additional evaluation is needed to assess the biological and functional significance of these single nucleotide polymorphisms, and larger confirmatory validation studies will be required to determine the clinical implications. © 2011 Elsevier Inc.

Breast cancer, Single nucleotide polymorphism, Local recurrence, 53BP1, Breast-conserving surgery.

INTRODUCTION

The majority of women presenting with early-stage breast cancer are treated with breast-conserving surgery and radiation. Local relapse of disease remains a significant pattern of treatment failure, particularly in younger women, which ultimately may compromise survival and can be associated with significant social and psychological consequences (1–6). Primary tumor factors, such as receptor status, margins, histologic subtype, grade, and more recently, molecular profiles, have been extensively evaluated as potential risk factors for local relapse, with some consistent but many conflicting results (1, 7–16). Host factors including age and race and, to a lesser extent, genetic factors such as *BRCA1*, *BRCA2*, and *CHEK2*

have also been evaluated, again with conflicting results (17–22). The most consistently reported risk factors to date for local relapse after breast-conserving surgery and radiation have been patient age, with younger age predicting for higher local relapse rates and positive margin status predicting for higher local relapse rates (1, 3, 15, 22–29).

Although there are multiple studies evaluating the high-penetrance *BRCA1/BRCA2* genes and local relapse, overall patient numbers have been relatively small because less than 1% of the population and fewer than 5% of breast cancer patients are carriers of deleterious mutations (30). There are even fewer data on other more rare high-penetrance genetic syndromes, where it is estimated that only 1% to 2% of familial cases are explained by mutations in other known cancer

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susceptibility genes, such as *P53*, *PTEN*, *ATM*, and *CHEK2* (30–33).

Although deleterious mutations in highly penetrant breast cancer susceptibility genes, such as *BRCA1/2*, are relatively rare, there is a wide spectrum of much more common low-penetrance genetic changes in genes that may be clinically relevant (32–34). Many of these genetic changes are single nucleotide substitutions, which may or may not affect the function of the gene, commonly referred to as single nucleotide polymorphisms (SNPs). For some of these polymorphisms, fairly large segments of the population may be affected. A recently discovered example of a clinically relevant common polymorphism is the *CYP2D6* gene, where approximately 10% of the population are homozygous carriers of a single nucleotide variation in the *CYP2D6* gene (35). Patients homozygous for the polymorphism have less efficient metabolism of tamoxifen to its active metabolite endoxifen and have been reported to have inferior outcomes compared with wild-type and heterozygous patients when treated with tamoxifen. This represents a novel approach for individualizing therapy based on the genetics of the host, as opposed to the clinical, pathologic, or molecular characteristics of the primary tumor.

It is evident that evaluation of SNPs of the host as risk factors for recurrence and response, as well as for normal tissue reactions in patients undergoing radiation therapy, is an exciting and novel area of investigation that has not been extensively explored. In this regard there are a large number of candidate genes, related to radiation response and deoxyribonucleic acid (DNA) damage repair, that have common polymorphisms that may be clinically relevant. One such candidate is the gene encoding for the tumor suppressor p53 binding protein 1 (*TP53BP1*) (36–39). 53BP1 participates in the early DNA damage response after radiation and is recruited rapidly to sites of DNA breaks, where it is required for efficient recruitment of other critical DNA repair proteins such as *BRCA1*. 53BP1 and *ATM* interact in irradiated cells, with *ATM* activation leading to phosphorylation of 53BP1 and intact 53BP1 being required for optimal *ATM* autophosphorylation. Polymorphic variants in *TP53BP1* are therefore excellent potential candidates for predicting response to radiation and for cancer susceptibility. Several common polymorphisms in the *TP53BP1* gene have been identified (36). Although the functional and biologic significance of these polymorphisms is unclear and the evidence linking polymorphisms in *TP53BP1* to breast cancer risk has been mixed, 53BP1 clearly plays a major role in response to DNA damage by radiation, and modest changes in the function of the gene can have significant consequences (36, 38, 40). Evaluation of *TP53BP1* polymorphisms on outcomes after radiation therapy in breast cancer patients has not been evaluated. The purpose of this study was to evaluate the prognostic significance of polymorphisms in the *TP53BP1* gene in premenopausal patients treated with breast-conserving surgery and radiation.

METHODS AND MATERIALS

The patient population consisted of 176 premenopausal women treated with breast conserving surgery and whole breast irradiation between 1985 and 2003. Patients were treated by lumpectomy and whole breast irradiation, with or without regional nodal irradiation and systemic therapy, in accordance with standard practice and as previously described (18, 41). Premenopausal early stage patients were selected because these patients are at highest risk for local relapse and represent a relatively homogeneous cohort. Patients were recruited from the radiation therapy treatment facilities during follow up, and after they provided informed consent, blood was drawn for genetic testing, DNA was stored, and patient data were deidentified with a unique identification code, in accordance with guidelines for this institutional review board approved protocol. All patient data including demographic information, clinical, pathologic, treatment, and outcomes were stored in a computerized data base for analysis. Because patients were recruited from follow up clinics after radiation treatment, median follow up from the date of original diagnosis was 12 years.

Three known polymorphisms in the *TP53BP1* gene, previously described by Frank *et al.* (36), were genotyped by standard methods: D353E, G412S, and K1136Q. These polymorphisms are associated with amino acid changes in the coding region of *TP53BP1*. To test whether the allele status of the three loci in the *TP53BP1* gene are highly correlated with each other, we performed a linkage disequilibrium analysis using a common methodology in population genetics described by Devlin and Risch (42). This method calculates a D' value and an r value, which quantifies the degree of linkage between the loci based on the distribution in the population of a specific loci. This methodology allows one to create a haplotype map of the three loci and quantifies the degree of association between loci based on the genetic analysis of the population tested (42).

Genomic DNA was extracted from 1 mL of peripheral blood, obtained through venipuncture, by use of a spin column based method according to the manufacturer's protocol (Qiagen, Valencia, CA). Genotyping was performed with a TaqMan assay on the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). In brief, reactions were performed with 5 to 10 ng of genomic DNA in a 25 μ L volume. Polymerase chain reaction cycling conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute.

For each of the three loci, alleles were classified as C, G, A, or T. In the case of D353E (rs560191), the alleles were either C or G. Genotyping for each patient was thus categorized as CC, CG, or GG; patients were classified as GG if they were homozygous for the variant G allele (GG) or CC/CG if they carried at least one copy of the common C allele (CC or CG). All clinical, pathologic, treatment, and outcomes data, along with the results of the SNP analysis, were entered into a computerized database, deidentified, and analyzed with standard statistical packages.

RESULTS

The linkage disequilibrium analysis of the three loci in *TP53BP1* showed a tight correlation, indicating a nonrandom association between these three loci. On the basis of the analysis described previously, the D' value between the all three loci was 1.0, indicating a tight linkage of all three loci in both white and African-American cohorts. Further linkage analysis of the correlation coefficient (r) showed a value of 1.0 between loci D353E and K1136Q among white patients and



Fig. 1. On the basis of the frequency of alleles in the white (Cauc) and African American (AfroA) populations in this study, an estimated haplotype map of the three loci in *TP53BP1* was generated.

0.98 among African Americans, whereas the *r* value between these loci and G412S was 0.73 in white patients and 0.82 in African Americans (42). These data confirm a very strong linkage between D353E and K1136Q and a strong but less complete linkage between these two loci and G412S. On the basis of this analysis, an estimated haplotype map of the three loci is given in Fig. 1. Correlations between the various variables and outcomes were essentially identical for K1136Q and, as expected, were not as strong for G412S, which was not as tightly linked. The linkage analysis between the three loci is shown in Table 1.

Because the three loci are so tightly linked and highly correlated, we report here on the results of the D353E locus (rs560191). Of the 176 premenopausal women, 124 (71%) were classified as CC CG and 52 (29%) as GG. As noted in Table 2, the mean age was 44 years for the GG patients vs. 38 years for the CC CG patients (*p* < 0.001), and GG was more common in African-American women than in white women (69% vs. 13%, *p* < 0.001). There were no significant correlations of rs560191 with other critical variables: T stage, N stage, margin status, use of adjuvant therapy, or family history. Although progesterone receptor status was evenly distributed between the populations, there was a slight predominance of estrogen receptor (ER) negative tumors in the GG group.

In addition, BRCA1/2 mutation status was known in 149 of the 176 patients, and there was no correlation between BRCA1/2 mutation status and the polymorphisms in *TP53BP1*.

Because younger age in a majority of studies is associated with higher rates of local relapse, it was hypothesized that the GG patients, who were older, would have a lower local relapse rate. Despite the fact that GG patients were older, the 10-year rate of local relapse was higher (22% GG vs. 12% CC CG, *p* = 0.04). As noted previously, there is no correlation between the TP53BP1 status and BRCA1/2 status, which would explain the difference in local control. Figure 2 shows the local relapse rate as a function of genotype over time. On multivariate analysis, when age, receptor status, margin status, and adjuvant therapy were taken into account, however, genotyping did not retain statistical significance (*p* = 0.12).

As noted previously, GG was more common in African-American women, and there was a predominance of ER-negative tumors in the GG group. However, in this sample there was no difference in the local relapse rate between white and African-American women (17% vs. 15% at 15 years), and there was no significant difference in local relapse as a function of ER status (17% ER negative vs. 20% ER positive at 15 years). The difference in local relapse rate between the GG and the CC CG groups could therefore not be explained by an imbalance in race, age, or ER status.

The frequency of the homozygous variant GG genotype in our white population of patients was 13%, and it did not differ significantly from the approximate 10% frequency of the GG genotype in white breast cancer patients or white control subjects reported by Frank *et al.* (36).

Table 1. Linkage disequilibrium of three loci in *TP53BP1*

D353E*	K1136*			G412S*		
	Homozygous WT	Heterozygous	Homozygous variant	Homo WT	Heterozygous	Homozygous variant
Homozygous WT	49	2	1	50	2	0
Heterozygous	3	60	1	29	25	0
Homozygous variant	0	3	40	6	16	21

Abbreviation: WT = wild type.

* Correlation between allele status of all three loci was highly significant at *p* < 0.00001.

Table 2. Patient population classified by status of D353E (rs560191)

Variable	CC	CG patients	GG patients	p Value
No. of patients		124	52	Not applicable
Age (mean) (y)		38	44	< 0.001
Race		30% AA 87% CA	69% AA 13% CA	< 0.001
T stage		57% T1 26% T2 17% Tis	52% T1 34% T2 13% Tis	0.34
N stage		78% node negative	71% node negative	0.37
ER status		51% ER positive	30% ER positive	0.03
PR status		46% PR positive	36% PR positive	0.29
HER2/Neu status		34% HER2 positive	31% HER2 positive	0.81
Margins		4% positive	6% positive	0.91
Family history		17% strong	25% strong	0.28
BRCA1/2 status		11% positive	8% positive	0.57
Adjuvant chemotherapy		50%	49%	0.90
Adjuvant hormones		23%	15%	0.25

Abbreviations: ER = estrogen receptor; PR = progesterone receptor.

To determine whether the higher frequency of GG genotype in our African-American breast cancer patient population was different from the frequency in control subjects, we obtained 100 age-matched control African-American samples without a diagnosis of breast cancer (Bioserve, Beltsville, MD) and conducted a 2:1 age-matched control. As shown in Table 3, the frequency of the GG genotype in our African-American patient population of 69% did not differ significantly from the GG genotype frequency of 81% in the African-American control population.

The rate of contralateral events in GG patients was slightly but not significantly higher at 10 years (13% vs. 8%, $p =$ not significant). This is shown in Fig. 3.

Breast Relapse Free Time by Status of SNP in 53BP1-D353E:1059C>G (rs560191)

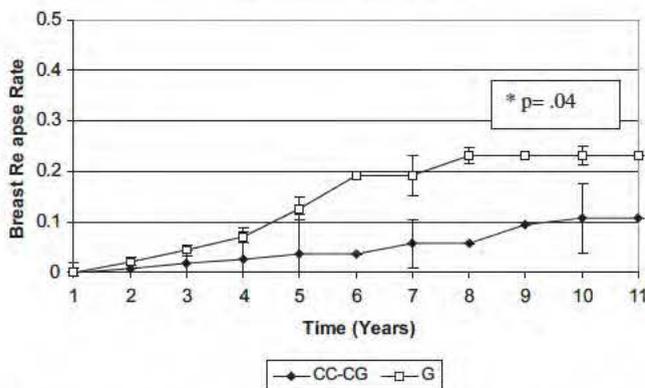


Fig. 2. Ipsilateral breast cancer rate by single nucleotide polymorphism (SNP) status in *TP53BP1*. The difference between the homozygous variant (GG) and the other cohorts (CC CG) was significant at $p = 0.04$.

Table 3. D353E (rs560191) in African American cancer patients vs. age matched African American control subjects

	CC	CG*	GG*	Total
Age matched control subjects	18 (19%)		79 (81%)	97
Patients	16 (31%)		35 (69%)	51
Total	34		114	148

* $p = 0.08$.

Because these patients were all alive and without evidence of disease at the time of recruitment, no attempt was made to correlate genotype with distant metastasis or overall survival.

DISCUSSION

Single nucleotide polymorphisms represent the most common type of genetic variation, and with the completion of the Human Genome and HapMap project, numerous common SNPs have been identified and characterized (33, 34, 43-46). Whether many of these common SNPs are clinically or biologically significant remains to be determined. However, because some of these polymorphisms can result in amino acid changes within the protein coding portion of genes critical to cellular function, they are candidates for potential predictors of variation in biological function. One limitation of our study is the lack of understanding of how these particular polymorphisms may affect the biological function of the gene. It has been established that the 53BP1 protein is a critical component of DNA repair. Although these polymorphisms do result in amino acid changes, how those changes affect the specific structure and function of the protein has not been elucidated. Future laboratory studies will need to be performed to further explore the biological significance of these SNPs.

Development of Contralateral Breast Cancer by Status of SNP in 53BP1-D353E:1059C>G (rs560191)

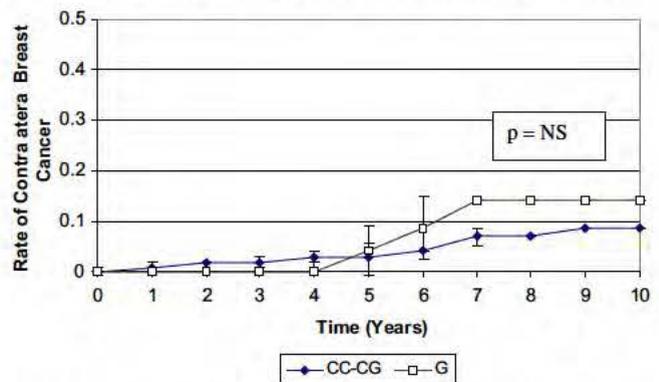


Fig. 3. Contralateral breast cancer rate by single nucleotide polymorphism (SNP) status in *TP53BP1*. There is no significant difference (NS) in contralateral events between the homozygous variant (GG) and the CC CG cohorts.

There are a number of genes involved in DNA repair and radiation response in which SNPs have been identified (47–50). The P53 binding protein is a critical component in response to DNA damage, and the gene encoding for this protein has been fully characterized and polymorphisms identified (36, 38, 40, 51, 52). Polymorphisms in *TP53BP1* have been evaluated with respect to the risk of breast cancer, with conflicting results (36, 38, 40, 51, 52). With respect to breast cancer risk, although *TP53BP1* may be a minor contributing factor, it is more likely that combinations of SNPs in this and other critical genes may be associated with breast cancer risk. Of note, we observed no correlation between *TP53BP1* polymorphisms and deleterious mutations in *BRCA1/2*.

Outcomes related to SNPs in genes related to DNA damage/repair response have not been extensively evaluated. There have been a few studies that evaluated short- and long-term normal tissue reactions as a function of polymorphisms, with mixed but promising results (53–56). Polymorphisms in *ATM* have been shown to correlate with chronic fibrosis in breast cancer patients, and a recent study evaluating polymorphisms in transforming growth factor β showed an association with radiation pneumonitis in lung cancer patients undergoing radiation therapy (56). Krupa *et al.* (57) showed a correlation between polymorphisms in repair genes *RAD51* and *XRCC3* and nodal metastasis, but they did not evaluate correlations with local or regional relapse. Skerrett *et al.* (58) also noted associations of transforming growth factor β with recurrence in breast cancer but did not evaluate local control.

Data correlating a number of polymorphisms with overall or disease-free survival and response to chemotherapy and other drugs continues to rapidly develop. One of the most well-described examples of this is *CYP2D6*, the gene associated with metabolism of tamoxifen to its active endoxifen metabolite (35). A specific polymorphism that is found in approximately 10% of the population is associated with slow metabolism and poorer response to tamoxifen therapy. Currently, a commercially available test can be used to identify patients with this polymorphism to guide medical recommendations regarding hormonal therapy.

Evaluation of locoregional control with radiation as a function of germline polymorphisms has not been extensively evaluated. Although there are a number of studies assessing local control as a function of germline *BRCA1/2* status, evaluation of local control as a function of SNPs in genes associated with radiation response is underexplored (17–21). One recent study reported a positive correlation in a functional polymorphism in the promoter of *BCL2* and disease-free and overall survival in oropharyngeal cancers treated with surgery and radiation (59). This polymorphism in the *BCL2* promoter has also been shown to correlate with rising prostate-specific antigen level after prostatectomy (60). In contrast to the rapidly increasing numbers of studies assessing polymorphisms associated with response to drug therapy, however, this area is largely underdeveloped (61–70).

The P53 binding protein is a key component in radiation response (51). Our laboratory has been evaluating 53BP1 expression and radiation response and hypothesized that polymorphisms in *TP53BP1* may be associated with outcomes in patients undergoing breast-conserving surgery and radiation. In a cohort of patients treated with breast-conserving surgery and radiation, we found that the homozygous GG genotype was associated with a significantly higher risk of local relapse on univariate analysis. Because the GG genotype carrying group of patients was older than the other CC and CG genotype carriers, one would expect a lower rate of local relapse, given the known association of young age and higher local relapse rates (3). Our GG genotype carriers were also more predominant among African-American women, and there were slightly more ER-negative tumors among the GG genotype carriers. However, there were no differences in the local relapse rates between the African-American and white women in this study and no differences between ER-negative and ER-positive patients in this cohort. In addition, there was no correlation between the status of *TP53BP1* polymorphisms and *BRCA1/2* in this cohort. Therefore the higher local relapse rate could not be explained by an imbalance in any of these factors. Although it is possible that the amino acid changes and protein structure alteration in 53BP1 result in altered radiation sensitivity, it is apparent that further studies will be required to determine the biological and functional significance of these polymorphisms. Though significant on univariate analysis, the correlation did not hold on multivariate analysis. Given that our patient numbers and number of events were relatively small, larger sample sizes and validation studies will be required to determine the clinical significance of our observations.

These results do show, however, the potential for further exploration of these types of studies, evaluating outcomes of patients undergoing radiation therapy as a function of SNPs. Genes associated with DNA response such as *TP53BP1*, *ATM*, *p53*, *BRCA1/2*, and others are all potential candidate genes with known polymorphisms that warrant further study. Although polymorphisms in any given candidate gene may show only modest effects on radiation outcomes, patterns of polymorphisms in multiple genes are likely to yield clinically relevant results. The availability of SNP chips, which allow simultaneous evaluation of thousands of genes, makes this type of research currently available and attractive (71).

Because genetic changes are present in both tumor tissues and normal tissues, polymorphisms can be associated with tumor response as well as normal tissue response. In this study we did not evaluate normal tissue reactions or cosmesis, but that is another potential endpoint that could be evaluated in future studies. In addition, we are evaluating polymorphisms in other genes associated with radiation response to assess their prognostic potential. In the era of increasing attention on personalized medicine, this area remains a unique and exciting avenue of investigation in translational research.

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Polymorphic variants in TSC1 and TSC2 and their association with breast cancer phenotypes

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Abstract TSC1 acts coordinately with TSC2 in a complex to inhibit mTOR, an emerging therapeutic target and known promoter of cell growth and cell cycle progression. Perturbation of the mTOR pathway, through abnormal expression or function of pathway genes, could lead to tumorigenesis. TSC1 and TSC2 expression is reduced in invasive breast cancer as compared with normal mammary epithelium. Because single nucleotide polymorphisms (SNPs) in regulatory genes have been implicated in risk and age at diagnosis of breast cancers, systematic SNP association studies were performed on TSC1 and TSC2 SNPs for their associations with clinical features of breast cancer. TSC1 and TSC2 haplotypes were constructed from genotyping of multiple loci in both genes in healthy volunteers. SNPs were selected for further study

using a bioinformatics approach based on SNP associations with drug response in NCI-60 cell lines and evidence of selection bias based on haplotype frequencies. Genotyping for five TSC1 and one TSC2 loci were performed on genomic DNA from 1,137 women with breast cancer. This study found that for TSC1 rs7874234, TT variant carriers had a 9-year later age at diagnosis of estrogen receptor positive (ER+), but not ER-, ductal carcinomas ($P = 0.0049$). No other SNP locus showed an association with age at diagnosis, nor any other breast cancer phenotype. TSC1 rs7874234 is hypothesized to be functional in ER+ breast cancer because the T allele, but not the C allele, may create an estrogen receptor element (ERE) site, resulting in increased TSC1 transcription and subsequent inhibition of mTOR.

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Keywords Breast cancer risk · SNP · TSC1/TSC2 · Case study

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Abbreviations

TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
SNP	Single nucleotide polymorphism
ER	Estrogen receptor
ERE	Estrogen receptor element
mTOR	Mammalian target of rapamycin

Background

Breast cancer is one of the most frequently diagnosed cancers in women [1], where etiology is postulated to be polygenic [2]. Polymorphisms in genes contributing to tumorigenesis are less penetrant, but may account for the majority of breast cancers due to their higher frequency in the general population [2]. In fact, single nucleotide polymorphisms (SNPs) have been implicated in risk and age at diagnosis of breast cancers [3, 4]. One such polymorphism in MDM2 was found to lead to increased risk for earlier age at diagnosis of estrogen receptor (ER) positive breast cancers in women carrying the variant allele of the SNP [5].

Although breast cancers are also associated with mutations in the highly penetrant breast cancer susceptibility genes, BRCA1 and BRCA2, fewer than 10% of cases can be attributed to this etiology [2]. What is known based on BRCA1 and BRCA2 is that these familial syndromes are associated with particular breast cancer subtypes [6]. Breast cancer is a heterogeneous disease that is reflective of its molecular features and histology [7]. Whereas BRCA1 carriers frequently develop hormone receptor negative disease, the phenotype in BRCA2 carriers reflects the distribution observed for spontaneous breast cancer [6]. Therefore, other genetic contributors, including SNPs, may also be reflected in tumor heterogeneity.

Tuberous sclerosis is an autosomal dominant disorder that results from mutations in the tumor suppressor genes, TSC1 or TSC2 [8–10]. Tuberous Sclerosis is characterized by benign hamartomas that affect multiple systems including the brain, skin, heart, and kidneys [8–10]. Lymphangiomyomatosis, a manifestation of tuberous sclerosis seen in 30–40% of affected women, shares some of the same characteristics of breast cancers including the following: gender-dimorphism, estrogen-modulation, increased cell proliferation and migration [11–13]. TSC1 acts to stabilize TSC2, preventing its degradation and both function together as the TSC1/TSC2 heterodimer [8, 10]. This complex inhibits the G protein, Rheb, which acts upstream of the mammalian target of rapamycin (mTOR) kinase [10, 14]. The serine threonine kinase mTOR plays a central role in the control of cell growth and proliferation through phosphorylation of its effector molecules, 4E-BP1

and S6K1 [15]. Activation of the pathway occurs in response to growth factors, amino acids, and nutrients, leading to mRNA translation and ribosome biogenesis [15]. TSC1/TSC2 complex when not functional, leads to uncontrolled mTOR activity causing uncontrolled cell growth and tumor formation. Rapamycin inhibits mTOR and has been used successfully to treat individuals with tuberous sclerosis [16]. Furthermore, analogs of rapamycin are currently being tested in breast and other cancer clinical trials [14].

Despite the implications highlighted by tuberous sclerosis and the key position of TSC1 and TSC2 in the mTOR pathway, few studies have examined the role of TSC1 and TSC2 in breast cancer [17]. We investigated a panel of 18 TSC1 and 14 TSC2 SNPs by first mapping their haplotypes and using bioinformatics approaches to further select potentially functional SNPs. Five germline TSC1 SNPs and one germline TSC2 SNP were evaluated from women with breast cancer and correlated with clinicopathologic characteristics. The data from these studies indicates that estrogen signaling may modulate the effect of a TSC1 SNP in age at diagnosis of breast cancer.

Methods

Subjects

The cohort consisted of 1,137 consecutively enrolled patients, invited to participate in this prospective study from 2004–present, through the Stacy Goldstein Breast Cancer Center at The Cancer Institute of New Jersey (CINJ). Over 95% of eligible individuals gave consent for participation. A history of biopsy-proven breast cancer was verified by pathology records and confirmed on review by our institutional breast pathologist. Fewer than 5% of cases were not available for review and for those pathological features were based on pathology reports from other institutions. Lobular carcinoma in situ (LCIS) was excluded. Negative estrogen receptor (ER) staining was defined as <10%. BRCA1/2 testing was performed where clinically indicated and patients with known BRCA1/2 mutations were then excluded from age at diagnosis analysis due to potential confounding bias. Investigations were performed with prior approval by the University of Medicine and Dentistry of New Jersey Institutional Review Board.

Determining the haplotype structure

A list of 18-tagged SNPs in TSC1 and 14-tagged SNPs in TSC2 (Table 1) was generated based on HapMap data, representing the minimum number of SNPs necessary to complete the haplotype for TSC1 and TSC2. The SNP

Table 1 TSC1 and TSC2 SNPs used to generate haplotype structure

Gene	rs#	Location	Allele	
TSC1	3761840	Intron 2	C/T	
	11243929	Intron 19	C/T	
	2809243	Exon 22, 3'UTR	A/G	
	739441	Exon 22, 3'UTR	C/T	
	2519757	Intron 16	C/T	
	739442	Exon 22, 3'UTR	A/G	
	11243940	5' end (not in gene)	A/G	
	10491534	3'UTR	C/T	
	2809244	3'UTR	A/C	
	7026607	Intron 1	A/G	
	1076160	Intron 19	C/T	
	7874234	Intron 1	C/T	
	7865232	Intron 8	C/T	
	6597584	Intron 19	A/C	
	13295634	Intron 5	G/T	
	7870151	Intron 13	A/C	
	4419933	Intron 1	C/T	
	1073123	Exon 10, missense mutation	A/G	
	TSC2	2074969	Intron 11	A/G
		17654678	Intron 15	G/T
30259		Intron 32	C/T	
13335638		Intron 37	C/T	
8063461		Intron 16	A/G	
2074968		Intron 10	C/G	
2073636		Intron 5	A/G	
8050755		Intron 16	C/T	
13331451		Intron 22	C/T	
7190284		Intron 16	A/C	
1800720		Intron 5	C/T	
12325450		Intron 20	C/T	
13337626	Exon 23	C/T		
8047396	Intron 26	C/G		

UTR untranslated region

tagging formalism employed an information-theoretic definition of haplotype diversity, and the optimal tag SNPs were chosen using a greedy procedure [18] that minimized the haplotypic uncertainty (unpublished). This SNP tagging algorithm was designed independently of Haploview Tagger. It outperforms Tagger giving similar but better results, requiring slightly fewer SNPs to tag the haplotypes. Genomic DNA isolated from lymphoblastoid cell lines (LCLs) was genotyped to determine frequency of haplotypes (demonstrated for one locus in each gene; Fig. 1 and Online Resource 1). LCLs were obtained from Coriell Institute for Medical research (Camden, NJ, USA) and represent DNA from healthy individuals. For both TSC1 and TSC2, only haplotypes occurring at greater than 2% are depicted.

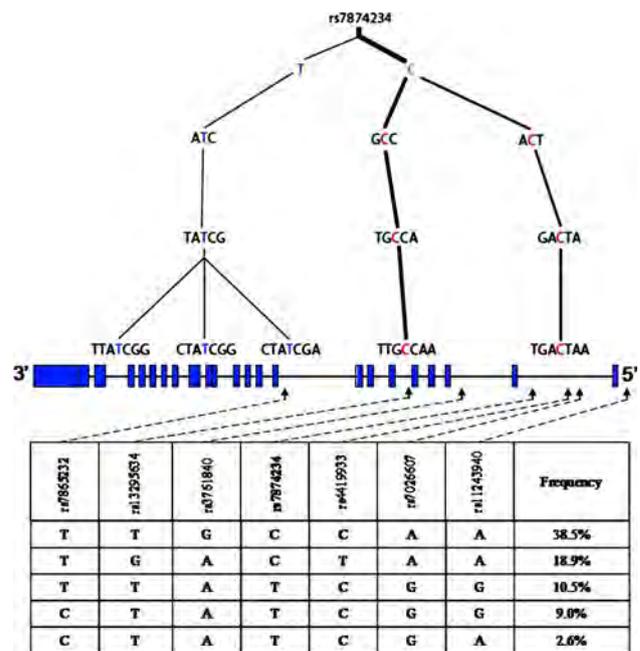


Fig. 1 Haplotype structure and gene map of TSC1 for Caucasians. The haplotype structure of TSC1 determined from analysis of LCLs is shown, along with the gene structure, haplotype block for rs7874234, and relevant haplotype frequencies. Below the haplotype trees, is the gene structure of TSC1; boxes indicate exons with intervening spaces representing intronic sequence. Below the gene structure is the haplotype block and corresponding haplotype frequencies. Dashed lines and arrows indicate the SNP position within these blocks and location on the corresponding gene map above. The haplotype structure and corresponding haplotype frequencies represent the SNPs immediately adjacent, either right or left of the reference loci, rs7874234

Candidate TSC1 and TSC2 SNPs for Clinical Study

Candidate SNP selection for further study in the breast cancer cohort was achieved by two different approaches. The first approach searched for SNPs manifesting signatures of natural selection in the pattern of genotype correlations with nearby SNPs. To identify genetic variants that deviate from the standard assumptions of selective neutrality, a previously described and publicly available map of recent positive selection of the human genome (Haplotter, <http://hg-wen.uchicago.edu/selection/haplotter.htm>) was utilized. In Haplotter, recent positive selection is determined using a haplotype-based approach that looks for enrichment of the classic signal for strong directional selection using the phase II data of the HapMap. Haplotter utilizes a test statistic called the integrated haplotype score (iHS), which is a measure that includes the degree of haplotype homozygosity around a given SNP [19]. Highly positive and negative iHS scores denote SNPs that harbor higher haplotype homozygosity, compared with other SNPs with similar allele frequencies in the genome. Using this methodology, SNPs in TSC1 (rs7874234, rs1076160,

rs1073123, and rs3761840) and SNPs in TSC2 (rs13335638) were identified.

The second approach was based on associations between the genotypes and the response of tumor derived cell lines to standard chemotherapeutic agents (Online Resource 2) [20, 21]. The mutational status of p53, the genotypes of 109,687 SNPs (Affymetrix 125K chip), and the GI50 data for the NCI60 cell panel of tumor derived cell lines was obtained from the NCI/NIH Developmental Therapeutics Program web site, <http://www.dtp.nci.nih.gov>. A univariate test was undertaken for 132 standard agents to evaluate allelic differences in the GI50s. Specifically, the average log GI50 [$X = -\log_{10}(\text{GI50})$] for cells for each of the three genotypes of a given locus (AA, Aa, and aa) were calculated for cells either wild-type or mutant for p53. Subsequently, the probability (P value) was computed that just by chance the difference for the following groupings either was equal to or larger than the actual measurement: (a) $X_a - X_{AA}$ or (b) $X_{aa} - X_A$, or (c) $X_{AA} - X_a$, or (d) $X_A - X_{aa}$, or (e) [$X_{aa} - X_{aA}$ and $X_{aA} - X_{AA}$], and (f) [$X_{AA} - X_{aA}$ and $X_{aA} - X_{aa}$]. These probabilities were estimated using a permutation test (10^6 permutations) that preserved the allele or genotype group sizes but permuted the samples among the groups. Results $P < 0.05$ were considered significant and $P < 0.1$ marginally significant. A multiple hypothesis test was performed for allelic differences in the GI50s across the entire panel of drugs. A Fisher's exact test to compute the statistical significance of observing h univariate hits for a SNP on a total of $D = 132$ drugs, given that overall H significant hits are observed after testing S reference SNPs on the D drugs. All 109,687 Affymetrix genotyped SNPs were chosen as a reference set. Using this methodology, TSC1 rs2809243 and TSC2 rs13335638 were identified (Online resource 2).

Therefore a total of five SNPs in TSC1 and one SNP in TSC2 were further analyzed for associations with breast cancer phenotypes, i.e. associations of age at diagnosis and breast cancer subtype (ductal versus lobular), ER status (ER-positive versus ER-negative) and menopausal status as well as recurrence.

Genotyping

Genomic DNA was extracted from 1 ml of peripheral blood, obtained through venipuncture, using a spin column-based method according to the manufacturer's protocol (QIAGEN). Genotyping for TSC1 and TSC2 SNPs was performed using Taqman assays on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Briefly, reactions were performed using 5–10 ng genomic DNA in 10 μ l volume. For TSC1 rs7874234, rs1076160, rs2809243, rs3761840, and rs1073123 and for TSC2 rs13335638, PCR cycling conditions were 50°C for 2 min, 95°C for 10 min,

followed by 45 cycles of 92°C for 15 s and 60°C for 1 min. Conditions were modified to optimize reactions for the following several loci: TSC1 rs11243940, conditions were identical except only 40 cycles (instead of 45 cycles) were performed; for TSC1 rs739442 and rs10491534, conditions were identical except the final annealing temperature was 58°C.

Statistical Analysis

A permutation test was performed to determine the statistical significance of differences in mean age at diagnosis between different genotype groups (e.g., wild-type homozygote or heterozygote vs. variant homozygote). This permutation test was chosen because it is non-parametric, with the assumption that all genotype groups, or categories, are equivalent and making no assumptions about the age of diagnosis distribution. Fisher's exact test was used to determine the statistical significance of the association between categorical values for each genotype group. The odds ratio and 95% confidence interval were then computed using a Bayesian estimate for the odds ratio posterior distribution.

Results

Analysis in LCLs and haplotype generation

Using genotype data generated by the Hapmap project, the minimal set of TSC1 and TSC2 SNPs sufficient to reconstruct their haplotypes was determined. These 18-tagged SNPs in TSC1 and 14-tagged SNPs in TSC2 were analyzed in LCLs, obtained from healthy individuals, and used to generate haplotype frequencies and are demonstrated for two loci (Fig. 1 and Online Resource 1). For TSC1 rs7874234, since genotype frequencies were similar between Caucasian and African American populations (Table 3), only the haplotype tree for the Caucasian population is shown (Fig. 1). However, since genotype frequencies differed between the Caucasian and African American populations, haplotype trees for both populations were generated for TSC2 rs13335638 (Online Resource 1).

The major TSC1 haplotypes generated, with rs7874234 as the reference locus, accounted for 38.5, 18.9, 10.5, 9, and 2.6% of the genotypes in the Caucasian population (Fig. 1) and was similar in African Americans (Table 3). The C allele was more prevalent and represented 57.4% of the haplotypes. The major TSC2 haplotypes generated, with rs13335638 as the reference locus, accounted for 63.0%, 7.6%, 6.1%, 3.2% and 3.0% of the genotypes in Caucasians, as depicted in Online Resource 1. In the Caucasian population, the major allele was the T allele for

this TSC2 SNP. Haplotype trees for both Caucasian and African American populations are shown for TSC2 (Online Resource 1). For African Americans, there was more heterogeneity, and the major haplotypes occurred at frequencies of 24.0, 22.2, 16.2, 11.0, 9.3, 5.1, and 2.0% (with six haplotypes occurring at 2.0%). In the African American population, both the C and the T allele are represented almost equally, with one C allele haplotype accounting for 22.2% of the genotypes, and one T allele haplotype accounting for 24.0% of the genotypes.

Demographics and SNP Frequencies in the Breast Cancer Cohort

The demographics for the breast cancer cohort are depicted in Table 2. The data shows that the majority of women were Caucasian and the majority of cancers were ductal in origin. The average age at diagnosis was 51.5 years, with patients ranging from 19–89 years of age. Nearly 75% of all breast cancers were ER positive.

Table 2 Demographics of the breast cancer cohort

	<i>n</i>	%
Ethnicity		
African American	63	5.6
Asian	72	6.4
Caucasian	869	77.5
Hispanic	78	7
Other	39	3.5
Type of Breast Cancer		
Ductal	807	83.9
Lobular	94	9.8
Other	61	6.3
Age at Diagnosis, years		
Mean	51.5	
Median	50	
Range	19–89	
ER Status		
Neg	235	25.4
Pos	692	74.6

Table 3 Genotype Frequencies for relevant TSC1 and TSC2 SNPs for all populations in the CINJ breast cancer cohort

SNP	Genotype	Caucasian, <i>n</i> (%)	African American, <i>n</i> (%)	Asian, <i>n</i> (%)	Hispanic, <i>n</i> (%)
TSC1 rs7874234	CC	503 (59.5)	35 (57.4)	54 (75.0)	45 (58.4)
	CT	306 (36.2)	20 (32.8)	17 (23.6)	26 (33.8)
	TT	36 (4.3)	6 (9.8)	1 (1.4)	6 (7.8)
TSC2 rs13335638	CC	30 (3.5)	18 (29.0)	0 (0)	7 (9.0)
	CT	266 (31.0)	26 (41.9)	5 (7.1)	19 (24.4)
	TT	563 (65.5)	18 (29.0)	65 (92.9)	52 (66.7)

Population-specific genotype frequencies were observed for TSC1 rs7874234 and TSC2 rs13335638 (Table 3). TSC1 genotype frequencies did not deviate from Hardy-Weinberg equilibrium (HWE) for any of the races in this cohort. TSC1 rs7874234 genotype frequencies were similar between the populations depicted, except for the Asian population. TSC2 rs13335638 genotypes were in HWE for all populations except the Hispanic subset. Both Asian and Hispanic populations represent a small number of individuals and heterogeneity in area of participant origin. In Caucasians, the TT genotype was observed in 65.5% of the population. However, in the African American population, both CC and TT genotypes were equally prevalent (each accounting for 29% of the population).

Association between TSC1 and TSC2 SNPs and breast cancer phenotypes

Six SNPs across TSC1 and TSC2 were evaluated for associations with breast cancer phenotypes (Table 4). For all comparisons, ancestral homozygotes versus variant homozygotes were used to determine odds ratios and *P* values. Associations for TSC1 and TSC2 SNPs with breast cancer subtype (ductal versus lobular), ER status (ER-positive versus ER-negative) and menopausal status at diagnosis were evaluated but no significant associations were found. A trend was observed for an association between ER status and TSC1 rs1073123 genotype, though it did not reach significance. Likewise, for rs7874234, the mean age at diagnosis between ancestral and variant homozygotes differed by 3.4 years, but this did not reach significance.

All SNP loci were evaluated for association with age at diagnosis. One TSC1 SNP, rs7874234, showed a significant association with age at diagnosis as depicted in Fig. 2. To reduce the heterogeneity in the study population and potential confounders, samples were stratified into groups with the same cancer subtype and ethnic background. In a case-only analysis of rs7874234, for Caucasian women who had ER+ ductal carcinomas, CC, CT, and TT carriers had an average age at diagnosis of 52, 55 years and 61 years, respectively. Homozygous variant TT carriers had a 9-year later age at diagnosis of ER+ ductal

Table 4 Association between TSC SNPs and breast cancer phenotypes

SNP	Ductal versus lobular		ER versus ER+		Pre versus post menopausal diagnosis		Δ Mean age at diagnosis ^a	P value
	OR	P value	OR	P value	OR	P value		
TSC1 rs7874234 CC vs. TT	0.21 (0.03 1.53)	0.09	0.73 (0.38 1.42)	0.36	1.52 (0.68 3.41)	0.31	+3.36	0.06
TSC1 rs1076160 CC vs. TT	0.85 (0.51 1.42)	0.53	1.01 (0.66 1.53)	0.98	1.02 (0.66 1.58)	0.91	0.14	0.44
TSC1 rs2809243 AA vs. GG	1.50 (0.82 2.76)	0.19	1.09 (0.69 1.73)	0.71	0.8 (0.50 1.28)	0.34	1.3	0.12
TSC1 rs1073123 AA vs. GG	0.70 (0.09 5.50)	0.74	0.39 (0.14 1.08)	0.06	0.72 (0.22 2.38)	0.58	+0.75	0.41
TSC2 rs13335638 TT vs. CC	1 (0.38 2.63)	1	1.23 (0.61 2.46)	0.56	1.22 (0.54 2.79)	0.63	1.18	0.47

^a Values reflect analysis of entire breast cancer cohort

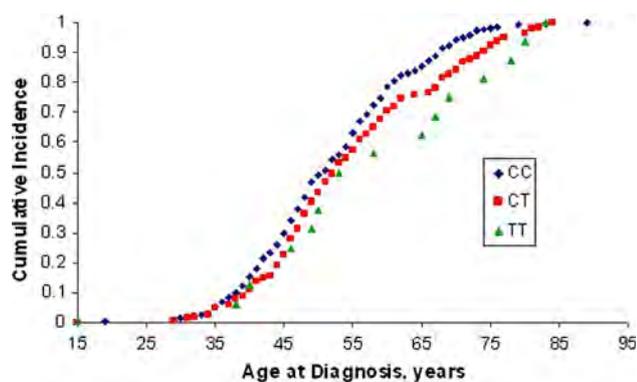


Fig. 2 TT genotype of TSC1 rs7874234 associates with a later age at diagnosis of ER+ ductal carcinomas in Caucasian women cumulative incidence as a function of age at diagnosis was evaluated for TSC1 rs7874234 to determine genotype specific effects. The breast cancer population demonstrates ductal ER+ carcinomas in Caucasian women. CC carriers are depicted by filled diamonds, CT carriers by filled squares, and TT carriers by filled triangles

carcinomas as compared with CC carriers ($P = 0.0049$). When comparing all three genotypes, the curve for heterozygotes fell in between the TT and CC curves, and associated with a 3-year later age at diagnosis than CC carriers and a 6-year earlier age at diagnosis than TT carriers ($P = 0.00036$). When stratifying the analysis by post-menopausal status, post-menopausal women with ER+ ductal carcinomas showed a similar pattern of genotypes for age at onset as was observed in Fig. 2 (data not shown). Specifically, an additive effect was observed in the analysis of post-menopausal women with ER+ ductal carcinomas. CC, CT, and TT carriers were diagnosed at 61, 64, and 68 years of age, respectively ($P = 0.00046$). None of the other five SNPs showed significant associations with age of diagnosis by any stratification.

Discussion

Due to the ubiquitous role of TSC1 and TSC2 in the mTOR pathway and the pathway in tumor biology [22], a

systematic evaluation of clinical associations with SNPs in TSC1 and TSC2 was undertaken. Analysis of TSC1 SNP rs7874234 in this breast cancer cohort showed that in Caucasian women, variant homozygote TT carriers developed ductal ER+ breast carcinomas on average 9 years later than CC carriers. Furthermore, the intermediate age of diagnosis of heterozygotes in comparison to either homozygote indicates an additive effect. The largest differential appears in the postmenopausal age group (assuming the average age of menopause in the US is 51 years of age). No other significant associations were observed for other TSC SNP loci and other clinico-pathologic variables, including ER status, breast cancer subtype and menopausal status.

The effect observed with TSC1 rs7874234 indicates a deleterious effect with the CC genotype and that the TT genotype confers protection against earlier development of ER+ ductal carcinomas. Alternatively, a later age at diagnosis for TT carriers could also mean that tumors in TT carriers are slower-growing and take longer to reach a threshold for detection than CC carriers. In silico analysis showed an estrogen receptor element (ERE) within the flanking sequence of rs7874234. Although there is high variability in ERE sequences, comparison of known EREs in other human genes, revealed sequence homology between “GTTAG” in TSC1 with the ERE identified for Human calbindin-D9k [23]. However, this homology only exists for the T allele. This is suggestive that different alleles in rs7874234 may affect ER binding.

Taken together, these findings support the possibility that the T allele may mediate estrogen-specific effects in risk towards later onset breast cancer. It is hypothesized that the T allele allows for ER to bind to this ERE in TSC1, activating TSC1 transcription and increased inhibition of mTOR, delaying breast cancer in TT carriers. The C allele, however, does not allow for ER binding, and therefore there is no increased inhibition of mTOR, furthering earlier tumorigenesis in CC carriers.

The post-menopausal effect observed fits nicely in our hypothesized mode of action for the T allele: if the T allele

increases binding affinity of activated ER and hence leads to increased TSC1 transcription, this would make TSC1 in carriers more sensitive to the low levels of circulating estrogens in post-menopausal women. In premenopausal women, because the circulating levels of estrogens are much higher, ER binding to the TSC1 ERE is not limiting. Furthermore, effects of estrogens on other cell pathways may be more critical in premenopausal women [24]. Other SNP association studies have demonstrated similar post-menopausal effects [25–27]. A recent study of SNPs in folate and alcohol metabolic pathway genes and breast cancer risk showed an association with a SNP in 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) and increased risk for breast cancer in postmenopausal but not premenopausal women [25]. Another study investigated the role of SNPs in ER-alpha and other estrogen-metabolizing genes and breast cancer risk in Chinese women and found that postmenopausal but not premenopausal breast cancer risk was associated with a heterozygous CYP17 genotype [26].

One advantage of using this study cohort is the availability of extensive clinico-pathologic information that was collected for all study participants. Analysis was enhanced by the ability to reduce heterogeneity within the study cohort. SNP selection which was supported by bioinformatic analysis reduced the number of SNPs to be analyzed, making this a cost-effective approach. This analysis was stratified by race, while other subgroup analysis was limited due to small numbers.

Further study in a larger cohort would allow the detection of associations in other subgroups. For example, analysis of this SNP in the African American population showed no significant association with age at diagnosis of ductal ER+ breast carcinomas; however, this may be due to the small number of African American patients with a TT genotype. Furthermore, defining the molecular mechanism of SNP functionality would further support the association observed for rs7874234. TSC1 has not been previously shown to be regulated by estrogen, and while informatics gave evidence for a plausible explanation for rs7874234 functionality, the possibility exists that another SNP, not rs7874234, could be the functional SNP. Therefore, the effect observed could be due to other SNP(s) in the haplotype represented by rs7874234. As a case-only study, a limitation of this study is that it only evaluated one aspect of risk, i.e., age at diagnosis, whereas overall risk for development of breast cancer would provide further clinical utility in potentially identifying individuals at risk for the disease and knowing the optimal time for screening and prevention.

In this cohort, no association was found between any TSC SNPs and recurrence (data not shown). Although analysis of drug response in the NCI-60 cell lines

demonstrated genotype-specific effects for another TSC1 SNP, rs2809243, these results were dependent on p53 status (Online Resource 2). In a p53 mutant background, the genotypes for rs2809243 significantly differed in their growth inhibition to several different chemotherapeutic agents ($P = 4.7 \times 10^{-7}$). However, the heat maps showed a divergent effect between a p53 mutant versus p53 wild-type background. For example, the TT genotype associated with a better response to alkylating agents in a p53 mutant background, while the CC genotype associated with better response to these same agents in a p53 wild-type background, indicating an opposing effect between p53 mutant and p53 wild-type backgrounds for the same class of drugs (Online Resource 2). Overall, the T allele associated with better response in the p53 setting, while the C allele correlated with better response in a mutant p53 background. Since upwards of 30% of tumors harbor p53 mutations in breast cancer [28], the absence of a recurrence-phenotype in the breast cancer cohort, where p53 status is unknown, may not reflect a true lack of association.

In summary, we found that the TSC1 rs7874234 variant associated with delayed age at diagnosis of ER-positive ductal carcinomas in Caucasian women. The observed findings are intriguing and bear confirmation in other breast cancer populations.

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Research Article

Germline Mutations and Polymorphisms in the Origins of Cancers in Women

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Several female malignancies including breast, ovarian, and endometrial cancers can be characterized based on known somatic and germline mutations. Initiation and propagation of tumors reflect underlying genomic alterations such as mutations, polymorphisms, and copy number variations found in genes of multiple cellular pathways. The contributions of any single genetic variation or mutation in a population depend on its frequency and penetrance as well as tissue-specific functionality. Genome wide association studies, fluorescence in situ hybridization, comparative genomic hybridization, and candidate gene studies have enumerated genetic contributors to cancers in women. These include p53, BRCA1, BRCA2, STK11, PTEN, CHEK2, ATM, BRIP1, PALB2, FGFR2, TGFBI, MDM2, MDM4 as well as several other chromosomal loci. Based on the heterogeneity within a specific tumor type, a combination of genomic alterations defines the cancer subtype, biologic behavior, and in some cases, response to therapeutics. Consideration of tumor heterogeneity is therefore important in the critical analysis of gene associations in cancer.

1. Inherited Mutations that Predispose to Cancers in Women

There is strong evidence that inherited genetic factors (mutations plus single nucleotide polymorphisms) can play a major role in breast cancer susceptibility [1]. Inherited mutations in a small number of genes account for about five to ten percent of women's cancers. These inherited variations, identified in breast, ovarian, and endometrial cancer susceptibility, can be characterized in the general population by their frequency and the magnitude of their impact upon a patient (Table 1). Some inherited variants occur rarely in the general population, but confer large risks to the individual. Examples of these genes are BRCA1 and BRCA2 in breast and ovarian cancers. A second class of inherited variants confers a lower risk, and these variants are also rare in the general population. An example of this class of genes is a mutation in the CHEK2 gene in breast cancer. The third class, composed of high-risk variants that are also common in the population, has never been identified by the

methods presently available and may in fact not exist because it may well be strongly selected against in populations. Finally, a fourth class of inherited variants includes those that confer low disease risk to the individual, but occur at higher frequencies in populations. These include some of the recent findings from genome-wide association studies (GWASs) mostly with breast cancers. A summary of the major findings to date for these genes is in Table 1 and is discussed in what follows.

Despite these advances made in identifying inherited breast cancer susceptibility genes, the vast majority of breast cancers are sporadic, that is, no identifiable mutation in one of the known breast cancer susceptibility genes. While this may reflect the fact that we have yet to identify the next BRCA gene, it may also reflect the polygenic nature of breast cancer susceptibility. Other contributors to genetic susceptibility, for example, polymorphisms, may have a higher relative contribution to risk, but their lower penetrance makes identification more difficult. Furthermore, modification of genetic susceptibility by environmental factors,

TABLE 1: Genetic loci implicated in hereditary, familial, and sporadic breast cancer susceptibility.

High penetrance, low frequency	Low penetrance, low frequency	Low penetrance, High frequency
BRCA1	CHEK2	FGFR2
BRCA2	ATM	LSP1
PTEN	PALB2	MAP3K1
p53	BRIP1	TGFB1
STK11		TOX3
		2q35
		8q

both endogenous and exogenous, may alter the degree of penetrance. Supporters of the polygenic nature of breast cancer suggest that the contributions from polymorphisms are very important because of their high frequency in the population.

1.1. High-Penetrance, Low-Frequency Inherited Variants. Although inherited mutations in a small number of genes account for only about five to ten percent of women's cancers, by far the BRCA1 and BRCA2 gene mutations are the most common examples of this observation (50–70% of familial breast cancers) [2]. In some populations BRCA1 and BRCA2 mutations can account for ten percent of all breast cancers (Ashkenazi Jewish populations) and ovarian cancers but in many ethnic groups and in all populations taken together these mutations are much rarer (reviewed in [3]). The BRCA1 and BRCA2 proteins appear to be scaffolding proteins that assemble DNA repair complexes of proteins at double-strand DNA breaks (mediating homologous DNA repair processes) (reviewed in [4]). Mutations in these genes result in a faulty repair process and a high mutation rate, especially during DNA replication, leading to cancers. The penetrance of these mutations for cancer occurrence and the age of onset of these cancers in women can be quite variable. There have been a number of other possible functions ascribed to the BRCA1 and BRCA2 proteins such as ubiquitin ligase activity and a modifier of transcription and it is certainly possible that these protein complexes act in several ways [5]. Breast cancers initiated in women who are heterozygous for BRCA1 or BRCA2 often have a reduction to homozygosity at the BRCA-locus eliminating its functions. This results in DNA damage in the tumor which should activate the p53 protein resulting in apoptosis, senescence, or cell cycle arrest. If this is the case, the p53 gene product would be a suppressor of this cancer phenotype and contribute to the variable penetrance of these breast cancer genes. Consistent with this is the observation that BRCA1/2-initiated breast cancers have very high rates (29–84%) of somatic p53 mutations compared to 14–35% in non-BRCA1/2-related breast cancer [6].

Inherited mutations in several other genes, such as PTEN and p53, can give rise to cancers in women. Cowden's Disease is a heterozygous deficiency in the PTEN gene that can result in breast, endometrial, and other cancers [3, 7]. The

PTEN protein is a lipid (PIP-3) phosphatase that modulates a growth factor pathway, in turn regulating metabolic pathways in cells, angiogenesis, mitochondrial functions and apoptotic functions [8]. Genetic alterations in this pathway are among the most common somatic mutations observed in breast and endometrial cancers [9, 10]. Mutations in LKB1 also predispose to breast and ovarian cancers as one of the phenotypes in Peutz-Jeghers syndrome [3, 11]. Inherited defects in one allele of the p53 gene give rise to Li-Fraumeni syndrome, where a subset of the cancers observed at an early age are breast cancers [12].

1.2. Low-Penetrance, Low-Frequency Inherited Variants. This class of inherited variants is difficult to detect with existing methods because the rarity of these variants and coupled with small effect sizes this means that most association studies will not be able to detect them due to limitations in population sizes under study. In the extreme, these variants may represent "private" mutations that confer a small degree of risk to very few individuals in this population, such that nearly every person would have a unique set of predisposing alleles. While it has been difficult to detect inherited variants of this type there are several examples of this type of variant which were uncovered by examining candidate genes that an investigator suspected played a role in a cancer. Inherited alterations in the CHEK2 gene which normally produces a protein kinase found in signal transduction pathways (p53 pathway and others), alerts the cell that there is DNA damage and its loss can have an impact upon several types of cancer [13]. Similarly the ATM protein kinase harbors genetic variants that detect single- and double-strand breaks in the DNA and signals to the p53 pathway and other DNA repair processes. Variants in this gene could lower or raise the sensitivity of this DNA damage detector and impact upon the efficiency of p53 and its tumor suppressor pathway and can predispose women to breast cancers [14]. The BRIP1 gene (BRCA1 interacting protein-1) encodes a protein that is a DNA/RNA helicase of the REC Q family that binds to the carboxy-terminus of BRCA1 protein conferring an activity involved in DNA repair and variants of this gene can predispose to breast cancers [15]. Interestingly this gene product is also a component of the Fanconi anemia gene pathway for DNA repair processes. Finally the PALB2 gene product (partner and localizer of BRCA2) is part of the BRCA2 protein complex and plays a role in DNA repair. It has recently been shown to be a genetic determinant of familial breast and other cancers primarily in the certain populations, but found at even lower frequency in other populations [16].

1.3. Low-Penetrance, High-Frequency Inherited Variants. Fewer than 10% of breast cancers are attributable to known mutations in breast cancer susceptibility genes BRCA1 and BRCA2. The multigenic susceptibility due to common, low-penetrance risk markers is yet to be defined [1, 17–20]. Both candidate gene [21] and genome-wide association studies have identified novel markers for susceptibility [22–25] and prognosis [26]. Genome-wide association studies

have become widely used to identify commonly occurring alleles at disease susceptibility loci. These studies use a large number of high-density markers to identify associations with disease that rely upon patterns of linkage disequilibrium in the human genome. GWASs have been successful in identifying genes for breast cancer, and GWASs for ovarian and endometrial cancers are underway although several investigators have validated findings from GWAS studies designed originally for breast cancer studies but employed for ovarian cancer [27]. Some of the more reproducible genes that GWASs studies have indicated can play a role in the risk for developing breast cancers include FGFR2, LSP1, MAP3K1, TGFB1, TOX3, 2q35, and 8q [17, 22, 24].

2. Somatic Mutations That Are Commonly Observed in Women's Cancers

Both gene amplifications and deletions can lead to common somatic mutations in women's cancers. Among the amplifications are the following. (1) HER-2/Neu, amplified in about 15% of the breast cancers, is a growth receptor that activates the Ras-MEK and the PI3K pathways in cancer cells [28]. (2) Cyclin D, amplified in about 10–12% of the breast cancers, is a subunit of the cyclin dependent kinase –4/6 that acts upon the Rb protein freeing the E2F transcription factor for entry into the cell cycle [28, 29]. (3) WIP1, amplified in about 13% of breast cancers, is a serine/threonine phosphatase that inactivates the ATM kinase and the p53 protein [30]. The GASC1 gene, which produces a histone demethylase activity, is amplified in about 5–10% of breast cancers but 20–25% of the basal breast cancers. This enzyme removes dimethyl and trimethyl groups from histone H-3 lysine-9 and 36 residues which results in altered transcriptional patterns in these cells. Inactivation of gene functions by deletion or other mechanisms commonly occurs in (1) PTEN in breast, ovarian, and endometrial cancers, and (2) p53 in HER2/neu positive breast cancers, triple negative breast cancers, and BRCA-associated breast and ovarian cancers. PI3K amplifications and activating mutations are common in breast and endometrial cancers [31, 32] and Ras activating mutations are common in endometrial cancers. Several genes such as AKT and STAT3 are often expressed at high activities in all of these cancers but without detectable amplifications of those genes. Epigenetic alterations, such as methylation of cytosine residues in CpG dinucleotides, can bring about the inactivation of genes (p16 gene in breast cancers) while mismatch repair defects have been observed to enhance the mutation rate of many genes in endometrial cancers. In addition to those somatic mutations discussed here, a large number of mutations in many oncogenes and tumor suppressor genes have been observed at lower rates in women's cancers.

Large copy number variations in genetic loci from tumor tissues have been observed using fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH), and a reduced heterozygosity of single nucleotide polymorphisms over large regions of a chromosome. This

type of genomic instability has been observed at many loci in all chromosomes in some breast tumors. Other breast tumors demonstrate little or no genomic instability (below the level of detection). As a generalization those individuals who have tumors that demonstrate very high levels of genomic instability have a poorer prognosis [33]. While some loci are repeatedly amplified, as occurs in Her2 overexpressing breast cancers, or deleted, as with PTEN in endometrial cancers, the heterogeneity of mutations in women's cancers is striking. There are many mutational paths to initiate and propagate a tumor.

Notably however, somatic mutations often occur in genes where germline mutations in those same genes are the etiologic factors in cancer susceptibility syndromes. Alternatively, somatic mutations occur in other genes involved in regulatory aspects of those vital pathways. Despite the number of mutational pathways to initiate and propagate tumors, several specific genomic alterations are associated with particular breast cancer phenotypes. These phenotypes are manifested in their molecular profile, biology, and prognosis. Patterns of transcriptional profiles obtained from breast tumors have permitted a fairly reproducible classification of breast cancers that are derived from different cell types or have evolved under the influence of different gene expression patterns [34–38]. These different transcriptional patterns correlate well with critical diagnostic criteria (ER+, PR+, HER-2/neu+, triple negative, BRCA1) that guide both diagnosis and treatment protocols for these types of breast cancers. The classification also correlates well with some mutations such as p53, but other causal mutations such as cyclin D and WIP1 amplifications, PI3K and STAT3 activations need to be explored. Classification based upon transcriptional profiles also associates well with several clinical parameters. For example, luminal A cancers are hormone receptor positive, are diagnosed primarily in older women, are low grade with low proliferative index, and have mainly wildtype p53 [35, 38]. Luminal B cancers also tend to retain wildtype p53 but have reduced or absent expression of progesterone receptor and are more likely to recur than luminal A cancers [35, 36, 38]. In contrast to luminal tumors, basal cancers are hormone receptor negative and Her2 negative, are more likely to be diagnosed in young, premenopausal women, are high grade with high proliferative index, and are associated with higher risk of recurrence [35–38]. Her2-amplified breast cancers, regardless of hormone receptor status, are of higher grade and proliferative index, have worse prognosis with higher recurrences in first five years after diagnosis, and commonly have p53 mutations [35, 36]. Like basal tumors, BRCA1-associated breast cancers predominantly occur in young, premenopausal women, are primarily hormone receptor negative, and the most likely to carry p53 mutations [34]. Unfortunately this type of detail and analysis does not yet exist for ovarian and endometrial cancers.

Thus, it is now clear that there are at least five types of breast cancer with characteristic transcriptional profiles that can harbor some subset of mutations that drive these cancers [39]. Importantly, each type of breast cancer calls for different treatment protocols and often results in different

outcomes. We have only partially established the critical mutational patterns in each type of breast cancer and we have only begun to extend this type of analysis to other women's cancers. However, it is apparent that breast cancer heterogeneity reflects underlying genomic alterations leading to different biology and phenotypes.

3. Single Nucleotide Polymorphisms (SNPs) and Their Phenotypes

Inherited mutations in genes involved in DNA repair processes (BRCA1, BRCA2), cell cycle checkpoints and apoptosis (p53, Rb), and gene products that regulate critical pathways (PTEN) clearly play a central role in predetermining the initiation of cancers, often with an incomplete penetrance. Polymorphic alleles in many additional genes, often in these same signal transduction pathways, can also contribute, albeit in a smaller quantitative fashion, to the origins of a cancer, the propagation of a cancer, and the treatment responses of a cancer. By definition a mutation in a gene occurs rarely in a population (below 1% of the population under study) while a polymorphism occurs more commonly. Because these polymorphic alleles can act cooperatively and many genes in the same signal transduction pathway can show epistatic relationships, single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) can have observable impact upon the incidence of a cancer in a defined population, the age of onset of a cancer, the response to treatment, the frequency of relapse, and the overall survival of a patient population. Thus in addition to inherited mutations, SNPs and CNVs in a population provide a genetic background that can influence the cancer cells harboring the inherited and somatic mutations that arise and cause a tumor. The phenotypes observed in people with inherited mutations in cancer causing genes are an increased incidence of cancers in a family or population and an earlier age of onset of a cancer than observed in the total population. Mutations in the p53 gene show this pattern and in addition multiple independent cancers in the same individual can be observed [3, 12]. Inherited mutations also often produce a limited set or tissue type of cancer such as BRCA1 or BRCA2 with breast and ovarian tumors [3]. It is thought that the BRCA1 and BRCA2 proteins function in many tissues to repair DNA damage, so the limited cancer causation to breast and ovary remains a mystery. Indeed all of the tumor suppressor genes demonstrate a tissue preference in the tumors they cause when they function as inherited alleles but somatic mutations in those same genes are often found in a much wider group of cancers of different tissues [40]. SNPs and CNVs will likely also have limited tissue impact upon cancers and like inherited mutations, functioning throughout development and life, can have cumulative impact over a lifetime.

The possible role of a SNP or an CNV in cancer is usually demonstrated by an association study correlating the presence of an "at-risk" allele with the incidence of a cancer or a related phenotype. This is fundamentally a statistical argument that provides correlation, not causality.

The situation gets better if the at-risk allele can be shown, in vitro or in vivo, to have a different level or activity that could lead to the population wide phenotype. Examples of these correlations are now being demonstrated for results from GWAS and are taking into account tumor subtypes [41]. Thus molecular and cellular studies can provide an important rationale for the population study results. In some cases it may also be possible to model the at-risk allele in another genetic system, such as a mouse carrying the alternate human alleles in the orthologous gene of the mouse. One would then explore the phenotypes observed in humans using such a mouse model. In this way it may be possible to move from correlation to causality. In the process of these studies one may learn about the details of the properties of an SNP or CNV that enlightens the population studies. A very good example of this is an SNP, SNP309, in the first intron of the MDM2 gene in humans. The MDM2 protein is a ubiquitin ligase which negatively regulates p53 levels in a cell by polyubiquitination of the p53 protein followed by its degradation. Thus MDM2 levels and activity in a cell regulate the p53 protein levels in a cell. SNP309 in the MDM2 gene comes in two forms, a G-allele and a T-allele. The first intron of the MDM2 gene contains sites for transcription factors that regulate the levels of the MDM2 mRNA. The G-allele binds a transcription factor, Sp-1, better than the T-allele [42]. Ten base pairs away from this Sp-1 site is an ER binding site and the Sp-1 and ER transcription factors can interact so that the highest levels of MDM2 mRNA and protein are produced in cells that are G/G homozygotes and ER+ and exposed to estrogen as shown in breast cancer cells in culture. Indeed the association of the G-allele of SNP 309 with an early age of onset of a cancer is most commonly observed in premenopausal women with ER+ tumors [43]. Thus if one analyzes all women with breast cancers for an association with the presence of the "at-risk" allele of this SNP the statistical test for an association commonly fails. Only when the association is tested with premenopausal females with ER+ tumors can a clear association be found. This is a good example of understanding the biology and genetics before one undertakes large association studies.

The human genome of any individual contains about three million SNPs that distinguish that person from another. In the population of humans there are an estimated fifty million SNPs. Most of these differences have no detectable phenotype. Because of this, large genome wide scans (GWAS) of SNPs now employ a million SNPs to test for an association with a disease [44]. This is clearly an exercise in multiple hypothesis testing and so one requires very large populations of cases (and controls) and a statistical significance (a type 1 error rate) that provides a $P = 10^{-7}$ value. Even then the number of false positives can be large and so repeated independent studies are required to refine the truly significant associations. This is in part why many SNP associations with cancers have been so poorly reproducible. Small study populations will often give lots of false positives not observed in independent repeat studies. In case-control studies where one is examining the different allele frequencies in a case and a control

group there is presently no mathematical test to prove that these two populations are equivalent. Allele frequencies can differ in racial groups or other populations and while it is easy to control for some parameters we do not know all of the variables. In spite of these difficulties several recent publications employing GWAS approaches with large populations have been reported for associations with breast cancers, colorectal cancers, lung cancers, melanomas and prostate cancers [17, 22, 24, 25, 45–51].

A different approach to uncovering active SNPs with associations to cancers is to examine a small number of candidate genes for the presence of SNPs that impact upon a phenotype. The criticism of this approach is that novel genes and SNPs that impact upon a cancer will not be discovered by this approach. Rather it is a chance to delve deeper into the diversity and properties of a gene, its protein and its phenotypes in a population. The most likely candidate genes that have functional SNPs are the ones that have mutations in some cancers or provide an inherited basis for cancer when they are mutated. Because SNPs are expected to be less deleterious than a mutation which inactivates or fully activates a function, it is helpful to look for candidate SNPs in genes that demonstrate haploinsufficiency. This encompasses those genes that have a cancer-related phenotype when an individual has only one wild type allele, and presumably half the activity and level of a protein. Interestingly the p53 gene, MDM2 gene, and the MDM4 gene (a second negative regulator of p53 that acts upon MDM2) are all haploinsufficient genes in mice and p53 is haploinsufficient in humans (there is presently no test for MDM2 or MDM4 haploinsufficiency in humans) [52, 53]. These three proteins make up the core of p53 regulatory activities in a cell (see Figure 1). There is a great deal of evidence demonstrating that the levels and/or activities of these proteins in a cell are tightly controlled by extensive feedback loops and that small changes in these proteins have phenotypes that are readily observed.

Another way to look for SNPs that have biological activity is to examine whether a mutant allele or a polymorphic allele is under negative or positive selection in a population. If that is the case then that allele must have a biological activity that impacts upon the organism. There are now a growing number of methods to look for regions of a genetic locus under positive or negative selection. Selection pressures in humans commonly result from genes that contribute to resistance to infectious diseases (about 20% of human cancers are caused by or associated with viruses), optimal use of nutritional opportunities (the IGF-PI3K-PTEN-mTOR pathways help to regulate this), or the highest levels of fecundity, leaving more offspring in a population (the p53 pathway can participate in this and is discussed below). Employing information theory-entropy based methods Atwal et al. have suggested that some alleles of MDM2 and MDM4 are under positive selective pressures in Caucasian populations [54, 55]. Based on identification of selected loci in MDM4, studies have now demonstrated associations between MDM4 SNP loci with risk of breast and ovarian cancers as well as age of onset of ovarian cancers and hormone receptor negative breast cancers [55, 56].

There is also evidence that an allele in the coding region of the p53 protein may also be under positive selective pressure in Caucasian populations. This SNP at codon 72 in the p53 protein (out of 393 amino acids) either encodes an arginine (Arg) or a proline (Pro) residue. The Pro-allele is the ancestral form and Africans near the equator have very high levels of the Pro-allele. As populations move to northern latitudes in Europe (Caucasians) and in Asia (Asians) there is an increasing frequency of the Arg-allele reaching 75–85% in Scandinavia. One explanation for this distribution comes from the observation that p53 induces the synthesis of pro-opiomelanocortin which regulates the tanning response. This could be thought of as a protective mechanism for light-skinned populations or helping to protect individuals of lighter skin color which was developed to enhance the production of vitamin D in northern climates. In a recent study in China, a correlation was found that implicates both temperature and ultra-violet light sources as the driving forces upon selection of the p53 Arg/Pro and SNP309 polymorphisms [57].

There is a growing body of evidence that the newly formed and selected Arg-allele in Caucasian and Asian populations has quite different properties than the Pro-allele. Cells in culture with the Arg-allele transcribe several proapoptotic genes at higher rates than the same cell lines with Pro-alleles. Several studies have demonstrated that cells with the Arg-allele undergo higher frequencies of apoptosis than the same cells with Pro-alleles. A deletion of the p53 protein proline rich domain, in which the Arg/Pro polymorphism resides, reduces the efficiency of apoptosis by that mutant p53 protein. These studies demonstrate at the cellular and molecular level that functional differences exist between these two alleles of the p53 gene [58–61].

Perhaps the best explanation for the selection of the p53 Arg allele in Caucasian populations is the observation that the cytokine leukemia inhibitory factor (LIF) is regulated at the transcriptional level by p53 and two times more LIF is produced in cells by the Arg-allele than the Pro-allele of p53 [62]. p53-mediated production of LIF in the uterus is required for implantation of mouse embryos after fertilization (LIF is also produced in humans for implantation) and so both p53 and LIF are required for high levels of fecundity [63]. Interestingly, the frequency of the p53 Pro-allele is quite enriched in women who are at an in vitro fertilization clinic and demonstrate lower levels of implantation of fertilized eggs [62]. This observation may explain the selective pressures on these alleles in Caucasians. Obviously there are not similar fertility difficulties in Africans with the Pro-allele, suggesting that the genetic background (other alleles in genes in the p53 pathway) is an important factor for this phenotype. Poor fertility was observed in p53 knockout mice and varied in different genetic backgrounds [63]. Some of the compensating MDM2 and MDM4 alleles are also under selection pressures in Caucasian populations. It has been these types of studies that have identified functional SNPs that can now be tested for their activities and associations with specific cancers.

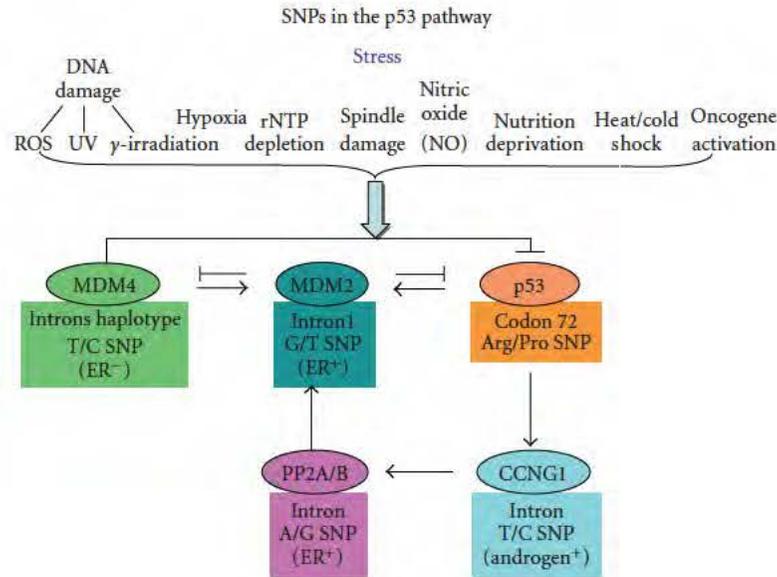


FIGURE 1

4. The p53 Pathway and Cancer Prevention

The p53 protein and its signal transduction pathway respond to a wide variety of stresses and act as a fidelity check point preventing mistakes leading to high mutation rates. Cellular stresses such as DNA damage, telomere shortening, hypoxia, nutrient deprivation, an interruption of ribosome biogenesis, errors in proper mitotic spindle functions, or even the mutational activation of selected oncogenes (*myc*, *Ras*) can activate the p53 protein so that it becomes an efficient transcription factor for selected genes. A wide variety of protein kinases, histone methylases, ubiquitin ligases, and so forth participate in detecting these stress signals and modifying the p53, MDM2, or MDM4 proteins. This results in shutting down the MDM2/4 negative regulation of p53, an increased half-life, and an increased concentration of the p53 protein in the cell (Figure 1). Higher levels of a modified p53 protein then give rise to a transcriptional response of p53 regulated genes. The most common outcomes of this signal transduction pathway are apoptosis, cellular senescence, or cell cycle arrest. Because these stresses upon a cell can cause a very high error rate in both DNA replication and cell division, p53 blocks progression through the cell cycle or eliminates clones that contain mutational events. In this way p53 acts as a tumor suppressor gene over a lifetime of stressful events. The presence of only one wild type p53 allele in mice or humans (Li-Fraumeni syndrome) leads to an early onset of tumors compared to the wild type population and often leads to multiple independent tumors in an individual with almost a one hundred percent penetrance [64, 65]. Four independent studies have now shown that the G-allele (at-risk allele) of SNP309 in the MDM2 gene, which raises the levels of this mRNA and protein inhibiting p53 activity, lowers the age of onset of tumors and increases the number of independent tumors observed in individuals with Li-Fraumeni syndrome [42, 66–68]. Those individuals with

Li-Fraumeni syndrome who do not have a p53 mutation (patients with a high frequency of tumors due to an unrelated mutation) are not affected by the SNP309 G-allele demonstrating the specificity and epistatic relationship between MDM2 and p53. Li-Fraumeni patients with a p53 mutation also have more rapid telomere erosion, demonstrating the role of p53 and SNP309 in this process [67]. It is well known that p53 senses the loss of telemetric DNA and will stop cell division or cause cellular apoptosis. One study examining both the p53 Arg/Pro and SNP309 polymorphisms suggested that the p53 Pro-allele can have an impact upon the age of onset of cancers (earlier) and survival (poorer) in patients with Li-Fraumeni syndrome and SNP309 can make the situation worse, but the number of patients with both genotypes was too small to obtain a statistically confident result [68].

5. SNPs in the p53 Pathway Associated with Breast and Ovarian Cancers

The literature examining the association of SNPs in genes in the p53 pathway is fraught with contradictions. Undoubtedly, this comes about for several reasons. First, studies involving small population sizes do not necessarily provide adequate statistical power. Second, studies may fail to stratify populations into groups that reflect the biology and clinical impacts of a cancer. For example, MDM2 SNP309, which acts preferentially in premenopausal females with ER+ tumors, is a smaller group in the total cohort. Third, there is a failure to understand that SNPs in different genes may have very different phenotypes in the context of a cancer (tissue specificity, the age of onset phenotype, etc.). Finally, there is a tendency to make comparisons of cases and controls that are not biologically or genetically equivalent.

At present, the structure of many association studies leads to false positives and negatives as well as uncovering an occasional functional SNP. For example, a very large population of women with breast cancer was analyzed in a GWAS but the patients were not stratified by ER status or for any of the clear differences between different types of breast cancers [22]. Clearly, this will dilute any signal that comes from just one of these cancer types. In addition, when SNPs in tumor suppressor genes or oncogenes are under study it may be the case that a mutation in that gene will eliminate the SNP from being identified or all SNPs in genes epistatic to the mutated gene may no longer score in the association study. Because of these difficulties we must rely upon the independent replication of results as well as a functional explanation for how an SNP is acting to uncover an association. In a formal large meta-analysis of published results from the literature van Heemst et al. [69] studied the impact of p53 Pro/Pro and Arg/Arg polymorphisms upon the frequency of developing cancers and upon the longevity of the population under study. They found that individuals with a Pro/Pro genotype had an increased risk of developing a cancer over their lifetimes when compared to individuals with an Arg/Arg genotype. In a prospective study of individuals 85 years and older, carried out with 1226 people over a ten-year period, they found that people with the Pro/Pro genotype had a 2.45 increased proportional mortality from cancer ($P = .007$). But this group also showed a longer longevity (a 41% increased survival in the population, $P = .032$). One interpretation of this result is that the Arg/Arg genotype has a higher apoptotic rate in response to stress and so protects against cancer better, but also kills stem cells more efficiently over a lifetime, reducing longevity [69]. This suggests that studying older patients may reveal a phenotype in this p53 SNP because it acts over a lifetime to protect the host from stresses. By the same token, older mice show declines in p53 activities with age and this lower level of p53 responses could uncover a phenotype at older ages that is too robust to measure in younger groups [70]. If this interpretation is correct one can see why the p53 Arg/Pro SNP has given rise to such contradictory responses when the ages of the case and control groups are not taken into account.

Similarly, studies with MDM2 SNP309 have produced contradictory associations with cancers. Some of this has to do with mixing both males and females into the cohort under study (SNP309 is regulated by the ER), some of this has to do with a failure to separate ER+ and ER- tumors in the analysis, and some studies just choose the wrong phenotype or cohort to measure. For example, the observation that the G-allele (the at-risk allele) of SNP309 is associated with an earlier age of onset in a variety of cancers has now been reproduced by many independent groups employing soft tissue sarcomas [43], lymphomas [43], leukemia [71], melanomas [72], head neck [73] and oral squamous cell carcinomas [74], gastric cancer [75], colon cancers [76, 77], lung cancers [78–80], endometrial cancer [81, 82], bladder cancers [83, 84], glioblastoma [85], neuroblastoma [86], and both breast cancers [43] and ovarian [87] cancers. In a study

of lung cancers, Lind and her colleagues [78] found that G/G homozygotes had a 1.62 odds ratio of developing cancer compared with T/T homozygotes. When only females in the study were considered the G/G to T/T odds ratio was 4.06. This is the same result observed in an independent study with large diffuse B-cell lymphomas where the G-allele of SNP309 was associated with cancer only in premenopausal females and not in postmenopausal females nor in men [43].

In a third recent study of melanomas, a similar association was found only in premenopausal females [72]. This pattern suggests that active estrogen receptors are present in a large number of tumors and can affect the outcome of the disease. This set of genetic observations opens up possible new routes for therapy with these tumors. In another large study with lung cancer patients carried out in China by Zhang et al. [80] they demonstrated an odds ratio of 1.83 for the G/G over T/T alleles and a 1.47 fold odds ratio for the p53 Pro/Pro over Arg/Arg individuals. Those patients who were G/G and Pro/Pro had an odds ratio of 4.36; whereas those smokers (a mutagenic stress that activates p53) who were Pro/Pro, G/G had an odds ratio of 10.41. Understanding the biology of the signal transduction pathway can permit one to study the relevant variables. Often combinations of SNPs that have an epistatic relationship can provide much more significant results.

Recently, biologically functional SNPs were detected in the MDM4 gene that appear to be under evolutionary selection pressures and have an impact upon fecundity in females at an IVF clinic [62, 63]. Association studies employing five different patient populations have indicated that selected alleles of these SNPs confer an increased risk for or early onset of breast cancers and ovarian cancers [55, 56]. The ethnic backgrounds of the cohorts under study made a difference in the ability to detect these associations so that once again a genetic background of other SNPs that reside in other genes in the same pathway could play an important role. The minor alleles of these same MDM4 SNPs demonstrated a clear enrichment in their frequencies in women at an IVF clinic who had difficulties with implantation of embryos. These diverse phenotypes suggest a functional consequence of these SNPs that can be selected for or against over recent (Caucasian and Asian) times of human evolution. It will be important to observe replications of these data in a wide variety of cancers.

Because many of the cancer treatments result in DNA damage and other stresses to a cell, the response (as determined by a combination of SNPs) to treatment and long-term survival could depend upon the combination of alleles in the p53 pathway SNPs. To test this notion association studies will have to assemble large groups of individuals who have experienced a defined cancer and treatment and record the outcomes over many years. Such cohorts are more difficult to assemble but are an important part of this effort. Characterizing the patient and the tumor genome prior to the selection of treatments is a growing concern and would be aided by the use of validated SNPs and mutations.

6. Limitations with SNP Studies

The success of these studies has been in part due to the use of large study samples (usually based around multicenter consortia) and replication data sets that have been designed into the gene discovery algorithm. Although this approach maximizes the identification of possible genes involved in contributing to breast cancers, these studies often give rise to a number of false positive findings due to multiple hypothesis testing with a very large number of SNPs in the GWAS scans. In addition, the GWAS approach tends to optimize the discovery of genes with statistically significant marginal effects. Therefore, it may miss significant genes. First, genes may not be detected whose effects are not significant on the margin but are significant in conjunction with other genes or exposures. Second, genes may not be identified if there is substantial genetic heterogeneity among cases, such that the proportion of individuals in the population whose disease is caused by a gene is so small that its effect is “washed out” in the total sample. Third, GWASs tend to favor large numbers over epidemiologically rigorous study designs. Although large samples are clearly required to detect small effects, and replication/validation of initial results maximizes the chances that reported associations are true positives, it is possible that unmeasured biases due to study design limitations may have resulted in high false negatives.

Likewise, meta-analyses, used to critically evaluate and statistically combine studies, have been performed for MDM2 SNP309. However, a caveat to such an analysis is that the studies are comparable. Population-specific effects and SNP functionality in independent racial genetic backgrounds may exist and limit the ability to combine heterogeneous study groups. To emphasize this, Shi et al. describe a causative selection of MDM2 SNP309 and p53 Arg72 associated with environmental stresses, that is, cold winter temperatures and UV intensity, wherein the two SNPs are not coselected [57]. This is further supported by two publications describing population-specific differences between African-Americans, Caucasians, and Caucasians of Ashkenazi Jewish descent for both MDM2 SNP309 and MDM4 haplotypes [54, 55]. A combined analysis for SNP309 was presented in Wilkening et al. [88]. Data from eleven breast cancer studies, five colorectal cancer studies, or seven lung cancer studies were each combined for a fixed meta-analysis. Based on their analysis, they concluded that the SNP309 variant did not have an impact on risk or colorectal cancers, but did exhibit increased risk in the homozygous state for lung cancer. They concluded that SNP309 alone has little effect on the risk of common cancers. In reviewing criteria of studies within the analysis, there is significant heterogeneity between study groups. Other studies have also previously concluded that the effects of SNP309 are evident in women but not in men and in hormone receptor-positive breast cancers [43]. Most studies do not differentiate between gender or hormone receptor positive diseases, both of which may dilute any effects. In contrast to the conclusions made by Wilkening et al. [88], those of Hu et al. [89] are that the homozygous variant is associated with increased risk of all types of tumors where tumor type and ethnicity contributed

to substantial heterogeneity. The latter publication by Hu et al. [89] describes in detail methods for identifying appropriate studies, data extraction and analysis. The majority of publications fail to reduce heterogeneity in their populations based on molecular markers, gender, and degree of disease heterogeneity. Therefore, a meta-analysis would be limited by these factors and should be interpreted with caution.

The majority of useful and reproducible reports involving SNP associations with breast, ovarian, and endometrial cancers have occurred in the context of candidate gene studies. These studies typically identify loci that are hypothesized to contain genetic variants that may be associated with disease risk. Using this approach, a number of putative susceptibility genes have been identified for these tumor sites that have been validated. Furthermore, candidate gene studies have tended to have used more rigorous study designs, collected useful epidemiological and confounder data, and have detailed information that may define etiologically heterogeneous groups of individuals that may provide a setting in which genes that are not easily detectable in the usual GWAS setting may be found. This comes about because we have a great deal of information about those genes already known to play a role in cancer causation that modifies the questions we ask and the associations we look for in a study. It appears that both the GWAS algorithm and the candidate gene algorithm may have value in identifying susceptibility genes, and these genes may be different because of the strengths and weaknesses of each approach.

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Effects of Fulvestrant on Mdm2 Expression and Sensitivity of Human Breast Carcinoma Cells to Chemotherapeutic Drugs

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Abstract

Purpose: MDM2 is overexpressed in several human malignancies and contributes to the development of cancer mainly through the inhibition of p53 tumor suppressor activity. MDM2 is an E3 ubiquitin ligase that catalyzes the polyubiquitylation of p53, marking p53 for proteasomal degradation. MDM2 overexpression is strongly related to the presence of estrogen receptor (ER) as the MDM2 promoter contains an estrogen response element. We tested the hypothesis that by blocking expression of MDM2 with antiestrogens, sensitivity to chemotherapeutic drugs could be restored in ER+ breast cancer cell lines. We focused on the antiestrogen fulvestrant since it is known to downregulate ER expression.

Experimental Design: We investigated the effects of fulvestrant on MDM2 expression and sensitivity of the ER+ human breast cancer cell lines T47D and MCF7 to chemotherapeutic drugs. MDM2 expression was measured at protein and mRNA levels by Western and qPCR. Cells were treated with either fulvestrant alone, chemotherapy alone, or in combination. Chemotherapeutic drugs included doxorubicin, etoposide or paclitaxel. Drug sensitivity assays (MTT assays) were performed. The CompuSyn computer program for quantitation of synergism and antagonism was used to determine if there was any change in the sensitivity of the breast cancer cells to these cytotoxic agents with fulvestrant.

Results: Fulvestrant down-regulated Mdm2 expression through increasing the turnover rate of this oncoprotein in the ER positive human breast cancer cell lines MCF7 and T47D. Fulvestrant not only blocked the up-regulation Mdm2

caused by estradiol, but also decreased Mdm2 protein to the level below that seen in the breast cancer cells cultured in the absence of estradiol. Fulvestrant had no effects on activity of p53 and level of MDM2 mRNA, but enhanced the turnover rate of MDM2 protein. Combination of fulvestrant with doxorubicin, etoposide or paclitaxel showed a synergistic effect on these chemotherapeutic drugs.

Conclusion: This study demonstrates that fulvestrant possesses a suppressive effect on Mdm2 expression and a synergistic effect with chemotherapeutic drugs in estrogen receptor positive human breast cancer cells. These results provide a rationale and support for testing the combination of fulvestrant with chemotherapy as a new therapeutic strategy for patients with advanced breast cancers.

INTRODUCTION

Fulvestrant, a new type of estrogen receptor (ER) antagonist that lacks estrogen agonist effect and cross-resistance with other hormonal agents, is currently used in clinic to treat patients with ER positive, advanced and metastatic breast cancers. Fulvestrant downregulates the intracellular ER levels resulting in abrogation of estrogen-sensitive gene transcription. One of the genes whose expression is upregulated in response to estrogen is *Mdm2*, first identified as an oncoprotein encoded by double minute chromosomes in murine sarcoma cells, and later found to be overexpressed in a variety of human cancers. Transcriptional activation of *Mdm2* by estrogen is mediated via an estrogen response element in breast cancer cells homozygous or heterozygous for the single nucleotide polymorphism in the *MDM2* promoter (SNP 309) (1).

MDM2 mainly acts as a negative regulator of p53 activity, thus prohibiting cells from entering into cell cycle arrest, senescence, or apoptosis. In addition, MDM2 also plays a regulatory role in cell-cycle progression independently of p53. For instance, MDM2 promotes the degradation of the phosphorylated retinoblastoma protein (pRB) (2) and p21 (3), thereby modulating their activities. MDM2 also interacts with the S-phase promoting factor E2F1 and increases its function (4). Due to its ability to determine the fate of critical regulators of cell cycle, the activity and expression of MDM2 have been shown to affect the sensitivity of cancer cells to chemotherapeutic agents (5,6).

In the current study, we sought to determine whether the anti-estrogen agent, fulvestrant, could suppress the expression of MDM2 and enhance the response of breast cancer cells to treatment with standard chemotherapeutic drugs. Our study shows that treatment of ER positive breast cancer cells with fulvestrant resulted in an increased turnover and down-regulation of MDM2 protein, and sensitized tumor cells to chemotherapeutic drugs doxorubicin, etoposide, and paclitaxel. These results suggest that combined use of fulvestrant with these cytotoxic drugs may enhance effectiveness of chemotherapy in patients with ER positive breast cancers.

MATERIALS AND METHODS

Cell lines and culture

T47D and MCF7 breast cancer cell lines were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂/95% air. For estrogen and anti-estrogen treatments, cells were cultured in phenol red-free RPMI supplemented with charcoal-stripped 10% fetal bovine serum for 48 hours prior to drug treatment. Cell lines were discarded after 3 months and new lines obtained from frozen stocks.

Reagents and antibodies

Etoposide (VP-16) (Sigma), paclitaxel (TAX) (Sigma) and fulvestrant (Fulv) (Sigma) were dissolved in DMSO. Doxorubicin hydrochloride (DOX) (Sigma) was dissolved in water. β-estradiol-water soluble was purchased from Sigma. Primary antibodies

used for Western blotting and immunoprecipitation were as follows: Mdm2 (SMP14), ER α (HC-20), p53 (DO-1), (Santa Cruz Biotechnology); p21 (Ab-1), Calbiochem; monoclonal anti- β -actin clone AC-15, Sigma. Proteins were visualized using enhanced chemiluminescence (ECL) detection (Amersham Pharmacia Biotech).

Real-time RT-PCR

Cells plated in 60-mm dishes were treated with fulvestrant as indicated. Total RNA was extracted from the treated cells with TriZol Reagent (Invitrogen Life Technologies) and quantified by UV absorbance spectroscopy. First strand cDNA synthesis and amplification were performed using Omniscript RT Kit (QIAGEN, Valencia, CA). The following human *Mdm2* primers were used: forward: 5'-ACCTCACAGATTCCAGCTTCG-3'; reverse: 5'-TTTCATAGTATAAGTGTCTTTTT-3' (7). The *β -actin* primers were as follows: forward: 5'-GCC AACACAGTGCTGTCTGG-3'; reverse: 5'-GCTCAGGAGGAGCAATGATCTTG-3'. The *RPL19* primers were: forward: 5'-CCATGAGTATGCTCAGGCTTCA-3'; reverse: 5'-CTGACGGGAGTTGGCATTG-3' (8). SYBR Green quantitative PCR amplifications were performed on the Stratagene 3005P Real-Time PCR system. Reactions were carried out in a 25- μ l volume containing 12.5 μ l of 2 \times SYBR Green PCR Master Mix (Bio-Rad). The thermal profile for the real-time PCR was 95 $^{\circ}$ C for 10 min followed by 40 cycles at 95 $^{\circ}$ C for 30 s, 53 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s. The threshold cycle (C_T) values were determined and the quantification data were analyzed using either *RPL19* or *β -actin* as housekeeping genes.

Western blot analysis

Cell lysates were prepared using the CellLytic™ MT Cell Lysis Reagent (Sigma) with Protease Inhibitor Cocktail. The lysates were clarified by centrifugation at 12,000 X g for 30 min at 4°C. Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay reagent (Bio-Rad). Lysates (50 µg proteins) were separated onto 8% SDS-PAGE gels followed by transfer to nitrocellulose membranes. Membranes were incubated in blocking solution consisting of 5% powdered milk in PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h, then immunoblotted with the indicated primary antibody overnight at 4°C. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology Inc., Rockford, IL). Quantification of protein bands was performed using ImageJ software (<http://rsb.info.nih.gov/ij>).

Co-Immunoprecipitation

T47D cells were plated in 100-mm dishes. After the respective treatment (fulvestrant 1 µM for 16 h or vehicle), cells were washed twice with ice-cold PBS, scraped off the dishes and pelleted at 1500 x g for 5 min. Cell pellets were then lysed in NETN buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% IGEPAL CA-630 (Sigma), 1 mM EDTA, 1 mM phenylmethylsulphonylfluoride and Protease Inhibitor Cocktail (Sigma)] for 30 min at 4°C in a rotating wheel. Lysates were clarified by centrifugation at 16,000 X g for 20 min at 4°C. Protein concentration was determined by Bradford assay and equal protein amounts were incubated with Mdm2 (SMP14)

antibody or normal rabbit serum for 6 h at 4°C. Protein A/G Plus-Agarose (Santa Cruz Biotechnology) was then added and incubated overnight at 4°C. Beads were then washed five times with lysis buffer containing 0.5% of IGEPAL and one time with PBS and boiled with 2x Laemmli sample buffer. Extracted proteins were loaded onto 8% SDS-PAGE gels followed by transfer to nitrocellulose membranes. Blots were assayed for the expression of Mdm2 and ER α .

Cycloheximide Treatment

Cells were treated with vehicle or 1 μ M of fulvestrant for 16 h, and then pulse-chased for MDM2 protein in the presence of 20 μ g/ml of cycloheximide (CHX). Cell extracts from the treated cells collected at the indicated times were analyzed by Western blotting.

Drug sensitivity assay

Cells were plated in 96-well tissue culture plates, allowed to attach for 5-6 h, and then treated with different drug combinations for 66 h. Fifty microliters of 2.5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma) in PBS were then added to each well, and cells were incubated at 37°C for 4 h. Formazan crystals were dissolved in DMSO. Absorbance was determined at 570 nm using a Wallac 1420 Victor³ plate reader (Perkin Elmer). Viability was expressed as a percentage of control by dividing the absorbance of each treated sample by the average of the untreated controls. Combination index (CI) for drug interaction (e.g., synergy) was calculated using the CompuSyn software. (ComboSyn, Inc.). CI values

at different effect and dose levels and isobolograms were generated using this software.

RESULTS

Treatment with fulvestrant down-regulates Mdm2 protein in human breast cancer cells

To test the effects of anti-estrogen on the expression of Mdm2, the ER positive human breast cancer cell lines, MCF7 and T47D, were treated with different concentrations of fulvestrant and then Mdm2 expression measured. Fig. 1 shows that fulvestrant treatment caused a decrease in Mdm2 expression in both of the cell lines and that the reduction of Mdm2 correlated with the decrease in ER expression. Treatment of MCF7 and T47D cells with estradiol increased Mdm2 expression. However, fulvestrant not only reduced basal Mdm2 expression (in the absence of estradiol), but also blocked the up-regulation of Mdm2 induced by estradiol (Fig. 2).

p53 activity is not affected by fulvestrant

Because Mdm2 is a p53-regulated gene and there are known interactions between ER and p53, the potential role of p53 in Mdm2 down-regulation with fulvestrant was investigated. The ER positive human breast cancer cell lines, MCF7 and T47D, were treated with different concentrations of fulvestrant and p53 expression measured (Fig. 3). Mdm2 depletion by fulvestrant did not correlate with an increase in p53, as might have been expected according to the regulatory role of Mdm2 on p53. Instead a slight decrease in p53 protein was observed. In addition, activation of p53 was not affected by fulvestrant as

measured by expression of p21, a gene that is tightly controlled by p53. Fulvestrant did not alter levels of p21.

Fulvestrant treatment does not alter Mdm2 mRNA level

To determine whether the down-regulation of Mdm2 caused by fulvestrant resulted from altered transcription of Mdm2 gene, Mdm2 mRNA in MCF7 and T47D cells treated with vehicle or fulvestrant was measured using quantitative PCR. This was performed at both 16 and 66 hours for several concentrations of fulvestrant in both MCF7 and T47D. The shorter time period was chosen as fulvestrant treatment can affect multiple transcriptional systems. While protein levels decrease with all doses of fulvestrant at 66 hours, mRNA levels increase for both cells lines (Fig. 4: upper panels). Similar patterns were noted at 16 hour treatment with fulvestrant in both cell lines (Fig. 4, lower panels). These results suggest that fulvestrant does not suppress transcription of *Mdm2* gene.

Fulvestrant increases the turnover rate of Mdm2 protein

As fulvestrant seemed to directly affect the protein levels of Mdm2 without down regulating the mRNA levels of this gene, the effect of fulvestrant was Mdm2 protein half-life was evaluated. Mdm2 protein turnover rate was evaluated in T47D and MCF-7 cells treated with fulvestrant or vehicle, in order to determine the effect of this anti-estrogen agent on stability of Mdm2 protein. The pulse-chase experiments demonstrated that fulvestrant facilitated degradation of Mdm2 protein, as reflected in the shortened half-life of this protein in the presence of fulvestrant (27 min vs. 42 min in T47D cells; 80 min vs. 180 min in MCF7 cells) (Fig. 5). Thus, the down-regulation of Mdm2 expression by fulvestrant appeared to be attributable to the enhanced Mdm2 turnover.

Fulvestrant treatment does not disrupt the ER α -Mdm2 complex

ER α is known to interact with other proteins. As fulvestrant results in decreased ER α as well as reduced Mdm2 protein half-life, co-immunoprecipitation was performed to identify ER α -Mdm2 interaction. T47D cells were cultured with or without 1 μ M of fulvestrant for 16 h after which incubation time immunoprecipitations were performed (Fig. 6). Fig. 6A (upper panel) confirms reduced expression of ER α as a function of fulvestrant treatment, i.e. there was an 84% decrease in ER α expression. As expected, Mdm2 is present in both immunoprecipitation lanes with and without fulvestrant treatment (panel B, lower). Mdm2 was also present in the input lanes when a longer exposure was done (data not shown). In both samples with and without drug treatment, ER α is immunoprecipitated with Mdm2. This suggests that an Mdm2-ER α complex remains intact with fulvestrant treatment and would not explain the altered Mdm2 half-life.

Fulvestrant enhances the sensitivity of human breast cancer cells to chemotherapeutic drugs

Inhibition of Mdm2 has been reported to potentiate cytotoxic effects of chemotherapeutic drugs such as paclitaxel (5). Therefore, using MCF7 and T47D breast cancer cell lines, it was evaluated whether down-regulation of Mdm2 by fulvestrant could enhance the effectiveness of cytotoxic drugs that are commonly used for treatment of breast cancer. Dose-response studies of doxorubicin, paclitaxel and etoposide in combination with fulvestrant were performed, and the data were

analyzed using the CompuSyn software. CompuSyn analyses showed that combined use of doxorubicin, paclitaxel or etoposide with fulvestrant resulted in different degrees of synergism in both of the breast cancer cell lines tested (Fig. 7A-C).

DISCUSSION

Because fulvestrant reduces ER α expression, we hypothesized that the effectiveness of fulvestrant would be mediated through direct effects on transcription of Mdm2. This study demonstrates that fulvestrant possesses a suppressive effect on Mdm2 expression. However, the effects on Mdm2 expression are exerted through altered protein half-life rather than on estrogen-regulated Mdm2 expression. The mechanism of reduced half-life of Mdm2 is not mediated through ER α -Mdm2 protein stabilization despite evidence through co-immunoprecipitation of protein-protein interaction. We tested the hypothesis that by blocking expression of MDM2 with antiestrogens, sensitivity to chemotherapeutic drugs could be restored in ER positive breast cancer cell lines. We focused on the antiestrogen fulvestrant since it is known to downregulate ER expression. Our data demonstrate that fulvestrant exerts synergistic effects in combination with chemotherapeutic drugs in ER positive human breast cancer cells.

Numerous studies have identified associations between MDM2 and ER α expression in breast tissue and breast cancer cell lines (9-11). *In vitro* data have demonstrated that MDM2 is an estrogen-responsive gene through action of activated ER α on the estrogen response element in the first intron of (11-14). However, the relevance of alternative mechanisms of regulation of ER-associated genes has clinical relevance.

For instance, Yang *et al.* (15) identified a novel role for MDM2 in regulating cell adhesion and cell motility through endosomal targeting of proteins. The mechanism supports observations correlating Mdm2 expression with breast cancer stage and outcomes (16-19).

The mechanism of fulvestrant therapeutic benefit is thought to be due to classic reduction in ER α and its resultant reduction in estrogen-regulated gene expression. As many estrogen regulated genes are pro-survival, pro-growth, and anti-apoptotic, reduced gene expression could contribute to the effectiveness of fulvestrant. However, this study describes a novel effect of fulvestrant in altering protein stability. In contrast to the lack of ER α -mediated effect on Mdm2 protein half-life, Mdm2 has been demonstrated to regulate ER α turnover through its ubiquitin-ligase activity with targeted ER α degradation and transactivation (20). This occurs through direct interaction with ER α and p53 in a ternary complex both in the absence or presence of estrogens. Mdm2 exerts its effects both dependent on and independent of p53. This study demonstrates that fulvestrant-induced reduction in ER α and Mdm2 may be independent of p53 expression. This effect is supported by the observation by Brekman *et al.* (13) that estrogen-induced breast cancer cell proliferation required a p53-independent role of Mdm2. Finally, data from Kim *et al.* (21) emphasizes the importance of protein-protein interactions between MDM2 and ER α leading to functional responses where this interaction results in enhanced ER α -mediated gene expression and estrogen responsiveness.

This study demonstrates that fulvestrant possesses a suppressive effect on Mdm2 expression and a synergistic effect with chemotherapeutic drugs in estrogen receptor positive human breast cancer cells. Cytotoxic drug-fulvestrant combinations demonstrating additive or synergistic interactions should be evaluated in *in vivo* models for breast cancer to determine their effectiveness. These results provide a rationale and support for testing the combination of fulvestrant with chemotherapy as a new therapeutic strategy for patients with advanced breast cancers.

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Figure Legends

Fig. 1 Fulvestrant decreases Mdm2 protein expression. MCF7 (A) and T47D (B) cells were cultured in the presence of different concentrations of fulvestrant for 66 h. The ER and Mdm2 levels were checked by Western Blot and normalized to the levels of β -actin. The decrease in protein expression (showed as percentage) after fulvestrant treatment was calculated for each drug concentration.

Fig. 2 Fulvestrant abolishes the effect of estradiol on Mdm2 expression. MCF7 (A) and T47D (B) cells were cultured in the presence of different concentrations of E2 for 72 h, with or without fulvestrant. Mdm2 protein levels were measured by Western Blot and normalized to the levels of β -actin.

Fig. 3 Mdm2 depletion by fulvestrant does not correlate with an increase in p53 activation. MCF7 cells (wild-type for p53) were cultured in the presence of different concentrations of estradiol (E2) for 72 h, with or without fulvestrant. The p53 (A) and p21 (B) protein levels were measured by Western Blot and normalized to the levels of β -actin.

Fig. 4 Fulvestrant does not reduce Mdm2 mRNA abundance. MCF7 and T47D cells were cultured in the presence of different concentrations of fulvestrant for 66 h (A and B, upper panels) or 16 h (C and D, bottom panels). Mdm2 expression was evaluated at both the protein and mRNA levels at two different fulvestrant incubation times. Protein levels were assessed by Western Blot (WB) using β -actin as the housekeeping gene. The mRNA levels were determined by quantitative PCR (qPCR) and the quantification data were analyzed following the standard curve method using *RPL19* as housekeeping gene.

Fig. 5 Fulvestrant decreases Mdm2 protein half-life. T47D (A) and MCF7 (B) cells were cultured with or without 1 μ M of fulvestrant for 16 h. After drug treatment, cells were exposed to cycloheximide (CHX) for different incubation times. Mdm2 protein was measured by Western Blot and normalized to the levels of β -actin.

Fig. 6 Fulvestrant does not disrupt the ER α -Mdm2 complex. T47D cells were cultured with or without 1 μ M of fulvestrant for 16 h after which co-immunoprecipitation was performed. Western Blot was performed to assess expression of Mdm2 and ER α . Panel A (upper panel) and B (lower panel) are the same filter with different exposure times for ER α : B was exposed for a longer time than A. IP: immunoprecipitation; IN: input; Fulv: fulvestrant. A, ER α expression in the input lanes is shown. The last two lanes show the decrease in ER α expression as a result of fulvestrant treatment (decrease of ~ 84 %). B, Mdm2 and ER α expression are shown.

Fig. 7A CompuSyn analysis of fulvestrant-chemotherapeutic drug interaction. MTT assays were carried out and the results of the drug combination analysis using CompuSyn software are shown. Three different drug combinations at constant and non-constant ratios were assessed for each cell line: MCF7, T47D. CI: Combination Index. CI < 1: synergism; CI = 1: additive effect (Add); CI > 1: antagonism. The different degrees of synergism (green) or antagonism (red) are shown: slight synergism (Syn-), moderate synergism (Syn*), synergism (Syn**), strong synergism (Syn***), very strong synergism (Syn****), slight antagonism (Ant-), moderate antagonism (Ant*), antagonism (Ant**), strong antagonism (Ant***) and very strong antagonism (Ant****). Isobolograms from three different drug combinations for doxorubicin, paclitaxel, and etoposide at two different constant ratios were assessed for each cell line and were used to calculate CI (Fig. 7B). In Fig. 7C, the same

analysis as for Fig. 7B was carried out for a *second* experiment where the same doses were used for both cell lines.

Figure 1

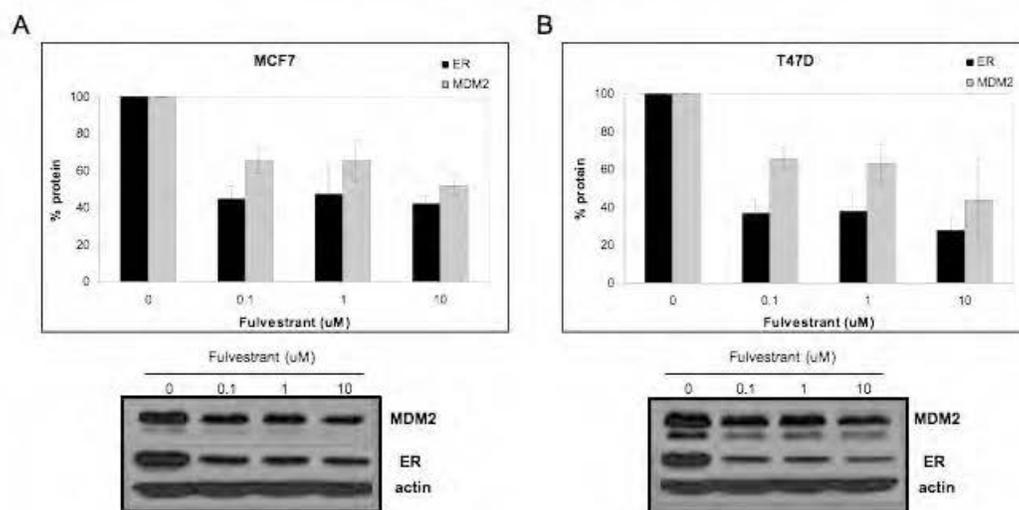
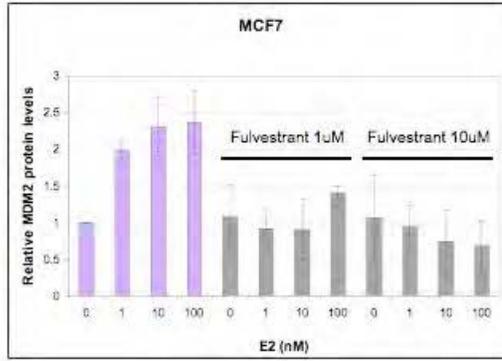


Figure 2

A



B

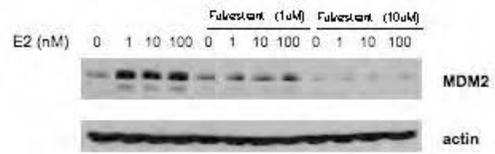
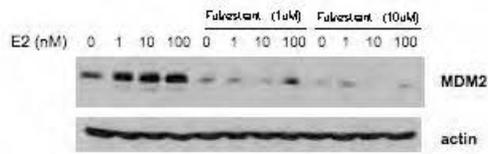
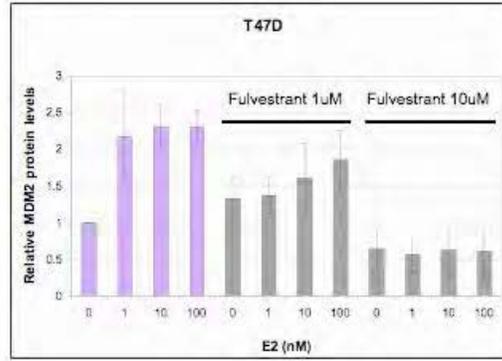
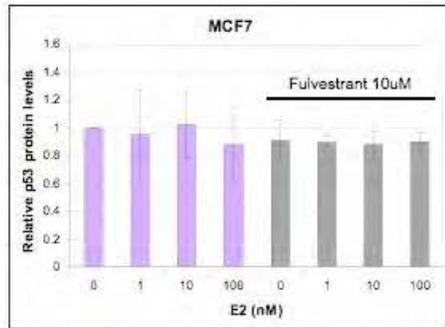


Figure 3

A



B

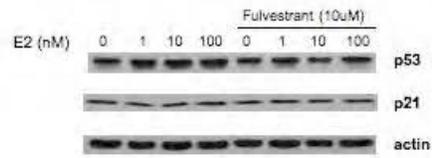
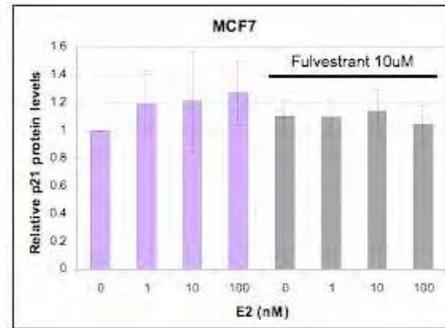


Figure 4

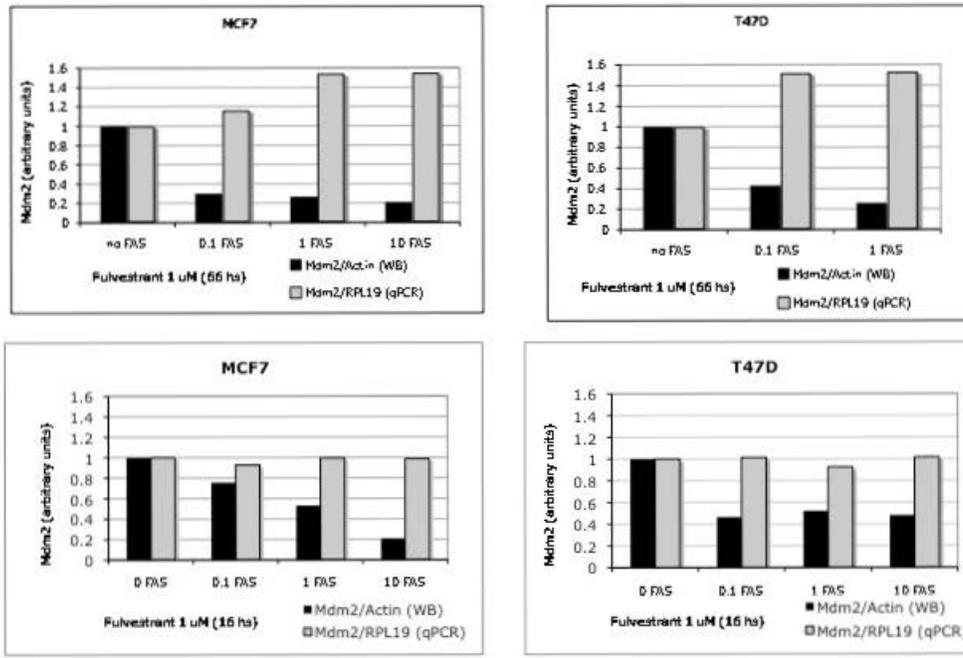


Figure 5

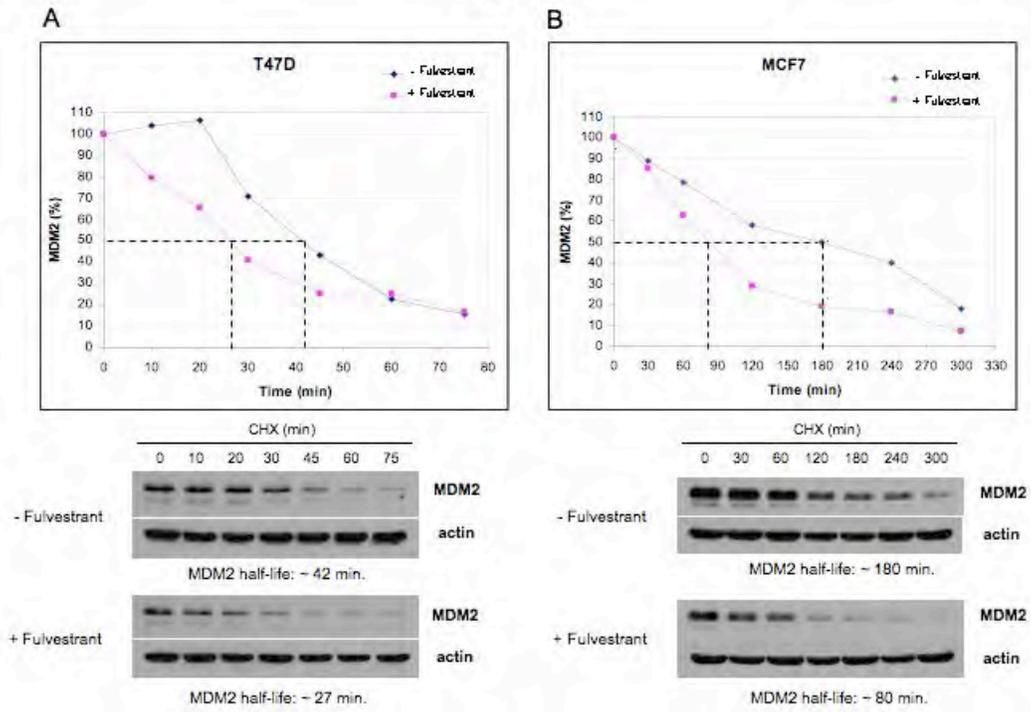


Figure 6.

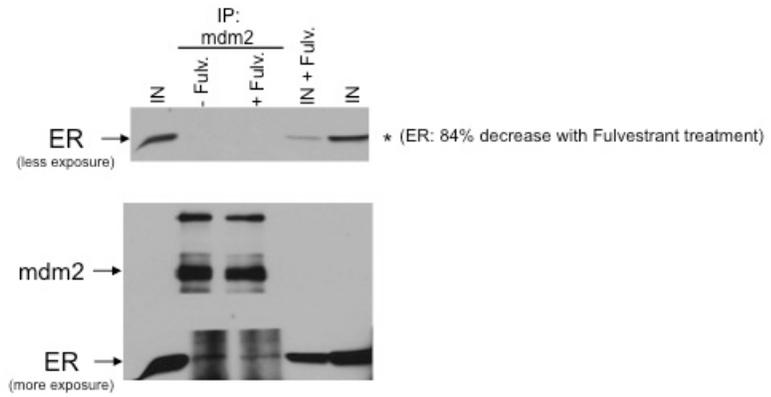


Figure 7A.

CompuSyn analysis for the experimental combination data points

		Constant ratio	CI values:	
MCF7	Fulvestrant : Doxorubicin	1 : 0.5	0.45; 0.63; 0.79	Syn *, Syn **
		1 : 0.015	0.34; 0.53; 0.84	Syn *, Syn **
	Fulvestrant : Paclitaxel	1 : 0.025	0.33; 0.71; 0.97	Syn ** / Add
		1 : 0.0005	0.31; 0.47; 0.51	Syn **, Syn ****
	Fulvestrant : Etoposide	1 : 1	0.1; 0.15; 0.23	Syn ***, Syn ****
		1 : 5	0.29; 0.32; 0.39	Syn **, Syn ****
T47D	Fulvestrant : Doxorubicin	1 : 0.25	0.68; 0.97; 1.04	Syn ** / Add
		1 : 0.0035	0.71; 0.79; 0.98	Syn * / Add
	Fulvestrant : Paclitaxel	1 : 0.0125	0.82; 0.89; 1.56	Syn *, Syn - / Ant **
		1 : 0.0002	0.71; 0.74; 3.02	Syn * / Ant **
	Fulvestrant : Etoposide	1 : 10	0.89; 0.89; 0.96	Syn - / Add
		1 : 0.07	0.69; 1.00; 1.32	Syn ** / Add / Ant *

CI < 1 Synergism: Syn (slight -, moderate *, syn **, strong ***, very strong****)
CI = 1 Additive effect: Add
CI > 1 Antagonism: Ant (moderate *, ant **)

Figure 7B.

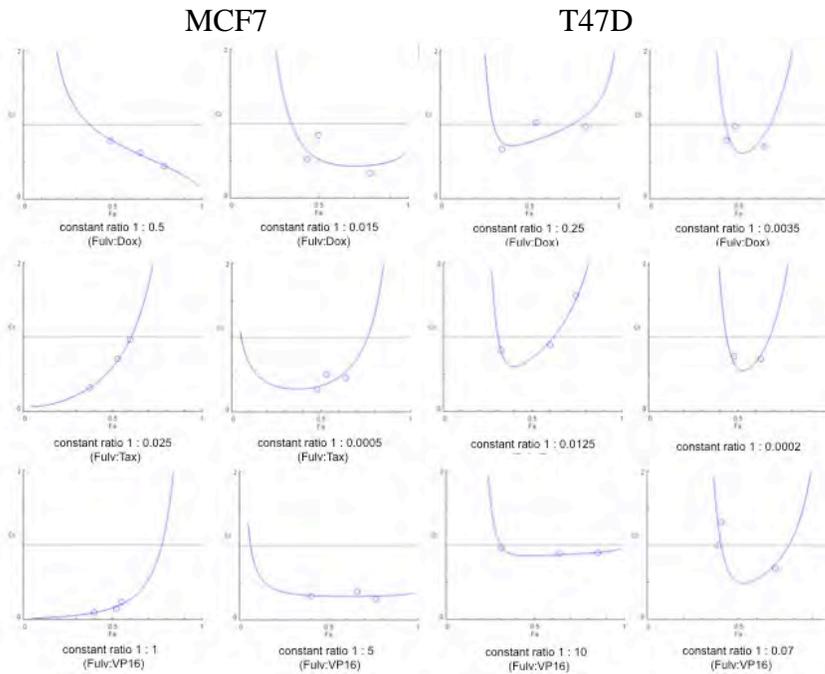


Figure 7C.

		CI values:	
MCF7	Fulvestrant : Doxorubicin at constant ratio	1 : 0.5	0.76; 0.76; 0.95; 1.02 <i>Syn / Add</i>
	Fulvestrant : Paclitaxel at constant ratio	1 : 0.025	0.15; 0.2; 0.76; 1.46 <i>Syn / Ant</i>
	Fulvestrant : Etoposide at constant ratio	1 : 1	0.25; 0.36; 0.37; 1.22 <i>Syn / Ant</i>
T47D	Fulvestrant : Doxorubicin at constant ratio	1 : 0.5	0.68; 0.96; 1.17; 1.37 <i>Syn / Add / Ant</i>
	Fulvestrant : Paclitaxel at constant ratio	1 : 0.025	0.80; 1.49; 1.58; 2.57 <i>Syn / Ant</i>
	Fulvestrant : Etoposide at constant ratio	1 : 1	1.33; 1.50; 7.57; 64.76 <i>Ant</i>

CI < 1 *Synergism: Syn*

CI = 1 *Additive effect: Add*

CI > 1 *Antagonism: Ant*

SUPPORTING DATA

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

Race	Number of patients	% of patients
African American	204	5.9
Asian	142	4.1
Caucasian	2676	77.3
Hispanic	204	5.9
Other	235	6.8

Tumor Type	% of patients
Colloid/Mucinous	1.3
DCIS	9.8
Invasive Ductal	74.5
Invasive Lobular	9.9
Medullary	0.6
Metaplastic	0.4
Other	3.4
Unknown	n/a

ER Status	% of Patients
Positive	74.9
Negative	25.1

PR Status	% of Patients
Positive	63.9
Negative	36.2

Her2/Neu Status	% of Patients
Not amplified or 0-2+ IHC	79.9
Amplified or 3+ IHC	20.1
(all 2+ by IHC were reflexed for FISH)	

Stage	% of patients
0	9.3
1	34.6
IIA	12.5
IIB	12.5
IIIA	7.0
IIIB	2.5
IIIC	2.0
IV	4
Unknown	15.6

Tumor	% of Patients
T0	5.2
T1	46.3
T2	24.7
T3	6.5

T4	4.6
Node status	
N0	47.6
N1	32.5
N2	3.7
N3	0.2
Metastatic Status	
M0	86
M1	4
Recurrence Status	
	% of patients
Yes	20.3
No	79.7
(excludes stage IV at diagnosis)	

Table 2. Time to recurrence of breast cancer from date of initial biopsy-proven disease.

Year(s) to recurrence	n	% of all recurrences
1	14	0.080
2	38	0.216
3	28	0.159
4	22	0.125
5	21	0.119
6	10	0.057
7	5	0.028
8-10	18	0.102
>10	20	0.114
unknown	16	

Table 3. Distribution of the adjuvant therapy received by breast cancer patients in this cohort.

Patients Receiving Each Treatment	No (%)	Yes (%)
Radiation	22.9	77.1
Chemotherapy	33.0	67.0
Hormonal therapy	27.7	72.3
Trastuzumab	87.9	12.1

Table 4. Rates of breast cancer recurrence as a function of hormone receptor status and use of adjuvant hormone therapy.				
	ER-/no hormone therapy		ER+/hormone therapy	
	No recurrence	Recurrence	No Recurrence	Recurrence
TT	49 (0.34)	21 (0.34)	170 (0.36)	33 (0.32)
TG	73 (0.50)	29 (0.47)	221 (0.47)	51 (0.50)
GG	24 (0.16)	12 (0.19)	76 (0.16)	18 (0.18)

Table 5. Rate of breast cancer recurrence in ER+ and ER- disease by MDM2 SNP309 genotype and use of adjuvant hormone therapy.				
	ER-/no hormone therapy		ER+/hormone therapy	
	No recurrence	Recurrence	No Recurrence	Recurrence
TT	39 (0.31)	11 (0.31)	141 (0.37)	19 (0.31)
TG	65 (0.52)	17 (0.49)	171 (0.45)	33 (0.53)
GG	21 (0.17)	7 (0.20)	65 (0.17)	10 (0.16)

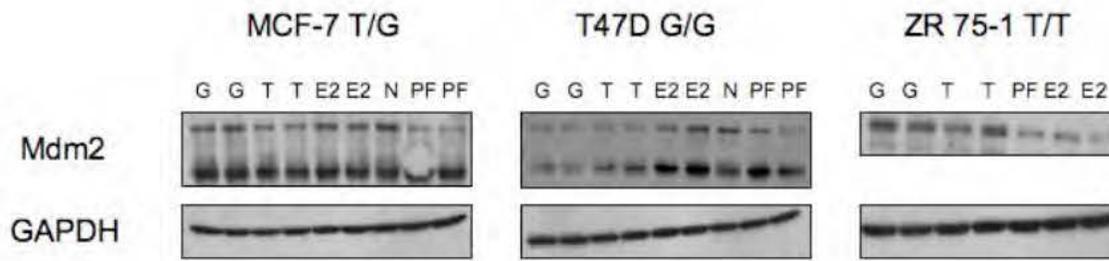


Figure 1. Western blot demonstrates mdm2 protein expression in three ER+ breast cancer cells lines representing the three SNP309 genotypes: ZR75-1 (TT), T47D (GG), MCF7 (TG). Cells were grown under different conditions: phenol-free, charcoal stripped media (PF), normal media (N), estradiol (E2), Tamoxifen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

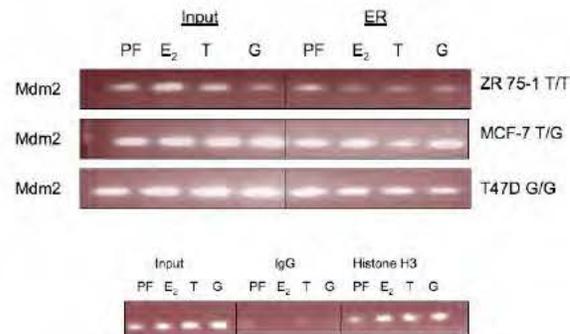


Figure 2. Chromatin immunoprecipitation using anti-ERalpha antibody with PCR of the mdm2 P2 promoter region was performed in the three ER+ breast cancer cell lines representing each of the three MDM2 genotypes.