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TITLE: MEKK1 is a novel regulator of the Dmp1-Arf-p53 pathway and prognostic indicator in breast cancer

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MEKK1 is a novel regulator of the Dmp1-Arf-p53 pathway and prognostic indicator in breast cancer

Breast cancer is the most common malignancy in women. Selection of patients for personalized therapy based on a risk-benefit assessment has not been improved. Dmp1 is a tumor suppressor that activates the p14ARF-p53 pathway to prevent breast cancer development. Regulation of Dmp1 at a transcription level by oncogenes is well understood; however, the role of Dmp1 phosphorylation is unknown. We identify a serine/threonine kinase of the MAPK pathway, MEKK1, which directly phosphorylates Dmp1. MEKK1 phosphorylates Dmp1 on multiple Serine/Threonine residues. Dmp1 and MEKK1 synergize on the Arf transactivation and the p53 pathway induction. Expression of MEKK1 in breast cancer cell lines leads to accumulation of ARF, p53, and p53 target genes. Analysis of MEKK1 locus in human breast tumor samples revealed that MEKK1 is frequently deleted and low expression of MEKK1 may be a new prognostic indicator of patient outcome.
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INTRODUCTION:

Breast cancer remains a significant public health issue among women in the industrialized world. Many current drugs are effective for treatment; however, the side-effects associated with the therapies make their use in the clinic limited. This is especially the case with early stage carcinomas, which have the potential to relapse into more aggressive metastatic disease following tamoxifen or aromatase inhibitor therapy. As a result, physicians have difficulty separating patients that could benefit from more aggressive cytotoxic therapy from low risk patients, sparing them from the toxic side-effects. Moreover, recent analysis provided evidence that extensive breast cancer screening in United States has contributed to over-diagnosis of early stage breast cancer, many of which would never have clinical symptoms (1). It is of interest to identify tumor biomarkers that predict prognostic and therapeutic outcome especially in the patients with early stage disease.

Recently, we have identified the transcription factor Dmp1 (Cyclin D-interacting myb-like protein 1; Dmtf1) as a critical tumor suppressor in breast cancer. Oncogenic Ras and ERBB2 (Her2) overexpression activate the Dmp1 promoter (2). Dmp1 directly binds to the p14ARF promoter, stabilizes p53, and induces growth arrest during tumorigenesis. Genomic analysis of hDMP1, INK4a/ARF, and p53 shows that hDMP1 locus is hemizygosely deleted in ~42% of patient tumor samples with wild-type INK4a/ARF or p53. The mutual exclusive inactivation among hDMP1-ARF-p53 provides evidence that DMP1 is a physiological regulator of the p53 pathway in human mammary epithelium (3).

Since Dmp1 is vital in blocking breast cancer development, it is important to understand how this molecule is regulated at the protein level. The Dmp1 protein is extensively post-translationally modified, specifically, via phosphorylation. It is unknown which kinases phosphorylate Dmp1 and what their functional significance in normal physiology or carcinogenesis may be. In our preliminary experiments, we identify a kinase, MEKK1 (MAP3K1) that directly binds and phosphorylates Dmp1. Dmp1 and MEKK1 were found to synergize in activating the Arf promoter, which was dependent on MEKK1 kinase activity. MEKK1 is a Serine/Threonine kinase of MAPK pathway located on human chromosome 5q11, a locus frequently deleted in breast cancer (4-5). More recently, it was found that ~10% of luminal breast cancers carry MAP3K1 inactivating mutations within its kinase domain (6-7). It remains unclear how MEKK1 contributes to initiation or progression of human cancer.

Here we extend our study to mechanistically understand the role of MEKK1-mediated phosphorylation of Dmp1 in tumor suppression and whether the MEKK1-DMP1-ARF-p53 axis could be used as a prognostic/predictive biomarker in breast cancer.
Dmp1 is a *bona fide* tumor suppressor that directly binds to the $p^{14\text{ARF}}$ promoter to activate p53-dependent cell cycle arrest. Although the Dmp1 protein is expected to migrate around 85 kDa on a SDS-PAGE gel, it is most often observed around 120-130 kDa. The difference in protein migration is due to extensive post-translational modification of Dmp1 protein. Using calf intestine phosphatase (CIP), we showed that Dmp1 is phosphorylated. The role of Dmp1 phosphorylation in normal physiology or carcinogenesis is unknown. In an $Arf$ promoter luciferase assay, we identified a serine/threonine kinase, MEKK1, which synergizes with Dmp1 to activate the $Arf$ promoter. The synergy observed was dependent on Dmp1. Furthermore, we analyzed 27 tumors and matched normal DNAs from breast cancer patients for $MEKK1$ deletion. In this small cohort of patients, we found that that MEKK1 was hemizygoeusely deleted in ~20% of our samples. From the preliminary data, we hypothesized that MEKK1 participates in tumor suppression via direct Dmp1 phosphorylation to increase Dmp1 activity on the $Arf$ promoter. We also hypothesized that MEKK1 is frequently deleted in breast cancer and that loss of MEKK1 is an indicator of poor patient outcome. The following specific aims will be tested: Determine the signaling pathway that links MEKK1 and Dmp1-Arf (Aim 1); Study upstream activators of MEKK1 and their biological significance in modulating Dmp1-Arf (Aim 2); and elucidate the involvement of MEKK1 in human breast cancer and determine its prognostic value (Aim 3).

**Specific Aim 1:** To determine the signaling pathway that links MEKK1 and Dmp1-Arf.

  a. **We will determine if MEKK1-mediated activation of Arf is dependent on its known downstream constituents (Months 1-3).**

  Using an $Arf$ promoter luciferase assay, we have shown that the constitutively active C-terminal domain of MEKK1 (CA-MEKK1) synergizes with Dmp1 on the $Arf$ promoter activation in the presence of MEK1 inhibitor (U0126) or JNK1/2 inhibitor (SP600125). However, Sek1 (MKK4) and MKK7, two other known MEKK1 substrates, have no commercially available inhibitors (4). To test their involvement, we cloned shRNAs for Sek1 and MKK7 into the pRETROSUPER-puro vector. Knockdown of Sek1 or MKK7 in NIH3T3 cells using shRNAs did not abolish MEKK1 synergy with Dmp1 on the $Arf$ promoter. Conversely, expression of constitutively active Sek1 (SEK1-ED) did not synergize with Dmp1. These findings further supported the hypothesis that MEKK1 directly acts upon Dmp1 to increase its transcriptional activity.

  b. **We will purify recombinant MEKK1, Dmp1, and Dmp1 phospho-mutants from Sf9 cells and carry out in vitro kinase assays (Months 1-6).**

  To assess whether MEKK1 directly phosphorylates Dmp1, we performed *in vitro* kinase assay. Constitutively active MEKK1 was subcloned with N-terminal Flag-tag into pVL-1392 baculovirus vector. The CA-MEKK1 was purified from Sf9 cells using anti-flag beads and eluted with Flag peptide. Enzymatic activity of recombinant purified CA-MEKK1 was confirmed using recombinant MEK1, a known substrate of MEKK1. Since bacteria lack the machinery necessary to endogenously modify proteins post-translation, a bacterial system was employed to express and purify His-tagged Dmp1. The *in vitro* kinase assay shows that MEKK1 can directly phosphorylate Dmp1 (Figure 3). Furthermore, we show that insertion of the kinase-abolishing point mutations (K1253M or D1369A) into CA-MEKK1 completely abrogates synergy between MEKK1 and Dmp1 on the $Arf$ promoter activity (Figure 1). Similarly, full length MEKK1 containing the N-terminal regulatory domain was unable to synergize with Dmp1. Interaction of MEKK1 and Dmp1 in NIH3T3 cells was shown by co-immunoprecipitation of one molecule and Western blotting by an antibody against the other (Figure 2).

  Besides functional synergy of Dmp1 and MEKK1 on the $Arf$ promoter and the requirement of MEKK1 kinase activity, we also showed that Dmp1 protein accumulates and physically shifts migration in a SDS-PAGE gel as a result of MEKK1 co-expression. The protein shift is indicative of Dmp1 phosphorylation as this can be reversed by treatment of immunoprecipitated Dmp1 with phosphatase (CIP). The protein accumulation is independent of Dmp1 expression since mRNA level remains unchanged. Again, kinase dead MEKK1 (K1253M) did not affect Dmp1 protein mobility.

  The findings from this aim provide evidence that MEKK1 synergy with Dmp1 on the $Arf$ promoter is dependent on MEKK1-kinase activity and that MEKK1 directly phosphorylates Dmp1.
Since CA-MEKK1 causes a shift in banding pattern of Dmp1 protein and the accumulation of an upper Dmp1 band on a SDS-PAGE gel, we wanted to investigate whether this effect was due to increased Dmp1 stability when CA-MEKK1 is co-expressed. Using NIH3T3 cells, we assessed Dmp1 protein half-life when MEKK1 is co-expressed. We show that MEKK1 does not extend Dmp1 half-life or increase binding of Dmp1 to the Arf promoter, suggesting that overall accumulation of Dmp1 protein and/or phosphorylation specific recruitment of transcriptional co-activators is responsible for increased Arf transcription (Figure 6-7).

c. We will map MEKK1-mediated phosphorylation sites on Dmp1 by in vitro mutagenesis using Arf promoter reporter assays and in vitro kinase assays (Months 7-12).

In the preliminary data, we showed that the mutation of several single Serines or Threonines (identified using mass spectrometry or computational algorithms) was able to reduce synergy between MEKK1 and Dmp1 on the Arf promoter activity. However, none of the mutated sites completely abolished the synergy supporting a hypothesis that MEKK1 phosphorylates Dmp1 on multiple sites to increase Dmp1 transcriptional activity. To address this idea, we began making compound phospho-mutations on Dmp1 to functionally identify Serines or Thrionines phosphorylated by MEKK1 that increase Dmp1 activity on the Arf promoter. The compound mutations chosen were based upon Dmp1 phospho-mapping data with mass spectrometry when CA-MEKK1 was co-expressed and responses that were observed with single mutations. Following Dmp1 compound mutations were made: S120A/T454A; S120A/T642A; S454A/T642A; and a triple mutation S120A/S454A/T642A. However, none of the compound Dmp1 mutants significantly abrogated synergy with MEKK1 on the Arf promoter. These data further support hypothesis that MEKK1 phosphorylates Dmp1 on multiple sites and possibly on Serine and/or Threonine residues that were not detected by mass spectrometry. Due to large number of Serines and Thrionines within the Dmp1 protein sequence, we opted to synthesize entire Dmp1 cDNA sequence where Serines and Thrionines followed by Proline (TP or SP motifs phosphorylated by kinases in the MAPK family) will be converted to Alanines. Total of 15 Serines or Thrionines were identified to be potential phosphorylation sites using data obtained by mass spectrometry or by computational algorithms. Dmp1 cDNA was synthesized in fragments that would allow us to create multiple combinations of Dmp1 S/T mutants. Expression of one compound mutant made thus far, Dmp1 12S/T with mutations in the C-terminus, shows increased migration in the SDS-PAGE gel. Such pattern is associated with reduced phosphorylation. We are currently testing all the Dmp1 mutants’ ability to activate the Arf-p53 and whether they synergize with MEKK1 co-expression.

d. We will determine whether MEKK1 induces Dmp1 and Arf in normal cells using qRT-PCR and Western blotting. This experiment will require cloning of MEKK1 into pBabe-puro-ER vector. Also, Dmp1-null and Arf-null cells will be used to implicated these molecules in MEKK1-mediated growth arrest (Months 4-12).

In the proposal, we set out to clone CA-MEKK1 into pBabe-puro-ER vector in order to achieve spacial and temporal activation of MEKK1 with 4-Hydroxytamoxifen (4HT) treatment. Following the proposed cloning, we determined that MEKK1 fused to the ligand binding portion of estrogen receptor was not able to activate p14ARF or the p53 pathway. We hypothesized that the addition of the bulky ER fusion onto MEKK1 may have affected its ability to bind substrate or traffic between nucleus and cytoplasm. Therefore, we changed our strategy by cloning CA-MEKK1 into the all-in-one doxycycline inducible pTRE-Tight-puro lentiviral vector. We established MDA-MB-361 and MDA-MB-175VII breast cancer cell lines with doxycycline inducible CA-MEKK1. CA-MEKK1 expression was detected at 24 hours post 1ug/ml Doxycycline treatment and maintained for 72 hours. Western blot analysis shows accumulation of p14ARF and p21Cip1, but not p16Ink4a. Further, we show that CA-MEKK1 induces senescence in wild-type MEFs, which was dependent on Dmp1. The Dmp1-null MEFs proliferated upon stable CA-MEKK1 expression and had low β-Galactosidase activity, an indicator of senescence.

In order to study synergy between MEKK1 and Dmp1 on activation of the endogenous Arf-p53 pathway, we established wildtype MEFs using standard protocols. Low passage MEFs were co-transfected with Dmp1 and CA-MEKK1 or individually and Arf-p53-p21 expression was assessed with Western blotting. We observed significant synergy between MEKK1 and Dmp1 on induction of Arf, p53, and p21 compared to Vector, Dmp1, or CA-MEKK1 alone. Similarly, lentiviral co-infection of Dmp1 and CA-MEKK1 in IMR90 cells significantly
increased p14ARF, p21, and hDM2 mRNA expression compared to either alone (Figure 5). These data further strengthen the hypothesis that MEKK1 synergizes with Dmp1 to transactivate the Arf promoter, which ultimately leads to activation of p53 and its target genes.

e. **Using luciferase reporter assays, we will study the mechanism by which the Dmp1 promoter is activated by MEKK1. (Months 13-15)**

In the initial proposal, we hypothesized that MEKK1 has ability to increase Dmp1 expression by activating the Dmp1 promoter. Since then, we have shown that MEKK1 mediated increase in Dmp1 protein is independent of transcription. Dmp1 protein accumulates in response to MEKK1 co-expression while Dmp1 mRNA level remain unchanged. Hence, we will no longer pursue experiments to study MEKK1 mediated activation of the Dmp1 promoter.

f. **Demonstration of transcription factor or MEKK1-binding to the Dmp1 promoter in response to MEKK1 by EMSA and chromatin immunoprecipitation. (Months 16-18)**

Since MEKK1 has no apparent effect on the Dmp1 mRNA expression, we have not studied MEKK1 ability to bind to the Dmp1 promoter. However, we have studied MEKK1-mediated effect on Dmp1 protein to determine mechanism by which MEKK1 increases Dmp1 transcriptional activity. Using EMSA, we have shown that MEKK1 does not increase Dmp1 ability to bind the Arf promoter and it has no effect on Dmp1 protein stability as measured by protein half-life (Figure 6-7). Next we will study whether CA-MEKK1 affects Dmp1 nuclear import since this protein has no consensus nuclear localization signal that regulates its nuclear import.

**Specific Aim 2: To study upstream activators of MEKK1 and their biological significance in modulating Dmp1-Arf.**

a. **We will study activation of MEKK1 by chemotherapeutic drugs (cisplatin, mitomycin C, etoposide) and their ability to activate Dmp1-Arf via an MEKK1 dependent mechanism. These experiments will utilize reporter assays in HEK293 cells and *in vivo* induction of Arf and/or Dmp1 in wild-type MEFs, HMECs, and Dmp1-null MEFs (Months 15-21).**

MEKK1 is a Serine/Threonine kinase of the Stress Activated Protein Kinase (SAPK) pathway that is activated by a variety of extra- and intracellular signals. Interestingly, several common chemotherapeutic drugs (Cisplatin, Etoposide, and Mitomycin C) used to treat cancer have been shown to activate and cleave MEKK1. Therefore, we hypothesized that Cisplatin, Etoposide, and Mitomycin C will activate Dmp1 via MEKK1-mediated phosphorylation. To determine if the drugs had an effect on Dmp1 protein mobility in a SDS-PAGE gel, we treated HEK293 and NIH3T3 cells that were transfected with Flag-Dmp1. All three drugs, Cisplatin, Etoposide, and Mitomycin C, caused a shift in Dmp1 protein mobility and accumulation of the upper band that is indicative of phosphorylation. Treatment of immunoprecipitated Dmp1 with phosphatase (CIP) reversed the drug-induced shift in a SDS-PAGE gel, indicating phosphorylation (Fig. 4). Similarly, endogenous Dmp1 protein shifted in gel following treatment with chemotherapeutic drugs. In order to show that phosphorylation of Dmp1 by chemotherapeutic drugs was due to MEKK1 activation, we knocked down MEKK1 in HEK293 cells using shRNA. Treatment of knockdown cells with Cisplatin, Etoposide, and Mitomycin C reduced the shift of Dmp1 in a SDS-PAGE gel compared to Vector control. However, the knockdown of MEKK1 achieved was ~50% by qPCR and Western blot, which suggests that the chemotherapy drugs are still able to activate MEKK1. We are in the process of designing more efficient shRNA to achieve complete MEKK1 knockdown.
b. We will study activation of MEKK1 by cytokines (TNF-α, IL-1) and their ability to activate Dmp1-Arf via MEKK1-dependent mechanism. These experiments will utilize reporter assays in HEK293 cells, in vivo induction of Arf and/or Dmp1 in wt MEFs, HMECs, and Dmp1-null MEFs. (Months 15-21)

We have tested ability of TNF-α and IL-1β ability to activate the Arf and Dmp1 promoter using luciferase reporter assays. Neither of the cytokines was able to increase activation of these promoters. Similarly, TNF-α and IL-1β failed to increase endogenous Dmp1, Arf, or p21 mRNA expression in human breast cancer cell lines (BT474, T47D, and MDA-MB-361). Hence, we conclude that TNF-α and IL-1β cytokines do not activate Dmp1-Arf-p53 pathway in the context of mammary epithelial cells. Even though these cytokines have been reported to activate MEKK1 in monocytes and macrophages, they fail to directly modulate p53 pathway in breast cancer cells. We still do not exclude that they may affect the p53 pathway in vivo as other cells in the tumor microenvironment may sense these cytokines and subsequently release additional factor that directly affect epithelial cells.

Specific Aim 3: To elucidate involvement of MEKK1 in human breast cancer and determine its prognostic value.

a. We will receive 100 paired normal and tumor breast cancer tissue samples from Advanced Tumor Bank at Wake Forest University Comprehensive Cancer Center. Genomic DNA will be isolated and LOH analysis for MEKK1, hDMP1, INK4a/ARF, and p53 will be conducted. We will also conduct immunohistochemistry of MEKK1 in breast cancer tumor samples. (Months 21-36)

We have isolated DNA from 110 matched normal and tumor breast tissues and conducted LOH analysis for hDMP1, INK4a/ARF, p53 and qPCR analysis for hDM2 amplification. The analysis showed that hDMP1 is hemizygoeusly deleted in ~42% of human breast tumors, which was mutually exclusive of INK4a/ARF or p53 deletion. The results of these findings have been published in Maglic, D., et al. Oncogene 2012. Furthermore, we have designed LOH primers in 5’ and 3’ microsatellite regions of MEKK1 locus. The 3’ primers generated two unique peaks that were used to quantitate hemizygous allele loss between normal and tumor tissue. Analysis of the 3’ MEKK1 locus shows ~20% hemizygous deletion in the tumor compared to matched normal tissue. Currently, we are performing LOH analysis with 5’ specific primers for MEKK1 locus. Moreover, we have compared MEKK1 mRNA expression in 43 patient tumors and matched normal tissue. Twenty-two patients expressed lower level of MEKK1 mRNA in tumor compared to surrounding normal tissue, while 12 patient tumors over-expressed MEKK1 (Figure 8). The patients with MEKK1 LOH(+) showed corresponding low mRNA expression. Hence, we hypothesize that other mechanism such as repression of the MEKK1 promoter, hypermethylation, or modulation of co-activators may contribute to decreased mRNA observed in tumors without deletion.

b. Using statistical analysis, we will correlate LOH of MEKK1, hDMP1, INK4a/ARF, and p53 alone and in combination to known prognostic indicators of breast cancer. (Months 30-36)

In our recent publication (Maglic, D., et al. Oncogene 2012), we compared hDMP1, INK4a/ARF, p53 LOH and hDM2 amplification with known prognostic markers (Ki67 index, DNA ploidy, percent S-phase, breast cancer sub-type, and age) and relapse-free survival. We found that hDMP1 hemizygous deletion is associated with longer relapse-free survival, diploid DNA karyotype, and lower Ki67 index compared to patients with intact hDMP1 locus. Conversely, patients with p53 deletion had shorter relapse-free survival and higher Ki67 index. Patients with hDMP1 loss (LOH positive) associated with luminal A sub-category of breast cancers. Hence, loss of hDMP1 offers better prognosis for breast cancer patients as it alleviates selective pressure to inactivate p53.

After completion of MEKK1 LOH analysis, we will analyze whether MEKK1 deletion provides prognostic value for breast cancer patients and whether it overlaps with hDMP1, INK4a/ARF, or p53 deletion.
KEY RESEARCH ACCOMPLISHMENTS:

- Provide evidence that MEKK1 directly phosphorylates Dmp1 in \textit{in vitro} kinase assay
- MEKK1 synergy with Dmp1 on the \textit{Arf} promoter is independent of other MEKK1 substrates (JNK1/2, MEK1, Sek1 (MKK4), and MKK7)
- Dmp1 and MEKK1 co-immunoprecipitate in NIH3T3 cells
- MEKK1 induces accumulation of Dmp1 and shift of Dmp1 protein mobility in a SDS-PAGE gel which can be reversed by phosphatase treatment
- MEKK1 appears to phosphorylate Dmp1 on multiple Serine/Threonines to modulate its function
- Dmp1 and MEKK1 synergize on activation of the endogenous Arf-p53-p21 in wildtype MEFs and IMR90 cells
- MEKK1 induces senescent and growth arrest in wild-type MEFs but not in \textit{Dmp1}-null MEFs
- Constitutively active MEKK1 activates endogenous ARF-p53-p21 pathway in breast cancer cell lines
- Cisplatin and Etoposide induce Dmp1 phosphorylation
- \textit{hDMP1} is frequently deleted in human breast cancer with mutual exclusivity from \textit{INK4A/ARF} or \textit{p53} inactivation
- \textit{hDMP1} loss is associated with luminal A type of breast cancer, low Ki67 index, and diploid DNA karyotype
REPORTABLE OUTCOMES:

Publications:


Poster Presentation:


CONCLUSION:

Over the last decade, approval and implementation of new effective therapies in the breast cancer clinic has been dismal. This is mostly due to poor therapeutic index of drugs and significant side-effects including potential for secondary malignancies. As a result, physicians have been limited to use the existing therapies with high cytotoxic effects. However, significant improvements have been made in the imaging modalities. The radiologists are able to diagnose women with early stage breast cancer and even with pre-malignant lesions. This poses a dilemma for oncologists how to determine a course of treatment since some patients may have indolent disease while others could develop metastatic cancer (1). Hence, it would be useful to develop an approach to better stratify low risk patients with indolent disease from those that need aggressive therapy to prevent metastasis.

Recently, we have shown that transcription factor Dmp1 is critical in preventing development of breast cancer in mice and humans. Dmp1 transactivates p14ARF and induces p53-dependent cell cycle arrest. Breast cancer associated oncogene, ERBB2 (Her2), activates Dmp1-ARF-p53 pathway and prevents cellular transformation (2). DMP1 locus is frequently deleted in human breast cancers and offers better prognosis for patients. Thus far, we have good understanding how Dmp1 is regulated at the transcription level; however, it is unknown whether and how Dmp1 is modulated post-translationally. We show that Dmp1 is phosphorylated and that a serine/threonine kinase, MEKK1, can directly phosphorylate Dmp1. Importantly, MEKK1 and Dmp1 synergize on transactivation of p14ARF (p19ARF in mice), which was dependent on MEKK1 kinase activity. Since MEKK1 was a potent activator of p53 tumor suppressor pathway, we hypothesized that it may be involved in human cancer. In fact, MEKK1 is located on human chromosome 5q11, a locus frequently deleted in lung and breast cancer (4). Our loss of heterozygosity (LOH) analysis shows that MEKK1 is hemizygotically deleted in ~20% of breast cancer tumor samples. In an independent cohort of patients, we observed that low mRNA expression of MEKK1 was associated with higher probability developing distant metastasis. All of our current data suggests that MEKK1 is a novel activator of the Dmp1-Arf-p53 pathway and it may be a useful predictor of patient outcome.

In the future, we will delineate mechanism of how MEKK1 activates the p53 pathway to block tumor development or progression. Understanding the signaling cascades that dictate how cancer behaves among different patient populations will provide us with a novel molecule that could be explored for therapy. The long term goal is to develop rational approach to patient stratification based on tumor genetic alteration that will guide physicians in selection of personalized therapy for each patient.
REFERENCES:


SUPPORTING DATA (APPENDICES):

Figure 1. MEKK1 and Dmp1 synergy on the Af promoter activation depends on MEKK1 kinase activity. (A) Af promoter fused upstream of luciferase gene was used as an indicator of Af transcription. Constitutively active or kinase dead MEKK1 (K225STM or D1169) was co-transfected with Dmp1 in NIH3T3 cells. The cells were serum starved for 48 hours and luciferase activity was measured 48 hours post transfection. SEAP (secreted endocrine alkaline phosphatase) was co-transfected as a measure of transfection efficiency. Error bars indicate standard errors of the mean from multiple experiments.

Western:

Dmp1
MEKK1

Figure 2. Dmp1 and MEKK1 co-immunoprecipitate in NIH3T3 cells. NIH3T3 cells were co-transfected with Dmp1 and CA-MEKK1 and total cell lysates were collected 48 hours post transfection. Dmp1 and MEKK1 were immunoprecipitated using specific antibodies against each molecule. Western blot analysis was used to detect efficiency of co-immunoprecipitation.

Figure 3. MEKK1 directly phosphorylates Dmp1 and changes of pattern of Dmp1 protein migration in SDS-PAGE gel. (A) Recombinant MEKK1 purified from Sf9 cells and Dmp1 purified from bacterial cells were incubated in kinase assay buffer containing γ32P-ATP. Dmp1 labeling by γ32P was visualized by running the samples on SDS-PAGE gel and exposing the gel to X-ray film. (B) NIH3T3 cells were co-transfected with Dmp1 and MEKK1 or vector alone. Flag-Dmp1 was immunoprecipitated with anti-flag beads and western blotted using Dmp1 specific antibody (RAX).
Figure 4. MEKK1 and chemotherapy drugs change pattern of Dmp1 protein migration in SDS-PAGE gel which can be reversed with phosphatase treatment. NR3T3 cells were co-transfected with Flag-Dmp1 and MEKK1 or treated with chemotherapy drugs (Chlorambucil 100μM or Toposine 50μM) for 18 hours. K125/3M kinase dead MEKK1 mutant was used as control. Dmp1 protein was immunoprecipitated with anti-Flag beads and followed with mock treatment or with 20 units of CIP for 1 hour at 37°C. Phosphatase treatment was stopped with 1X sample buffer and proceeded with western blot analysis using Dmp1 (RAX) antibody. Two images show short and long exposure to X-ray film.

Figure 5. MEKK1 increases Dmp1 transactivation of the Arf-p53-p21 pathway. (A) Low passage wild-type MEFs were co-transfected with Dmp1 and CA-MEKK1 as indicated. Western blot analysis was performed for endogenous p16Arf, p53, and p21. (B) Low passage NIH/3T3 cells were co-transfected with lentiviral vectors carrying Dmp1 and/or CA-MEKK1. After 48 hours, total RNA was isolated, reverse transcribed to cDNA, and analyzed using qPCR for p16Arf, p21, p53, and p16INK4A.

Figure 6. MEKK1 has no effect on Dmp1 ability to bind Arf promoter. NR3T3 cells were co-transfected with Dmp1 and CA-MEKK1. Nuclear extracts (NE) were isolated and EMSA was performed using an Arf promoter containing Dmp1 consensus sequence. Non-specific probe (4A4 probe) or unlabeled Arf probe (cold) was used as controls. Dmp1 specific antibodies (MA1 and MA6) and MEKK1 antibodies (C-22) were used for detecting super-shift or disruption of complex.
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Figure 7. MEKK1 does not affect Dmp1 protein half-life. Dmp1 was co-transfected with constitutively active MEKK1 (CA-MEKK1), kinase dead MEKK1 (K1253M), or alone in NIH3T3 cells. 24 hours post transfection, cells were treated with Cycloheximide to inhibit novel protein synthesis. Total cell lysates were collected following Cycloheximide treatment at time points indicated.

![Graph](image7)

Figure 8. MEKK1 mRNA expression in breast cancer patients. Total RNA was extracted from 43 breast cancer patient tumor and matched normal tissue. The RNA was reverse transcribed to cDNA and analyzed using TaqMan primers for MEKK1. β-Actin primer was used as endogenous control. The bar graph presents each patient tumor MEKK1 mRNA compared to matched normal tissue expression.
Prognostic value of the hDMP1-ARF-Hdm2-p53 pathway in breast cancer

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Our recent study showed critical roles of Dmp1 as a sensor of oncogenic Ras, HER2/neu signaling and activation of the Arf-p53 pathway. To elucidate the role of human DMP1 (hDMP1) in breast cancer, one hundred and ten pairs of human breast cancer specimen were studied for the alterations of the hDMP1-ARF-Hdm2-p53 pathway with follow up of clinical outcomes. Loss of heterozygosity (LOH) of the hDMP1 locus was found in 42% of human breast carcinomas, while that of INK4a/ARF and p53 were found in 20 and 34%, respectively. Hdm2 amplification was found in 13% of the same sample, which was found independently of LOH for hDMP1. Conversely, LOH for hDMP1 was found in mutually exclusive fashion with that of INK4a/ARF and p53, and was associated with low Ki67 index and diploid karyotype. Consistently, LOH for hDMP1 was associated with luminal A category and longer relapse-free survival, while that of p53 was associated with non-luminal A and shorter survival. Thus, loss of hDMP1 could define a new disease category associated with prognosis of breast cancer patients. Human breast epithelial cells/cancer cells with wild-type p53 were sensitive to growth inhibition by activated Dmp1:ER while those that delete p14ARF or p53, and/or Hdm2 amplification showed partial or nearly complete resistance, indicating that p53 is a critical target for hDMP1 to exhibit its biological activity.

Oncogene advance online publication, 8 October 2012; doi:10.1038/onc.2012.423

Keywords: Dmp1 (Dmtf1); breast cancer; loss of heterozygosity; relapse-free survival; Ki67; prognostic marker

INTRODUCTION

Breast cancer is the most common malignancy in women and remains significant health issue in industrialized countries.1–3 Strong evidence supports the idea that breast cancer is initiated by defined genomic alterations, many of which are currently used as therapeutic targets or biomarkers.4 However, it is still unclear which and how genomic alterations in human breast cancer contribute to its biology. Furthermore, it is unknown whether they drive progression of the disease, response to therapy, or if they could be used as prognostic/predictive markers for better patient stratification and molecular subtyping. Recently, the potential of DNA copy number aberrations for molecular subtyping of breast cancer patients apart from analysis of other macromolecules, and warrants future clinical implementation.5 Dmp1, a cyclin D binding myb-like protein 1 (also called Dmtf1), was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait.6–7 Dmp1 shows its activity as a tumor suppressor by directly binding to the Arf promoter to activate its gene expression and, thereby, induces Arf and p53-dependent cell cycle arrest.8 The activity of the Arf-p53 pathway is significantly attenuated in Dmp1-deficient cells since those cells can easily give rise to immortalized cell lines that retain wild-type p19ARF and functional p53 and are transformed by oncogenic Ras alone.9,10 The murine Dmp1 promoter is efficiently activated by oncogenic Ras, as well as by constitutively active MEK1/2 and/or ERK1/2 in primary culture cells.11 Thus, Dmp1 is a key mediator between Ras-Raf-MEK-ERK mitogenic signaling and the Arf-p53 tumor suppressor pathway. Dmp1-deficient mice are prone to tumor development. Tumors induced by the E11-Myc or K-Ras transgene were greatly accelerated in both Dmp1+/− and Dmp1−/− backgrounds with no differences between groups lacking one or two Dmp1 alleles.9,10,12 Indeed, nearly all tumors from Dmp1−/− mice retained and expressed the wild-type Dmp1 allele, and most expressed wild-type Dmp1 mRNA and protein, suggesting typical haploid-insufficiency of Dmp1 in tumor suppression.10,12,13,14–15

We recently characterized the signaling pathway between HER2/neu and Dmp1 using MMTV-neu mice as a model.16 Both Dmp1 and p53 were induced in pre-malignant hyperplastic lesions from MMTV-neu mice, and mammary carcinogenesis was significantly accelerated in both Dmp1+/− and Dmp1−/− mice.16 We also observed selective deletion of Dmp1 in >50% of wild-type HER2/neu carcinomas, while the involvement of Arf, Mdm2, or p53 was rare. Tumors from Dmp1-deficient mice showed significant downregulation of Arf and p21CIP1, showing p53 inactivity and more aggressive phenotypes than tumors without Dmp1 deletion.16 Thus, our study shows the pivotal roles of Dmp1 in HER2/neu-p53 signaling and breast cancer development.

The human DMP1 (hDMP1; hDMTF1) gene is located on chromosome 7q21, a region often deleted in human breast/lung cancers and hematopoietic malignancies.17–19 We recently analyzed 51 human non-small cell lung carcinoma (NSCLC)
samples and found that loss of heterozygosity (LOH) of hDMP1 was present in ~35% of lung cancers\(^2\)\(^-\)\(^5\) in a mutually exclusive fashion with that of INK4a/ARF and/or p53 in the same samples. This raised the possibility that hemizygous hDMP1 deletion might define a new disease entity with different response to therapy.\(^1\)\(^2\)\(^,\)\(^3\)\(^5\)\(^,\)\(^1\(^2\)\(^,\)\(^1\(^5\)\) The current study was conducted to demonstrate the frequency and pattern of genes involved in the hDMP1-ARF-Hdm2-TP53 pathway in human breast cancer. We analyzed 110 pairs of normal and cancer tissues from breast cancer for LOH of hDMP1, INK4a/ARF, p53 and gene amplification of Hdm2,\(^6\)\(^\_\)\(^2\)\(^,\)\(^3\)\(^\_\)\(^1\(^5\)\) and correlated the results of LOH/gene amplification with disease-free survival and known prognostic markers for human breast cancer (reviewed in Masood\(^2\)\(^2\)\(^,\)\(^,\)\(^Taneja et al.\(^1\)\(^8\)).

**RESULTS**

The human DMP1 gene (hDMP1; hDMTF1) is often deleted in human breast cancer

To determine the frequency and patterns of inactivation of the hDMP1-ARF-Hdm2-p53 pathway in human breast cancer, we extracted DNA from 110 pairs of clinical samples and conducted LOH analyses for hDMP1, INK4a/ARF, p53, and gene copy number assay for Hdm2 (exon 4). Representative patterns for LOH-positive cases for each locus are shown in Figure 1. The results from a total of 110 patients are summarized in Table 1 (66 cases with promoter methylation assays for hDMP1) and Supplementary Table S1 (the other 44 cases). LOH for hDMP1 was found in 27 samples with the 5' probe (#92465, 24.5%), 30 cases (#198004, 27.3%) with the 3' probe, and 46 of 110 cases (41.8%) with either the 5' or 3' probes. None of the 61 samples we studied showed methylation of the hDMP1 promoter (Table 1, the 4th column). None of the 15 randomly chosen breast cancer samples showed mutation(s) for the hDMP1 gene except for the polymorphisms at codon 91 (data not shown). Detailed mapping of the genomic fragment deleted in breast cancer showed that gene deletion was limited to the hDMP1 locus (from #69164 to #251945)\(^1\)\(^2\)\(^,\)\(^3\)\(^2\)\(^,\)\(^1\(^5\)\) in 30 of 32 cases of LOH (93.8%) (Supplementary Figure S1), a higher percentage than hDMP1 deletion in human NSCLC (78.9%).\(^1\)\(^3\)\(^\_\)\(^1\(^5\)\) In one case, the hDMP1 deletion was not detectable by the regular LOH assays since the gene deletion was limited to the exons 8 – 20 (case #2005-930) (Table 1).

With INK4a/ARF probes, LOH (including homozygous deletion in #2003-226) was detectable in 19 cases with the 5' probe #33647 (17.3%), 10 cases (9.1%) with the 3' probe #27251, and 22 of 110 (20.0%) with either the 5' or 3' probe. Likewise, LOH for the TP53 locus was detectable in 22 cases (20.0%) with the 5' probe #15811, 30 with the 3' probe #89737 (27.3%), and 37 of 110 (33.6%) with either the 5' or 3' probes. This percentage was higher that the reported percentage of p53 mutations in sporadic breast cancers (20%.\(^1\)\(^4\)). We then sequenced the DNA-binding domain of the p53 gene in 10 p53 LOH(+) samples and found that the remaining p53 allele was mutated in 4 of 10 p53 LOH(+) cases (Table 1, Supplementary Figure S2). We then stained tissue blocks from breast cancer (13 p53 LOH(+) cases and 8 p53 LOH(−) cases) with a specific antibody to p53 (DO-1) and found overexpression of p53 in 6 of 13 p53 LOH(+) cases (46.2%), but not in any of the 8

![Figure 1](image_url)  
*Figure 1. Representative patterns of LOH for hDMP1, INK4a/ARF, and p53 in human breast carcinoma. Genomic DNA was extracted from paired normal and malignant breast cancer specimen and PCR was conducted with 6-FAM-labeled primers that amplify the dinucleotide repeats within (or close to) each locus.\(^2\)\(^,\)\(^3\)\(^1\(^5\)\) The area peaks of the PCR products were quantitated by ABI 3730xl DNA analyzer. The qLOH values were determined through the following equation: qLOH = Area Peak 1/Area Peak 2 (normal tissue) divided by Area Peak 1/Area Peak 2 (tumor tissue). The arrows indicate the peak that was lost in tumor cells. The sample was considered to have LOH when the value was >2.0 or <0.5. (a) Genomic locus of the hDMP1 gene. The two different primer sets were designed to amplify the dinucleotide repeat sequences located on the 5’ and 3’ end of the hDMP1 gene. The non-coding exons were colored silver and the coding exons were colored gold. (b) Genomic structure of the human INK4a/ARF locus. The two sets of PCR primers were designed to detect the dinucleotide repeats within 500 bps of Exon 1β (#33647) and those between Exon 1β and Exon 1α (#27251). The inverted triangles shown in red indicate the location of high-affinity hDMP1-binding sites. (c) Genomic structure of the human p53 gene and the location of the PCR primers used for LOH analyses. (d) LOH analysis of breast cancer with hDMP1 primer sets. 5’: #2006-1202, qLOH = 0.31; 3’: #2004-817, qLOH = 2.05. (e) LOH analysis with INK4a/ARF primer sets. 5’: #2008-1476, qLOH = 0.44; 3’: #1999-84, qLOH = 11.25. (f) LOH analysis with p53 primer sets. 5’: #2008-1272, qLOH = 0.48; 3’: #2008-26, qLOH = 0.33.*
cases with p53 LOH (−) breast cancers (Supplementary Figure S2). Importantly, all breast cancers with p53 mutation as demonstrated by sequencing showed overexpression of the p53 protein (Supplementary Figure S2). Conversely, none of the 8 samples without LOH for p53 showed high expression of p53 as studied by immunohistochemistry. These results are consistent with the previous report that showed frequent association of p53 mutations with loss of the other p53 allele in breast cancer.25 Thus, the hDMP1 locus was more frequently deleted in our breast cancer samples than the INK4a/ARF or p53 locus.

LOH for hDMP1 and INK4a/ARF was found to be mutually exclusive in 65 of 65 cases (95.4%, \( P = 0.0027, \chi^2 = 8.977; 95\% \text{ confidence interval}, 89.8–100\% \) (Table 1 and Supplementary Table S1). Likewise, LOH for hDMP1 and p53 was also mutually exclusive in 63 of 73 cases (86.3%, \( P = 0.025, \chi^2 = 5.013; 95\% \text{ confidence interval}, 78.4–94.2\% \). On the other hand, LOH for INK4a/ARF and p53 was exclusive only in 31 of 45 cases (68.9%, \( P = 0.0009, \chi^2 = 11.088 \) against mutually exclusive hypothesis; 95\% confidence interval, 55.4–82.4\%). The Hdm2 gene amplification (more than 6 copies) was found in 14 of 110 samples (12.7\%, Table 1 and Supplementary Table S1). The Hdm2 gene amplification and LOH for hDMP1 appeared to occur independently of the other loci (93.0%, \( P = 0.282, \chi^2 = 1.157\), not exclusive; 95\% confidence interval, 87.1–98.9\%). Thus, our data demonstrate that (1) LOH for hDMP1 is typically found in human breast cancers with wild-type INK4a/ARF and p53 genomic loci, (2) LOH for INK4a/ARF

Table 1. LOH analyses of 66 pairs of human breast carcinomas for the hDMP1, INK4a/ARF, and p53 loci.
and p53 occur simultaneously, and 3) LOH for hDMP1 and Hdm2 amplification occur at random with respect to one another.

We next studied the correlation between LOH for hDMP1 and known prognostic factors for breast cancer: HER2, estrogen receptor (ER), progesterone receptor (PR), Ki67, DNA ploidy, clinical stage, and age (data not shown). Setting the cut-off level at 20%, we found significantly more cases with low Ki67 (that is, Ki67 < 4) in the $h$DMP1 group in comparison to the LOH+ group ($P = 0.0204$, $\chi^2 = 5.74$) while LOH for $p53$ was associated with low Ki67 ($P = 0.0056$, $\chi^2 = 7.25$). We then classified all the breast cancer cases based on the data from histochemical studies for ER, PR, HER2, Ki67, cytokeratin, and morphology of tumor cells as proposed from the Komen Website http://ww5.komen.org/BreastCancer/SubtypesofBreastCancer.html into luminal A, luminal B, HER2, triple-negative, and unclassified/normal-type. The Ki67 positivity ratio of 14% was used to differentiate luminal A and luminal B subtypes, and breast cancers with HER2 (+++) was categorized into HER2 subtype. According to these criteria, 29.1% (32 of 110) of total cases were classified into luminal A, 20.9% (23 cases) were luminal B, 20.0% (22 cases) were HER2 type, 16.4% (18 cases) were triple-negative/basal-type, and 8.2% (9 cases) were unclassified/normal-type (Table 2), which were close to those that had been shown in the literature. Six of 110 cases could not be classified due to lack of paraffin sections. We then conducted statistical analyses and found that hDMP1 LOH (+) breast cancers were significantly associated with luminal A subtype ($P = 0.0084$, $\chi^2 = 5.88$) while LOH for $p53$ was associated with ploidy of tumor DNA ($P = 0.0015$, $\chi^2 = 5.88$). On the other hand, LOH for $ink4a/arf$ or $p53$ was associated with aneuploidy of DNA ($P = 0.0517$, $\chi^2 = 5.88$; $P = 0.0141$, $\chi^2 = 6.03$, respectively). Conversely, Hdm2 amplification was not associated with ploidy of tumor DNA ($P = 0.701$). HER2 protein overexpression (2 + 3 +) was found in both hDMP1 LOH (+) (10/41, 24.4%) and (-) (25/59, 42.4%) without a statistically significant difference ($P = 0.064$). This finding is in agreement with the fact that MMTV-neu tumor development was accelerated in both $Dmp1$-null16 and p53-mutant24 backgrounds. There was no statistically significant difference in ER, PR, clinical stage, patients’ age and LOH for hDMP1.

We then classified all the breast cancer cases based on the data from histochemical studies for ER, PR, HER2, Ki67, cytokeratin, and morphology of tumor cells as proposed from the Komen Website http://ww5.komen.org/BreastCancer/SubtypesofBreastCancer.html into luminal A, luminal B, HER2, triple-negative, and unclassified/normal-type.27 The Ki67 positivity ratio of 14% was used to differentiate luminal A and luminal B subtypes, and breast cancers with HER2 (+++) was categorized into HER2 subtype. According to these criteria, 29.1% (32 of 110) of total cases were classified into luminal A, 20.9% (23 cases) were luminal B, 20.0% (22 cases) were HER2 type, 16.4% (18 cases) were triple-negative/basal-type, and 8.2% (9 cases) were unclassified/normal-type (Table 2), which were close to those that had been shown in the literature.27 Six of 110 cases could not be classified due to lack of paraffin sections. We then conducted statistical analyses and found that hDMP1 LOH (+) breast cancers were significantly associated with luminal A group of breast cancers ($P = 0.0085$, $\chi^2 = 6.92$) while p53 LOH (+) breast cancers were significantly associated with non-luminal A subtype ($P = 0.0234$, $\chi^2 = 5.14$) (Table 2). Since LOH for hDMP1 is associated with low Ki67 index, higher incidence of a diploid karyotype, and luminal A subcategory, it was expected that deletion of hDMP1 would be a favorable prognostic factor for breast cancer patients.

Correlation of DMP1 protein expression with hDMP1 LOH and HER2 status in human breast cancer

We then studied whether LOH for hDMP1 affects protein expression in breast cancer samples by immunohistochemistry with specific antibodies.28,29 The nuclear hDMP1 expression levels were categorized into four grades, 0 to 3+++ (Figure 2a). Breast cancer samples without LOH for hDMP1 showed more intense

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>hDMP1 LOH</th>
<th>INK4a/ARF LOH</th>
<th>p53 LOH</th>
<th>Hdm2 Amplification</th>
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<td>INK4a/ARF #33647</td>
<td>INK4a/ARF #27251</td>
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<td>n.d.</td>
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All samples including Table S1

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<th>n=110</th>
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<td>95% CI</td>
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</table>

Positive results for LOH (qLOH > 2.0 or < 0.5) are shown in bold red type. When one of the two markers (‘+’ or ‘−’) showed qLOH value > 2.0 or < 0.5, the sample was considered positive for LOH for the tumor suppressor locus. Cases of mutually exclusive inactivation of hDMP1 and INK4a/ARF or hDMP1 and p53 are shown ‘yes’ in bold blue type. Exclusive of hDMP1: LOH for INK4a/ARF (or p53) or amplification of Hdm2 is not overlapping with LOH of the hDMP1 locus in the same sample. Light brown shading indicates cases with LOH for hDMP1. Detailed analysis by real-time PCR showed that case #2005-930 had an internal deletion for hDMP1 (that (darker brown shading). The hDMP1 gene was sequenced in samples with %. The p53 gene was sequenced in samples with * (no mutation), and ** (with mutation). Abbreviations. H.D.: hemizygous deletion as determined by real-time PCR; No del: no deletion by real-time PCR; Un: unmethylated. Homo Del: homozygous deletion; single, LOH was not evaluated due to a single peak result; n.d.: not determined.

nuclear staining for hDMP1 (mostly grades 2–3) while tumors with LOH showed weaker staining (mostly grades 0–1) \( (P = 0.0006) \), Figure 2). Normal breast epithelial cells also showed weak (1 +) hDMP1 staining (data not shown). We found a significant increase in hDMP1 staining in breast carcinomas that showed HER2 overexpression (2 + or 3 +) \( (P = 0.0038) \), Figure 2b), regardless of LOH for hDMP1. Together, our data show that: 1) hDMP1 protein is downregulated in clinical samples that showed LOH for hDMP1 and 2) HER2 and hDMP1 expression levels are positively correlated.

Impact of LOH for hDMP1, INK4a/ARF, p53, and Hdm2 amplification on breast cancer survival

We then studied the impact of LOH for hDMP1, INK4a/ARF, p53, and Hdm2 amplification in stage I to III patients \( (n = 108; 2 \) cases of stage IV patients were eliminated from the survival study, Figure 3). Breast cancers with LOH for DMP1 had longer relapse-free survival than those without LOH \( (P = 0.0092; \chi^2 = 6.79; \) 70% survival 1987 vs 1036 days) \( (Figure 3a) \). LOH for INK4a/ARF had no impact on patients’ survival \( (P = 0.591; \chi^2 = 0.289; \) 70% survival 1121 vs 1830 days) \( (Figure 3b) \). Conversely, breast cancer with Hdm2 amplification showed significantly shorter survival than those without gene amplification \( (P = 0.0217; \chi^2 = 5.27; \) 70% survival 499 vs 1830 days) \( (Figure 3c) \). Likewise, LOH for p53 had significantly negative impact on patients’ disease-free survival \( (P = 0.0211; \chi^2 = 5.41; \) 70% survival 1036 vs 1932 days) \( (Figure 3d) \) consistent with the finding that ~50% of p53 LOH cases showed simultaneous mutation of the remaining p53 allele (Supplementary Figure S2). The survival of breast cancer patients without LOH for the three loci and absence of Hdm2 amplification was not significantly different from those with involvement of the pathway (Supplementary Figure S3). Together, our data indicate that the more downstream the molecule is localized in DMP1-ARF-Hdm2-p53 signaling, the more negative impact the marker shows on breast cancer.

Growth inhibition of human breast epithelial cells by Dmp1:ER

Finally, we studied whether conditional activation of Dmp1:ER affects the growth of human breast epithelial and cancer cells. Normal immortalized human mammary epithelial cells (MCF10A), human mammary epithelial cells (HMEC) and breast carcinoma cell lines (MCF7, MDA-MB-175VI, ZR-75-1, BT-549, and HCC1569) were infected with Dmp1:ER or empty vector virus, and puromycin-resistance cells were cultured under the presence of 2 μM 4-hydroxytamoxifen (4-HT).\(^{9,12}\) The genomic statuses for p14ARF, Hdm2, p53, and HER2 for human breast epithelial or cancer cells are summarized in Supplementary Table S2. Cell growth was completely inhibited by expressing Dmp1:ER in both MCF10A and tert-immortalized HMEC (Figures 4a and b). Significant inhibition of cell growth by Dmp1:ER was also observed in ZR-75-1 (Figure 4e) and MDA-MB-175VI (data not shown) breast cancer cells with wild-type ARF and p53 although the effect was significantly weaker in breast cancer cells than in HMEC or MCF10A. Western blotting (and real-time PCR in HMEC) analyses showed significant accumulation of P14ARF, p53, p21\(^{CIP1}\), and Hdm2 in response to activation of Dmp1:ER in HMEC and ZR-75-1 cells (Figures 5a and e; Supplementary Figure S4). In MCF10A cells, significant accumulation of p53 and p21\(^{CIP1}\) was observed at 12–36 h in response to Dmp1:ER (Figure 5b) although p14ARF did not accumulate due to gene deletion. This data is consistent with our recent findings that Dmp1 physically interacts with p53 to neutralize the activities of Mdm2 in ARF-null cells.\(^{30}\) β-gal staining showed that ~40% of MCF10A cells underwent senescence by Dmp1 while ~70% of HMEC became senescent suggesting mixed growth inhibitory response (Supplementary Figure S5). The growth of MCF7 cells (ARF-null, p53 wild-type) was partially inhibited by Dmp1:ER (Figure 4c). Conversely HCC1569 cells with p53 deletion or BT-549 cells with p53 mutation did not slow down their growth by Dmp1:ER (Figures 4d and f). Indeed lysate analyses showed consistently high levels of p14ARF and undetectable p53 targets p21\(^{CIP1}\) or Hdm2 in these cells (Figures 5d and f).

We studied the growth of breast epithelial/cancer cells depleted of DMP1 by shRNA.\(^{12}\) Western analyses showed more than 90% downregulation of the hDMP1 protein in all of these three breast cancer cells and inactivation of the p53 pathway in MCF10A (Supplementary Figure S6). Depletion of hDMP1 by shRNA accelerated the growth of MCF7 cells (Supplementary Figure S7), but not T47D or MDA-MB-361 (wild-type ARF, mutant p53), suggesting that endogenous DMP1 is inhibiting the growth of p53 wild-type cells, but not in cells with mutant p53. The growth of p53 mutant cells by shRNA were retarded, possibly because shRNA to hDMP1 affected the function of other splicing variants\(^{31}\) or hDMP1 interacts with mutant p53 for stabilization.

Table 2. Subclassification of breast cancers studied and relationship with hDMP1 and p53 LOH

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<tr>
<th></th>
<th>hDMP1 LOH(+)</th>
<th>hDMP1 LOH(-)</th>
<th>Percentage</th>
<th>Reported percentage</th>
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<tbody>
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<td>19</td>
<td>13</td>
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</tr>
<tr>
<td>Luminal B</td>
<td>8</td>
<td>15</td>
<td>19.0</td>
<td>0.3530</td>
</tr>
<tr>
<td>HER2</td>
<td>5</td>
<td>17</td>
<td>21.1</td>
<td>0.0573</td>
</tr>
<tr>
<td>Triple-negative</td>
<td>6</td>
<td>12</td>
<td>11.9</td>
<td>0.5026</td>
</tr>
<tr>
<td>Normal/unclassified</td>
<td>4</td>
<td>5</td>
<td>9.5</td>
<td>0.7951</td>
</tr>
<tr>
<td>Not evaluated total</td>
<td>46</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 LOH(+)</td>
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<td>13</td>
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</tr>
<tr>
<td>p53 LOH(-)</td>
<td>8</td>
<td>15</td>
<td>19.0</td>
<td>0.9648</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All the breast cancer cases \( (n = 104, \text{ enough information was not available in 6 cases}) \) have been subclassified into luminal A, luminal B, HER2, triple-negative/basal type, and unclassified/normal-type based on the data from histochemical studies for ER, PR, HER2, Ki67, cytokeratin, and morphology of tumor cells as described in the Materials and Methods. The percentage of our breast cancer samples in each category was very close to those reported in the literature. hDMP1 LOH(+) breast cancers were significantly associated with luminal A category while p53 LOH(+) breast cancers were associated with non-luminal A subtype. Significant association of hDMP1 LOH and luminal A subtype and that of p53 LOH with non-luminal A subtype are shown in bold. The percentage of each breast cancer subtype in our samples are also shown in bold.
Then we conducted cell invasion assay using MCF7 cells with or without depletion for hDMP1 (see Supplementary Materials and Methods). Our results show 3.31 ± 0.603 MCF7 cells with hDMP1 downregulation invaded from upper to lower chamber while only 1.57 ± 0.970 cells migrated to the lower chamber in mock infected cells (P = 0.048). Conversely there was no significant effect of DMP1 expression in invasion assay with p53 mutant BT549 cells (55.2 ± 9.25 vs 64.5 ± 14.1). Together, our data indicate that 1) both non-transformed human mammary epithelial cells and breast cancer cells with wild-type ARF and/or p53 (HMEC, MCF10A, MDA-MB-175VII, and ZR-75-1) are sensitive to growth inhibition/senescence by Dmp1 while breast cancer cells that delete ARF or deleted/mutant p53 show partial (MCF7) or nearly complete (HCC1569, BT-549) resistance to growth inhibitory effect by Dmp1, 2) Endogenous hDMP1 inhibits the growth of breast cancer cells with wild-type p53, and 3) DMP1-loss is associated with invasive phenotypes of breast cancer cells.

**DISCUSSION**

In this study we analyzed 110 pairs of human breast cancer samples and demonstrated that hDMP1 is deleted in 42% of the samples. This percentage is even higher than the involvement of INK4a/ARF (~20%) or p53 (~35%) of the samples we analyzed, and importantly, was found in mutually exclusive fashion from LOH for INK4a/ARF or p53. On the other hand, LOH for INK4a/ARF and p53 were apparently overlapping, suggesting collaboration of these two loci, possibly through the synergism of p16INK4a loss and p53 inactivation. Deletion of hDMP1 was limited to the hDMP1 locus in 94% cases showing specificity of hDMP1 deletion in breast cancer. Importantly, deletion of the hDMP1 locus resulted in significant downregulation of the nuclear expression of the hDMP1 protein in breast cancer cells, signifying that the gene deletion significantly affected hDMP1 function and contributed to breast carcinogenesis. DMP1 protein expression was significantly higher in HER2(+) tumors than HER2(-) or negative samples indicating the presence of the signaling pathway between HER2 and hDMP1 in breast cancers. Two different intensity values for hDMP1 indicate that the staining pattern for hDMP1 was mosaic; the average values (DMP1 scores) were used for statistical analyses.

Figure 2. Histological grading of hDMP1 in human breast carcinoma. (a) human breast cancer tissues were stained with Dmp1-specific RAX antibody and the intensity of the nuclear staining was graded from 3(++), 2(+), 1(±), and 0 (negative). The scale bar is 100 μm. (b), correlation between LOH for hDMP1 and immunohistochemical grading of breast cancers. Breast cancer samples without LOH for hDMP1 showed significantly stronger nuclear signals for hDMP1. The hDMP1 signals were significantly higher in HER2 3 + or 2 + samples than in HER2 1 + or negative samples indicating the presence of the signaling pathway between HER2 and hDMP1 in breast cancers. Two different intensity values for hDMP1 indicate that the staining pattern for hDMP1 was mosaic; the average values (DMP1 scores) were used for statistical analyses.
p53 to continue the study to investigate the impact of single allelic cancer patients. Currently, we do not have enough samples for survival analyses of p53 for those that have been reported in the literature (20%) in sporadic breast cancers. Our data also indicate that approximately half of those that have been reported in the literature (20%) in sporadic breast cancers.24 Our data also indicate that approximately half of the cases retain one p53 allele without p53 mutation. It has been reported that p53 heterozygous mice develop tumors at a mean latency of 70 weeks without losing or mutating the wild-type p53 allele in mice suggesting that loss of one allele of p53 contributes to tumorigenesis in vivo. Although we currently do not have enough samples for survival analyses of p53−/− breast cancers, with or without p53 mutation, we plan to continue the study to investigate the impact of single allelic p53 loss with or without p53 mutation on survival of breast cancer patients.

Since hDMP1 is a transactivator for the ARF promoter and p14ARF indirectly regulates the activity of p53 through Hdm2, there is a gradient of prognosis of breast cancer patients from (fair) hDMP1 LOH→INK4a/ARF LOH→Hdm2 amplification→or→p53 LOH (poor) possibly because: i) the closer the molecule is to p53, the more seriously p53 function will be affected, ii) LOH of p53 may be associated with a gain-of-function mutation of p53,36 and iii) Hdm2 has multiple interacting partners other than p53 (e.g., E2F1, YY1, RB, ribosomal proteins) that explain its oncogenic potential.37 Furthermore, depending on which therapies were used to treat our cohort of patients, it is possible that loss of hDMP1 spared deletion of p53 gene, increased effectiveness of chemotherapy and radiation treatment and, thereby, extended time to relapse.

It should be noted that loss of hDMP1, INK4a/ARF, p53, or Hdm2 amplification did not exclusively correlate with currently used prognostic markers for breast cancer (ER, PR, HER2).23 Thus, LOH studies for hDMP1, INK4a/ARF, p53, and real-time PCR assay for Hdm2 will be independent laboratory tests to predict the prognosis of breast cancer patients. Although hDMP1-loss is a favorable prognostic factor associated with longer relapse-free survival of patients than hDMP1 intact cases, 35% of breast cancer patients relapsed during the observation period of 8 years. Thus, it is likely that other genetic alteration(s) collaborate with DMP1-loss to accelerate recurrence of the disease. Further molecular genetic studies are required to clarify which molecular events collaborate with hDMP1-loss in breast cancer progression.

Our data show that shRNA to hDMP1 stimulated proliferation of breast cancer cells with wild-type p53, but inhibited cell growth of cells with mutant p53. There are two possible explanations why significantly longer (1987 vs 1036 days) for hDMP1 LOH(+) cases than (−) patients. On the other hand, p53 LOH(+) breast cancer was associated with non-luminal A subtypes, both Hdm2 amplification and LOH for p53 were associated with shorter disease-free survival. Of note, although breast cancers with LOH for hDMP1 was associated with relatively low Ki67 index in comparison to p53 LOH samples, the former samples still showed higher Ki67 index (mean 19% in our samples) than normal breast epithelial cells (~2% Shetty et al), indicating that loss of hDMP1 is associated with proliferation of normal breast epithelial cells, which can collaborate with other genetic alterations to develop breast cancer.

Our study shows that 35% of human breast cancers have LOH for p53 and 46% of such cases have mutation(s) of p53. This means ~16% of breast cancers have mutation(s) for p53 in our samples. Interestingly this percentage of p53 mutation is close to those that have been reported in the literature (20%) in sporadic breast cancers.24 Our data also indicate that approximately half of p53 LOH cases retain one p53 allele without p53 mutation. It has been reported that p53 heterozygous mice develop tumors at a mean latency of 70 weeks without losing or mutating the wild-type p53 allele in mice suggesting that loss of one allele of p53 contributes to tumorigenesis in vivo. Although we currently do not have enough samples for survival analyses of p53−/− breast cancers, with or without p53 mutation, we plan to continue the study to investigate the impact of single allelic p53 loss with or without p53 mutation on survival of breast cancer patients.

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It should be noted that loss of hDMP1, INK4a/ARF, p53, or Hdm2 amplification did not exclusively correlate with currently used prognostic markers for breast cancer (ER, PR, HER2).23 Thus, LOH studies for hDMP1, INK4a/ARF, p53, and real-time PCR assay for Hdm2 will be independent laboratory tests to predict the prognosis of breast cancer patients. Although hDMP1-loss is a favorable prognostic factor associated with longer relapse-free survival of patients than hDMP1 intact cases, 35% of breast cancer patients relapsed during the observation period of 8 years. Thus, it is likely that other genetic alteration(s) collaborate with DMP1-loss to accelerate recurrence of the disease. Further molecular genetic studies are required to clarify which molecular events collaborate with hDMP1-loss in breast cancer progression.

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patients with hDMP LOH(−), half of which harbor p53 mutation, further suggesting that hDMP1 may promote breast cancer progression by stabilizing mutant p53. Thus, it would be of great interest to understand whether DMP1 affects the function of mutant p53.

In conclusion, we have characterized the frequency and the pattern of alteration of the hDMP1-ARF-Hdm2-p53 pathway in human breast cancer. Each component in the signaling pathway can define a different disease entity associated with prognosis. Hemizygous deletion of DMP1 is found in nearly half of human breast carcinomas that often retain the wild-type p53 and INK4a/ARF loci. This finding is significant as we move closer towards personalized therapy for each breast cancer patient based on their tumor genetic alterations. Our data suggests that patients with hDMP1 LOH should be selected for current and future therapies whose efficacy is dependent on an intact p53 gene. On the other hand, patients with wild-type hDMP1 (~50% of all breast cancer patients in this study) in their tumor biopsy should be spared toxic side-effects from treatments that would be ineffective with p53 LOH. Alternatively, further research is necessary to develop small molecules that specifically activate hDMP1 promoter or protein which will be a feasible approach to treat human breast cancer patients with DMP1 LOH since their tumors maintain a second wild-type DMP1 allele without mutation or promoter methylation.

MATERIALS AND METHODS

The protocols for LOH assay, statistical analyses, immunohistochemical studies of breast cancer samples, cell invasion assay, and real-time PCR are described in Supplementary Materials and Methods.

Human breast cancer samples and cell lines

One hundred and ten pairs of human breast carcinomas and their normal counterparts were obtained from the Tissue Procurement Core Facility of Wake Forest University. The patients’ profiles are as follows. Age: 37–89 years old, mean 57 years; stage I: 30%; stage II: 45%; stage III: 23%; stage IV: 2%; histology, ductal carcinoma (ca): 87%; lobular ca: 6%; metastatic ca: 3%; mucinous ca: 2%; papillary ca: 2%; HER2, 3+: 22%; 2+: 15%. These cases comprise a population-based cohort treated at Wake Forest Baptist Medical Center from 1999–2008. Standard of care treatments included hormone therapy (i.e., tamoxifen monotherapy), chemotherapy (anthracyclines, taxanes), no systemic therapy, and local radiation. Disease-free survival events were defined as local, regional or distant recurrence during the time interval from diagnosis to last follow-up.

Classification of human breast cancers

Breast cancer samples were classified into 5 types (luminal A, luminal B, HER2, triple-negative/basal, and normal/unclassified) based on the data from histochemical studies for ER, PR, HER2, Ki67, cytokeratin, and morphologic alterations have been conducted at Wake Forest University. The patients’ profiles are as follows. Age: 37–89 years old, mean 57 years; stage I: 30%, stage II: 45%, stage III: 23%, stage IV: 2%; histology, ductal carcinoma (ca): 87%; lobular ca: 6%; metastatic ca: 3%; mucinous ca: 2%; papillary ca: 2%; HER2, 3+: 22%; 2+: 15%. These cases comprise a population-based cohort treated at Wake Forest Baptist Medical Center from 1999–2008. Standard of care treatments included hormone therapy (i.e., tamoxifen monotherapy), chemotherapy (anthracyclines, taxanes), no systemic therapy, and local radiation. Disease-free survival events were defined as local, regional or distant recurrence during the time interval from diagnosis to last follow-up.

Western blotting

Proteins were extracted with ice-cold EBC buffer with proteinase inhibitors. After gel electrophoresis and transfer to nitrocellulose membranes, proteins were visualized by immunoblotting with affinity-purified polyclonal antibodies to Dmp1 (RAX), p53 (sc-6243G, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Hdm2 (ab16895 [2A10], Abcam, Cambridge, MA, USA), p14ARF (sc-53639, 53640), p21CIP1 (sc-6246), or β-Actin (sc-6151), followed by incubation of the filters with HRP-conjugated secondary antibodies, and reaction with the enhanced ECL detection kit (PerkinElmer, Boston, MA, USA).
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Figure 5. Western blotting analyses of (breast epithelial or cancer cells expressing activated Dmp1:ER or empty vector. Lysate analyses were conducted by Western blotting with specific antibodies to Dmp1, p14ARF, p53, Hdm2, and p21CIP1. (a) HMEC, (b) MCF10A, (c) MCF7, (d) HCC1569, (e) ZR-75-1, and (f) BT-549 cells. Arrows show the position of specific band(s) of each protein. Bottom axis shows hours after addition of 2 mM 4-HT.


Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)