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Prognostic value of the hDMP1-ARF-Hdm2-p53 pathway in breast cancer

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Abstract

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 *p14^{ARF}* 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.

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

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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 has been re-evaluated. It suggests that specific DNA deletions and/or amplifications may be independent predictors of patient outcomes 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-53 pathway is significantly attenuated in *Dmp1*-deficient cells since those cells can easily give rise to immortalized cell lines that retain wild-type p19^{Arf} 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 *Eμ-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, 13-15).

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 *p21*^{Cip1}, 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* (h*DMP1*; h*DMTF1*) 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 h*DMP1* was present in ~35 % of lung cancers (12) in a mutually exclusive fashion with that of *INK4a/ARF* and/or *p53* in the same samples. This raised the possibility that hemizygous h*DMP1* deletion might define a new disease entity with different response to therapy (12, 15). The current study was conducted to demonstrate the frequency and pattern of genes involved in the h*DMP1*-ARF-Hdm2-TP53 pathway in human breast cancer. We analyzed 110 pairs of normal and cancer tissues from breast cancer for LOH of h*DMP1*, *INK4a/ARF*, *p53* and gene amplification of *Hdm2* (20, 21), and correlated the results of LOH/gene amplification with disease-free survival and known prognostic markers for human breast cancer (reviewed in 22, 23).

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 cancers, 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) (12) in 30 of 32 cases of LOH (93.8 %) (Supplementary Figure S1), a higher percentage than *hDMP1* deletion in human NSCLC (78.9 %) (12). 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 than the reported percentage of *p53* mutations in sporadic breast cancers (20%, 24). 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 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 62 of 65 cases (95.4 %, $p = 0.0027$, $\chi^2 = 8.977$; 95 % 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% 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 locus (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* 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 expression (i.e., $Ki67+ < \text{or} = 20\%$) in the *hDMP1* LOH (+) group in comparison to the LOH (-) group ($p = 0.0266$, $\chi^2 = 4.92$). Conversely, breast cancers with LOH for *p53* were associated with high Ki67 ($> 20\%$) ($p = 0.0153$, $\chi^2 = 5.88$) while LOH for *INK4a/ARF* or *Hdm2* amplification was not associated with this proliferation marker ($p = 0.196$ and $p = 0.522$ respectively). We also found that breast cancers with LOH for *hDMP1* more often had diploid DNA content than LOH (-) cases ($p = 0.0463$, $\chi^2 = 3.97$). On the other hand, LOH for *INK4a/ARF* or *p53* was associated with aneuploidy of DNA ($p = 0.0217$, $\chi^2 = 5.08$; $p = 0.0141$, $\chi^2 = 6.03$, 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*-null (16) and *p53*-mutant (26) 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://www5.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, 30.8% (32 of 104) of total cases were classified into luminal A, 23.1% (24 cases) were luminal B, 19.2% (20 cases) were HER2 type, 17.3% (18 cases) were triple-negative/basal-type, and 9.6% (10 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 ($p = 0.0085$; $\chi^2 = 6.924$) while *p53* LOH(+) breast cancers were significantly associated with non-luminal A subtype ($p = 0.0234$; $\chi^2 = 5.141$) (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* will 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 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 1,987 vs. 1,036 days) (Figure 3a). LOH for INK4a/ARF had no impact on patients' survival ($p = 0.591$, $\chi^2 = 0.289$; 70% survival 1,121 vs. 1,830 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. 1,830 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 1,036 vs. 1,932 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. Non-transformed human mammary epithelial cells (MCF10A, human mammary epithelial cells [HMEC]) and breast carcinoma cell lines (MCF7, MDA-MB-175VII, 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) (8, 12). The genomic statuses for p14^{ARF}, Hdm2, p53, p16^{INK4a}, and HER2 for human breast epithelial or cancer cell lines are summarized in Supplementary Table S2. Cell growth was completely inhibited by expressing Dmp1:ER in both MCF10A and *tert*-immortalized HMEC (Figure 4a, b). Significant inhibition of cell growth by Dmp1:ER was also observed in ZR-75-1 (Figure 4e) and MDA-MB-175VII (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 p14^{ARF}, p53, p21^{CIP1}, and Hdm2 in response to activation of Dmp1:ER in HMEC and ZR-75-1 cells (Figure 5a, e; Supplementary Figure S4). In MCF10A cells, significant accumulation of p53 and p21^{CIP1} was observed at 12-36 hours in response to Dmp1:ER (Figure 5b) although p14^{ARF} 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 (Figures 4c). Conversely HCC1569 cells with *p53* deletion or BT-549 cells with p53 mutation did not slow down their growth by Dmp1:ER (Figure 4d, f). Indeed lysate analyses showed consistently high levels of p14^{ARF} and undetectable p53 targets p21^{CIP1} or Hdm2 in these cells (Figure 5d, 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.

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 \pm 0.603 MCF7 cells with *hDMP1* downregulation invaded from upper to lower chamber while only 1.57 \pm 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 \pm 9.25 vs. 64.5 \pm 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 cases. 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 *p16^{INK4a}* 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(-), consistent with our recently published data showing that HER2/neu induces *Dmp1* in mouse model of breast cancer and that HER2 activates *hDMP1* promoter in human mammary epithelial cells (16).

Our study shows that LOH of *hDMP1* is associated with relatively low Ki67 index and increased frequency of diploid DNA, both of which are indicators for favorable prognoses of breast carcinomas (23,32,33). In agreement, *hDMP1* LOH(+) breast cancer was associated with luminal A subtype, and relapse-free survival was significantly longer (1,987 vs. 1,036 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%; 34), 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 (35) 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 continue the study to investigate the impact of single allelic *p53* loss with or without *p53* mutation on survival of breast cancer patients.

Since h*DMP1* is a transactivator for the *ARF* promoter and p14^{ARF} indirectly regulates the activity of p53 through Hdm2, there is a gradient of prognosis of breast cancer patients from (fair) h*DMP1* 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 h*DMP1* 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 h*DMP1*, *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 h*DMP1*, *INK4a/ARF*, *p53*, and real-time PCR assay for *Hdm2* will be independent laboratory tests to predict the prognosis of breast cancer patients. Although h*DMP1*-loss is a favorable prognostic factor associated with longer relapse-free survival of patients than h*DMP1* 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 h*DMP1*-loss in breast cancer progression.

Our data show that shRNA to h*DMP1* 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 *p53* mutant cells proliferate slower with h*DMP1* knockdown. First, the shRNA used downregulates all the three *DMP1* splicing variants including the tumor suppressor DMP1 α . The function of other two transcripts is unknown although published study suggested the β and γ variants might be blocking the activity of hDMP1 α (31). Development of splicing isoform-specific shRNA will be needed to elucidate the function of each variant on cell growth. The second possibility is that hDMP1 may directly interact with mutant p53 and h*DMP1* knockdown may affect p53 gain-of-function, and thereby, reduce proliferative capacity of cells with specific *p53* mutation. In support of later, patients with h*DMP1* LOH(+) tumors have favorable prognosis compared to patients with h*DMP1* 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 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 h*DMP1* 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 h*DMP1* (~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 the *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/Subjects 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 %, metaplastic 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 morphology of tumor cells as proposed from the Komen Website <http://ww5.komen.org/BreastCancer/SubtypesofBreastCancer.html>. These pathological examinations have been conducted at Wake Forest University Breast Cancer Center of Excellence. The Ki67 positivity ratio of 14% was used to differentiate luminal A and luminal B subtypes (27), and breast cancers with HER2 (+++) was categorized into HER2 subtype.

Western blotting

Proteins were extracted with ice-cold EBC buffer with proteinase inhibitors (7). 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), Hdm2 (ab16895 [2A10], Abcam, Cambridge, MA), p14^{ARF} (sc-53639, 53640), p21^{CIP1} (sc-6246), or β -Actin (sc-1615), followed by incubation of the filters with HRP-conjugated second antibodies, and reaction with the enhanced ECL detection kit (PerkinElmer, Boston, MA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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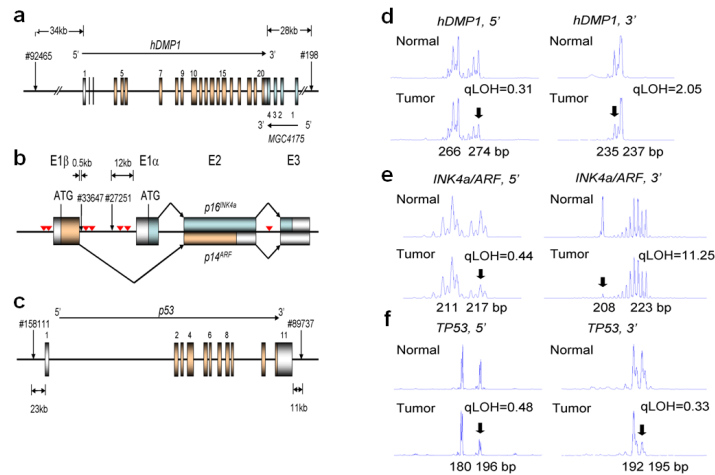


Figure 1. Representative patterns of LOH for *hDMP1*, *INK4a/ARF*, and *p53* in human breast carcinoma

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 (12, 15). The area peaks of the PCR products were quantitated by ABI 3730xl DNA analyzer. The qLOH values were determined through the following equation: $qLOH = \text{Area Peak 1} / \text{Area Peak 2 (normal tissue)} \div \text{Area Peak 1} / \text{Area Peak 2 (tumor tissue)}$. 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 noncoding 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 1 β (#33647) and those between Exon 1 β and Exon 1 α (#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

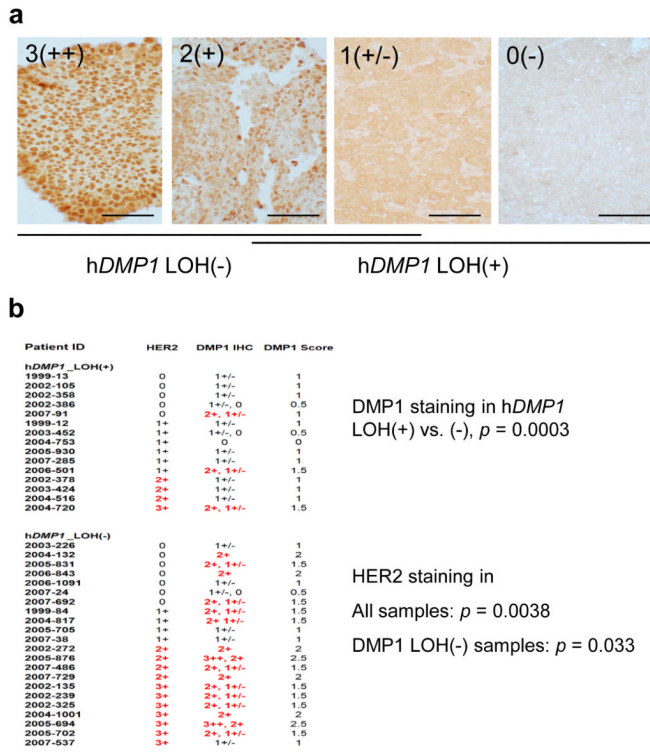


Figure 2. Histological grading of *hDMP1* in human breast carcinoma

a, human breast cancer tissues were stained with Dmp1-specific RAX antibody (28) and the intensity of the nuclear staining was graded from 3(++), 2(+), 1(+/-), and 0 (negative). The scale bar is 100 μ m.

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.

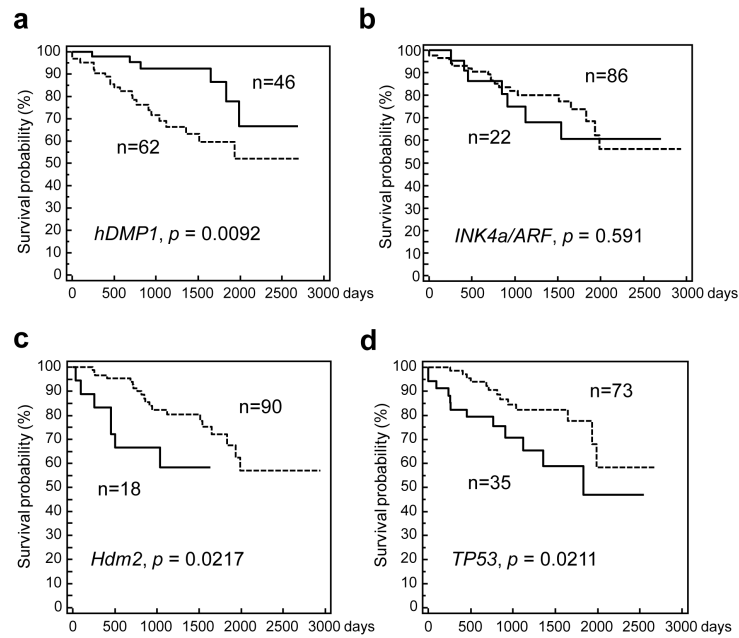


Figure 3. Relapse-free survival of 108 cases of human breast carcinoma dependent on LOH for *hDMP1*, *INK4a/ARF*, *p53* or *Hdm2* amplification

Kaplan-Meier analyses have been conducted to study the impact for the impact of loss or gain of each locus on breast cancer patients' disease-free survival up to 3,000 days. Only patients with stage I to III disease have been analyzed. Positive cases for gene deletion or amplification are indicated in solid lines and negative cases are shown in discontinuous lines. LOH for *hDMP1* (a) has significantly positive impact (i.e. fair prognosis) on patient's relapse-free survival while *Hdm2* amplification (c) or LOH for *p53* (d) had significantly negative impact. LOH for *INK4a/ARF* (b) had little influence on breast cancer patients' long-term survival.

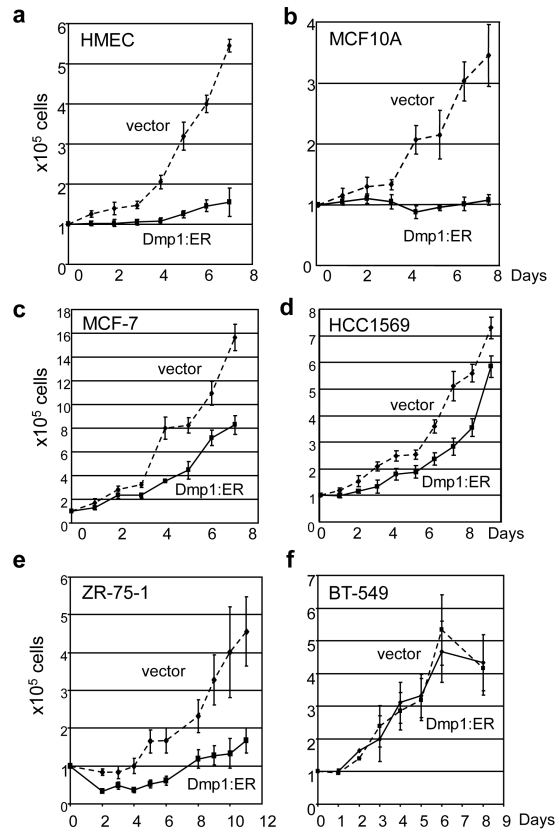


Figure 4. Proliferation assay of non-transformed human breast epithelial cells and breast carcinoma cell lines that overexpress Dmp1:ER

(a) HMEC (human mammary epithelial cells); $HER2^{low}$, ARF^+ , $p53^+$

(b) MCF10A; $HER2^{low}$, ARF^- , $p53^+$

(c) MCF7; $HER2^{low}$, ARF^- , $p53^+$

(d) HCC1569; $HER2^{++}$, ARF^+ , $p53^{del}$

(e) ZR-75-1; $HER2^{high}$, ARF^+ , $p53^+$

(f) BT-549; $HER2^{low}$, ARF^+ , $p53^{mut}$

solid lines show the growth curves of Dmp1:ER virus-infected cells treated with $2 \mu\text{M}$ 4-HT, discontinuous lines show those of mock-infected cells with 4-HT.

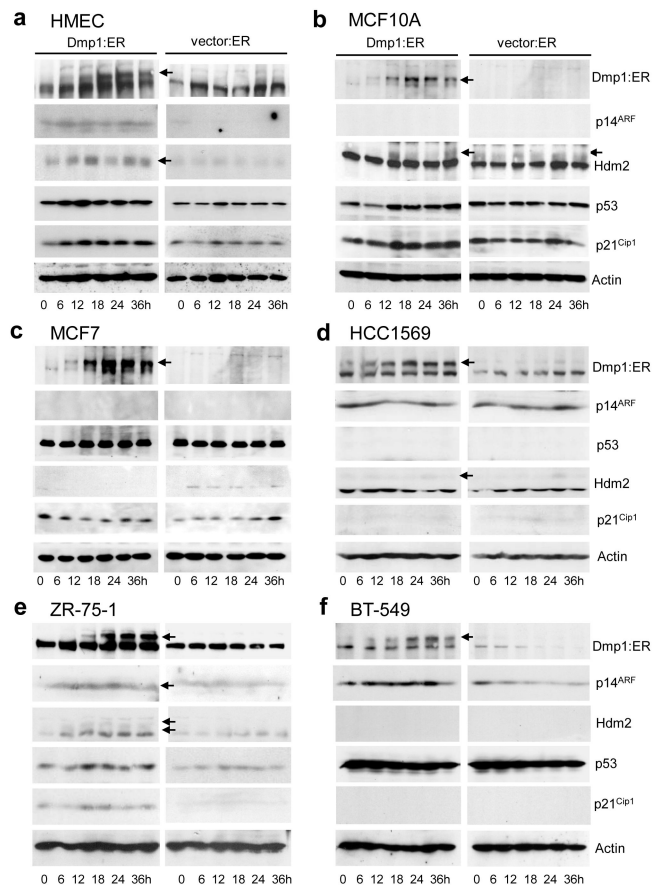


Figure 5. Western blotting analyses of (breast epithelial or cancer cells expressing activated Dmp1:ER or empty vector

Lysate analyses were conducted by Western blotting with specific antibodies to Dmp1, p14^{ARF}, p53, Hdm2, and p21^{CIP1}. (a) HMEC, (b) MCF10A, (c) MCF7, (d) HCC1569, (e) ZR-75-1, and (f) BT-549 cells. Bottom axis shows hours after addition of 2 μ M 4-HT.

Table 1
LOH analyses of 66 pairs of human breast carcinomas for the *hDMP1*, *INK4a/ARF*, and *p53* loci

Positive results for LOH (qLOH >2.0 or <0.5) are shown in bold red type. When one of the two markers (5' or 3') 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 of *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.

Patient ID	<i>hDMP1</i> LOH				<i>INK4a/ARF</i> LOH				<i>p53</i> LOH				<i>Hdm2</i> Amplification	
	<i>hDMP1</i> #92465 5'	<i>hDMP1</i> #198004 3'	<i>hDMP1</i> MSP #33647 5'	<i>hDMP1</i> MSP	<i>INK4a/ARF</i> #27251 3'	<i>INK4a/ARF</i> MSP #14 MSP	<i>INK4a/ARF</i> MSP #16 MSP	Exclusive to <i>hDMP1</i> LOH	<i>p53</i> #15811 5'	<i>p53</i> #89737 3'	Exclusive to <i>hDMP1</i> LOH	Exclusive to <i>INK4a/ARF</i> LOH	<i>Hdm2</i> Ampl.	Exclusive to <i>hDMP1</i> LOH
1999-12	1.21	2.57	Un	Un	1.17	Un	Un	yes	0.92	0.49**	no	yes	No	yes
1999-13	0.36	1.04	Un	Un	0.90	Un	Un	yes	0.89	1.53*	yes	yes	No	yes
2000-210	13.82	0.88	n.d.	n.d.	single	n.d.	n.d.	yes	1.34	0.82	yes	no	No	yes
2002-105	0.15	1.27	Un	Un	single	Un	Un	yes	single	7.68**	no	yes	No	yes
2002-358	1.17	3.19	Un	Un	1.06	Un	Un	yes	0.63	0.95	yes	no	No	yes
2002-378	0.42	2.05	Un	Un	1.25	Un	Un	yes	1.68	1.51	yes	no	No	yes
2002-386	0.15	0.49	Un	Un	0.67	Un	Un	yes	0.04	8.64	no	yes	No	yes
2003-424	>10	4.81	Un	Un	1.52	Un	Un	yes	0.89	1.01	yes	no	No	yes
2003-452	2.72	1.34	Un	Un	0.71	Un	Un	yes	0.84	1.10	yes	yes	No	yes
2004-516	8.11	2.02	Un	Un	1.15	Un	Un	yes	0.83	1.13	yes	yes	No	yes
2004-720	1.16	>10	Un	Un	0.90	Un	Un	yes	0.98	single	yes	yes	No	yes
2004-753	0.96	2.08	Un	Un	0.72	Un	Un	yes	1.03	1.14	yes	yes	4.66	no
2004-780	1.31	0.32	Un	Un	0.94	Un	Un	yes	1.18	0.98	yes	yes	No	yes
2004-817	0.97	0.49	Un	Un	1.00	Un	Met	yes	1.13	1.23	yes	yes	No	yes
2004-850	2.53	No del	Un	Un	1.02	Un	Un	yes	0.75	1.75	yes	yes	No	yes
2004-857	2.03	1.25	Un	Un	0.96	Un	n.d.	yes	0.70	1.25	yes	yes	No	yes
2005-78	2.06	1.52#	Un	Un	1.19	Un	Un	yes	0.78	0.97	yes	yes	No	yes
2005-483	0.92	2.04#	Un	Un	0.36	Un	Un	no	0.40	0.49*	no	no	No	yes
2005-686	No del	2.94	Un	Un	0.92	n.d.	Un	yes	1.56	0.97	yes	yes	No	yes

Patient ID	hDMPI LOH				INK4a/ARF LOH				p53 LOH			Hdm2 Amplification			
	hDMPI #92465 5'	hDMPI #198004 3'	hDMPI MSP	hDMPI MSP	INK4a/ARF #33647 5'	INK4a/ARF #27251 3'	p14 MSP	p16 MSP	Exclusive to hDMPI LOH	p53 #15811 5'	p53 #89737 3'	Exclusive to hDMPI LOH	Exclusive to INK4a/ARF LOH	Hdm2 Ampl.	Exclusive to hDMPI LOH
2005-823	1.01	H.D.	n.d.	n.d.	1.10	1.01	Un	Un	yes	1.06	0.97	yes		3.09	no
2005-930	1.20	1.03#	Un	Un	0.84	1.80	Un	Un	yes	0.93	1.02	yes		3.21	no
2005-958	2.18	H.D.	Un	Un	0.64	1.01	Un	Un	yes	1.57	0.56	yes		No	yes
2005-972	2.85	H.D.	Un	Un	0.92	0.65	Un	Un	yes	0.93	0.93	yes		No	yes
2006-501	0.47	H.D.#	Un	Un	1.34	0.70	Un	Un	yes	0.94	1.08	yes		No	yes
2006-545	0.37	No del	Un	Un	0.88	1.02	Un	n.d.	yes	0.79	0.95	yes		No	yes
2006-819	3.08	No del	Un	Un	1.08	0.77	Un	Un	yes	1.03	0.89	yes		No	yes
2006-1169	H.D.	0.96#	Un	Un	1.20	1.03	Un	Un	yes	2.17	1.15	no	yes	11.58	no
2007-88	1.31	H.D.	Un	Un	1.04	1.01	Un	partial	yes	0.98	1.10	yes		No	yes
2007-91	0.48	H.D.	Un	Un	0.98	0.95	Un	Un	yes	1.10	1.73	yes		No	yes
2007-127	>10	No del	Un	Un	0.14	6.02	Un	Un	no	0.24	0.92	no		No	yes
2007-245	1.01	H.D.	Un	Un	1.16	0.98	Un	Un	yes	1.01	0.95	yes		No	yes
2007-285	Del	No del	Un	Un	0.98	1.00	Un	Un	yes	1.57	1.84	yes		No	yes
2007-404	0.46	No del#	Un	Un	1.10	1.06	Un	Un	yes	0.93	1.12	yes		No	yes
2007-425	1.32	2.12	Un	Un	0.60	1.59	Un	Un	yes	2.87	2.70*	no	yes	No	yes
1999-84	0.64	No del	Un	Un	1.30	11.25	Un	Met	yes	0.59	4.08**	yes		No	no
2002-135	1.05	0.72	Un	Un	1.07	0.79	Un	Un	yes	1.49	0.43	yes		No	no
2002-239	0.52	1.19	Un	Un	0.83	0.21	Un	Un	yes	0.20	single	yes		No	no
2002-272	1.01	No del	Un	Un	1.75	0.69	Un	Un	yes	0.98	7.14	yes		No	no
2002-325	1.05	No del	Un	Un	0.87	0.85	Un	Un	yes	1.55	single	yes		67.75	yes
2003-226	0.64	0.57	Un	Un	1.12	Homo Del	Un	Un	yes	1.39	0.61	yes	yes	4.37	yes
2004-132	0.88	0.68	Un	Un	4.66	2.77	Un	Met	yes	3.37	0.23*	yes	no	3.01	yes
2004-738	0.89	No del	Un	Un	1.06	0.95	Un	Met	yes	1.03	0.93*	yes		No	no
2004-851	0.72	No del	Un	Un	1.38	0.75	n.d.	Un	yes	1.77	0.33	yes		No	no
2004-1001	1.70	No del#	Un	Un	0.23	1.16	n.d.	Un	yes	2.93	2.76	yes		No	no
2004-1033	1.11	No del#	Un	Un	0.78	1.05	Un	Un	yes	0.61	1.45	yes		No	no
2005-173	0.84	No del	Un	Un	0.23	2.01	partial	Un	yes	6.23	0.30	yes	no	No	no
2005-448	1.14	No del	Un	Un	0.68	1.18	Un	Un	yes	0.84	single*	yes		No	no

Patient ID	hDMPI LOH				INK4a/ARF LOH				p53 LOH			Hdm2 Amplification					
	hDMPI #92465 5',	hDMPI #198004 3',	hDMPI MSP	hDMPI MSP	INK4a/ARF #33647 5',	INK4a/ARF #27251 3',	p14 MSP	p16 MSP	Exclusive to hDMPI LOH	p53 #15811 5',	p53 #89737 3',	Exclusive to hDMPI LOH	Exclusive to INK4a/ARF LOH	Hdm2 Ampl.	Exclusive to hDMPI LOH		
2005-546	0.77	No del#	n.d.	n.d.	0.94	1.15	Un	Un	0.92	0.92	No	No	No	No			
2005-627	0.80	No del#	Un	Un	2.75	0.70	Un	Un	5.86	0.12	No	no	No	No			
2005-694	0.94	No del#	Un	Un	1.03	1.06	Un	Un	0.89	0.73*	3.02	yes	3.02	No	yes		
2005-702	1.17	No del	Un	Un	1.51	1.08	Un	Un	0.79	0.57	No	yes	No	No			
2005-705	1.59	1.16#	Un	Un	0.42	1.49	Un	Un	1.11	0.92	No	yes	4.12	No	yes		
2005-787	1.05	No del#	Un	Un	1.03	0.99	Un	Un	0.97	1.14	No	no	No	No			
2005-831	1.12	1.00#	Un	Un	2.11	1.22	Un	Un	1.05	0.42	No	yes	no	No			
2005-876	0.90	0.83#	Un	Un	2.01	1.73	Un	Un	0.73	0.47	No	yes	no	No			
2006-438	0.88	No del	Un	Un	1.01	0.89	n.d.	Un	1.15	2.13	No	yes	yes	No			
2006-633	0.97	1.09	Un	Un	1.00	1.05	Un	Un	0.92	0.09	No	yes	yes	No			
2006-777	0.93	No del	Un	Un	1.00	1.03	n.d.	Un	0.98	0.99	No	yes	3.79	No	yes		
2006-843	0.96	0.57#	Un	Un	1.09	0.79	Un	Un	0.97	1.01	No	yes	No	No			
2006-1091	0.78	0.99	Un	Un	3.75	0.29	Un	Un	0.99	1.06	No	yes	No	No			
2007-24	1.23	0.78	Un	Un	0.98	1.01	Un	Un	1.01	0.89*	No	yes	No	No			
2007-38	1.06	No del	Un	Un	1.12	1.11	Un	Un	1.08	0.34*	No	yes	yes	No			
2007-486	No del	No del	Un	Un	0.99	1.00	Un	Un	1.02	0.68*	No	yes	No	No			
2007-537	1.07	No del	Un	Un	1.09	1.07	Un	Un	1.01	0.71	No	yes	No	No			
2007-692	1.40	No del	n.d.	n.d.	0.98	0.91	partial	Un	0.40	2.34*	No	yes	yes	No			
2007-729	1.03	No del	n.d.	n.d.	single	0.79	n.d.	Un	0.46	2.35**	No	yes	yes	No			
All samples including Table S1																	
n=110	41.8%				20.0%				33.6%			68.9%		12.7%		93.0%	
95% CI									89.8-100			78.4-94.2		55.4-82.4		87.1-98.9	
									Exclusive			Exclusive		Non-exclusive		Exclusive	
									p=0.0027			p=0.025		p=0.0009		p=0.282	

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

All the breast cancer cases (n=104, 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.

	<i>hDMP1</i> LOH(+)	<i>hDMP1</i> LOH(-)	Percentage	<i>p</i> values
Luminal A	19	13	45.2	0.0085
Luminal B	8	15	19.0	0.5350
HER2	5	17	21.1	0.0573
Triple-negative	6	12	11.9	0.5026
Normal/unclassified	4	5	9.5	0.7951
Not evaluated	4	2		
total	46	64		
	<i>p53</i> LOH(+)	<i>p53</i> LOH(-)	Percentage	<i>p</i> values
Luminal A	6	26	16.7	0.0234
Luminal B	8	15	22.2	0.9848
HER2	10	12	27.8	0.2288
Triple-negative	8	10	22.2	0.3342
Normal/unclassified	4	5	11.1	0.5546
Not evaluated	2	4		
total	38	72		
	All cases	Percentage	Reported percentage	
Luminal A	32	29.1	28	
Luminal B	23	20.9	19	
HER2	22	20.0	17	
Triple-negative	18	16.4	27	
Normal/unclassified	9	8.2	8	
unknown	6	5.4		
total	110			