Interaction between p53 Mutation and a Somatic HDMX Biomarker Better Defines Metastatic Potential in Breast Cancer

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Abstract

TP53 gene mutation is associated with poor prognosis in breast cancer, but additional biomarkers that can further refine the impact of the p53 pathway are needed to achieve clinical utility. In this study, we evaluated a role for the HDMX-S/FL ratio as one such biomarker, based on its association with other suppressor mutations that confer worse prognosis in sarcomas, another type of cancer that is surveilled by p53. We found that HDMX-S/FL ratio interacted with p53 mutational status to significantly improve prognostic capability in patients with breast cancer. This biomarker pair offered prognostic utility that was comparable with a microarray-based prognostic assay. Unexpectedly, the utility tracked independently of DNA-damaging treatments and instead with different tumor metastasis potential. Finally, we obtained evidence that this biomarker pair might identify patients who could benefit from anti-HDM2 strategies to impede metastatic progression. Taken together, our work offers a p53 pathway marker, which both refines our understanding of the impact of p53 activity on prognosis and harbors potential utility as a clinical tool. Cancer Res; 75(4); 1-11. ©2015 AACR.

Introduction

The tumor suppressor p53, a central node of the cellular stress response pathway, regulates transcriptional programs important in suppressing tumor formation and progression, and the cellular response to certain therapies. The role of p53 in activating apoptosis, cell-cycle arrest, and the DNA damage response is well established, associating not only with p53’s tumor-suppressing properties, but also the cellular response to DNA damage-inducing cancer therapies (1–3). In recent years, however, the less well understood role of p53 in controlling cell migration and invasion has emerged, whereby both wild-type (wt) and mutant p53 are involved in processes of cell migration, cellular adhesion, cytoskeletal organization, and angiogenesis, which are important steps of metastatic progression (4, 5). Given p53’s roles in controlling the key cellular processes associated with the prevention of malignant transformation and tumor progression, it is not surprising that the loss of p53’s function is common in many cancers. In approximately 50% of all tumors, wt p53 activity is lost by mutation of the TP53 gene (6, 7). The majority of these mutations are point mutations leading to single amino acid substitutions and resulting in the expression of a mutant p53 protein. Mutant p53 can have dominant-negative effects over wt p53, but can also acquire oncogenic gain of functions (5, 8).

Many attempts have been made to translate our vast knowledge of the p53 tumor suppressor into personalization strategies and targeted therapies to improve patient survival. One such approach is to use p53’s high rate of mutation as a prognostic biomarker for overall survival, and a predictive marker for response to DNA damaging therapies, such as radiotherapy and many standard chemotherapeutics (8–10). Indeed, multiple studies have demonstrated that patients with mutant p53 in their cancers do have poorer outcomes (11). To date, breast cancer is one of the cancers wherein TP53 mutational status demonstrates the most significant prognostic impact (12). For example, in a study of 1,794 European patients with breast cancer, it was noted that the presence of p53 mutations in tumors conferred an increased relative risk (RR) of tumor-related death of 2.27 (13). The prognostic value of p53 mutations was shown to be independent of other known prognostic factors, such as tumor size, node status, and hormone-receptor status.

Although very significant, the prognostic value of TP53 mutational status in isolation is too small to dramatically affect clinical decisions for breast cancer or other cancers (14). One key reason is clearly the fact that there are many ways in which a cancer cell can...
inhibit p53’s activity and still retain a wt gene. These include common mutations of crucial upstream or downstream pathway genes that also result in attenuation of p53-mediated tumor suppression (8, 15). Thus, additional biomarkers that can identify carriers with wt TP53, but attenuated p53 signaling, will be required to increase the prognostic value and maximize clinical utility.

A crucial upstream pathway gene is HDMX (MDM4). The HDMX protein can bind to the N-terminal transactivation domain of p53 and thereby suppresses its transactivating function (16). In addition, it has been shown that HDMX can modulate the translocation of p53 by HD2 from the nucleus to the cytoplasm (16) and can stimulate HDM2-mediated ubiquitination and degradation of p53 (17). The crucial role of HDMX in the regulation of p53 is further highlighted by the fact that germline inactivation of mdm4 in mice leads to embryonic lethality through an increased activity of p53 during the early stages of development (18–21). Importantly, this phenotype is completely rescued by concomitant inactivation of the p53 gene (18–21). Recently, it was demonstrated that a biomarker for HDMX expression (HDMX-S alternatively spliced transcript levels compared with HDMX full-length transcript levels, HDMX-S/FL ratio) associates with multiple common somatic genetic lesions connected with p53 inhibition in cell line panels and sarcomas. The somatic lesions included TP53 mutation and HDM2 overexpression, a key negative regulator of p53 (22). Specifically, cancer cell lines and sarcomas with a high HDMX-S/FL ratio associated with both lower levels of HDMX protein and an enrichment of cell lines or tumors with an attenuated p53 pathway, either by direct TP53 gene mutation or overexpression of HDM2, a key inhibitor of p53. A model was proposed that higher HDMX-S/FL ratios, and therefore lower HDMX protein levels, can arise in cancer cells that already have inhibited p53 signaling through alterations of other key p53 pathway genes (22). Consistent with this model, patients, whose sarcomas contained higher HDMX-S/FL ratios metastasized faster and had poorer survival rates.

Families who inherit an attenuated p53 stress response by ways of a mutant TP53 in their germlines, develop tumors at an alarmingly high rate (23, 24). The two most frequent tumor types developed in these families are sarcomas and breast cancer. Thus, a biomarker such as the HDMX-S/FL ratio, which further defines p53 pathway attenuation and prognoses in sarcoma, is a good candidate prognostic marker for breast cancer. In this study, we provide supportive evidence for this hypothesis, and demonstrate that these two p53 pathway biomarkers could offer similar prognostic utility for breast cancer survival as microarray-based molecular subtyping. Unexpectedly, we demonstrate that the prognostic utility is independent of DNA-damaging treatments, but due to differential metastatic potentials of patients, thereby offering further support for an important role of the p53 pathway in metastasis. Finally, we provide evidence that the p53 pathway biomarkers identify patients for whom anti-MDM2 agents could serve as metastatic preventative therapies.

**Materials and Methods**

**Patient material**

Clinicopathologic data of 190 patients with breast cancer from Oslo MicroMetastasis Project (MicMa) and Ullevål University Hospital (ULL) cohorts are shown in Supplementary Table S5. Tumor material was obtained before adjuvant radio- and/or chemotherapeutics were administered. The ULL cohort consists of 78 women with primary breast cancer recruited at the Ullevål University Hospital between 1990 and 1994, and was first described by Bukholm and colleagues (25). All patients were treated according to Norwegian national guidelines at the time of diagnosis. Patients receiving adjuvant systemic therapy were given nine courses of CMF (cyclophosphamide, methotrexate, 5-fluorouracil) and/or tamoxifen for 2 years. Dosage of radiation given as adjuvant treatment was dependent on indication; after breast conserving therapy, the mammary gland was given 50 Gy (2 Gy × 25). The MicMa breast cancer cohort consists of 112 women with early-stage breast cancer from the Oslo MicroMetastasis Project, and was first described by Wiedswang and colleagues (26). Routine selection of patients to adjuvant treatment was based upon prevailing National Guidelines, where postmenopausal hormone receptor (HR)—positive patients received tamoxifen only, postmenopausal HR-negative patients received CMF and premenopausal patients, if HR positive, received CMF followed by tamoxifen. Five patients received high-dose chemotherapy and another five, preoperative chemotherapy due to large tumor size. After completing primary therapy, the patients were followed at 6 to 12-month intervals.

**Gene expression**

The RNA from patients with breast cancer was extracted from primary tumors using the TRizol reagent (Invitrogen) from fresh frozen tumor material. The RNA from breast cancer cell lines was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer’s protocol. The RNA extraction was followed by cDNA synthesis following standard protocols. The qRT-PCR amplification was performed in triplicate duplex reactions according to the manufacturer’s recommendations using FAM-labeled TaqMan probes, MDM4-FL (Hs00967241_m1; exon 5–6), and custom designed probes for MDM4-S, HDM2-P1, and HDM2-P2:

<table>
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<th>Forward</th>
<th>Reverse</th>
</tr>
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<td>MDM4-S_F:</td>
<td>5′-GCCCTTCTCTATGATATGCTAAGAAAGAATC-3′</td>
<td>5′-TTCTGTAGTTCTTTTTCTGGAAGTGGAA-3′</td>
</tr>
<tr>
<td>MDM4-S_R:</td>
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<td>5′-GCCACATTTGCCTGCTCCTC-3′</td>
</tr>
<tr>
<td>MDM4-S_M_FAM:</td>
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</tr>
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</tr>
<tr>
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<td>5′-CAAATGACCGACATGT-3′</td>
</tr>
<tr>
<td>HDM2-P2_R:</td>
<td>5′-CACTACATAGTGATCAGACATGT-3′</td>
<td>5′-CGATCCAGCGACATGT-3′</td>
</tr>
</tbody>
</table>

Relative gene expression was normalized according to expression of GAPDH measured in the same reaction with VIC-labeled TaqMan probe (4326317E; Applied Biosystems). The qRT-PCR with breast cancer cell lines cDNA was performed with the Applied Biosystems 7500 detector. The qRT-PCR with breast cancer patients’ cDNA was performed with the Applied Biosystems 7900HT detector where a Human Breast Total RNA (Ambion) was used as a reference to generate a standard curve. The Sequence Detection Systems Software v2.3 was used to calculate the amount of transcript expressed for each sample from the standard curve.

Expression profiling of 50 classifier genes and 5 control genes (PAM50; ref. 27) defined five molecular subtypes in both the MicMa and ULL cohort. Gene expression was measured, as
previously reported, using the Stanford 42K cDNA microarray and Agilent catalog design whole human genome 4 × 44K one color oligo array for the ULL and MicMa cohorts, respectively (28, 29).

Cell culture and lentiviral transductions

Breast cancer cell lines were a gift from Mieke Schutte/John Martens (Erasmus MC, Rotterdam, the Netherlands). The origin of all breast cancer cell lines and verification of their individual identity has been described previously, including a full description of the TP53 gene sequencing (30, 31). MCF-10A1, MCF-10AT, and MCF-10CA1a were obtained from Dr. F. Miller (Karmanos Cancer Center, Detroit, MI). These cell lines were authenticated by STR typing within the last 6 months; STR profile was found to match the profiles as shown on the ATCC website. All cell lines were maintained in DMEM/F-12 medium supplemented with 5% horse serum (HS) and antibiotics (all purchased from Agilent Technology, 100 μg/mL cholera enterotoxin, 5 μg/mL hydrocortisone, 10 μg/mL insulin (all purchased from Sigma-Aldrich). Cells were treated with 10 μM/L Nutilin-3 (Cayman Chemical) where indicated. The lentiviral shRNA expression vector targeting p53 was described previously (32). For lentiviral transductions, cells were seeded 24 hours before infection and a multiplicity of infection of 2.0 was used. Cells were transduced overnight in the presence of 8 μg/mL polybrene (Sigma-Aldrich). The next day, medium was replaced with medium containing 0.5 μg/mL puromycin. Transduced cells were seeded for experimental purposes 3 to 4 days after transduction.

Spheroid invasion assay

The spheroid invasion assay was performed as described previously (33). Briefly, spheroids were formed from 1,000 cells and embedded in a 1:1 mixture of Type 1 collagen (PureCol; Advanced BioMatrix) and methylcellulose (Sigma-Aldrich) onto a collagen-coated 96-well flat-bottom plate. Invasion was monitored for 2 days and images were quantified by measuring the area occupied by cells using the ImageJ software.

Statistical analysis

Survival analyses were performed using the Kaplan–Meier analysis with log-rank test and the Cox multivariate proportional hazards regression model with the SPSS 21.0 software (SPSS Inc.; IBM). The Shapiro–Wilk test was used to assess a normal distribution of the sample. The ANOVA test with a Bonferroni correction for multiple comparisons, Kruskal–Wallis test, Mann–Whitney test, and unpaired t test were applied to determine the statistical significance in the differences between the means. The Fisher exact test was used to compare the differences in frequency distributions. Statistical significance was regarded as P < 0.05.

Results

The HDMX-S/FL ratio interacts with TP53 mutational status to further define breast cancer survival

We began to explore whether the HDMX-S/FL ratio can interact with TP53 mutational status to improve the prognostic value in two different breast cancer cohorts, namely 78 patients from Ulleval University Hospital (28) and 112 patients from the Oslo MicroMetastasis Project (26, 34). TP53 mutational status was determined in all tumors: 19 (24%) ULL and 42 (37%) MicMa tumors were found to have a TP53 mutation. Specifically, 80% of all TP53 mutations in both cohorts were missense mutations located in exon 4, 10% frame-shift deletions, 5% nonsense mutations, 3% splice-site mutations, and 2% were in-frame insertions.

We first determined the prognostic value of TP53 mutational status in both the ULL and MicMa cohorts, whereby we compared the breast cancer survival of patients whose tumors had wt TP53 with those whose tumors had mutant TP53. The breast cancer survival of the 19 patients from the ULL cohort whose tumors had mutant TP53 was significantly shorter than those 59 patients whose tumors had wt TP53 (P = 0.005, log-rank test, Fig. 1A). Indeed, mutant TP53 tumors associated with a 2.8-fold higher RR of tumor-related death, as derived from a Cox multivariate regression analysis adjusted for known breast cancer prognostic factors: pathologic node status and adjuvant systemic therapy (P = 0.015, Table 1). Similarly, the breast cancer survival of the 42 patients from the MicMa cohort with mutant TP53 was shorter than those 70 patients whose tumors had mutant TP53, although this failed to reach statistical significance (P = 0.111, log-rank test, Fig. 1A, Table 1).

To begin to explore a potential interaction with TP53 mutational status and the HDMX-S/FL ratio, we determined HDMX-FL and HDMX-S expression levels by performing qRT-PCR using TaqMan gene expression assays designed to detect full-length (FL) and splice isoform S transcripts of HDMX. The HDMX-FL probe detects the exon 5–6 boundary of the HDMX gene, which is present in HDMX-FL transcript and only one out of six alternative transcripts, namely HDMX-A. The HDMX-S assay was designed to recognize the exon 5–7 boundary created by the deletion of exon 6, which is specific to the HDMX-S transcript. Next, we divided patients from both cohorts into two groups of high and low HDMX-S/FL ratios. High HDMX-S/FL ratio was defined as tumors with HDMX-S/FL ratios above the mean levels of HDMX-S/FL found in all 78 and 112 tumors from ULL and MicMa cohorts, respectively. Twenty-two patients from the ULL cohort and 41 patients from the MicMa cohort had tumors with high HDMX-S/FL ratios, whereas 56 patients from the ULL cohort and 71 patients from MicMa cohort had low HDMX-S/FL ratios. Interestingly, and in both cohorts, the TP53 mutational status associated with differential breast cancer survival only in patients with low HDMX-S/FL ratios. Specifically, in ULL patients with low HDMX-S/FL ratios, patients with wt p53 tumors associated with an almost 15-fold better survival rate than patients with p53-mutant tumors (P = 0.003 log-rank test, Fig. 1B; RR, 14.8; P = 3.6 × 10−4, Cox analysis, Table 1). In the MicMa cohort, patients with wt TP53 tumors associated with better survival rate than patients with mutant TP53 tumors (P = 0.027 log-rank test, Fig. 1B; Table 1). However, in both cohorts, there were no significant differences in survival rates between patients with either wt and mutant TP53 tumors when the tumors also had high HDMX-S/FL ratios (Fig. 1C, Table 1).

Another biomarker, estrogen receptor (ER) status, and a clinical parameter, adjuvant systemic treatment, have been suggested to interact with TP53 mutational status to increase its prognostic value (35). Thus, we next compared the effects of these factors on breast cancer survival in our patients and explored potential interactions of them with the HDMX-S/FL ratio. To do this, we first combined all patients from both cohorts and defined the high and low HDMX-S/FL ratios, as
described above, whereby 62 tumors were noted to contain high HDMX-S/FL ratios and 128 tumors low. As expected, the TP53 mutational status associated with differential breast cancer survival rates in all 190 patients \((P = 0.005, \text{log-rank test, Supplementary Fig. S1, Supplementary Table S1})\) and this association was greater in those 128 patients whose cancer had low HDMX-S/FL ratios \((P = 1.44 \times 10^{-4}, \text{log-rank test, Supplementary Fig. S1, Supplementary Table S1})\) and absent in those 62 patients with cancers with high HDMX-S/FL ratios \((P = 0.996, \text{Supplementary Fig. S1 and Supplementary Table S1})\). Interestingly, the HDMX-S/FL ratio was the only factor that significantly interacted with TP53 mutational status to affect breast cancer survival as measured by the Cox proportional hazards model \((P = 0.031, \text{Table 2})\) and no additional interactions with the HDMX-S/FL ratio and these factors were noted.

The HDMX-S/FL ratio and TP53 mutational status define breast cancer survival in a similar manner to microarray-based molecular subtypes

When the 190 patients are stratified into four groups based on the two p53 pathway biomarkers (Fig. 2A), it becomes clear that these four groups fall into three different categories of prognoses with the wtTP53-lowHDMX-S/FL having good prognoses, wtTP53-highHDMX-S/FL and mutTP53-highHDMX-S/FL having intermediate prognoses, and mutTP53-lowHDMX-S/FL having poor prognoses \((P = 0.003, \text{log-rank test, Fig. 2B})\). As expected, those 102 patients without either biomarker for p53 pathway attenuation (good prognosis, wtTP53-lowHDMX-S/FL) had the longest survival times compared with the 62 patients from the intermediate group (wtTP53-highHDMX-S/FL and mutTP53-highHDMX-S/FL; \(P = 0.039, \text{log-rank test, Figure 1.}\)
The interactions of TP53 and the HDMX-S/FL ratio with other known prognostic factors are known to alter breast cancer survival as the more complex microarray-based molecular subtyping. To begin to assess the potential prognostic strength of these biomarkers, they still fell into the same three different categories of prognoses as seen in the breast cancer survival analyses. Specifically, the wtTP53-lowHDMX-S/FL group had the best prognoses, the wtTP53-highHDMX-S/FL and mutTP53-highHDMX-S/FL groups had intermediate prognoses, and the mutTP53-lowHDMX-S/FL group had the poorest prognoses ($P = 0.017$, log-rank test, Fig. 3C, Supplementary Table S2). These results suggest that the association of these p53 biomarkers with differential times of survival after breast cancer diagnosis is not due to differential responses to DNA-damaging therapies.

We next explored potential associations of these p53 pathway biomarkers with differential times to metastasis after diagnosis. For the 190 patients from the ULL and MicMa cohorts, we had information on the occurrence of distal metastases after diagnosis for 185 patients. When we separated the 185 patients into the four groups based on both p53 pathway biomarkers, they still fell into the same three different categories of prognoses as seen in the breast cancer survival analyses. Specifically, the wtTP53-lowHDMX-S/FL group had the best prognoses, the wtTP53-highHDMX-S/FL and mutTP53-highHDMX-S/FL groups had intermediate prognoses, and the mutTP53-lowHDMX-S/FL group had the poorest prognoses ($P = 0.028$, log-rank test, Fig. 3D, Supplementary Table S1). These same trends in MFS were fully determined the molecular subtype for the tumors from RNA derived from the tumors of 176 patients using the PAM50 assay (27). As expected, the five different breast cancer molecular subtypes fell into three different categories of prognoses ($P = 0.016$, log-rank test, Fig. 2E). Interestingly, the differences in survival times between the different categories were remarkably similar to differences found in the groupings based on the two p53 pathway biomarkers. For example, the good prognosis group (Luminal A subtype) had a 3-fold lower RR of tumor-related death compared with the poor groups (Luminal B, Basal, and ERBB2+; $P = 0.001$, Cox analysis, Fig. 2F). This association is very similar compared with the 4-fold lower RR noted between the good and poor prognoses groups as defined by TP53 mutational status and the HDMX-S/FL ratio.

To begin to assess the potential prognostic strength of these two p53 pathway biomarkers, we compared them to the well-utilized breast cancer molecular subtyping. Microarray-based gene expression profiling has been very successful in developing a molecular classification system for breast cancer and prognostic multigene classifiers (36, 37). Briefly, expression profiling of 50 classifier genes and 5 control genes (PAM50) is able to define minimally five different subtypes of breast cancer, which are known to have either good (Luminal A), intermediate (normal-like), or poor prognoses (Luminal B, Basal, and ERBB2+; Fig. 2D; ref. 27). We were able to successfully determine the molecular subtype for the tumors from RNA derived from the tumors of 176 patients using the PAM50 assay (27). As expected, the five different breast cancer molecular subtypes fell into three different categories of prognoses ($P = 0.016$, log-rank test, Fig. 2E). Interestingly, the differences in survival times between the different categories were remarkably similar to differences found in the groupings based on the two p53 pathway biomarkers. For example, the good prognosis group (Luminal A subtype) had a 3-fold lower RR of tumor-related death compared with the poor groups (Luminal B, Basal, and ERBB2+; $P = 0.001$, Cox analysis, Fig. 2F). This association is very similar compared with the 4-fold lower RR noted between the good and poor prognoses groups as defined by TP53 mutational status and the HDMX-S/FL ratio.

Together, these results suggest that these two p53 pathway biomarkers could offer a similar prognostic utility for breast cancer survival as the more complex microarray-based molecular subtyping.

### Table 1. Cox proportional regression analysis to predict the risk of tumor-related death for patients from individual MicMa and ULL cohorts

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<tr>
<th></th>
<th>df</th>
<th>$P$</th>
<th>RR</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
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<tr>
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<td>ULL: high HDMX-S/FL ratio</td>
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Abbreviations: CI, confidence interval; df, degrees of freedom; pN, pathologic node status.

The **HDMX-S/FL ratio interacts with TP53 mutational status to further define metastasis-free survival**

Mutant p53 is known to increase a cancer’s ability to both metastasize and become resistant to DNA-damaging therapies (38). To begin to assess whether the associations of the p53 pathway biomarkers with differential breast cancer survival are a consequence of differential metastatic progression or differential responses to the chemo- and radiotherapy, we performed the breast cancer survival analyses on patients who did not receive any type of adjuvant treatment or radiotherapy. Of the 181 patients from the ULL and MicMa cohorts for whom the treatment status was known, 110 patients had been treated with adjuvant- and/or radiotherapies (Fig. 3A). The remaining 71 patients, who did not receive any form of adjuvant- or radiotherapies, were, as expected, enriched for node-negative and early-stage cases, but did not significantly differ in the p53 biomarker grouping (Fig. 3B). Interestingly, when we separated the 71 patients into the four groups based on both p53 pathway biomarkers, they still fell into the same three different categories of prognoses as seen in the breast cancer survival analysis of all patients. Specifically, the wtTP53-lowHDMX-S/FL group had the best prognoses, the wtTP53-highHDMX-S/FL and mutTP53-highHDMX-S/FL groups had intermediate prognoses, and the mutTP53-lowHDMX-S/FL group had the poorest prognoses ($P = 0.017$, log-rank test, Fig. 3C, Supplementary Table S2). These results suggest that the association of these p53 biomarkers with differential times of survival after breast cancer diagnosis is not due to differential responses to DNA-damaging therapies.

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found for all four groups in those patients that did not receive adjuvant treatments or radiotherapy ($P = 0.048$, log-rank test, Fig. 3E, Supplementary Table S2). Together, these observations suggest that the associations of the p53 pathway biomarkers with differential survival are a consequence of differential metastatic progression, and not differential responses to the chemo- and radiotherapy.

**High HDMX-S/FL ratios associate with p53 inhibition in breast cancer**

As mentioned above, it was previously demonstrated that the HDMX-S/FL ratio associates with multiple common somatic genetic lesions connected with p53 inhibition in cell line panels and sarcomas (22). The somatic lesions included TP53 mutation and HDM2 overexpression, a key negative regulator of p53. Specifically, cancer cell lines and sarcomas with a high HDMX-S/FL ratio associated with both lower levels of HDMX protein and an enrichment of cell lines or tumors with an attenuated p53 pathway, either by direct TP53 gene mutation or overexpression of HDM2. To gain a better understanding of the molecular underpinnings of these highly significant associations of the HDMX-S/FL ratio and TP53 mutational status in the metastatic progression of breast cancer, we explored if similar associations could be noted in the breast tumors of the ULL and MicMa cohorts, as well as in a breast cancer cell panel consisting of 36 well-characterized cell lines, wherein it had previously been shown that the HDMX-S/FL ratio was a biomarker for overall HDMX protein levels (22).

HDM2 levels in tumors were determined through qRT-PCR measurements of mRNA transcripts from both HDM2 promoters, namely the P1 promoter and the p53-responsive P2 promoter. The average levels of HDM2-P1 transcript were 0.062 and ranged from 0.06 to 1.8, whereby 113 tumors had HDM2-P1 below average and 76 tumors had HDM2-P1 above. The average levels of HDM2-P2 transcript in wt TP53 tumors were 1.08 and ranged from 0.1 to 15.5, whereby 98 tumors had HDM2-P2 below average and 32 tumors had HDM2-P2 above average. The HDM2 levels of the cell line panel had been previously reported (32). With these data and together with the TP53 mutational status of both tumors (Supplementary Table S3) and cell lines (Supplementary Table S4), we were able to determine that like in sarcomas, higher levels of HDMX splicing associate with biomarkers for p53 pathway attenuation in breast tumors and breast cancer–derived cell lines. Specifically, we observed that the 62 tumors with high HDMX-S/FL ratios were significantly enriched for TP53 mutation or high expression of HDM2 in wt TP53 tumors. Specifically, 81% of tumors with high HDMX-S/FL ratios had either mutant TP53 or above-average HDM2 transcripts levels, whereas only 66% of tumors with low HDMX-S/FL ratios had mutant TP53 or above-average levels of either HDM2 transcripts ($P = 0.0359$, Fisher exact test, Table 3). Interestingly, we observed the similar enrichment of p53 pathway attenuation biomarkers in the 18 breast cancer cell lines with high HDMX-S/FL ratios. Specifically, 100% of the cell lines with high HDMX-S/FL ratios had either mutant TP53 or wt TP53 and the highest levels of HDM2 (the top 4), in contrast with only 78% in those cell lines that had low HDMX-S/FL ratios ($P = 0.033$, Fisher exact test, Table 3).

Overexpression of HDM2 and subsequent attenuation of p53 signaling in p53 wt cancers is frequently observed. These observations have motivated a large effort to develop small molecules to inhibit the HDM2-p53 interaction and reactive the pathway to promote tumor clearance (10, 39, 40). Our data would suggest that, together with the TP53 mutational status, the HDMX-S/FL ratio could help identify those patients for whom anti-HDM2 agents could serve as metastatic preventative therapies. As a test of this hypothesis, we studied the effects of HDM2 inhibition by the small molecule Nutlin-3, and its effects on an invasive phenotype of a breast cancer cell line with wt TP53 (MCF-10A). The invasive phenotype was measured using a spheroid invasion assay (33). Importantly, reducing p53 levels using short hairpin RNA promoted invasion of normal (MCF-10A1), premalignant (MCF-10A1T), and metastatic (MCF-10CA1a) breast epithelial cells (Fig. 4A and B), thereby underlining the importance of wt p53 in inhibiting metastatic progression of breast cancer. In contrast, the stabilization and activation of p53 by addition of Nutlin-3 inhibited invasion (Fig. 4C and D). Together, these data lend support to the hypothesis that anti-HDM2 agents could serve as metastatic preventative therapies in those patients with breast cancer with shorter MFS times, such as those defined by a wt TP53 gene and high HDMX-S/FL ratios.

**Discussion**

There is great heterogeneity between individuals in their cancer risk, progression, and responses to therapy. This heterogeneity is a major obstacle in designing uniformly effective prevention, screening and treatment strategies, and motivates the large effort to personalize them. The utilization of multiple pathologic and molecular biomarkers, particularly HR status, has improved breast cancer treatment management and survival. However, the vast majority of patients have yet to benefit from the existing biomarkers and still either succumb to their disease or are overtreated, underlining the need for additional prognostic and predictive biomarkers (36). A potential prognostic and/or predictive breast cancer biomarker is certainly the mutational status of the TP53 tumor-suppressor gene. As mentioned above, the prognostic value of TP53 mutational status alone is too small to dramatically affect clinical decisions for breast cancer or other cancers (14). Here, we demonstrated in two different breast cancer cohorts that an additional p53 pathway biomarker, the HDMX-S/FL ratio, can interact with TP53 mutational status to further identify patients with significantly different prognoses. We provided evidence that the associations are independent of DNA-damaging treatments, but due to differential metastatic potentials of patients. Indeed, there is a growing body of evidence that the p53 pathway can play key roles in suppressing metastatic progression (41–43). For example, p53 can activate E-cadherin, the key cell adhesion mediator and suppressor of EMT-dependent cancer cell invasion, through the facilitation of HDM2-mediated ubiquitination and the subsequent degradation of pRb and the transcriptional suppressor of E-cadherin (41).

It was previously demonstrated that the HDMX-S/FL ratio associates with multiple common somatic genetic lesions connected with p53 inhibition in cell line panels and sarcomas (22). The somatic lesions included TP53 mutation and HDM2 overexpression, a key negative regulator of p53. In this report, we offer evidence that like in sarcomas, higher HDMX-S/FL ratios associate with these biomarkers for p53 pathway...
Biomarkers in the p53 Pathway and Metastatic Breast Cancer

A

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>Gene Expression Levels</th>
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<tbody>
<tr>
<td>Luminal A</td>
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<tr>
<td>Luminal B</td>
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<tr>
<td>Basal</td>
<td></td>
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<tr>
<td>ERBB2+</td>
<td></td>
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<tr>
<td>Normal-like</td>
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B

C

D

E

F
attenuation in breast tumors and breast cancer–derived cell lines, namely TP53 mutation and HDM2 overexpression. Consistent with this, we observed that patients with wt TP53 and low HDMX-S/FL ratios associated with the longest overall and MFS times in both cohorts. Together, these data support a model, whereby tumors with wt TP53 and low HDMX-S/FL ratios, and therefore lower levels of p53 inhibitors like HDM2, will have retained greater p53 pathway-dependent suppression of metastasis, resulting in better outcomes for the patients.

Intriguingly, our observations suggest that the p53 attenuation associated with higher HDMX-S/FL ratios could also be relevant to patients with breast cancer whose tumors contain mutant p53. Indeed, an even larger body of literature has described a gain-of-function for mutant p53 that results in more aggressive/metastatic cancers (4, 44). The most well-described mechanism involves the inhibition of the p53 family member, p63. Specifically, it is well known that loss of p63 expression associates with metastatic phenotypes in a number of tumors, and, indeed, p63 expression serves as a marker for noninvasive epithelial tumors (45). Subsequently, it has been shown that mutant p53 can directly inhibit p63, resulting in lower expression levels of p63 target genes, including antimetastatic genes, such as the miRNA regulator Dicer, SHARP1, and cyclin G2, which both oppose TGF-β–mediated metastasis (46–48). It is therefore reasonable to hypothesize that our observation that patients with mutant p53 and low HDMX-S/FL ratios have the worst prognoses is due to the fact that the gain-of-function activity of mutant p53 is less

Figure 3.
The HDMX-S/FL ratio interacts with TP53 mutational status to further define MFS. A, pie chart depicting the frequency distribution of adjuvant treatments and radiotherapy of the 181 patients from MicMa and ULL cohorts. B, bar graphs depicting the frequency distributions of node status, stage, and p53 biomarkers in the 110 patients who received adjuvant treatment and/or radiotherapy, and the 71 patients who did not. C, Kaplan–Meier plot depicting the breast cancer survival of the 71 patients from the ULL and MicMa cohorts that did not receive any type of adjuvant treatment or radiotherapy and that were stratified into the four groups based on p53 biomarkers. D and E, Kaplan–Meier plots depicting the MFS of the 185 from the ULL and MicMa cohorts (D), and the 68 patients that did not receive any type of adjuvant treatment or radiotherapy (E), both stratified into four groups based on the p53 biomarkers. The P values noted are derived from a log-rank test.

Figure 2.
The HDMX-S/FL ratio and TP53 mutational statuses define breast cancer survival in a similar manner to microarray-based molecular subtypes. A, schematic representation of the combinations of p53 biomarkers. B, Kaplan–Meier plot depicting the breast cancer survival of 190 patients divided into four groups depending on the TP53 mutational status and the HDMX-S/FL ratios, namely wt TP53 with low HDMX-S/FL ratio (n = 102), wt TP53 with high HDMX-S/FL ratio (n = 27), mutant TP53 with low HDMX-S/FL ratio (n = 26), and mutant TP53 with high HDMX-S/FL ratio (n = 35). C, predicted survival curves for patients with different prognoses associated with TP53 mutational status and different HDMX-S/FL ratios derived from a Cox multivariate regression analysis that was adjusted to the known prognostic factors: pathologic lymph node status and adjuvant therapy treatment. Also noted is the calculated RR. D, schematic representation of the molecular subtypes and their gene expression signatures. E, Kaplan–Meier plot depicting the breast cancer survival of 176 patients divided into four molecular subtypes based on microarray analysis using the PAM50 assay. The P value noted is derived from a log-rank test. F, predicted survival curves for the different molecular subtypes derived from a Cox multivariate regression analysis that was adjusted to the independent prognostic factors: pathologic lymph node status and adjuvant therapy treatment (n = 156).
attenuated by the associated higher expression of p53 inhibitors, like HDM2. Indeed, further support of this hypothesis is provided by Terzian and colleagues, who clearly demonstrated that a loss of mdm2 in murine models resulted in the stabilization of both mutant p53 and a gain-of-function metastatic phenotype (49). However, we cannot rule out the possibility that the observed associations are due to the p53-independent effects of higher levels of HDMX (50).

Clearly, further elucidation of the p53 pathway biomarkers is needed in additional breast cancer patient cohorts and model systems to fully understand how they interact with each other to better predict and create the aggressive phenotype of primary tumors. However, it is likely that p53 pathway biomarkers could contribute to better prognostication, and decrease the overtreatment of patients with nonaggressive cancers, such as those that retain high levels of p53 signaling. Moreover, both wt and mutant p53 signaling directly affect malignant progression of tumors, therefore prognostic biomarkers in the pathway will not only serve to foresee prognoses, but also offer potential nodes of intervention. For example, our observations lend support to the hypothesis that anti-HDM2 agents could serve as metastatic preventative therapies in those patients with breast cancer with shorter MFS times, such as those defined by a wt TP53 gene and high HDMX-S/FL ratios.

Table 3. Higher HDMX-S/FL ratios associate with the p53 pathway alterations in breast cancer tumors and cell lines

<table>
<thead>
<tr>
<th>Mutant TP53 or wt TP53-high HDM2, %</th>
<th>Low HDMX-S/FL ratio (n = 129)</th>
<th>High HDMX-S/FL ratio (n = 62)</th>
<th>Fisher exact test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant TP53 or wt TP53-high HDM2, %</td>
<td>66</td>
<td>81</td>
<td>0.0359</td>
</tr>
<tr>
<td>wt TP53-low HDM2, %</td>
<td>34</td>
<td>19</td>
<td>0.033</td>
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Figure 4.
Breast cancer cell invasion is inhibited by p53 activation. A and B, MCF-10A1, MCF-10AT, and MCF-10CA1a expressing sh ctrl or sh p53 were harvested for total protein extraction and p53 expression was analyzed by Western blot using USP7 as loading control (A), or spheroids were formed and embedded into collagen (B). Invasion was determined by calculating the measured invaded area relative to the area on day 0. Results are expressed as mean ± SD of at least three spheroids and are representative of three independent experiments; *, P < 0.05 compared with sh ctrl of the same cell line on the same day. C and D, MCF-10A1, MCF-10AT, and MCF-10CA1a were mock-treated or treated with 10 μmol/L Nutlin-3 and harvested after 24 hours and analyzed for protein expression by Western blot (C), or spheroids were formed and embedded into collagen for 48 hours (D). Results are expressed as mean ± SD of at least three spheroids and are representative of three independent experiments; *, P < 0.05 compared with mock-treated cells of the same cell line.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Grawenda, S. Lam, A.-L. Borresen-Dale, V.N. Kristensen, H. Edvardsen
Writing, review, and/or revision of the manuscript: A.M. Grawenda, E.K. Möller, A.-L. Borresen-Dale, V.N. Kristensen, A.G. Jochemsen, H. Edvardsen, G.L. Bond
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.F.A.S. Teunisse, G.I.G. Alnæs, G.L. Bond
Study supervision: C.R. Goding, A.G. Jochemsen, G.L. Bond

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References


Interaction between p53 Mutation and a Somatic HDMX Biomarker Better Defines Metastatic Potential in Breast Cancer

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