

Corrections

CELL BIOLOGY. For the article “p600, a unique protein required for membrane morphogenesis and cell survival,” by Yoshihiro Nakatani, Hiroaki Konishi, Alex Vassilev, Hisanori Kurooka, Kei-ichiro Ishiguro, Jun-ichi Sawada, Tsuyoshi Ikura, Stanley J. Korsmeyer, Jun Qin, and Anna M. Herlitz, which appeared in issue 42, October 18, 2005, of *Proc. Natl. Acad. Sci. USA* (**102**, 15093–15098; first published October 7, 2005; 10.1073/pnas.0507458102), the caption for the issue cover on page *iii* appeared incorrectly. The online version has been corrected. The corrected cover caption appears below.

Cover image: Immunofluorescence localization of p600 (green), microtubules (red), and actin (blue) in human fibroblasts. Actin and microtubules appear to develop underneath the p600 meshwork, suggesting roles of p600 in cytoskeletal organization. See the article by Nakatani *et al.* on pages 15093–15098.

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GENETICS. For the article “An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival,” by Lance D. Miller, Johanna Smeds, Joshy George, Vinsensius B. Vega, Liza Vergara, Alexander Ploner, Yudi Pawitan, Per Hall, Sigrid Klaar, Edison T. Liu, and Jonas Bergh, which appeared in issue 38, September 20, 2005, of *Proc. Natl. Acad. Sci. USA* (**102**, 13550–13555; first published September 2, 2005; 10.1073/pnas.0506230102), the breast cancer microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus database (GEO, www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession no. GSE3494.

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An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival

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Perturbations of the p53 pathway are associated with more aggressive and therapeutically refractory tumors. However, molecular assessment of p53 status, by using sequence analysis and immunohistochemistry, are incomplete assessors of p53 functional effects. We posited that the transcriptional fingerprint is a more definitive downstream indicator of p53 function. Herein, we analyzed transcript profiles of 251 p53-sequenced primary breast tumors and identified a clinically embedded 32-gene expression signature that distinguishes p53-mutant and wild-type tumors of different histologies and outperforms sequence-based assessments of p53 in predicting prognosis and therapeutic response. Moreover, the p53 signature identified a subset of aggressive tumors absent of sequence mutations in p53 yet exhibiting expression characteristics consistent with p53 deficiency because of attenuated p53 transcript levels. Our results show the primary importance of p53 functional status in predicting clinical breast cancer behavior.

microarray | expression analysis | tumor profiling | class prediction

The p53 tumor suppressor is a critical regulator of tissue homeostasis, and its inactivation at the gene or protein level confers cellular properties conducive for oncogenesis and cancer progression. Mutations in p53 occur in >50% of human cancers (1, 2), and the mutational status of p53 is prognostic in many malignancies (3). In breast cancer, p53 mutations are associated with worse overall and disease-free survival, independent of other risk factors (4), and have been implicated in resistance to anticancer therapies (5–11). These observations, however, have been inconsistent (12, 13), owing, in part, to the variable accuracy of the methods to ascertain p53 status, variation in disease severity attributable to the different forms of p53 mutation, and studies of insufficient size (8, 11, 14). Further confounding the association between p53 status and patient risk is the growing number of alternative molecular mechanisms (e.g., MDM2) that compromise p53 function.

In this study, we explored the possibility that a gene-expression signature, derived from differences between p53 mutant (mt) and wild-type (wt) breast tumors, could provide a more accurate measure of the functional configuration of p53, thereby improving its prognostic utility. Using oligonucleotide microarrays covering >30,000 genes, we analyzed the global transcript levels of 251 primary invasive breast tumors for which we have detailed information on p53 status, as determined by cDNA sequencing (6) and pursued a validation strategy of intersecting alternative array data sets. We found that, in most cases, tumors with mt and wt p53 can readily be distinguished by their expression profiles and that a 32-gene p53 signature is consistently associated with patient survival in different patient subsets, independent of other risk factors, and is a superior prognostic and predictive indicator, compared with p53 mutation status alone.

Methods

Patients and Specimens. Frozen tissue was collected from 315 consecutively presented primary breast cancers representing 65% of all those resected in Uppsala County, Sweden, from January 1, 1987 to December 31, 1989 (6). Of these tissues, 251 were comprised predominantly of diseased tissue, were sequenced for p53 (6), and yielded sufficient RNA for array analysis. Clinicopathological variables measured at diagnosis were obtained from patient records and are described in detail in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. This microarray study was approved by the ethical committee at the Karolinska Institute, Stockholm, Sweden.

Expression Profiling. Total RNA was extracted from samples by using RNEasy Mini kit (Qiagen, Hilden, Germany) and evaluated on a 2100 Bioanalyzer (Agilent Technologies). *In vitro* transcription products were prepared from 2–5 μ g of total RNA, hybridized to the Affymetrix U133 A and B arrays and washed and scanned according to the manufacturer's instructions.

Microarray Data Processing. Raw data were normalized by using the global mean method. Probe-set signal values were natural log transformed and scaled by adjusting the mean intensity to a target signal value of log 500. Samples with suboptimal average signal intensities (i.e., scaling factors >3.5) or GAPDH 3'/5' ratios >3.5 were relabeled and rehybridized on new arrays. If visible artifacts were observed, the same cRNA was rehybridized on new chips.

Class Prediction. For gene selection, we fit a linear model to the expression data with expression level as the response and p53 status, estrogen-receptor (ER) status, and grade status as the predictor variables. As an initial filter, we excluded genes with a *P* value for model fit >0.001 and ranked genes in decreasing order of the absolute value of the p53 status coefficient. For class prediction, we evaluated several supervised learning methods, including diagonal linear discriminant analysis (15), *k* nearest neighbors (16), and support vector machines (17), as described in *Supporting Materials and Methods*.

Data Analysis. For all hierarchical cluster analyses, log expression values of each gene were mean centered, and genes and tumors were clustered by using Pearson correlation and aver-

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Abbreviations: DFS, disease-free survival; ER, estrogen receptor; mt, mutant; wt, wild type.

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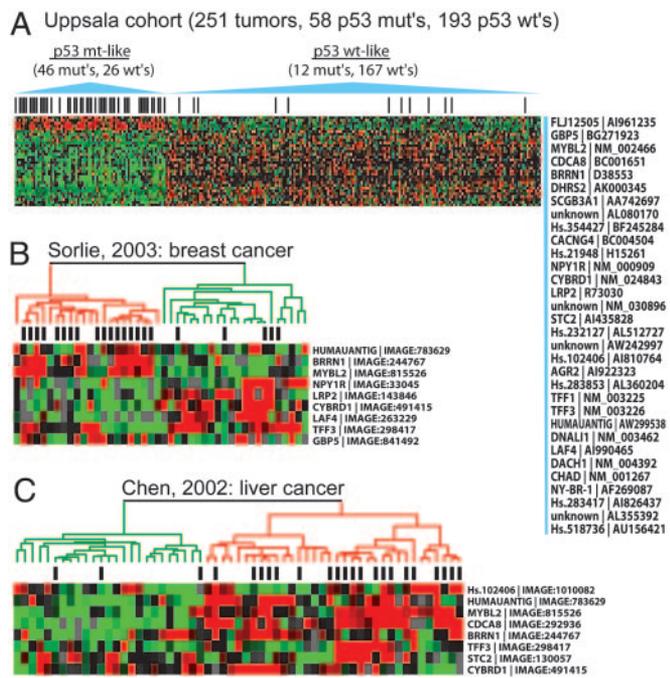


Fig. 1. The p53 signature is associated with p53 status in independent data sets. Clustergrams are oriented as outlined in Fig. 5. (A) Expression profiles of the Uppsala tumors segregated by the 32-gene signature. Unigene symbols and GenBank IDs are listed to the right. (B) P53 mt and wt breast tumors from Sorlie *et al.* (18) were clustered by using a nine-gene subset of the p53 signature. (C) P53 mt and wt liver tumors (predicted by immunohistochemistry) from Chen *et al.* (19) were clustered by using an eight-gene subset of the p53 signature. Green dendrogram branches denote tumors with the wt-like configuration; red branches indicate those with mt-like profiles. Probe IMAGE clone IDs from the original studies are listed. Black bars denote mt p53 status.

and where each tumor contained data for >50% of the genes. We observed that even this eight-gene subset was able to cluster the liver cancers into two primary clusters significantly correlated with p53 levels: 87% of the IHC-positive (predicted mts) in one cluster, and 61% of the predicted wts in the other ($P = 0.00035$) (Fig. 1C). Again, the probability of this clustering occurring by random chance was $P = 0.009$ by Monte Carlo P value estimation. Taken together, these observations suggest that the genes comprising the p53 signature are robust in their ability to classify not only breast tumors but also liver cancers

according to their p53 mutational status and, therefore, may have generalizable utility in predicting p53 status in a range of cancer types.

Transcript Analysis of p53 Pathway Genes Corroborates Tumor Classifications. We hypothesized that the p53 expression signature may better reflect the relative intactness of p53 function in the tumor than sequence mutation status alone, implying that p53 sequence-wt tumors “misclassified” as mt-like may, in fact, be p53 deficient by other means. First, we considered the possibility that p53 deficiency could result from reduced p53 transcript levels. We compared the transcript levels of p53 among the different tumor classes (Fig. 2). We observed that the overall expression level of p53 was significantly reduced in the 26 wt tumors with mt-like signatures (referred to henceforth as the “26 mt-like” tumors), compared with the remaining 167 wt tumors classified as wt-like ($P = 1.8 \times 10^{-4}$), strongly suggesting that reduced p53 transcripts can result in biological consequences *in vivo*.

We further hypothesized that known transcriptional targets of p53 would show altered transcription in p53-deficient tumors. Indeed, a number of p53 target genes demonstrated expression patterns consistent with a mutant p53 status (Fig. 2). The TP53-inducible genes *TP53INP1*, *SEMA3B*, *PMAIP1* (*NOXA*), *FDXR*, *CCNG1*, and *LRDD*, which all contain functional p53-binding sites in their promoters, showed significantly lower expression in the 26 mt-like tumors, compared with the other wt (all at $P < 0.05$). In a consistent manner, all but one of these genes were also significantly reduced in the p53 mt tumors, compared with all wt tumors. Furthermore, in all but two cases, these genes showed significantly higher expression in the set of 12 sequence-mt tumors classified as wt-like when compared with the other mts, suggesting that the p53 mutations in these 12 tumors may have a more benign effect, with respect to p53 functionality. *CHEK1* and *CHEK2* are both upstream effectors of p53 function known to be transcriptionally repressed by p53. Significantly, their mRNA levels were elevated in both the p53 mt and p53 mt-like classes. Again, the 12 mts classified as wt-like showed a reversed pattern, i.e., displaying significantly lower expression of these genes, compared with the other 46 p53 mutants. Together, these observations suggest that the “misclassified” tumors more correctly reflect the active/inactive status of the p53 pathway and are consistent with the notion that reduced p53 levels in breast tumors result in downstream transcriptional changes similar to those found in p53 mutations.

Of note, the canonical marker of p53 activity, *CDKN1A*

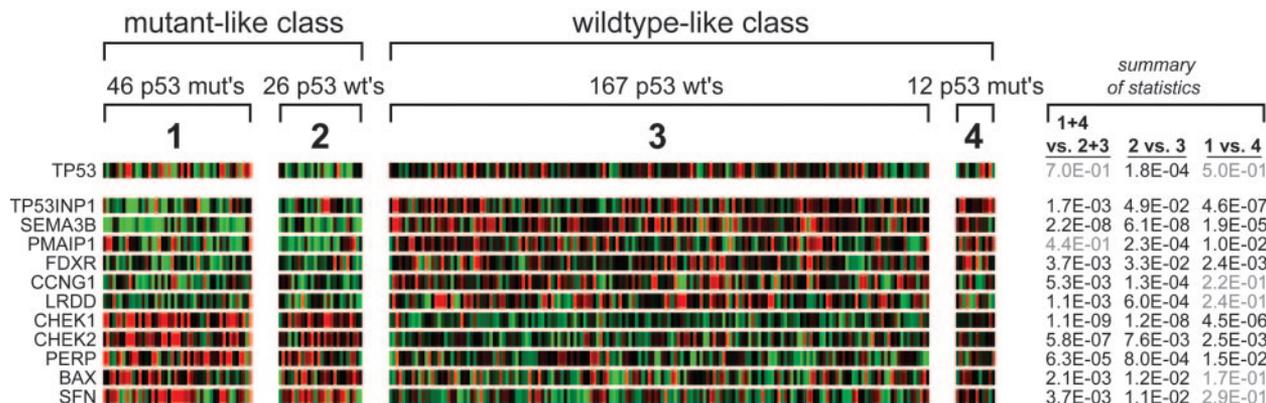


Fig. 2. Transcript levels of p53 and its transcriptional targets are consistent with classification results. Expression levels of p53-pathway-relevant genes were examined in different tumor subgroups. The four tumor subgroups are defined as follows: (i) p53 mt tumors classified as mt-like ($n = 46$), (ii) p53 wt tumors classified as mt-like ($n = 26$), (iii) p53 wt tumors classified as wt-like ($n = 167$), and (iv) p53 mt tumors classified as wt-like ($n = 12$). Differences in transcript levels were determined by t test and are shown in a summary table to the right; P values > 0.05 are shown in gray.

(p21/WAF1), was only moderately higher in p53 wt tumors, compared with those with sequence mutations ($P = 0.02$), and not significantly lower in the 26 mt-like tumors, compared with the other wts ($P = 0.09$; data not shown). Furthermore, the known p53-inducible genes *PERP*, *BAX*, and *SFN* (14–3–3 sigma) were, paradoxically, all expressed at higher levels in the p53 mutants and the 26 mt-like tumors rather than the expected lower levels (Fig. 2). These observations may reflect cross-talk among different transcriptional regulators in the consensus of primary tissues, as compared with dynamic changes in single cell lines. For example, the p53 target genes *p21* and *BAX* are also directly regulated by the breast cancer oncogene, *c-Myc*, in a manner independent of, and antagonistic to, p53 (25, 26). The regulation of p53 target genes by alternative transcriptional modifiers acting independently of p53 or in the context of p53 deficiency (e.g., *PERP*, *BAX*, and *SFN*) may have implications for p53 tumor-suppressor activity.

We next asked whether the mutational spectrum of p53 in our tumors could explain the different functional consequences, as measured by the expression profiles. Of the 46 p53 mt tumors correctly classified as mts, 43% (20 of 46) possessed “severe” mutations, defined as insertions ($n = 2$), deletions ($n = 11$), and stop codons ($n = 7$) resulting in frame shifts and truncations, whereas in the 12 p53 mutants classified as wt-like by the expression signature, only 1 contained a severe mutation, a 3-bp insertion in the DNA-binding domain, resulting in the in-frame addition of a glycine residue. Notably, this difference was statistically significant at $P = 0.02$. Using the IARC TP53 mutation database (ITMD) (27), we cross-compared the missense point mutations (mpms) in each tumor group with the ITMD’s index of 418 mutants previously analyzed for dominant-negative function. Only 1 of the 11 mpms among the 12 wt-like mutants had been demonstrated previously to have dominant-negative activity, compared with 12 of 27 within the mt-like group ($P = 0.039$). Together, these data suggest that, at the sequence level, the 12 p53 mutants classified as wt-like may, in fact, represent p53 mutant forms that have less biological effect.

The p53 Signature Predicts Outcome Better Than p53 Mutation Status Alone. We next asked whether the p53 signature could predict disease-specific survival in the patients of the Uppsala cohort. The classifier separated patients into low and high risk groups with a much higher statistical significance than the sequence-based p53 status alone ($P = 0.0006$ versus $P = 0.01$, respectively) (Fig. 3 *A* and *B*). More interestingly, when the classifier was tested on the subset of women with wt p53 by sequence, we again observed a significant separation of patients by survival ($P = 0.02$; Fig. 3*C*), indicating that women with p53 sequence-wt tumors, yet exhibiting the mt-like expression signature, have a greater likelihood of dying from their cancer. Fig. 3*D* shows that the survival curve for this tumor type is highly similar to that of p53 mt tumors classified as mt-like (blue and green curves, respectively), whereas the 12 individuals with p53 mt tumors classified as wt-like do not have significantly unique outcomes.

To further test the clinical utility of the p53 signature, we analyzed its prognostic performance on therapy-specific treatment groups. In a subpopulation of the Uppsala cohort consisting of 67 ER⁺ patients who received only adjuvant hormonal therapy after surgery, the signature was a significant predictor of disease-specific survival ($P = 0.05$), whereas p53 mutation status alone was not ($P = 0.4$) (Fig. 3 *E* and *F*). Importantly, by multivariate Cox regression analysis, the p53 classifier remained significantly associated with survival in the hormone-treated group ($P = 0.02$), the complete cohort ($P = 0.02$), and the p53 wt group ($P = 0.002$), even when controlling for the classical predictors (ER and progesterone receptor) and prognostic factors (lymph node status, Elston grade, tumor size, and patient age), whereas the p53 mutation status, as determined by se-

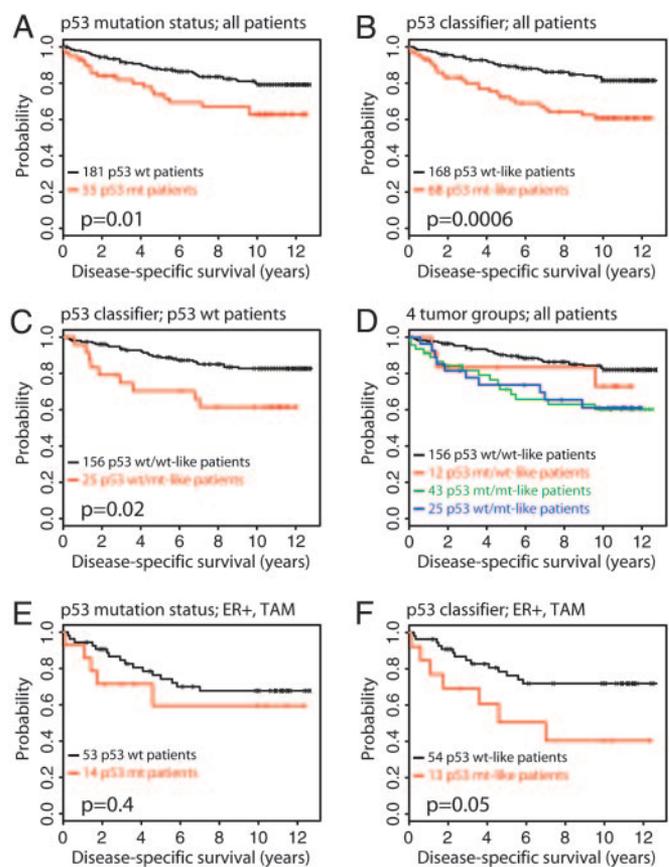


Fig. 3. The p53 classifier has greater prognostic significance than p53 mutation status alone. Kaplan–Meier survival plots for disease-specific survival are shown for patients classified according to p53 mutation status (*A* and *E*), the p53 classifier (*B*, *C*, and *F*), or both (*D*). All patients were assessed in *A*, *B*, and *D*. Only the patients with p53 wt tumors were assessed in *C*. Sixty-seven ER⁺, hormone-treated (TAM) patients were assessed in *E* and *F*.

quencing, did not. This demonstrates that the expression classifier is more directly prognostic of patient survival than is p53 mutation status alone.

The p53 Signature Predicts Outcome in Independent Therapy-Specific Data Sets. We next assessed the prognostic capability of the p53 signature genes in therapy-specific cohorts by using independent microarray data sets from the public domain (Fig. 4; and see Fig. 7, which is published as supporting information on the PNAS web site). First, we evaluated whether the signature genes were prognostic of tumor recurrence in the Ma *et al.* (20) data set of 60 breast tumors derived from patients treated with postoperative radiation and adjuvant tamoxifen monotherapy. In this cohort, patients with and without recurrent disease were matched with respect to tumor grade and tumor node metastasis stage. Twenty-two of the p53 signature genes mapped to 27 probes on the Ma *et al.* spotted oligonucleotide array. Hierarchical cluster analysis with these genes revealed two to three primary tumor clusters with expression profiles that resembled the mt-like and wt-like configurations (Fig. 4*A*). Using these tumor clusters to define patient survival groups, we analyzed disease-free survival (DFS) by the Kaplan–Meier estimate. As shown in Fig. 4*B* and *C*, the clusters were significantly associated with tumor recurrence [$P = 0.01$ (two clusters, C1 and C2) and $P = 0.005$ (three clusters, C1, C2, and C3)]. Thus, concordant results in two independent studies suggest that functional p53

p53 signature can predict p53 status with significant accuracy. As a predictor of disease-specific survival, we found that the signature significantly outperformed p53 mutation status in a large patient cohort with heterogeneous treatment. Importantly, the p53 signature could significantly distinguish patients having more or less benefit from specific systemic adjuvant therapies and locoregional radiotherapy. Recently, Ma *et al.* identified by microarray analysis two genes (*HOXB13* and *IL17RB*) whose expression ratio was predictive of tamoxifen response. Notably, we found that these genes were also predictive of disease-specific survival in the 67 Uppsala patients treated with tamoxifen monotherapy ($P < 0.01$; data not shown). However, these genes were not prognostic of recurrence in the van't Veer data set, nor were the van't Veer 70 genes prognostic of recurrence in the Ma data set (20), suggesting that tumor stage and/or therapeutic context is an important determinant of the prognostic capacity of some genes. In contrast, we demonstrate that the p53 signature genes are robustly prognostic of survival and recurrence in both early and late stage disease and in different therapeutic settings.

Although the p53 pathway may be compromised at some level in most human cancers, our analysis of genes involved in the p53 pathway suggests that the p53 expression signature defines some operational configuration of this pathway in breast tumors (more so than p53 mutation status alone) that impacts patient survival and therapeutic response. Recent evidence suggests that tumor sensitivity to some anti-cancer agents may depend largely on the relative intactness of p53-dependent mechanisms of apoptosis (7, 8, 10, 11) and that taxols (microtubule stabilizers), in particular, may have greater efficacy against p53-mt breast tumors than anthracycline-based (genotoxic) compounds (9). Whether the p53 classifier genes identified here are involved in some aspect of this p53 function or will have robust clinical utility as a predictor of therapeutic response warrants further investigation.

Other studies have elucidated gene expression signatures prognostic of breast cancer outcomes (21, 31). Although a 21-gene subset of our p53 signature could significantly distinguish patients with recurrent and nonrecurrent disease in the van't Veer study (21), none of these genes were found to overlap with the 231 genes identified as prognostic discriminators in the van't Veer set; and only one of the classifier genes, *MYBL2*, was found in the Sotiriou 485 survival-correlated genes (31). Similarly, none of the p53 signature genes were found in the top 25 relapse-associated genes reported by Ma *et al.* Thus, the p53 signature genes identified here represent a previously unrecognized prognostic cassette.

In cancer, it is clear that not all p53 mutations have equal effects; some simply confer loss of function, whereas others have a dominant-negative effect (such as transdominant suppression of wt p53 or oncogenic gain of function), whereas still others show only a partial loss of function where, for example, only a fraction of p53 target genes are dysregulated (32, 33). For these reasons, no single molecular assessment of p53 status appears to provide an absolute indication of the complete p53 function. Although the p53 classification method developed here seeks to categorize all tumors as either p53-deficient or not, it is likely that intermediate types exist with partial p53 functionality, distinguished by expression patterns that fall between those of the predominant mt-like and wt-like classes. Further investigation will be required to resolve the biological and clinical implications of such intermediate tumor classes.

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