Altered-Function p53 Missense Mutations Identified in Breast Cancers Can Have Subtle Effects on Transactivation


Abstract
Mutations of the sequence-specific master regulator p53 that alter transactivation function from promoter response elements (RE) could result in changes in the strength of gene activation or spectra of genes regulated. Such mutations in this tumor suppressor might lead to dramatic phenotypic changes and diversification of cell responses to stress. We have determined “functional fingerprints” of sporadic breast cancer–related p53 mutants, many of which are also associated with familial cancer proneness such as the Li-Fraumeni syndrome and germline BRCA1/2 mutant-associated cancers. The ability of p53, wild-type and mutants, to transactivate from 11 human target REs has been assessed at variable expression levels using a cellular, isogenic yeast model system that allows for the rapid analysis of p53 function using a qualitative and a quantitative reporter. Among 50 missense mutants, 29 were classified as loss of function. The remaining 21 retained transactivation toward at least one RE. At high levels of galactose-induced p53 expression, 12 of 21 mutants that retain transactivation seemed similar to wild-type. When the level of galactose was reduced, transactivation defects could be revealed, suggesting that some breast cancer–related mutants can have subtle changes in transcription. These findings have been compared with clinical data from an ongoing neoadjuvant chemotherapy treatment trial for locally advanced breast tumors. The functional and nonfunctional missense mutations may distinguish tumors in terms of demographics, appearance, and relapse, implying that heterogeneity in the functionality of specific p53 mutations could affect clinical behavior and outcome.

Introduction
The tumor suppressor p53 is a master regulatory gene that regulates the differential expression of target genes in a sequence-specific manner in response to cellular and environmental insults (1-3). Cell cycle regulation, apoptosis, angiogenesis, replication, and repair are processes interconnected within the p53 transcriptional network. p53 exerts itself as a transcription factor by binding as a homotetramer, or dimer of dimers, to a consensus response element (RE) sequence composed of two decamer half-sites [RRRCWWGYYY]$_2$ (where R = purine; W = A/T; Y = pyrimidine), which vary between target genes. Although the canonical consensus sequence allows for spacing between the half-sites of up to 13 nucleotides, we recently showed that transactivation was greatly reduced, as the separation between decamers is increased beyond 2 nucleotides (4). Activation of a p53 target gene is dependent on a matrix of factors, including cell type, stimuli, posttranslational modifications, and transcriptional cofactors (5).

The importance of p53 as a tumor suppressor and sequence-specific transcription factor in human cells is highlighted by the occurrence of p53 mutations in the majority of cancers (6). Interestingly, p53 is unique in comparison with other transcription factors in that >75% of mutations that occur in this tumor suppressor are single amino acid changes that result in missense mutations (7). These missense mutations predominantly occur in the DNA-binding domain (DBD) of the protein (>80%; ref. 7). At the molecular level, p53 mutations found in cancers, including breast cancer, are usually associated with
loss of the ability to maintain proper cell cycle checkpoints, suppress transformation caused by oncogenes, induce apoptosis, and maintain the integrity of the genome (1, 8).

Specific mutations in p53 are known to denature the native protein or abrogate its ability to bind DNA, thus completely abolishing its function. Such mutations are thought to provide a selective advantage within cancerous cells by forming a heterotrimer with wild-type (WT) p53 and functioning in a dominant-negative fashion (9, 10). Alternatively, gain-of-function mutations can potentiate tumorigenesis through oncogenic mechanisms, including aberrant transcriptional regulation of either known or novel target genes, presumably through structure-selective DNA binding or protein-protein interactions (9-11). Many p53 missense mutations have been described that retain function as sequence-specific transcription factors such that the ability to regulate cellular responses is altered but not completely lost (2, 12).

Functional mutations that alter the transcriptional capacity of the p53 master gene have been identified as supertransactivating, change in spectrum, or overall downward modulation of transactivation. For example, change-in-spectrum mutants may be capable of regulating genes containing a strong RE, such as p21, but unable to regulate those with a weak RE, such as Bax. This is consistent with the observation of mutant p53s that still induce cell cycle arrest yet lose the ability to activate apoptosis (13-15). In addition, p53 mutations may alter the active binding sites of potential transcriptional cofactors, thus diminishing the potential maximal level of transcriptional response. Modifications in the transcriptional network due to altered-function mutations may result in cellular responses that affect genome stability, repair, replication, and programmed cell death. Varying patterns of cellular responses, including apoptosis and survival, have been elicited in human cells as a consequence of distinct altered-function p53 mutations (16). Furthermore, the aberrant biological consequences of specific altered-function mutations can be influenced by specific cell type and activating stimuli.

Mutations in p53 are associated with ~25% of sporadic cases of breast cancer, a frequency lower than that in other sporadic cancers, such as lung and colorectal carcinomas. However, sporadic p53 mutations occur at much higher frequencies in BRCA1/2 germline-associated breast cancers possibly due to a decreased efficiency to repair damage (17, 18). BRCA1/2 and p53 are involved in maintaining genome stability by controlling aspects of homologous recombination and repair, centrosome regulation, cell cycle checkpoints, and transcription (19), where loss of either increases the likelihood of cancer (20). Interestingly, BRCA1-associated cancers have an altered spectrum of p53 mutations that may reflect changes in mutagenesis and/or selection for the acquired mutations (17). Whereas BRCA1 mutations are largely absent in somatic breast tumors, silencing of the gene through hypermethylation has been reported in sporadic cases (21). Such epigenetic changes have been reported to associate with estrogen receptor–negative (ER−) tumors and occur concomitantly with p53 mutations (21).

At the clinical level, p53 mutations in breast cancer have been associated with poor prognosis, earlier onset, increased aggressiveness of tumors, aneuploidy, and adverse responses to chemotherapeutic treatments (22). Studies that classify breast cancers based on gene expression profiling have shown that p53 mutations are more frequent in the hormone receptor–negative subtypes [such as the human epidermal growth factor-2–positive (HER2+)/ER−] and the basal-like subtypes [ER−, progesterone receptor negative (PR−), HER2−, cytokeratin 5/6+, and/or HER1−; refs. 23-25]. Based on a recent population-based study, these subtypes were prevalent among African American and/or premenopausal women and correlated with a more aggressive disease and shortened survival, irrespective of lymph node status (25). Regardless of subtype, p53 status (WT or mutant) also displays a signature expression profile in breast tumors, which is a prognostic indicator of patient survival, where WT p53 associates with a more favorable outcome (23, 26, 27).

We have used a newly developed model system in diploid yeast (4) to analyze the functional consequences of p53 missense mutations found in breast cancers on gene activation end points in the p53 transcriptional network at various levels of p53 expression. Transactivation capacities of WT and mutant p53 have been determined using a qualitative and a quantitative reporter, and a “functional fingerprint” was established for each p53 variant toward a subset of human REs that are representative of p53-dependent cellular responses. We have determined that p53 missense mutations found in sporadic and familial breast cancers can retain function, and the alterations in transactivation are often subtle where differences can be exaggerated by changes in p53 levels. Although patient numbers are limited, the separation of missense p53-associated breast cancer mutations into functional (which include fully functional and altered function) versus nonfunctional classes seems to associate with prognostic factors and outcome in a largely locally advanced patient population treated with chemotherapy before surgery. Functional fingerprinting of cancer-associated p53 mutants may thus be a useful tool for understanding tumor biology and behavior.

**Materials and Methods**

*Isogenic diploid yeast strains.* Two panels of isogenic haploid yeast strains, a “p53-host” and “RE reporter” strains, were developed in the budding yeast *Saccharomyces cerevisiae* with the *delitto perfetto* site-directed mutagenesis system as previously described (Fig. 1; refs. 4, 28). Each p53-host strain, yAT-iGAL::p53 (MATa leu2-3,112 trpl-1 his3-11,15 can 1-100 ura3-1, trp5::pGALI-p53:cyc1-Ter, lys2::HygroB), contains the WT or mutant p53 cDNA controlled by the inducible, “rheostatable” GALI promoter (2) integrated at the TRP5 locus on chromosome VII. p53 mutations were constructed using a derivative of the previously described p53-host strain containing a CORE cassette.
(CO, counterselectable, KLURA43; RE, reporter, KanMX4 resistance gene) integrated at various nucleotide positions spanning the p53 cDNA (4). Modifications of the p53 cDNA were done using the delitto perfetto approach so that CORE cassettes were replaced with an oligonucleotide containing the mutation of interest to generate a full-length mutant p53 cDNA. (Oligonucleotide sequences are available on request.) Replacement of the CORE was confirmed by selection on 5-fluoroorotic acid and kanamycin sensitivity. Specific p53 alterations were confirmed by colony PCR and sequencing (BigDye, Applied Biosystems). The second panel of isogenic strains, constructed previously, contains human target p53 REs upstream of the CYC1 minimal promoter and either the ADE2 or firefly luciferase reporter (2, 4). The RE reporter strains are also isogenic with the p53-host strains but LYS2 and Hygro'. Mating of the reporter and p53-host strains, followed by selection for diploid cells on Lys- Hygro+ plates, results in isogenic yeast that enable the assessment of the transactivation potential for WT or mutant p53 proteins toward individual REs in the p53 transcriptional network.

**Quantitative ADE2 color assay.** Single-colony isolates of the p53-inducible RE-ADE2 reporter strains were streaked onto a YPDA control plate containing glucose and high levels of adenine and grown to equivalent amounts at 30°C. The plates were then replica plated onto a series of nine plates containing selective media with low levels of adenine and galactose (0%, 0.001%, 0.002%, 0.004%, 0.008%, 0.010%, 0.012%, 0.016%, 0.020%, 0.024%, 0.028%, or 0.032%). These cultures were grown overnight (0–18 h) at 30°C by the ability of the mutant to produce growth at 30°C by the ability of the mutant to produce a change in colony pigmentation. Transactivation of the ADE2 gene, which is a direct readout of p53 interaction with the specific RE, results in white colonies, where decreased or loss of transactivation of ADE2 results in pink and red colonies, respectively (29). Colony pigmentation was manually scored on a scale of 1 to 5, where 1 is no apparent transactivation (red colonies) and 5 is strong transactivation (white colonies; Supplementary Fig. S2).

**Quantitative luciferase assay.** Diploid yeast strains containing GAL1::p53 (WT or mutant) crossed with a specified RE-luciferase reporter were grown overnight in 5-ml YPDA plus adenine (200 mg/L)–rich media. Overnight cultures were diluted 1:50 in H2O. For each measurement, 1 ml of the diluted culture was spun down, washed of residual glucose with H2O, and resuspended in 2-ml synthetic complete LYS media plus 2% raffinose supplemented with increasing amounts of galactose (0%, 0.002%, 0.004%, 0.008%, 0.010%, 0.012%, 0.016%, 0.020%, 0.024%, 0.028%, or 0.032%). These cultures were grown overnight (∼18 h) at 30°C to ∼2 × 10^7 to 4 × 10^7 per ml. In late log early stationary. The 2-ml cultures were spun down and the supernatant was aspirated. The remaining pellet was resuspended in 100 μl reporter lysis buffer (Promega), and an equivalent amount of 425- to 600-μm acid-washed, glass beads was added (Sigma). Samples were homogenized for 30 seconds in the mini-bead beater (Biospec Products, Inc.), briefly incubated on ice, and spun for 20 minutes at 16K relative centrifugal force in an Eppendorf 5415R centrifuge to separate the soluble protein fraction. The standard protocol

![Diagram](https://example.com/diagram.png)
recommended by the manufacturer (Promega) was done for the luciferase assay system starting with 10 μL of protein extract. Luciferase activity was measured from 96-well white optiplates (Perkin-Elmer) in a Wallac Victor2 multilabel counter (Perkin-Elmer). Light units were standardized per μg protein as determined by a Bio-Rad protein assay.

**Western blot analysis.** Diploid yeast strains containing GALI::p53 (WT or mutant) crossed with the p21-S' RE-luciferase reporter were grown as described above. Overnight cultures containing 0.024% galactose were harvested, lysed in 35 μL reporter lysis buffer (Promega) plus 2% protease inhibitors (cocktail for use with fungal and yeast extracts; Sigma), and processed in the same fashion as those used in the luciferase assay. Protein concentrations were measured with the Bio-Rad protein assay according to the standard protocol (Bio-Rad). Total protein (50 μg) was run on 4% to 12% Bis-Tris NuPAGE and transferred as previously described (29). The p53 protein was detected with a mix of DO7 (BD Biosciences Pharmingen) and pAb1801 (Santa Cruz Biotechnology) antibodies unless otherwise specified according to the manufacturer’s protocol. Bands were detected using horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) and the enhanced chemiluminescence detection system (Amersham). Membranes were stained with Ponceau S to determine efficiency of protein loading.

**Mutation analysis from patients participating in a neoadjuvant trial.** p53 mutation analysis was done on untreated breast cancer tissues obtained from women participating in two clinical-translational trials, a single-institution study from the University of North Carolina–Lineberger Comprehensive Cancer Center (UNC-LCCC 9819), and a multi-institutional cooperative group trial sponsored by the National Cancer Institute (CALGB 150007). In these correlative studies, women with locally advanced breast cancer were treated first with an anthracycline–taxane–based chemotherapy (with or without trastuzumab, depending on HER2 status). Both trials involved acquisition of breast cancer core biopsy tissue before treatment with any chemotherapy and were designed to examine molecular markers predicting response to cytotoxic chemotherapy (30). These studies were approved by the institutional review boards of the University of North Carolina at Chapel Hill and participating institutions through the Cancer and Leukemia Group B (CALGB). All study subjects gave written informed consent to participate in the clinical trial and the correlative science studies. p53 gene mutations were assessed using a two-tiered screening strategy. A prerelease version of the p53 AmpliChip array (Roche Molecular Systems) was first used to detect point mutations and 1-bp deletions. Samples identified as potentially positive by the AmpliChip was sequenced to confirm the mutation. Specimens that were mutation negative by AmpliChip were evaluated by single-strand conformational polymorphism analysis in p53 exons 2 to 11 to detect deletions larger than 1 bp and insertions (31). Mutations identified as potentially positive by single-strand conformational polymorphism analysis were sequenced to identify the mutation. To rule out the possibility that mutations occurred due to PCR errors, we reamplified and resequenced all mutation–positive specimens.

**Results**

**Functional fingerprinting of p53 missense mutations associated with breast cancers.** Budding yeast lack endogenous p53 and have been used as an *in vivo* test tube to analyze directly interactions between p53 and REs in a cellular environment (2, 3). Recently, we developed a diploid yeast model system to address the contribution of RE sequence, organization and level of human p53, as well as the consequences of mutations on p53-mediated transactivation (Fig. 1; ref. 4).

We sought to define how specific p53 missense mutations found in breast cancers interfere with p53 function by assessing the ability of mutant p53 to transactivate from a panel of REs associated with p53–dependent downstream target genes (Table 1). Fifty missense mutations were chosen for examination if they were identified in cases of sporadic breast cancers. Importantly, 20 were identified in patients undergoing neoadjuvant treatment for locally advanced breast tumors and participating in clinical trials examining biomarkers predicting response to sequential anthracycline- and taxane-based chemotherapy before surgery (30, 32). Furthermore, 18 also associate with familial BRCA1/2 cancers and/or are found as germline mutations in Li-Fraumeni syndrome (LFS), Li-Fraumeni–like syndrome (LFL), and/or familial history (FH) cancer patients (Table 1). Of particular interest were mutations present in the L2 loop, L3 loop, or zinc-binding regions of the protein, which have been correlated with breast cancers that are often nonresponsive to chemotherapeutic treatments, including doxorubicin, tamoxifen, and/or combined therapies of 5-fluorouracil and mitomycin (19, 33, 34).

The yeast ADE2 plate color assay (29) was used to determine functional fingerprints for WT and p53 missense mutations based on their ability to transactivate from 11 different human REs at variable levels of protein expression (Fig. 2; Supplementary Figs. S1 and S2). Briefly, single-colony isolates of yeast strains containing the mutation and RE of interest were replicated onto plates containing increasing concentrations of galactose, where the ability of the p53 variant to drive transactivation from a specific sequence could be assessed based on colony pigmentation (see Materials and Methods and Supplementary Fig. S2). The REs analyzed are associated with known human p53 target genes involved in cell cycle, DNA repair, apoptosis, angiogenesis, and p53 regulation (Supplementary Table S1). Mutations were categorized as fully functional if they were indistinguishable from WT p53 in transactivation capacity or altered function if the allele retained the ability to function from at least one RE, but deviated from
WT p53 in transactivation capacity from the REs examined at any of the levels of p53 expression examined. Among the 50 missense mutations, 29 were classified as loss of function due to their inability to transactivate from any RE (Table 1). The remaining 21 (42%) mutations were able to function from at least one RE, where the transcriptional capacities varied from different levels of functionality to fully functional. Among the 21 functional mutations, 9 were clearly altered in transactivation capacity at high levels of galactose (0.128% galactose; Supplementary Fig. S1) and displayed a change in spectrum for REs transactivated, as exemplified by reduced or complete lack of transactivation from the 14-3-3 REs only at low levels of galactose (low p53 expression), whereas other mutations (i.e., L194P) displayed a decreased ability to transactivate from the p21-5′ RE at all levels of induction and corresponding p53 expression. Of the six fully functional mutations examined that were similar to WT p53 in terms of transactivation from the strong p21-5′ RE in both the plate and luciferase assays, one (H214R) showed an altered transactivation potential when assessed for transactivation from the weaker GADD45 RE in the luciferase assay (Fig. 4). H214R had an increased ability to transactivate from the GADD45 RE in comparison with WT p53, whereas the remaining fully functional mutations, including A138V and R174W, remained indistinguishable from WT p53. Change-in-spectrum mutations, which retained transactivation function from some REs but were devoid of function from others, were also verified with the luciferase assays. As shown in Fig. 5, Y220C was able to transactivate from the strong p21-5′ RE but to reduced levels; the maximal level of transactivation was comparable with that for WT p53 transactivating from the weaker 14-3-3σ RE. The Y220C mutant was actually unable to transactivate from the 14-3-3σ RE.

The transactivation profiles with increasing levels of galactose (i.e., increased p53 expression) were similar between the DBD mutants and WT p53, where initial induction occurred between 0.004% and 0.008% galactose and maximal levels of transactivation were between 0.016% and 0.024% galactose (Figs. 3–5). This was not observed for the tetramerization domain mutant R337H (Fig. 6). Although maximal levels of transactivation seemed similar to WT p53, the R337H mutation clearly altered transactivation from the p21-5′, 14-3-3σ, and GADD45 REs, requiring higher levels of p53 expression than WT to initiate transactivation (Fig. 6). The requirement for increased p53 levels necessary for initial transactivation by the tetrameric mutants seemed dependent on the strength of the RE. The R337C mutation resulted in overall reduced in transactivation.

**Luciferase assays confirm transcriptional anomalies.** The functional status of the 21 missense mutants that retained function and several loss-of-function mutants was examined with a luciferase reporter assay that provides the opportunity to quantitate transactivation from REs in late log phase growing cells. The transactivation capacities from the p21-5′, GADD45, and 14-3-3σ REs were comparable with those with the color plate assay in terms of classifying functional status; however, the assay provides greater discrimination between mutant and WT p53 transactivation. Assessment of the altered-function mutants with the luciferase assay showed varying degrees of functionality from the p21-5′ RE, where the maximal level of transactivation was dependent on the specific mutation (Fig. 3). Similar to the results with the plate assay, several mutants (i.e., R267Q) differed from WT p53 in their ability to transactivate from the p21-5′ RE only at low levels of galactose (low p53 expression), whereas other mutations (i.e., L194P) displayed a decreased ability to transactivate from the p21-5′ RE at all levels of induction and corresponding p53 expression. Of the six fully functional mutations examined that were similar to WT p53 in terms of transactivation from the strong p21-5′ RE in both the plate and luciferase assays, one (H214R) showed an altered transactivation potential when assessed for transactivation from the weaker GADD45 RE in the luciferase assay (Fig. 4). H214R had an increased ability to transactivate from the GADD45 RE in comparison with WT p53, whereas the remaining fully functional mutations, including A138V and R174W, remained indistinguishable from WT p53. Change-in-spectrum mutations, which retained transactivation function from some REs but were devoid of function from others, were also verified with the luciferase assays. As shown in Fig. 5, Y220C was able to transactivate from the strong p21-5′ RE but to reduced levels; the maximal level of transactivation was comparable with that for WT p53 transactivating from the weaker 14-3-3σ RE. The Y220C mutant was actually unable to transactivate from the 14-3-3σ RE.

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**Altered-function mutants can maintain protein levels comparable with WT p53.** Protein levels were analyzed at 0.024% galactose (within the range of expression where transactivation was shown to plateau in the luciferase assays) by Western analysis for the 21 mutations that retained function and a representative loss-of-function mutant (Supplementary Fig. S3). Of the functional mutants, 13 displayed similar levels of protein to WT p53, as did the loss-of-function missense mutation. However, seven mutants (C141W, L194P, Y220C, M237I, P278A, E285K, and R337C) had reduced expression compared with WT p53. Surprisingly, several of these mutants were shown to efficiently function from multiple REs in the plate and luciferase assays. Detection of the p53 protein with additional antibodies that recognize different epitopes was consistent with most of these mutants having...
Table 1. p53 missense mutations associated with breast cancers: functional status, frequency, and features

<table>
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(Continued on the following page)
reduced levels of protein in comparison with WT p53 (Supplementary Fig. S4).

**Functional status and clinical response.** To address how different p53 mutations might influence response to chemotherapeutics, we examined transcriptional functional status of p53 missense mutations found in breast cancers in relation to clinical manifestations. Twenty-nine unique p53 missense mutations analyzed for functionality in this study (20 mutations) or in a related haploid yeast system (14, 35) have been identified in 46 patients with locally advanced breast tumors (primarily ductal carcinomas). The patients were participants in clinical trials that monitored clinical and pathologic responses to neoadjuvant treatment before surgery (Supplementary Table S3). As summarized in Table 2 and Supplementary Table S3, among the 46 patients, 10 of the missense mutations (11 patients) resulted in p53s that retained function (9 altered and 1 fully functional mutant) and 19 were loss of function (35 patients). This corresponded to ~24% of patients (11 among 46) having functional mutations, suggesting a significant group that might be approached differently about treatments.

Although the number of p53 missense mutations examined is limited, there are emerging trends that may differentiate patients with functional (altered or fully functional) versus nonfunctional p53 missense mutants (Table 2; Supplementary Table S3). Among the tumors with somatic p53 missense mutations where HER2, ER, and PR status could be assessed, functional missense mutations were more common in HER2* tumors (7 of 25, 28%) compared with HER2* (2 of 15, 13%). Among triple-negative (ER, PR, and HER2) tumors with p53 missense mutations, 5 of 16 (31%) carried functional mutations. There seemed to be a higher frequency of functional/total missense mutations among Caucasians and Asians (7 of 29 and 2 of 3, respectively) than among African American patients (2 of 14, 14%). Functional mutations seem to be associated with good prognostic factors, such as low incidence of nodal involvement (2 of 11, 18%). Conversely, compared with functional mutations, patients carrying nonfunctional mutations were more likely to be stage III at diagnosis (53% versus 27%), have high-grade tumors (59% versus 36%), and to relapse in distant sites (34% versus 10%) as well as local sites (12 versus 0%), although the numbers are small. In terms of responsiveness to chemotherapy, the pathologic complete response (i.e., eradication of tumor) was similar between patients with functional p53 mutations and nonfunctional missense mutations (21% and 27%, respectively). However, women with nonfunctional mutations were more likely to die (36% versus 20%) at 3 years after treatment than those with functional mutations. Although suggestive of trends, none of these differences reached statistical significance.

### Discussion

Single amino acid changes in the p53 master regulator protein that differentially affect transactivation may result in the selective advantage of specific mutations in certain tissue types or stages of neoplastic transformation, as well as alter the responsiveness to or the efficacy of chemotherapeutic agents. We have used a diploid yeast system to analyze the potential transactivation capacity for...
a set of p53 missense mutations associated with breast cancers. The 50 missense mutants examined represent 
18% of all somatic p53 mutations reported in the IARC TP53 mutation database and 20% of all p53 mis-
sense mutations documented in breast tumors (7). Among the mutations analyzed, 1 (K305M) occurs at
an acetylation site in a nonstructured portion of the pro-
tein, 2 (R337H and R337C) are in the tetramerization
domain, and the remaining 47 are distributed across the
sequence-specific DBD.

Functional fingerprinting established that 21 of the 50
missense mutants associated with breast cancer can retain
p53 function. The effect on transactivation seems depen-
dent on the specific amino acid alteration such that even
different missense mutations at the same residue can vary
in the effect on p53 functionality. This is exemplified at
codon 194, where changing the leucine residue to an argi-
nine (L194R) results in loss of function, whereas a proline
(L194P) results in altered function.

The majority of the altered-function mutations analyzed
in this study do not seem to be the result of a general
reduction in transactivation from all REs. Rather, they
seem to be change-in-spectrum mutants that affect the
REs differentially. Because each functional p53 missense
mutation had a unique functional fingerprint (Supple-
mentary Fig. S1) ranging from severely altered to fully
functional, there may be diverse cellular effects. The func-
tional consequences of mutations seem not to be predict-
able simply by assessments of conservation, topology, or
structural models, emphasizing the need to address the func-
tion of p53 mutants in vivo (Supplementary Table S2).

FIGURE 2. Functional fingerprints of p53 mutants reveal subtle transactivational differences. The ADE2 plate color assay was used to assess the
transactivation capacity of WT and mutant p53 toward 11 human target REs at various protein concentrations. Transcription of ADE2 is dependent
on the ability of p53 (WT or mutant) to interact with and transactivate from the specific RE sequence upstream of the minimal CYC1 promoter and the
reporter. The ADE2 color assay scores p53 transactivation capacity from a RE based on colony pigmentation, which ranges from red (no transactivation)
to pink (weak to moderate) to white (strong) depending on the extent of ADE2 transcription (35). The level of p53 expression was controlled by replica
plating strains onto plates containing rich media, raffinose (2%) as the carbon source, plus various amounts of galactose (0–0.128%) in the presence
of low levels of adenine (5 mg/L). Shown are examples of functional fingerprints for WT p53 and several change-in-spectrum mutations at four levels of
protein expression. At high expression levels (0.128%), several mutations were found to display an altered spectrum of REs regulated in comparison
with WT p53 (e.g., P151A and R283P). However, several mutants, such as L130V, seem indistinguishable from WT p53 in transactivation capacity.
Reducing the levels of expression with the rheostatable promoter exaggerated the subtle transcriptional effects of these mutations and distinguished
them from WT p53 and other mutations in transactivation capacity.
Importantly, the differences in transactivation for all of the mutations could not be attributed simply to protein stability because most of the p53 mutants were expressed at levels comparable with that for WT protein. Of the mutations that had a reduced level of protein expression, the C141W and E285K mutants were only modestly compromised for transactivation capacity in comparison with WT p53, whereas others, such as M237I and Y220C, were.

![Graph showing transactivation vs galactose concentration](Image)

**FIGURE 3.** Assessment of WT and mutant p53 transactivation toward the p21-5' RE using a luciferase assay. Diploid yeast strains containing GAL1::p53 (WT or mutant) and the p21-5' RE-luciferase reporter strain were grown overnight in complete medium, diluted, washed, and inoculated into selective medium containing either raffinose (2%) or raffinose (2%) plus increasing concentrations of galactose (0-0.024%) for an additional night at which point the cultures in late logarithmic growth. Protein lysates were obtained and a quantitative luciferase assay was used to determine the transactivation capacity for the p53 variants from the p21-5' RE. The strength of transactivation was calculated as relative light units/μg protein. Points, mean of seven independent experiments; bars, SE. The transactivation responses to increasing galactose can be described as basal, linear increase, and plateau. Maximal transactivation is dependent on the p53 variant. Many p53 missense mutations associated with breast cancers (i.e., R174W and R267Q) do not affect the maximal level of transactivation toward the strong p21-5' RE in comparison with WT p53. However, transactivation can be altered at low levels of galactose, which correspond to low levels of p53 expression. Several mutants, such as L194P, were shown to modulate the levels of transactivation at all concentrations of expression. C242S is a loss-of-function mutation.

![Graph showing transactivation vs galactose concentration](Image)

**FIGURE 4.** Transactivation from the GADD45 RE distinguishes altered-function mutants from WT p53. The ability of p53 (WT and mutant) to transactivate from the GADD45 RE was measured 24 h after inoculation into increasing concentrations of inducing media with a quantitative luciferase assay. The strength of transactivation was calculated as relative light units/μg protein. Transactivation from the GADD45 RE can differentiate mutant p53 alleles that looked similar to WT p53 in the ADE2 plate assay. H214R has an increased ability to transactivate from the GADD45 RE in comparison with WT p53 at low and high levels of expression, whereas A138V and R174W had similar transactivation potentials. Points, mean of six independent experiments; bars, SE.
severely compromised. It is possible that reduced levels of protein may be due to an increased level of degradation within the cell due to conformation changes. This could also explain associated temperature sensitivity of some alleles, specifically Y220C, M237I, and E285K (37-39). However, about these low expressing mutants, the issue arises as to whether cellular p53 protein expression is comparable between yeast and tumor cells. There are examples where expression in yeast is matched by low or undetected levels in breast cancer cell lines [i.e., E285K in BT474 and MDA-MD-134VI cells (40, 41) and Y220C in HCC1419 cells (42, 43)] or alternatively being accumulated in the cells [i.e., M237I in SUM149PT cells (40)]. Regardless of the amount of p53 protein expressed in yeast, the results are informative for those cases where the mutant proteins retain transactivation ability, as this indicates the potential for mutant protein to function in mammalian cells.

Breast cancer–associated mutations in the DBD can modulate p53 transactivation. The DBD of p53 consists of a β-sandwich, which provides a scaffold for two large β-loops, L2 and L3, which are stabilized by a zinc ion and a loop-sheet-helix motif (44, 45). Mutations in the DBD have been postulated to affect the binding affinity of p53 toward REs by abolishing DNA contacts, decreasing the thermodynamic stability of the protein, causing local distortions in the DNA-binding surface, or enhancing the loss of the zinc ion (46, 47). Mutations in the L2/L3 loops are predicted to be highly destabilizing to the tertiary structure of p53, can cause chemical shifts that alter the DNA-binding surface, and/or can alter the response to chemotherapeutics (7, 19, 33, 34, 48).

Of the DBD mutations analyzed for transactivation potential, 16 correspond to residues in the L3 loop and 10 are within the L2 loop; among these 26 mutants, 6 are also zinc-binding residues. All six mutations (C176F, H179R, C238F, C242F, C242S, and C242Y) that interfere with the histidine or cysteine side chains involved in coordination of the zinc ion rendered the protein nonfunctional in terms of transactivation, emphasizing the vital role of zinc in sequence-specific DNA binding and stabilization of the p53 protein (46, 49). Similarly, all the missense mutations analyzed in the L3 loop, which binds the
minor groove of DNA and partakes in the dimerization interface between core domains (47), were loss-of-function mutations, with the exception of M237I, which was very weak for transactivation. However, four mutants (R174K, R174W, P190L, and L194P) in the L2 loop retained function, of which several (R174K, R174W, and P190L) displayed subtle alterations in transactivation capacity. These results suggest that mutations in the L2 loop, which functions as a support for the L3 loop, may be less detrimental to transactivation potential than those of zinc binding or in the L3 loop.

**Tetramerization mutations may alter the level of p53 required for transactivation.** Although the majority of p53 missense mutations occur in the DBD, several missense mutations have been found in the tetramerization domain that are associated with germline syndromes and are found in sporadic breast tumors. R337C is a partial function mutation associated with LFS (50). R337H has been associated with pediatric cases of adrenocortical carcinoma; however, it may be a low-penetrant LFS or LFL allele as well (51-54). The functional fingerprints varied between these two altered-function mutations in that R337C had a greater effect on p53 transactivation displaying an overall dampening effect from the various REs. This reduced transactivation may reflect a greater instability of the protein as depicted in the protein analysis (Supplementary Figs. S1-S4). Contrary to previous reports, the p53 missense mutation R337H was not a silent mutation but displayed altered function when examined in the ADE2 phenotypic assay (Supplementary Fig. S1). At high levels of galactose induction (i.e., high levels of p53 expression from the GAL1 promoter), R337H looked identical to WT p53 in the color assay. At low levels of galactose, R337H had a reduced ability to transactivate from REs in comparison with WT p53, presumably due to its reduced ability to form tetramers. Interestingly, the luciferase assay revealed a pattern of transactivation that was unique to R337H, where higher levels of protein expression were required to detect transactivation. This altered pattern of transactivation may be a manifestation of the novel features of R337H, which include a pH-dependent instability and formation of amyloid-like fibrils (55, 56).

**Subtle variation in transactivation capacity of altered-function missense mutations.** Especially interesting is the observation that many of the mutants looked similar to WT p53 when examined at high levels of galactose (i.e., high p53 expression) yet were altered function at lower levels of galactose, consistent with our earlier findings (29). Importantly, these mutations would not have been distinguished from WT p53 in typical functional

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### Table 2. Clinical characteristics and response to therapy in patients with p53 functional or nonfunctional missense mutants

<table>
<thead>
<tr>
<th>Clinical factor</th>
<th>Functional mutants (10 mutations among 11 patients)</th>
<th>Nonfunctional mutants (19 mutations among 35 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (y)</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>7/11 (64%)</td>
<td>22/35 (63%)</td>
</tr>
<tr>
<td>African-Americans</td>
<td>2/11 (18%)</td>
<td>12/35 (34%)</td>
</tr>
<tr>
<td>Asian</td>
<td>2/11 (18%)</td>
<td>1/35 (3%)</td>
</tr>
<tr>
<td>Node positive</td>
<td>2/11 (18%)</td>
<td>17/34 (50%)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>7/11 (64%)</td>
<td>12/34 (35%)</td>
</tr>
<tr>
<td>III</td>
<td>3/11 (27%)</td>
<td>18/34 (53%)</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>1/11 (9%)</td>
<td>4/34 (12%)</td>
</tr>
<tr>
<td>Overall grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1/11 (9%)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3/11 (27%)</td>
<td>8/34 (23%)</td>
</tr>
<tr>
<td>3</td>
<td>4/11 (36%)</td>
<td>20/34 (59%)</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>3/11 (27%)</td>
<td>6/34 (18%)</td>
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<tr>
<td>Immunohistochemical subtype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 negative</td>
<td>7/9 (78%)</td>
<td>18/31 (58%)</td>
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<tr>
<td>Triple negative (ER, PR, HER2)</td>
<td>5/9 (56%)</td>
<td>11/23 (48%)</td>
</tr>
<tr>
<td>Pathologic complete response to therapy</td>
<td>3/11 (27%)</td>
<td>7/34 (21%)</td>
</tr>
<tr>
<td>Clinical response to chemotherapy</td>
<td>8/10 (80%)</td>
<td>29/33 (88%)</td>
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<tr>
<td>Recurrence (at ~3 y follow-up)</td>
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<td></td>
</tr>
<tr>
<td>Local</td>
<td>0/10</td>
<td>4/34 (12%)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>1/10 (10%)</td>
<td>12/35 (34%)</td>
</tr>
</tbody>
</table>

**NOTE:** There were 29 unique breast-cancer–associated p53 mutations identified among 46 patients.
assays where p53 is expressed at high levels from a constitutive promoter. Thus, by reducing the level of transcription with the rheostatable promoter, it was possible to unmask subtle transcriptional discrepancies. These mutants might have unique properties in terms of biological consequences. Possibly, the mutants function similarly to WT p53 for gene expression from target genes under high stress and chemotherapeutic conditions but differently under conditions of low p53 expression. In addition, other transcription factors could further modify the response (3).

Although mutations in p53 are often associated with nuclear accumulation of the protein, recent evidence indicates that this may not always apply in vivo. For example, Tsuda and Hirohashi (57) showed with immunohistochemical analysis of >50 human breast cancer tissue specimens that nuclear accumulation of the p53 protein was dependent on the type and position of the mutation, where missense mutations did not always result in stabilization of the protein. Similarly, in a study comparing immunohistochemical analysis with cDNA-based sequencing using >300 primary breast tumor samples (58), in >30% of the cases where a mutation was observed through sequencing, there was not a corresponding accumulation of p53 protein.

Recent results with knock-in mouse models indicate that levels of mutant p53 can be regulated in both normal and some tumor cells (59, 60). The accumulation of mutant protein does not occur until additional mutations are acquired in genes that may disrupt the p53 degradation pathway, such as MDM2 or p16INK4a (61). Although the subtle altered-function mutations identified in the present study retain the ability to function from the MDM2 RE, Lukashchuk and Vousden (62) have shown that the ability of p53 to transactivate MDM2 is not essential for degradation of the mutant p53 protein. Rather, additional E3 ubiquitin ligases, such as CHIP (COOH terminus of Hsc70-interacting protein), can target mutant p53 for degradation independent of ubiquitination by Mdm2, where Mdm2 plays a role in delivering the ubiquitinated proteins to proteasomes.

The functional relevance of p53 at low expression levels is beginning to be elucidated and highlights the need to understand mutants under such conditions. Espinosa et al. (63) observed that p53 occupies some target REs, including p21, before overall p53 stabilization. There is transcriptional initiation from these REs, but the transcriptional machinery stalls before elongation. Such regulation may be required for a rapid response to cellular stress. In addition, there is a transcriptional-dependent role for p53 in promoting cell survival, as well as modulating glucose metabolism and reactive oxygen species at basal levels of p53 expression (64, 65). For example, p53 has been found to target the sestrins (i.e., SESN1 and SESN2) and TIGAR (TP53-induced glycolysis and apoptosis regulator) to stimulate antioxidant and prosurvival signals (64, 65). The loss of transactivation function at low levels, as observed for some of the subtle, altered-function mutants, might lead to alternative modes of promoter selectivity and/or provide the opportunity for competing transcription factors to bind promiscuously to p53 target elements. Furthermore, p53 has also been found to promote, presumably in a transcriptional-independent fashion, global chromatin relaxation (66). This seems to influence genomic repair in response to UV stress exposures that are lower than those required for its activation as a transcription factor.

It is possible that mutations that subtly affect transactivation are acquired early in tumor development and, when combined with mutations in other genes, contribute in an additive fashion to the complex cancer disease. For example, the altered-function P190L and H214R mutants that were indistinguishable from WT at higher levels of galactose-induced expression are associated with germline BRCA1 and BRCA2 mutations, respectively. Such mutations in p53 may be an underlying contributor to the genomic instability observed in BRCA1-associated breast cancer cases, and the functional status of individual p53 missense mutations may affect the degree of genetic imbalance. Interestingly, a recent hierarchical clustering analysis using immunohistochemistry profiling to determine the relatedness of tumors has established that the extent of genomic instability correlates with specific breast cancer subtypes, where the basal subtype (the subtype in which p53 mutations are frequently observed) had the highest number of genomic aberrations in both sporadic and familial BRCA-associated cases (67–69).

In addition, inherited p53 mutations may influence tumor type and penetrance of the disease (59, 60, 70, 71). LFS and LFL germline disorders, which often harbor a p53 mutation, display an array of early-onset, tissue-specific tumors, of which breast tumors are among the most frequently observed (7, 70). Recent studies that have assessed the functional status of p53 germline mutations using yeast-based assays have related severity of inherited p53 missense mutations in terms of transcription functionality with clinical manifestations (7, 72). Partial deficiency alleles, defined by the ability to transactivate from at least one RE to 25% of the levels obtained by WT p53, are associated with a less severe family history, lower number of tumors, later onset of disease in comparison with severe deficiency (loss of function) alleles, and a higher risk of breast tumors.

In another recent study (73), the frequency and average size (base pair deletion or duplication) of DNA copy number variation are enriched in carriers of germline TP53 mutations within LFS families in comparison with those with WT p53 or in a healthy population. The clinical phenotypes that arise in later generations may correlate with greater genomic instability, as well as specific germline p53 mutation. Thus, the wide spectrum of transcription potentials of the 12 p53 missense mutations associated with germline disorders in the current study (ranging from nonfunctional to altered and subtle, or fully functional) can be expected to result in varying phenotypes, where the severity of the disease is likely influenced by the extent of p53 functionality.
**Functional status and clinical response.** We examined transcriptional functional status of p53 missense mutations found in breast cancers in relation to clinical manifestations (Table 2; Supplementary Table S3). Although the number of breast cancer–associated functional p53 mutants was small, there were trends described in Results that suggest differences in presentation and outcome between functional versus nonfunctional missense mutations. The nonfunctional missense mutations are associated with clinical responses similar to those in our studies with null mutations (data not shown), which are known to have poorer prognosis and reduced survival compared with patients with tumors that are WT for p53 (74). We have found that functional p53 mutations seem to have better disease-free survival compared with loss-of-function mutations (Table 2).

In terms of clinical response to chemotherapy, we observed in the present study a higher response rate for tumors expressing loss-of-function p53 mutations, which may seem counterintuitive. However, tumors with p53 mutations in general are more chemosensitive possibly because the breast cancer subtypes that usually have a higher proportion of p53 mutations (e.g., the basal-like) are more highly proliferative or have aberrant DNA repair functions (23, 25). In addition, the cumulative response to anthracycline/cyclophosphamide followed by taxane therapy was determined in this study. Although there is some indication from the literature that p53 mutant status could confer different responses to these single agents (75-77), it is difficult to predict responses to the sequential treatment. Although previous studies have investigated correlations between p53 mutations and pathologic variables in breast cancers, few studies have attempted to correlate the transcriptional activity of specific mutations with clinical phenotypes. In a large-scale study of a cohort of ~1,800 women (74), the presence of a p53 mutation was associated with high grade, positive node status, loss of hormone receptors, and greater risk of death due to breast cancer within a 10-year follow-up. However, when the functional status of the missense mutations was taken into consideration, no correlation was found between p53 transcriptional activity and patient survival. Importantly, the functional status of the missense mutations was determined with another yeast-based functional assay developed by Kato et al. (12) that uses a high p53 expression plasmid along with a high copy RE reporter plasmid system.

Functional analysis based on the Kato et al. system is available at the IARC p53 mutation database for 49 of the 50 mutations examined in the present study (7). Interestingly, a comparison between the two data sets shows ~65% agreement in terms of overall transactivation functionality (Supplementary Table S2). Only 8 of 49 or 16% of the missense mutations were previously identified as functional compared with 21 mutations in the present study system. The large discrepancy between the two systems may be in part due to the method of classifying a mutation as retaining function (Supplementary Table S2; refs. 7, 12). For example, we concluded that R337C was an altered-function missense mutation, whereas within the IARC database it is considered to be a nonfunctional mutation, although it did display transactivation for three of the eight REs analyzed (WAF1, MDM2, and P53R2; ref. 7). Furthermore, in a separate analysis, the Shiraishi et al. group (39) found that >140 missense mutations were temperature sensitive for transactivation capacity toward at least one RE; this indicated that there may be cellular conditions under which these mutations may become functional. Interestingly, five of these temperature-sensitive mutations that were reported as loss of function in the IARC database were classified as altered function in the present study (P151A, H214R, V272L, R283P, and E285K). Another mutation, Y220C, had also been categorized as loss of function; however, we have diagnosed this as a weak altered-function protein, and previously, it was reported as temperature sensitive in mammalian cell assays (37).

**Conclusions**

Given the heterogeneity of breast cancers, understanding the consequences of altered-function mutations on the p53 transcriptional network will help elucidate how specific mutations predispose and/or contribute to the development, penetrance, and phenotype of breast cancers. Among 50 p53 mutations identified in breast cancers, 21 had altered function—not simply complete loss—toward at least one RE. Although the transcriptional effects associated with these mutations are often subtle, we found that by reducing the level of transcription with the rheostatable promoter, it is possible to address the retained functions of mutant p53 and novel features in transcriptional networks. It is important to emphasize that the yeast-based results indicate the potential for transactivation and that many factors can come into play in p53-mediated transactivation in human cells. Because of the wide range of p53 expression, the present study also provides greater opportunity to identify change-of-spectrum mutants, as well as subtle changes in transactivation.

Although beyond the scope of the present study, this system may also be used to address the biological activity of the multiple p53 isoforms from specific REs. Given that p53 isoforms are differentially expressed in breast tumors in comparison with normal breast tissue (78), the ratios at which these isoforms are expressed, which can change with the presence of a mutation, may alter the transactivation profiles of WT and/or mutant p53. For example, similar to the p53 family members p63 and p73, specific p53 isoforms can influence promoter selection or functionality from specific REs through either synergistic or antagonistic mechanisms (78, 79).

Results obtained with the yeast functional assay seem predictive of whether a mutation will also have a biological effect in mammalian cells (16). Although the yeast-based assay can predict the potential for specific p53 mutations
to display altered function, assays in mammalian cells can ascertain the full effect of the functional mutations in the presence of p53 transcriptional cofactors and in the endogenous chromatin context of the RE. However, we propose that assessments of functional fingerprints for p53 missense mutations associated with breast cancer in yeast provide diagnostic value and with further study may also be used as a prognostic tool for implementing chemotherapeutic treatment. This would be particularly relevant to the tailoring of individual therapies, especially when the treatment agents affect p53-dependent biological responses. Furthermore, the technique of functional fingerprinting will be useful in determining if p53 dysfunction as a transcription factor can also be corrected by agents that reverse its structural stability, such as the carbazole derivative PhiKan083 acting on Y220C. (80).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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