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Altered-function p53 missense mutations identified in breast cancers can have subtle effects on transactivation

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Abstract

Mutations of the sequence-specific *master regulator* p53 that alter transactivation function from promoter response elements (REs) 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, isogenomic 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 towards at least one RE. At high levels of galactose induced p53 expression, 12/21 mutants that retain transactivation appeared similar to WT. 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 impact clinical behavior and outcome.

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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 comprised of two decamer half-sites $[RRRCWWGYYY]_2$ (where R= purine; W= A/T; Y=pyrimidine) which vary between target genes. While 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, post-translational modifications and transcriptional co-factors (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 to other transcription factors in that over 75% of mutations that occur in this tumor suppressor are single amino acid changes which result in missense mutations (7). These missense mutations predominantly occur in the DNA binding domain (DBD) of the protein (>80%) (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 hetero-tetramer with 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 super-transactivating, 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 impact 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 approximately 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 which may reflect changes in mutagenesis and/or selection for the acquired mutations (17). While 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 on-set, increased aggressiveness of tumors, aneuploidy, and adverse responses to chemotherapeutic treatments (22). Studies that classify breast cancers based on gene expression profiling have shown p53 mutations are more frequent in the hormone receptor-negative subtypes such as the HER2+/ER− [human epidermal growth factor-2 positive/estrogen receptor negative] and the basal-like subtypes [ER−, PR− (progesterone receptor), HER2−, cytokeratin $5/6^{+}$, and/or $HER1^{+}$] (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 employed 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 endpoints 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 towards 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. While 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 appears to associate with prognostic factors and outcome in a largely locally-advanced patient population treated with chemotherapy prior to surgery. Functional fingerprinting of cancer-associated p53 mutants may thus be a useful tool for understanding tumor biology and behavior.

Materials and Methods

Isogenomic diploid yeast strains

Two panels of isogenic haploid yeast strains, a “p53-host” and “response element (RE) reporter” strains, were developed in the budding yeast, *S. cerevisiae* with the *delitto perfetto* site-directed mutagenesis system as previously described (Figure 1) (4,79). Each “p53-host” strain, yAT-iGAL::p53 (MAT**a** leu2-3,112 trpl-1 his3-11,15 can 1-100 ura3-1, trp5::pGAL1:p53:cyc1-Ter, lys2::$Hygro^{R}$), contains the wild type or mutant p53 cDNA controlled by the inducible, “rheostatable” *GAL1* 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, KL*URA3*; RE,

reporter, KanMX4 resistance gene) integrated at various nucleotide positions spanning the p53 cDNA (4). Modifications of the p53 cDNA were performed 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 upon request.) Replacement of the CORE was confirmed by selection on 5-FOA and kanamycin sensitivity. Specific p53 alterations were confirmed by colony PCR and sequencing (Big dye, Applied Biosystems, Foster City, CA). The second panel of isogenic strains, constructed previously, contain 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^{s}. Mating of the reporter and p53-host strains followed by selection for diploid cells on Lys− Hygro+ plates, results in *isogenomic* yeast that enable the assessment of the transactivation potential for WT or mutant p53 proteins towards individual REs in the p53 transcriptional network.

Qualitative *ADE2* color assay

Single colony isolates of the p53-inducible RE-*ADE2* reporter strains were streaked onto an 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 9 plates containing selective media with low levels of adenine [5 mg/L], 2% raffinose and increasing galactose (0, 0.001, 0.002, 0.004, 0.008, 0.016, 0.032, 0.064 and 0.128%). Transactivation capacities for the p53 mutants were determined after three days of 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 the *ADE2* results in pink and red colonies, respectively (32). Transactivation capacities for the p53 WT and mutants were determined after three days of growth at 30°C by the ability of the mutant to produce a change in colony pigmentation. 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) (Supplemental figure 2).

Quantitative luciferase assay

Diploid yeast strains containing *GAL1*::p53 (WT or mutant) crossed with a specified RE-luciferase reporter were grown overnight in 5ml 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 re-suspended in 2mL 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 hr) at 30°C to ~$2 - 4 \times 10^7$ per ml (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, Madison, WI) and an equivalent amount of 425-600 micron acid-washed, glass beads was added (Sigma, St. Louis, MO). Samples were homogenized for 30 seconds in the Biospec Products, Inc. mini-bead beater (Bartlesville, OK), briefly incubated on ice and spun for 20 minutes at 16k relative centrifugal force (rcf) in an Eppendorf 5415R centrifuge (Batavia, IL) to separate the soluble protein fraction. The standard protocol recommended by the manufacturer (Promega; Madison, WI) was performed for the luciferase assay system starting with 10 μl of protein extract. Luciferase activity was measured from 96-well, white optiplates (Perkin Elmer, Waltham, MA) in a Wallac Victor2 multilabel counter (Perkin Elmer, Waltham, MA). Light units were standardized per μg protein as determined by a Bio-Rad protein assay (Bio-Rad; Hercules, CA).

Western analysis

Diploid yeast strains containing *GAL1*::p53 (WT or mutant) crossed with the p21-5′ RE-luciferase reporter were grown as described above. Overnight cultures containing 0.024% galactose were harvested, lysed in 35 μl reporter lysis buffer (Promega, Madison, WI) plus 2% protease inhibitors (cocktail for use with fungal and yeast extracts; Sigma, St. Louis, MO) 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; Hercules, CA). 50 μg of total protein was run on 4-12% BisTris NuPAGE and transferred as previously described (32). The p53 protein was detected with a mix of DO7 (BD BioSciences Pharmenigen) and pAb1801 (Santa Cruz) antibodies unless otherwise specified according to the manufacturer's protocol. Bands were detected using horseradish peroxide-conjugated secondary antibodies (Santa Cruz) and the enhanced chemiluminescence (ECL) detection system (Amersham, Cleveland, OH, USA). Membranes were stained with Ponceau S to determine efficiency of protein loading.

Mutation analysis from patients participating in a neoadjuvant trial

p53 mutational analysis was performed 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 multiinstitutional 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 generally followed by 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 (29). 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 pre-release version of the p53 AmpliChip array (Roche Molecular Systems) was first used to detect point mutations and 1 base pair 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 (SSCP) analysis in p53 exons 2-11 to detect deletions larger than 1 base pair and insertions (80). Mutations identified as potentially positive by SSCP were sequenced to identify the mutation. To rule out the possibility that mutations occurred due to PCR errors, we re-amplified and re-sequenced 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 upon p53-mediated transactivation (Figure 1) (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 prior to surgery (28, 29). 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-flurouracil and mitomycin (19,30, 31).

The yeast *ADE2* plate color assay (32) 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 (Figure 2 and Supplemental Figures 1 and 2). 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 Supplemental Figure 2). The REs analyzed are associated with known human p53 target genes involved in cell cycle, DNA repair, apoptosis, angiogenesis, and p53 regulation (Supplementary Table 1). 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(s), 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) (Supplemental Figure 1) and displayed a change-in-spectrum for REs transactivated, as exemplified by reduced or complete lack of transactivation from the 14-3-3σ and PCNA REs by P151A and R283P, respectively, in Figure 2. Of these 9 mutations, 3 (Y220C, M237I and P278A) retained the ability to transactivate from only the strongest REs, p21-5′ and P53R2 when p53 expression was induced with high levels of galactose (and, in the case of P278A, MDM2 which contains two full-site REs) (Supplemental Figure 1).

At high levels of galactose, the remaining 12 of the 21 mutants (L130V, A138V, C141W, P151H, R174K, R174W, P190L, H214R, R267Q, V272L, E285K, and R337H) looked similar to WT in their transactivation capacity (*i.e.*, L130V in Figure 2, Supplemental Figure 1). Transactivation from three biological replicates was either indistinguishable from WT p53 or nearly identical with the exception of 1 or 2 REs at 0.128% galactose. However, when the levels of galactose were reduced, subtle transactivation defects were revealed that further differentiated 6 of the 12 mutants (underlined) from WT p53 at 3 or more REs. Similar to previous studies (2), the deviation from WT p53 at lower levels of induction consisted of both reduced and enhanced transactivation capacities from specific REs, such as the ability of L130V to transactivate from the Cyclin G and AIP1 REs at 0.008% galactose, respectively (Figure 2). The remaining 6 mutants remained similar to WT p53 in transactivation capacity even at low levels of galactose with the exception of subtle variation at 1 or 2 REs. For example, A138V was only observed to be slightly reduced in transactivation compared to WT p53 towards PCNA at 0.008% galactose (Supplemental Figure 1).

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 to 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 (Figure 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 6 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 (Figure 4). H214R had an increased ability to transactivate from the GADD45 RE in comparison to 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 Figure 5, Y220C was able to transactivate from the strong p21-5′ RE, but to reduced levels; the maximal level of transactivation was comparable to 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 – 0.008% galactose and maximal levels of transactivation were between 0.016% - 0.024% galactose (Figures 3-5). This was not observed for the tetramerization domain mutant R337H (Figure 6). Although maximal levels of transactivation appeared 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 (Figure 6). The requirement for increased p53 levels necessary for initial transactivation by the tetrameric mutants appeared dependent on the strength of the RE. The R337C mutation resulted in overall reduction in transactivation.

Altered-function mutants can maintain protein levels comparable to 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 (Supplemental Figure 3). 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 to 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 reduced levels of protein in comparison to WT p53 (Supplemental Figure 4).

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 (14,33) have been identified in 46 patients with locally advanced breast tumors (primarily ductal carcinomas). The patients were participants in clinical trials that monitor clinical and pathologic responses to neoadjuvant treatment prior to surgery (Supplemental Table 3). As summarized in Table 2 and Supplemental Table 3, 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 regarding treatments.

While 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 and Supplemental Table 3). 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-negative tumors (7 of 25, 28%) compared with HER2-positive (2 of 15, 13%). Among triple negative (ER, PR, and HER2) tumors with p53 missense mutations, 5 of 16 (31%) carried functional mutations. There appeared to be a higher frequency of functional/total missense mutations among Caucasians and Asians (7/29 and 2/3, respectively) than among African American patients (2/14, 14%). Functional mutations appear 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 vs 27%), have high grade tumors (59 vs 36%) and to relapse in distant sites (34 vs 10%), as well as local sites (12 vs 0%) although the numbers are small. In terms of responsiveness to chemotherapy, the pathologic complete response (pCR; *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% vs 20%) at 3 years post-treatment than those with functional mutations. While suggestive of trends, none of these differences reached statistical significance.

Discussion

Single amino acid changes in the p53 master regulator protein that differentially impact 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 approximately 18% of all somatic p53 mutations reported in the International Agency for Research on Cancer (IARC) TP53 mutation database and 20% of all p53 missense mutations documented in breast tumors (7). Among the mutations analyzed, one (K305M) occurs at an acetylation site in a non-structured portion of the protein, 2 (R337H and R337C) are in the tetramerization domain and the remaining 47 are distributed across the sequence-specific DNA binding domain.

Functional fingerprinting established that 21 out of the 50 missense mutants associated with breast cancer can retain p53 function. The effect on transactivation appears dependent upon the specific amino acid alteration such that even different missense mutations at the same residue can vary in the impact on p53 functionality. This is exemplified at codon 194 where changing the leucine residue to an arginine (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 appear to be change-

in-spectrum mutants that impact the REs differentially. Since each functional p53 missense mutation had a unique functional fingerprint (Supplemental Figure 1) ranging from severely altered to fully functional, there may be diverse cellular effects. The functional consequences of mutations appear not to be predictable simply by assessments of conservation, topology, or structural models, emphasizing the need to address the function of p53 mutants *in vivo* (Supplemental Table 2). For example, a recent structure-based analysis of mutations in the DBD does not appear indicative of the *in vivo* transactivation capacity. Over 45% of the predictions based on computational geometry (which assessed residual score profiles derived from Delaunay tessellations) (7, 34) differed with results from our transactivation assays (Supplemental Table 2).

Importantly, the differences in transactivation for all of the mutations could not be attributed simply to protein stability since most of the p53 mutants were expressed at levels comparable to 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 to WT p53, whereas others such as M237I and Y220C were 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 (35-37).

However, regarding 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 (38,39) and Y220C in HCC1419 cells (40,41)) or alternatively being accumulated in the cells (*i.e.*, M237I in SUM149PT cells (38)). 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 that are stabilized by a zinc ion and a loop-sheet-helix motif (42,43). Mutations in the DBD have been postulated to affect the binding affinity of p53 towards 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 Zn ion (44,45). Mutations in the L2/L3 loops are predicted to be highly destabilizing to the tertiary structure of p53, can cause chemical shifts which alter the DNA binding surface and/or can alter the response to chemotherapeutics (7,19,30,31,46).

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 6 mutations (C176F, H179R, C238F, C242F, C242S, C242Y) that interfere with the histidine or cysteine side chains involved in coordination of the Zn ion rendered the protein nonfunctional in terms of transactivation, emphasizing the vital role of Zn in sequence-specific DNA binding and stabilization of the p53 protein (44,47). 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 (45), were loss-of-function mutations with the exception of M237I which was very weak for transactivation. However, 4 mutants (R174K, R174W, P190L and L194P) in the L2 loop retained function of which several (underlined) 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 (48). R337H has been associated with pediatric cases of adrenocortical carcinoma (ACC); however, it may be a low penetrant LFS or LFL allele as well (49-52). The functional fingerprints varied between these two altered-function mutations in that R337C had a greater impact 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 (Supplemental Figures 1-4). 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 (Supplemental Figure 1). 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 to 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 (53,54).

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 (32). Importantly, these mutations would not have been distinguished from WT p53 in typical functional 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. Also, 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 this may not always apply *in vivo*. For example, Tsuda and Hirohashi (55) showed with immunohistochemical analysis of over 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 (56), in over 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 levels of mutant p53 can be regulated in both normal and some tumor cells (57,58). The accumulation of mutant protein does not occur until additional mutations are acquired in genes which may disrupt the p53 degradation pathway such as MDM2 or $p16^{INK4a}$ (59). Although the subtle altered-function mutations identified in the present study retain the ability to function from the MDM2 RE, Lukashchuck and Vousden (60) 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 (C 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*. (61) observed that p53 occupies some target REs, including p21, prior to overall p53 stabilization. There is transcriptional initiation from these REs, but the transcriptional machinery stalls prior to 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 (62,63). For example, p53 has been found to target the sestrins (*i.e.*, SESN1 and SENS2) and TIGAR (TP53-induced glycolysis and apoptosis regulator) to stimulate antioxidant and pro-survival signals (62,63). 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 (64). This appears to influence genomic repair in response to UV stress at 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 impact 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 (65-67).

In addition, inherited p53 mutations may influence tumor type and penetrance of the disease (57,58,68,69). 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 frequent observed (7,68). Recent studies which 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 (7,70). 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 to severe deficiency (loss-of-function) alleles and a higher risk of breast tumors.

In another recent study (71), the frequency and average size (bp deletion or duplication) of DNA copy number variation is enriched in carriers of germline TP53 mutations within LFS families in comparison to those with WT p53 or in a healthy population. The clinical phenotypes which 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 and Supplemental Table 3). While the number of breast cancer-associated functional p53 mutants was small, there were trends described in the 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 as compared to patients with tumors that are WT for p53 (72). We have found that functional p53 mutations appear 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 chemo-sensitive, possibly because the breast cancer subtypes which 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). Also, the cumulative response to anthracycline/cyclophosphamide followed by taxane therapy was determined in this study. While there is some indication from the literature that p53 mutant status could confer different responses to these single agents (73-75), it is difficult to predict responses to the sequential treatment. While 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 approximately 1,800 woman (72), 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 transactivation 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 less than 65% agreement in terms of overall transactivation functionality (Supplemental Table 2). Only 8/49 or 16% of the missense mutations were previously identified as functional as compared to 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 (Supplemental Table 2) (7,12). For example, we concluded that the 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 3 of the 8 REs analyzed (WAF1, MDM2 and P53R2) (7). Furthermore, in a separate analysis, the Kato *et al*. group (37) found that greater than 140 missense mutations were temperature sensitive for transactivation capacity towards at least one RE; this indicated there may be cellular conditions under which these mutations may become functional. Interestingly, 5 of these temperature sensitive mutations which 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 (35).

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 fifty p53 mutations identified in breast cancers, 21 had altered function—not simply complete loss—towards 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.

While 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 response elements. Given that p53 isoforms are known to be differentially expressed in breast tumors in comparison to normal breast tissue (76), the ratios at which these isoforms are expressed, which can change with the presence of a mutation, may alter the transactivation profiles of wild type 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 response elements through either synergistic or antagonistic mechanisms (76,77).

Results obtained with the yeast functional assay appear predictive of whether a mutation will also have a biological impact in mammalian cells (16). While the yeast-based assay can predict the *potential* for specific p53 mutations to display altered function, assays in mammalian cells can ascertain the full impact 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 impact 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 (78).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACC	adrenocortical carcinoma

BRCA1/2	breast cancer susceptibility gene
CORE	COunterselectable REporter cassette
DBD	DNA binding domain
ER	estrogen receptor
FH	familial history
HER2	human epidermal growth factor-2
IARC	International Agency for Research on Cancer
LFL	Li-Fraumeni-like Syndrome
LFS	Li-Fraumeni Syndrome
pCR	pathologic complete response
PR	progesterone receptor
REs	response elements
WT	wild-type

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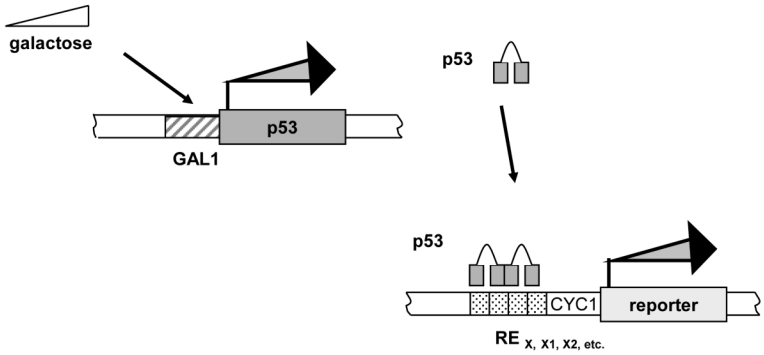

Figure 1. Evaluation of transactivation potential towards REs by WT and mutant p53s at variable levels of expression

The budding yeast *S. cerevisiae* is used as an *in vivo* test tube to assess the consequences of p53 missense mutations associated with breast cancer on transactivation capacity from human RE targets. Isogenomic diploid yeast strains were generated by mating haploid "p53-host" strains with "RE-reporter" strains of opposite mating types. Each haploid strain was constructed with the *delitto perfetto in vivo* mutagenesis system where p53 and the REs were integrated into the chromosomes as previously reported (4,79). Importantly, all of the conditions within the diploid yeast are constant except for the p53 mutation of interest or a few nucleotide differences in the RE sequence. The *rheostatable GAL1* promoter allows for tight regulation of p53 expression where increasing levels of galactose allow for over 150- fold induction in protein expression (4). Regulation of protein expression allows for ascertainment of functional discrepancies between WT and mutant p53 transactivation at variable expression levels. Since p53 is not endogenous in yeast and human p53 can utilize the yeast transcriptional machinery, placement of the reporter downstream of the minimal *CYC1* promoter provides the opportunity to directly monitor p53 interactions with RE sequence.

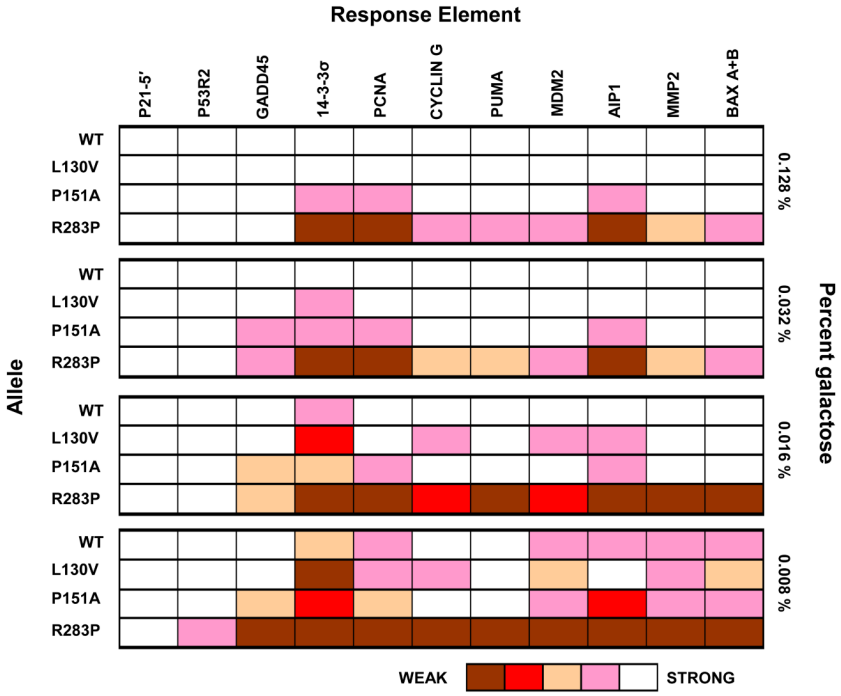

Figure 2. Functional fingerprints of p53 mutants reveal subtle transactivational differences

The *ADE2* plate color assay was used to assess the transactivation capacity of WT and mutant p53 towards 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 (33). 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 (5mg/L). Shown are examples of functional fingerprints for WT p53 and several change-in-spectrum mutations at 4 levels of protein expression. At high expression levels (0.128%) several mutations were found to display an altered spectrum of REs regulated in comparison to WT p53, for example, P151A and R283P. However, several mutants, such as L130V, appear 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.

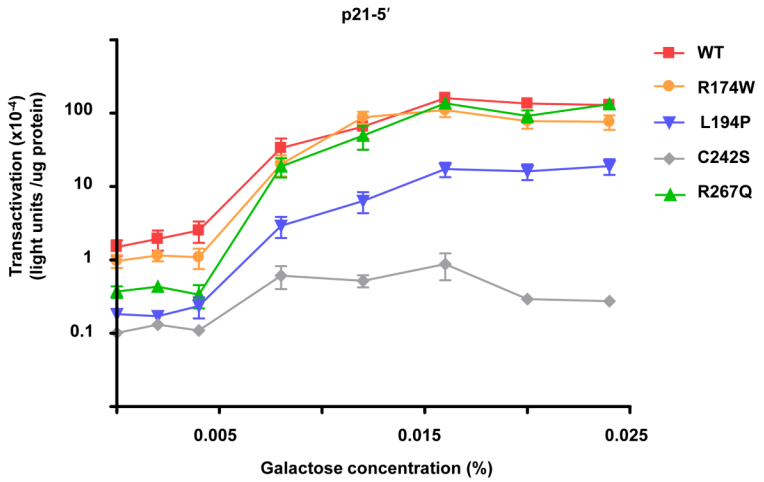

Figure 3. Assessment of WT and mutant p53 transactivation towards the p21-5′ RE using a luciferase assay

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/ug protein. Depicted are the mean and standard error of measurement (SEM) for 7 independent experiments. 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 towards the strong p21-5′ RE in comparison to 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.

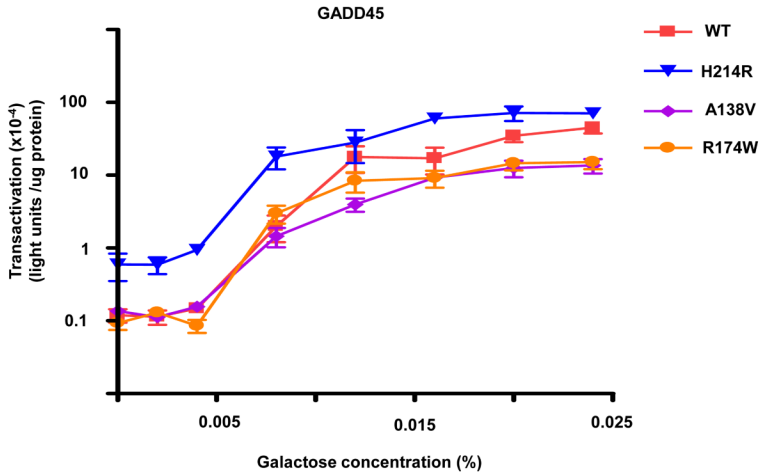

Figure 4. Transactivation from the GADD45 RE distinguishes altered-function mutants from WT p53

The ability of p53 (WT and mutant) to transactivate from the GADD45 RE was measured 24 hours after inoculation into increasing concentrations of inducing media with a quantitative luciferase assay (see Figure 4.2). 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 to WT p53 at low and high levels of expression, whereas A138V and R174W had similar transactivation potentials. Presented are the mean and standard error of measurement (SEM) for 6 independent experiments.

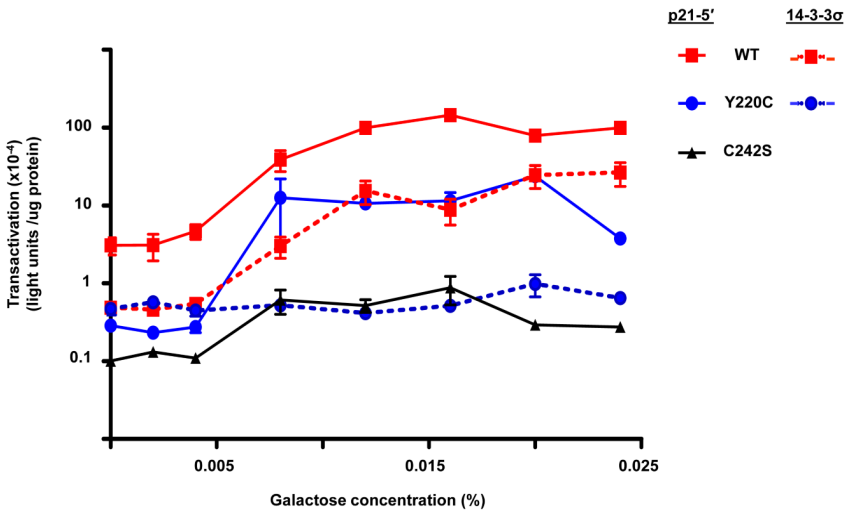

Figure 5. Change-in-spectrum p53 missense mutations can eliminate REs from the p53 transcriptional network

To establish that change-in-spectrum mutations are capable of altering the transcriptional network as a result of eliminating transcription from several REs, transactivation was assessed for WT and Y220C p53 from the p21-5′ and GADD45 REs. Diploid yeast strains were grown overnight in complete medium, diluted, washed and inoculated into induction media. Protein lysates were obtained from overnight cultures and quantitative luciferase assays were used to determine the transactivation capacity of the p53 variants from the p21-5′ RE. Y220C is capable of transactivating from the p21-5′ RE to levels comparable to WT p53 transactivation from the weaker 14-3-3σ RE. However, Y220C was not capable of transactivating from the 14-3-3σ RE suggesting this target gene may be eliminated from the p53 transcriptional network for this particular mutant. The strength of transactivation was calculated as relative light units/μg protein. Depicted are the mean and standard error of measurement (SEM) of 6 independent experiments.

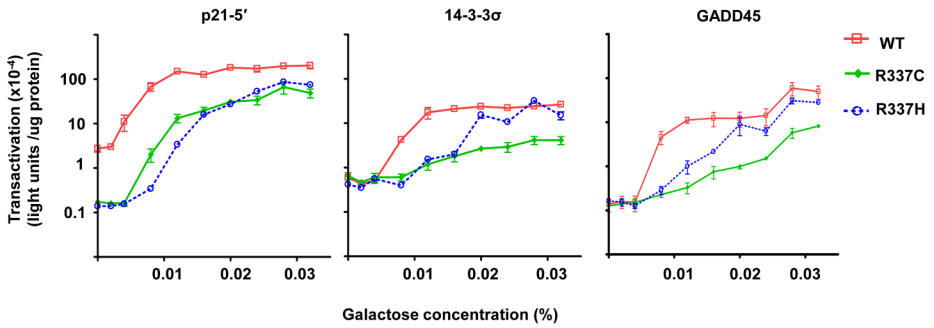

Figure 6. Altered-function mutations at the same codon in the tetramerization domain display different transactivation capacities

Transactivation capacities were obtained for the R337C and R337H mutants towards the p21-5′, 14-3-3σ, and GADD45 REs at increasing levels of expression with the quantitative luciferase assay. The levels of transactivation were severely diminished with R337C. R337H required an increased amount of p53 expression to stimulate transactivation. The magnitude of effect for R337C and R337H was dependent on the RE examined.

Table 1

p53 missense mutations associated with breast cancers: functional status, frequency, and features.

p53 MUTATION	FUNCTIONAL[a] STATUS	IARC DATABASE[b]			FEATURES[c]
		SOMATIC		GERMLINE FAMILIES	
		TOTAL	BREAST		
T125R	ALTERED	2	1	0	
L130V	ALTERED	21	3	0	Neo.
C135F	LOSS	49	3	0	
C135Y	LOSS	70	11	0	Neo.
A138V	FUNCTIONAL	48	7	0	
C141W	ALTERED	14	1	0	Neo.
P151A	ALTERED	17	3	0	BRCA1
P151H	FUNCTIONAL	35	4	0	Neo.
G154S	ALTERED	11	0	0	Neo.
V173L	LOSS	85	10	0	L2; Neo.
R174K	ALTERED	9	1	0	L2
R174W	FUNCTIONAL	14	2	0	L2
C176F	LOSS	181	7	0	L2-Zn
H179R	LOSS	139	16	0	L2-Zn; Neo.
R181P	LOSS	22	2	1	L2; FH
S183L	LOSS	3	1	0	L2
P190L	ALTERED	48	4	0	L2; BRCA1
L194P	ALTERED	14	1	0	L2; BRCA
L194R	LOSS	55	9	0	L2
H214R	ALTERED	72	5	0	BRCA2
Y220C	ALTERED	315	41	4	LFS; Neo.
M237I	ALTERED	172	26	1	L3; LFL
C238F	LOSS	38	5	0	L3-Zn; BRCA1
N239D	LOSS	43	5	0	L3; Neo.
N239T	LOSS	9	2	0	L3

p53 MUTATION	FUNCTIONAL[a] STATUS	IARC DATABASE[b]			FEATURES[c]
		SOMATIC		GERMLINE FAMILIES	
		TOTAL	BREAST		
S241C	LOSS	33	4	0	L3
C242F	LOSS	86	7	0	L3-Zn
C242S	LOSS	32	5	0	L3-Zn; BRCA1
C242Y	LOSS	51	5	3	L3-Zn; FH; Neo.
M243T	LOSS	8	2	0	L3
G244V	LOSS	23	2	1	L3
G245S	LOSS	396	35	18	L3; LFS, LFL, FH; Neo.; BRCA1
M246A	LOSS	0	0	0	L3; Neo.
M246T	LOSS	12	3	0	L3
R248L	LOSS	112	6	0	L3, Neo.
R249G	LOSS	43	7	0	L3
R249S	LOSS	374	14	0	L3, Neo.
D259V	LOSS	17	2	0	Neo.
G266R	LOSS	65	5	0	Neo.
R267Q	ALTERED	12	3	3	LFS, FH
V272L	FUNCTIONAL	47	1	1	LFS
R273C	LOSS	611	28	9	LFS, LFL, FH; Neo.
R273H	LOSS	733	76	16	LFS, LFL; Neo.; BRCA1/2
P278A	ALTERED	24	5	0	Neo.
D281E	LOSS	48	3	0	
R283P	ALTERED	34	3	0	Neo.
E285K	FUNCTIONAL	156	20	0	Neo.
K305M	LOSS	2	2	1	FH
R337C	ALTERED	17	4	3	LFS
R337H	ALTERED	2	0	69	LFL, FH; ACC

[a] Based on transactivation capacity from 11 human p53 target REs assessed with the plate color assay and the luciferase assay. Mutations were classified as loss-of-function if they were unable to transactivate from any RE at any protein concentration. Mutations were categorized as functional if they were equivalent to WT p53 in transactivation capacity or altered function if the allele retained the ability to function from a RE(s), but deviated from WT p53 in transactivation capacity from several REs at any level of p53 expression.

[b] Number of reported allele-specific mutations in the R12 release of the IARC p53 mutation database (7): total somatic mutations reported 24,810; missense mutations occurring in breast tumors, 1984; germline mutations, 399; however, families may have multiple tumors in various tissues with the same mutation. The single mutation not reported, M246A, was identified in a patient participating in a multi-institutional prospective trial. p53 status in the neoadjuvant treatment patients was confirmed by single-stranded conformational polymorphism analysis (SSCP) and validated with direct sequencing (71).

[c] Criteria used to select the specific missense mutation for transcriptional analysis. BRCA, associated with BRCA1/2 cancers; LFS, Li-Fraumeni syndrome; LFL, Li-Fraumeni-like syndrome; FH, familial history; L2, L2 loop; L3, L3 loop; Zn, zinc binding; Neo, identified in patients undergoing neoadjuvant treatment for locally advanced breast tumors.

Table 2

Clinical characteristics and response to therapy in patients with p53 functional or nonfunctional missense mutants. There were 29 unique breast cancer-associated p53 mutations identified among 46 patients.

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