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TITLE: P53 Mutation Analysis to Predict Tumor Response in Patients Undergoing Neoadjuvant Treatment for Locally Advanced Breast Cancer

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14. ABSTRACT
Studies suggest that p53 mediates responsiveness to chemotherapy . In an ongoing multiinstitutional prospective trial that is not supported by this award, breast cancer patients receiving neoadjuvant chemotherapy have serial response assessments and tumor sampling for research purposes. The project that is supported by this award involves analyzing the banked tumor specimens for p53 mutations using the GeneChip method, SSCP, and sequencing. We hypothesize that p53 status of the primary tumor will predict response to anthracycline-based and taxane-based chemotherapy given at different times in the same patient. A yeast-based functional assay is examining the impact of specific p53 mutations upon transactivation function. Progress to date includes optimizing the GeneChip method of p53 mutation analysis for core biopsy specimens, successful scaling down of the DNA requirements for such assays allowing evaluation of small tumor biopsy samples, and optimizing methods for p53 amplification within 1-2 large fragments so that SSCP and sequencing analysis will be feasible despite the small amount of DNA available. P53 mutation analysis upon the study samples is underway. Implementation of the yeast-based functional assay for assessing the effect of specific p53 mutations has been successful with altered transactivation function found in mutations from neoadjuvantly treated patients.

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P53, single strand conformational polymorphisms, clinical biomarkers, chemotherapy response prediction

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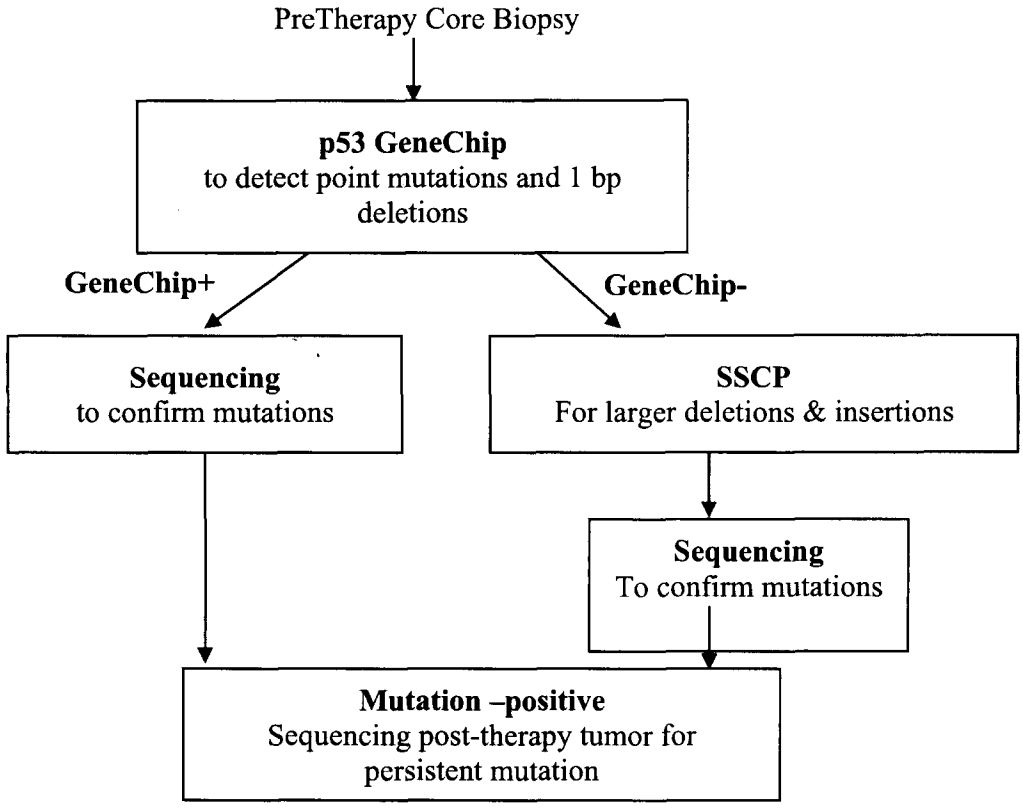
INTRODUCTION

The two most effective classes of chemotherapeutic drugs in breast cancer are the anthracyclines and the taxanes, which differ in mechanisms of action and resistance. Responsiveness to anthracyclines and taxanes may be mediated in part by the p53 mutational status of the tumor. P53 mutation status has had limited usefulness as a predictive tumor marker given the technical complexity of previous methods to determine it, however the development of p53 GeneChip technology has made high-throughput mutation analysis more feasible. This technology has been successfully applied to human tumor specimens [1,2]. Dr. Conway Dorsey's laboratory has previously determined the spectrum of expected p53 mutations in breast cancer[3] using sequencing, and is performing the GeneChip analysis and sequencing in this study.

In an ongoing multiinstitutional prospective trial, breast cancer patients who are receiving neoadjuvant chemotherapy have serial response assessments performed and undergo sampling of their tumor for research purposes at three time points. These timepoints are obtained prior to any chemotherapy and following treatment with an anthracycline-containing regimen. Those that receive a subsequent chemotherapy have another sample obtained after that regimen. This project involves analyzing the banked specimens for p53 mutation status using the GeneChip method. Specific mutations identified will be further examined for functional impact in a yeast-based assay to be compared with the clinical response. We hypothesize that p53 status of the primary tumor will predict response to anthracycline-based and taxane-based chemotherapy given at different times in the same patient.

Our approach has been to first screen time point 1 (pre-therapy) cores by the Affymetrix p53 GeneChip microarray method, which detects point mutations and single base deletions, then to evaluate GeneChip-negative cores from time point 1 for larger deletions and insertions using single strand conformational polymorphism analysis (SSCP). All cores that are identified as potentially positive by the GeneChip or SSCP are then sequenced to confirm and specifically identify the mutation.

Mutation results are correlated with response to anthracycline and taxane chemotherapy. Dr. Resnick's laboratory has developed a yeast-based assay for identifying the functional consequence of specific mutations. Including the p53 sequences determined in neoadjuvantly treated breast cancer patients, they look for variable effects on transactivation function of p53 by mutation.



BODY

This award is for performance of laboratory assays upon banked tumor specimens obtained from ongoing correlative science trials that are funded through alternative mechanisms. The performance of those trials, however, is crucial to the outcome of this project, so is summarized here. The trials include Lineberger Comprehensive Cancer Center (LCCC) Project 9819 and a multiinstitutional trial, Cancer and Leukemia Group B (CALGB) Protocol 150007, which is a joint effort of CALGB, the American College of Radiology Imaging Network (ACRIN), and the National Cancer Institute Specialized Programs of Research Excellence (SPORE). These trials are both open and enrolling patients. All participating patients are required to have received an anthracycline-based chemotherapy and have ascertainment of clinical and pathologic response to therapy in order to be included. LCCC 9819 at this time has enrolled 59 similarly treated patients and serves as a training set. As of mid-August, 2005, the CALGB 150007 multiinstitutional trial had enrolled 195 patients. At the current rate of 12 patients/month, this study should complete accrual approximately December 2005. Of the 195 enrolled patients, 18% are African American, and approximately 6% are Hispanic.

Statement of Work

Progress upon the approved statement of work is outlined below in the format used in the original application. Notably, Dr. Conway Dorsey's laboratory moved locations from the old School of Public Health building at UNC to a new UNC location, which introduced a 3

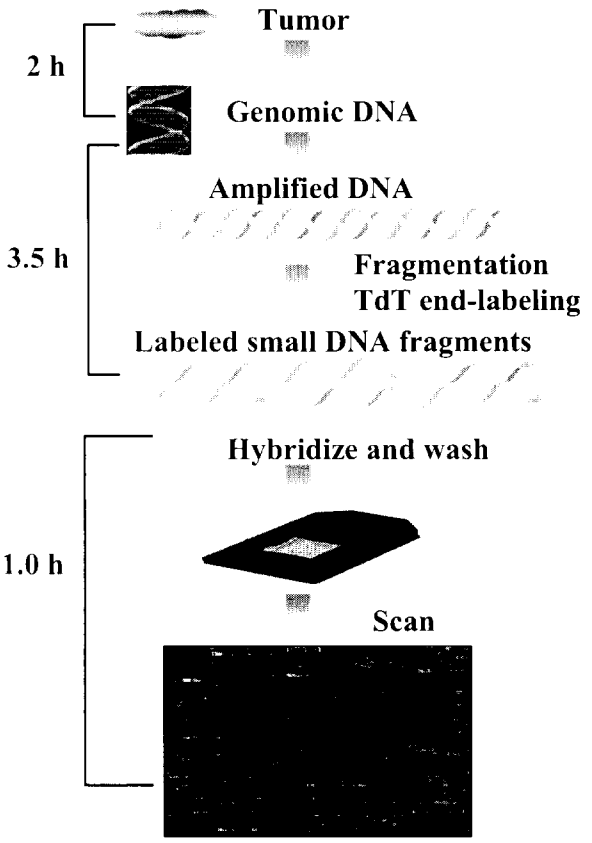
month delay in sample analysis in the spring of 2005. This has affected completion of the tasks planned between months 24-36.

Task 1. To optimize the GeneChip method of p53 mutation analysis in the UNC Molecular Epidemiology Core Laboratory (months 1-6)

Optimization of GeneChip method

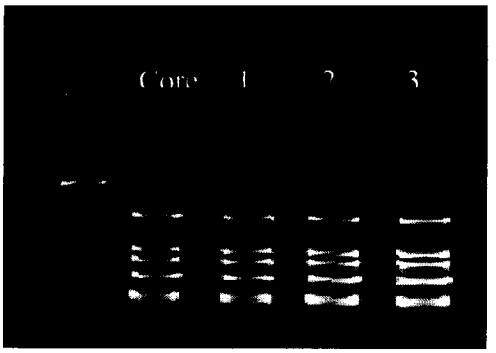
This portion of the research involved the establishment of multiplex PCR conditions to co-amplify all p53 exons from within one reaction, and the optimization of the p53 GeneChip hybridization conditions and analysis of microarray data. Figure 1 illustrates the GeneChip method. The p53 GeneChip assay was optimized using the Affymetrix normal control DNA (human placental DNA) and cell lines (BT549, Bt474, MDA-MB-231, and MDA-MB-435), and was successfully applied to human breast cancer core biopsy specimens (Figure 2).

Figure 1. p53 GeneChip method



Because of the very small quantities of DNA obtained from the breast core biopsies, our first priority in establishing assay conditions for the p53 GeneChip assay was to determine the smallest amount of DNA that could be reasonably amplified from the cores, but that would provide a valid p53 mutation result. This is crucial because only 8-10mg of total tissue is obtained from each core biopsy, with nucleic acid-based studies planned by several collaborating laboratories. Using the above cell lines, the DNA concentration required for the multiplex p53 PCR reaction, which amplifies each p53 exon 2-11 in individual fragments, has been successfully reduced from an original amount of 250ng DNA down to 50ng DNA. This reduction of DNA has not compromised our ability to identify mutations within cell line DNA. The research plan includes single strand conformational polymorphism (SSCP) and sequencing analysis to

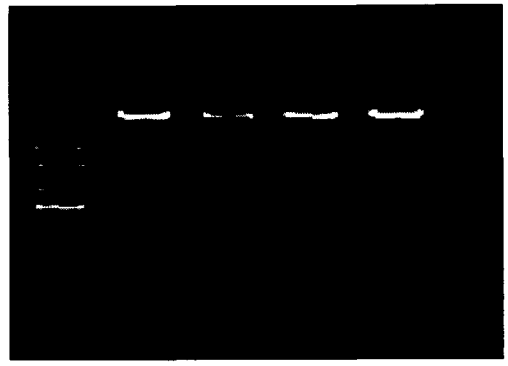
Figure 2. Initial step GeneChip: Multiplex PCR amplification of exons 2-11 using 50ng DNA.



comprehensively identify p53 mutations in GeneChip-negative samples. This technique of large-fragment PCR upon these limited tissue samples allows the SSCP and mutation analysis to be performed with a minimum of required DNA (Figure 3). The optimization of the GeneChip method was completed in 2003.

Figure 3. Initial step SSCP: PCR amplification of a single 1.5 kb fragment of p53 exons 4-8 using 50ng DNA.

Task 2. To determine the p53 mutational status of the primary breast cancers before any chemotherapy. In cases whose tumors exhibited p53 mutations pre-chemotherapy, determine if the same mutations are detectable after anthracycline then again after taxane with or without trastuzumab (Months 6-36).



Nucleic acids processing from core biopsies.

Dr. Conway-Dorsey and Dr. Perou are performing complementary assays (p53 mutation analysis and gene expression array, respectively) upon the frozen tissue in the UNC institutional trial. In the multiinstitutional trial, Dr. Joe Gray (UCSF) and Chris Haqq (UCSF) are also performing comparative genomic hybridization and additional RNA-based assays, so it has been crucial to optimize the nucleic acid retrieval method for this study. For this reason, a great deal of effort has been made to minimize the tissue, DNA, and RNA needs of each group so that all the planned assays may be performed. In order to optimize the conditions for maximal nucleic acid retrieval from these limited tissue resources, a training set of biopsies was obtained and tested. Several approaches to maximize RNA and DNA acquisition from core biopsies were tested, and we found that the optimal method remained the simplest. In this schema, tumor enrichment is performed by examination of an H&E-stained longitudinal section followed by manual dissection of non tumor-containing areas of the core. The remaining portion is divided evenly and one part is processed for RNA while the other part processed for DNA Prior to analysis of protocol core biopsies, 34 test core biopsies were successfully analyzed, providing DNA of good quality and, with only one exception, of adequate quantity.

The GeneChip assay is designed to detect point mutations and single base deletions in exons 2-11 of p53. After p53 Genechip microarray assays, tumors are then sequenced (for GeneChip-positive) or undergo SSCP (for GeneChip-negative).

UNC- LCCC 9819

To date, we have received DNA from 43 timepoint 1 core biopsies from the 9819 study; 26 have been evaluated for p53 mutations, and the remaining samples are currently being assessed (Table 1). Seven of 26 (27%) core samples were p53 mutation-positive, with the following mutations being identified: R273H (hotspot), R283P, N239D (L3), R249S (L3, hotspot), D281G, C141W, R213stop. As in the I-SPY trial, the mutations were concentrated within the DNA-binding region, particularly within hotspot codons and the L3 loop domain (see below).

Table 1. UNC institutional trial LCCC 9819 study core biopsies.

Sample #	DNA Quality	GeneChip Exon Location	AA Change	Codon Change	Sequence (Chip+)	Final Mutation status*	Type
02-0053-B3	3	wt	-	-	-	Pend	
02-0106-B4	0	poor amp	-	-	-	Pend	
02-0115-B2	3	wt	-	-	-	Pend	
01-0134-B	3	wt	-	-	-	Pend	
00-0129B	3	wt	-	-	-	Pend	
00-0315B	3	exon 8	R273H	cGt>cAt	R273H	Y	Missense
00-0392B	3	Wt	-	-	-	Pend	
00-0426B	3	Wt	-	-	-	Pend	
01-0080B	3	Wt	-	-	Δ	N	
01-0080B	3	exon 8	R283P	cGc>cCc	R283P	Y	Missense
01-0249B	3	exon 7	N239D	Aac>Gac	N239D	Y	Missense
01-0262B	3	Wt	-	-	-	Pend	
01-0319B	3	Wt	-	-	-	Pend	
01-0372B	3	Wt	-	-	-	Pend	
01-0432B(1)	3	exon 7	R249S	agG>agC	R249S	Y	Missense
01-0514B	3	exon 8	D281G	gAc>gGc	D281G	Y	Missense
03-0301	3	exon 8	G266R	gga>Aga	-	Y	Missense
02-0472	3	Wt	-	-	-	Pend	
02-0475	3	exon 5	C141W	tgc>tgG	C141W	Y	Missense
02-0484	3	Wt	-	-	-	Pend	
02-0349	3	Wt	-	-	-	Pend	
02-0342	3	defective chip-redo				Pend	
02-0542	3	Wt	-	-	-	Pend	
03-0054	3	exon 8	R213stop	cga>Tga	R213stop	Y	Nonsense
02-0135	3	Wt	-	-	-	Pend	
02-0181	3	Wt	-	-	-	Pend	
02-0149	3	Wt	-	-	-	Pend	

* Final mutation status depends upon GeneChip and SSCP results; Δ = abnormal chip, sequenced normal.

CALGB 150007/I-SPY multiinsitutional trial. We have received DNA from 78 patients. 68 core DNAs were from time point 1, 44 were from time point 2, and 22 were surgical tumor samples. GeneChip analyses have been completed on 66 of 68 time point 1 cores, and on 20 of the 44 time point 2 cores. The quantity of multiplex PCR product, produced as the first step in the GeneChip assay, is used as an indicator of DNA quality. Of the 88 time point 1 and 2 cores analyzed, only 2 failed to yield a robust PCR product from 50 ng DNA.

Table 2: I-SPY Samples Obtained for p53 analyses	
Total core DNA samples received	134
Total patients	78
Time point 1 cores	68
Time point 2 cores	44

Surgical samples	22

Table 3: p53 mutations identified in time point 1 and/or 2 breast cores from I-SPY

Case	Time Point 1			Time Point 2	Mutation Type	p53 Functional Domain
	% Tumor	*GeneChip*	*Sequencing*	*GC &/or Seq*		
1	70	intron 4	intron 4	nd	splicing	--
2	60	wt	G105V*	no core	missense	DNA binding domain
3	20	L130V	L130V	no core	missense	DNA binding domain
4	40	P151H	P151H + 11 bp del	no core	frameshift	DNA binding domain
5	40	wt	nd	G154S	missense	DNA binding domain
6	20	V173L	V173L	nd	missense	L2
7	75	wt	R175H	R175H	missense	L2
8	70	R175H	R175H	R175H	missense	L2
9	50	R175H	R175H	R175H	missense	L2
10	70	R175H	R175H	R175H	missense	L2
11	85	H179R	H179H	nd	missense	L2
12	30	R213stop	R213stop	no core	nonsense	DNA binding domain
13	90	Y220C	Y220C	no core	missense	DNA contact
14	90	E221stop	E221stop	nd	nonsense	DNA binding domain
15	20	C242Y	C242Y	nd	missense	L3
16	50	G245C	G245C	G245C	missense	L3
17	50	G245S	G245S	nd	missense	L3
18	65	M246A	M246A	no core	missense	L3
19	80	R248L	R248L	no core	missense	DNA contact/L3/hotspot
20	40	R249S	R249S	nd	missense	L3/hotspot
21	60	D259V	D259V	wt	missense	DNA binding domain
22	50	wt	E271G*	no core	missense	DNA binding domain
23	1	R273C	R273C	R273C	missense	DNA contact/hotspot
24	85	R273C	R273C	no core	missense	DNA contact/hotspot
25	80	R273H	R273H	R273H	missense	DNA contact/hotspot
26	45	R273H	R273H	no core	missense	DNA contact/hotspot
27	10	P278A	P278A	no core	missense	DNA binding domain
28	80	E285K	E285K	E285K	missense	DNA binding domain
29	75	R306stop	R306stop	no core	nonsense	DNA binding domain

*Need to be confirmed in second PCR and sequencing.
Wt; wildtype, Nd, not determined. DNA contact loops L2 (codons 163-195) and L3 (codons 236-251). Other codons outside of L2/L3 but making direct contact with DNA are 273 and 220.

Of 66 time point 1 cores successfully analyzed for mutations, 25 (38%) were mutation-positive by the GeneChip and 28 (42%) were positive by the combination of GeneChip and SSCP/sequencing. Five of the mutation-positive time point 1 cores were estimated to contain 20% or less tumor, demonstrating the sensitivity of this combined GeneChip/SSCP and sequencing approach. Of 20 time point 2 cores analyzed, 9 (45%) were mutation-positive by the GeneChip. The mutations identified in time point 1 and/or time point 2 cores are shown in Table 3. Notably, four patients carried missense mutations at codon 175 and four carried missense mutations at codon 273.

For the core samples that were GeneChip-negative, SSCP/sequencing analyses were conducted for p53 exons 2-11; these analyses are nearly complete. Although we expected to identify a substantial number of deletions and insertions in this series of breast tumors based on results of p53 analyses in the population-based Carolina Breast Cancer Study (CBCS) which showed 29% del/ins, we have thus far detected mostly point mutations (Conway, Edmiston et al. 2002). Only one deletion (11 bp) has been identified, and this occurred in a sample that also carried a missense mutation in codon 151. The disproportionate number of missense mutations in this study may be due to the highly-selected patient population. The patients enrolled in this neoadjuvant chemotherapy trial presented with large breast tumors. By comparison, 89% of cases in the CBCS had smaller stage 1 and 2 tumors. The frequent presence of mutations in the p53 hotspot codons 175 and 273, the concentration of mutations within the central DNA binding domain (exons 5-8), and in particular, in the L2 and L3 loop domains, may also be related to the large size of the tumors in this trial. We plan to evaluate the relationship between mutation type/location and clinical characteristics when laboratory analyses are complete.

Task 3. To correlate p53 status with response to anthracycline chemotherapy, then taxane with or without trastuzumab in the same patient (months 30-36):

Of 59 patients included in the institutional trial LCCC 9819, 81% responded to sequential anthracycline followed by taxane-based chemotherapy. P53 mutation status has been completed in 27. Of 18 wildtype patients, 3 (17%) responded to the anthracycline while of 7 patients with p53 mutations, 3 (43%) responded. The response to taxane at this time does not look dissimilar between the two groups, with 5 (42%) of 12 evaluable wildtype p53 responding, and 1 (33%) of 3 p53 mutant (Carey, Harden et al. 2004). These data, although preliminary, suggest that p53 mutations may be important in response to some neoadjuvant therapies.

Currently, we are evaluating the surgical samples (taken after all therapies) from mutation-positive patients to determine whether the original mutation persists or if new mutations have arisen. We also plan to evaluate

Table 4. LCCC 9819 clinical characteristics and response to anthracycline-, then taxane-based chemotherapy.

Median age	45
African-American	25 (42%)
ER-positive	29 (49%)
Premenopause	38 (65%)
Pretreatment stage	
II	19 (32%)
III	34 (58%)
IV	6 (10%)
Overall response	48 (81%)
RR anthracycline	36 (61%)
RR taxane	31 (60%)

core biopsies after AC treatment but before taxane from mutation-positive patients to specifically determine the nature of the persistence of p53 mutations during the AC component of therapy.

Furthermore, for the multiinstitutional trial, the National Cancer Institute has developed a database that allows the input of the multiple laboratories involved in this study to cross-examine the relationships of various markers to each other and to outcome. As the cohort matures, we will examine response not oly by chemotherapy type but also by mutation type and within defined subtypes.

Task 4. To compare p53 status with results of other planned assays within the larger correlative science trial such as bcl-2, estrogen receptor, and gene expression array analysis (months 1-36).

In preliminary analysis using immunohistochemical surrogates for breast cancer subtypes, we have found that p53 mutations segrated differently by subtype.. Of 25 assessable tumors, 3 (50%) of 6 Basal-like, 3 (30%) of 10 HER2, but only 1 (10%) of 9 luminal tumors had p53 mutations. This is consistent with our results from the p53 mutation analysis of the population-based Carolina Breast Cancer Study, in which p53 mutations were significantly more frequent among Basal-like (44%) and HER2 (43%), while Luminal B (23%) and Luminal A (15%) had fewer mutations (Carey, Perou et al. 2004).

The Perou laboratory has received 167 CALGB 150007 multiinstitutional total RNA samples, and all have been run on an Agilent Bioanalyzer as a first step in the assessment of RNA quality. Of the 167, 86 gave adequate RNA. RNA was extracted from the 2nd core biopsy for 7 samples that had previously failed with successful array in 3. Thus, many samples that failed previously may be recovered by accessing the 2nd core. For this reason, the fraction of tissue allocated to RNA analyses was increased in order to increase the yield of RNA/sample while leaving enough DNA for the array-CGH and p53 assays. Hierarchical clustering analysis of 45 of the ISPY samples showed that the expected subtype patterns (i.e. basal gene profile, proliferation profile and luminal gene profile) are clearly present in the successfully run microarrays, thus showing that the samples that did give good arrays showed patterns of expression that are seen in all breast tumor microarray experiments. Analysis of the relationship of gene expression profile with p53 mutation status is underway.

Determination of genome copy number aberrations using array CGH have been completed in the Gray laboratory for 61 samples from 39 patients. These include 29 samples from timepoint 1 and 2 complete sets of three time points (baseline, post-anthracycline, and at surgery). The quality of copy number profiles was poor in about 25%, which was found to be due to a technical error that has been corrected. Copy number abnormalities were seen in 35 stage III tumors. Minor differences between time points were observed in few cases and may be the consequence of treatment-induced selection. These analyses and correlations with p53 mutation status are underway, and will continue as the multiinstitutional trial completes accrual in late 2005.

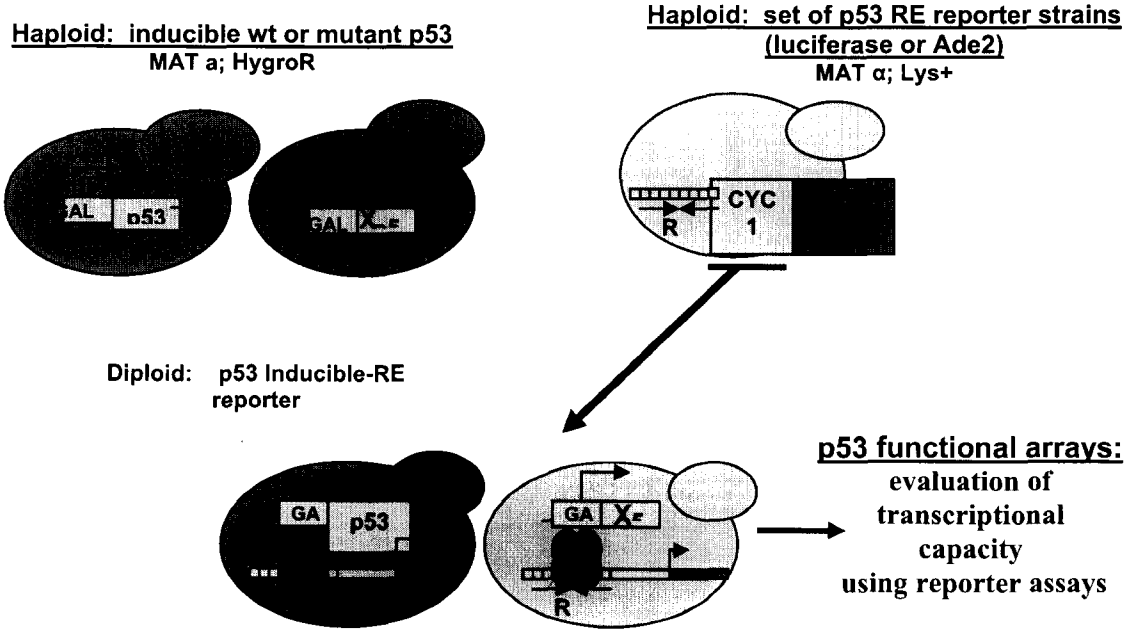
Task 5. To functionally classify the p53 mutants identified in breast cancer using established

and newly developed yeast-based assays (Months 12-36).

Establish system for functional characterization of various p53 mutations associated with breast cancer.

A yeast model system was utilized to analyze the functional consequences of p53 missense mutations towards many p53 response elements derived from human genes (Figure 4). Evaluation of transactivation capacity was based on a qualitative, visual assay using an *ADE2* color system and a quantitative luciferase reporter. Both reporters exploit a "rheostatable" promoter for p53 expression and utilize the "*delitto perfetto*" *in vivo* mutagenesis approach for rapid inclusion of REs upstream of a reporter and the development of mutant p53s (Inga, Monti et al. 2001; Inga and Resnick 2001; Storici, Lewis et al. 2001; Inga, Nahari et al. 2002; Inga, Storici et al. 2002). This system compares p53 variants at variable expression levels in a constant, isogenic chromatin environment.

Figure 4. Determining mutant p53 transactivation potentials. Transactivation capacity of p53 is evaluated using diploid cells where both p53 and human target response elements (REs) are integrated into specific chromosomal loci. Two panels of modified S. cerevisiae strains are generated. The first is a set of "p53-host" strains in which p53 (wildtype or mutant) is controlled by a "rheostatable" GAL 1,10 promoter. The second contains promoter REs upstream of the either the Ade2 or firefly luciferase reporter. Mating of these strains results in isogenic, isogenomic diploid yeast that enable the assessment of the transactivation potential for wt or mutant p53 proteins towards individual REs in the p53 transcriptional network. Each strain differs only by the mutation of interest and the 4-5 bases a particular RE varies from the consensus.



Twenty-seven mutations associated with breast cancer and Li Fraumeni syndrome (LFS) have been studied so far in the *ADE2*-based visual reporter system using twelve different

REs from a variety of p53-dependent biological responses. Among 27 non-hotspot mutants associated with breast cancers, 10 showed altered function--not complete loss--towards at least one RE (Table 5). At high expression levels, several of the mutant p53 functional fingerprints looked identical to wt p53 since they were capable of strongly transactivating from all the REs. However, reducing the expression of p53 revealed subtle transactivation defects in these mutations that were masked at higher expression thus differentiating themselves from wt p53 and other p53 missense mutations (Figure 5). One partial function mutant (L194P) also showed a change in transactivation specificity.

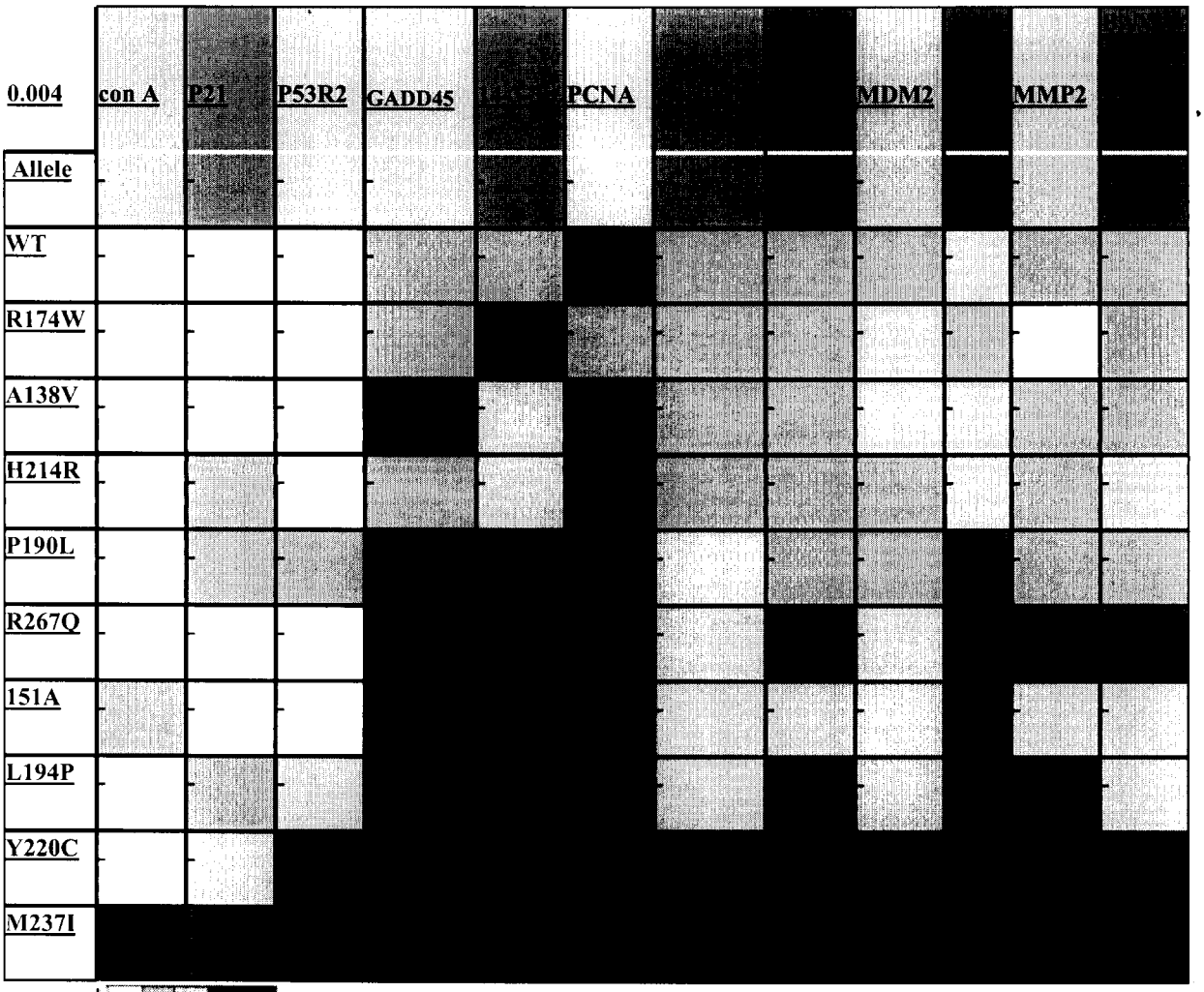
Table 5. Non-hotspot missense mutations associated with breast cancer. Functional fingerprinting on non hotspot mutations, shows approximately 40 % of the p53 missense mutations that are associated with breast cancer that we have examined have altered transcriptional capacities. The semi-quantitative *ADE2* reporter assay was used to classify the functional status of p53 missense mutations based on their ability to transactivate from 13 different REs of known target genes. The following criteria were invoked to decide which breast cancer associated mutants to examine: 1) present among Li Fraumeni Syndrome (LFS), Li Fraumeni-like Syndrome (LFL) or familial cancers (FH); 2) in the L2, L3 or zinc binding region of the protein, 3) associated with familial BRCA1/2 cancers and/or 4) multiple amino acid changes occurring at the

Mutation	IARC DATABASE * Total mutations			Features	Functional Status
	Somatic [breast tumors]		Germline		
C135F	42	[2]	0		LOSS
C135Y	64	[10]	0		LOSS
A138V	44	[7]	0		**ALTERED**
A138S	0	[0]	5	LFS, LFL, FH	LOSS
P151A	12	[3]	0	BRCA1	**ALTERED**
R174W	12	[3]	0	L2 loop	**ALTERED**
C176F	169	[7]	0	L2 loop	LOSS
S183L	3	[1]	0		LOSS
P190L	34	[1]	0	L2 loop; BRCA1	**ALTERED**
L194P	13	[1]	0	L2 loop; BRCA1	**ALTERED**
L194R	48	[8]	0	L2 loop	LOSS
H214R	66	[5]	0	BRCA1-associated	**ALTERED**
Y220C	263	[35]	17	LFS, LFL, FH	**ALTERED**
M237I	150	[23]	6	L3 loop; LFS, LFL,FH	**ALTERED**
C238F	34	[5]	0	L3 loop; BRCA1	LOSS
N239T	9	[2]	0	L3 loop	LOSS
S241C	28	[2]	0	L3 loop	LOSS
C242F	70	[6]	0	L3 loop	LOSS
C242S	31	[5]	0	L3 loop	LOSS
M243T	7	[2]	0	L3 loop	LOSS
G244V	22	[2]	0	L3 loop	LOSS
R249G	39	[7]	0	L3 loop	LOSS
R249S	351	[13]	0	L3 loop	LOSS
R267Q	9	[3]	20	LFS, LFL, FH	**ALTERED**
V272L	37	[1]	7	LFS, LFL, FH	LOSS
D281E	43	[2]	0	tumor (Perou lab)	LOSS
R337C	12	[3]	18 14	LFS, LFL, FH	**ALTERED**

same codon.

The quantitative luciferase-based assay was used to characterize the altered function mutants in greater detail. These quantitative assays showed altered function p53 missense mutations associated with breast cancers reduce the levels of transactivation *in vivo* (Figure 6). The induction patterns were consistent with the functional mutations simply modulating transactivation levels.

Figure 5. Nine p53 missense mutants within the central DNA binding domain of p53 have been identified as having an altered transactivation capacity. At high expression levels, many of these mutations appear to be silent mutations (data not shown). However, the rheostatable promoter provides the ability to use low p53 levels to address the potential of subtle defects in mutants that would be masked in typical, transient over-expression assays. Low p53 expression levels (0.004% galactose) reveal p53 mutations are distinct from wt p53 and other missense mutations in transactivation capacity towards several REs and even cause changein spectrum (see L194P). The missense mutation, Y220C, was identified in a breast cancer patient participating in the neoadjuvant treatment studies at the UNC hospitals.



strong weak; green = cell cycle; pale blue = DNA repair; purple = apoptosis; salmon = other.

Functional characterization of p53 mutations found in patients undergoing neoadjuvant chemotherapy treatment for locally advanced breast cancer.

Twenty-three breast cancer patients undergoing neoadjuvant chemotherapy for locally advanced breast cancer were determined to harbor a p53 missense mutation (Table 6). At NIEHS, twelve of the 23 mutations were assessed for the ability to transactivate from over 12 response elements (REs) of human downstream target genes with the phenotypic *ADE2* color assay (Table 5) Eleven of the twelve were classified as loss-of-function mutations based on the inability to transactivate the reporter from any RE. Similarly, functional analysis of mutants in IARC database also indicated that the mutants in the present neoadjuvant study are predominantly loss-of-transactivation. However, p53 Y220C was shown to have an altered transactivation capacity in comparison to wt p53 where it could only transactivate several strong REs. The quantitative luciferase assay revealed p53 Y220C reduced the maximal levels of transactivation from the p21::luciferase reporter in comparison to wt p53 (Figure 6).

Table 6. p53 missense mutations found in tumor samples of breast cancer patients undergoing neoadjuvant chemotherapeutic treatment. Currently, 23 breast cancer patients undergoing neoadjuvant treatment at UNC hospitals harbor a p53 missense mutation that can be assessed for functional status. We found that between the IARC database and results obtained at NIEHS that 16 of the mutations had been analyzed in yeast and/or human cells for transactivation capability from at least one response element. All but one of the mutants appeared to be complete loss of transactivation. However, in the IARC study, several mutants were only tested against a small number of REs. Twelve p53 missense mutations have been evaluated at NIEHS for transactivation capacity by the phenotypic *ADE2* color assay towards 13-15 response elements from human p53 target genes. Y220C was assessed as having altered functional ability. The NIEHS results agree with those in the IARC database. The mutants identified with a red asterisk (*) will also be characterized for functionality using the *ADE2* color assay at various levels of p53 expression. The "-" indicates that the p53's were not tested.

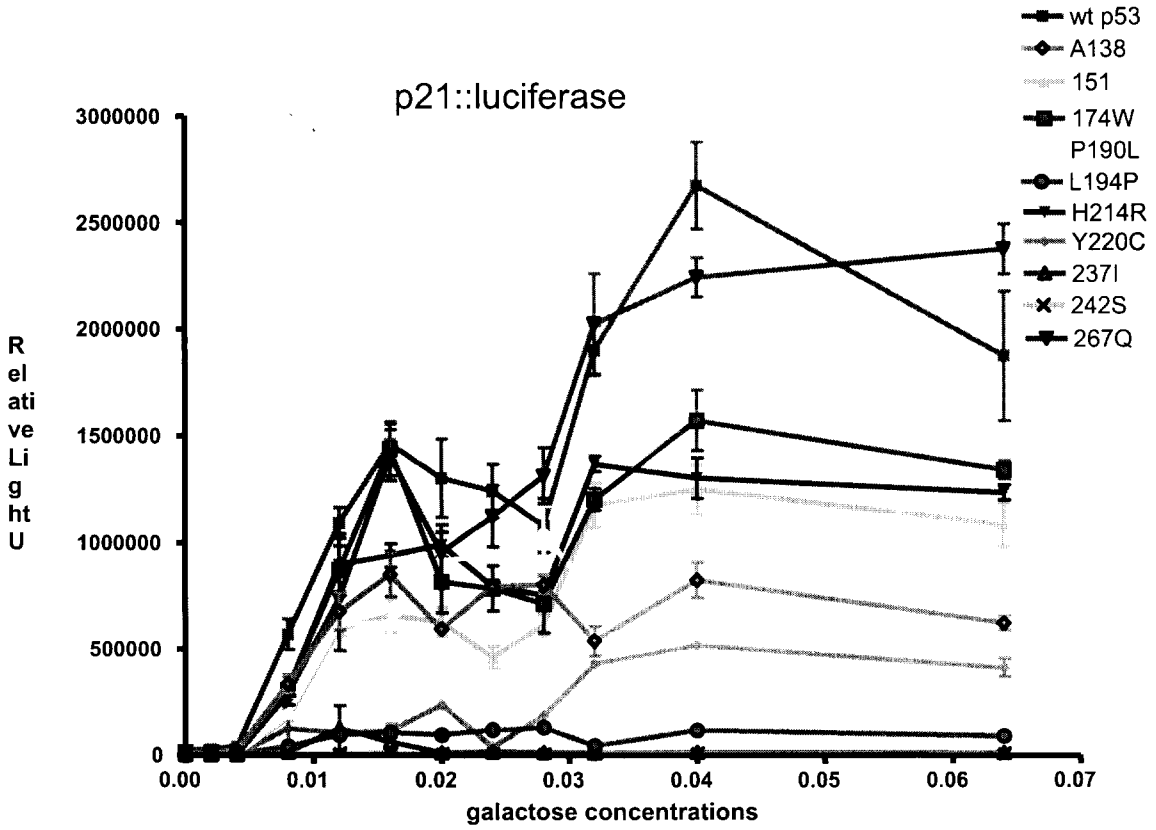
Mutation	IARC Database *			Transactivation Studies			
				IARC Database			NIEHS
	Total mutations			# REs examined		Assessment	Assessment (10 -15 REs in yeast)
	Somatic [breast tumors]		Germline	Yeast	Human cells		
130V	15	[2]	0	-	-	-	-*
141W	11	[0]	0	-	-	-	-*
151H	31	[4]	0	**3**	**0**	LOSS	LOSS
154S	10	[0]	0	-	-	-	-*
173L	77	[10]	0	-	-	-	LOSS
175H	hotspot	hotspot	hotspot	**13**	**13**	LOSS	LOSS
179R	129	[16]	0	-	-	-	-*
220C	263	[35]	17	**8**	**0**	ALTERED	ALTERED
239D	36	[5]	0	-	-	-	LOSS
242Y	44	[5]	10	**3**	**0**	LOSS	LOSS
245C	75	[3]	3	**0**	**2**	LOSS	-

245S	343	[33]	51	**4**	**1**	LOSS	LOSS
246A	0	0	0	-	-	-	-*
248L	hotspot	hotspot	0	**0**	**1**	LOSS	-*
249S	351	[13]	0	**9**	**4**	LOSS	LOSS
259V	16	[2]	0	**4**	**0**	LOSS	LOSS
266R	61	[5]	0	**3**	**0**	LOSS	LOSS
273C	hotspot	hotspot	hotspot	**6**	**3**	LOSS	LOSS
273H	hotspot	hotspot	hotspot	**11**	**7**	LOSS	LOSS
278A	19	[4]	0	-	-	-	-*
281G	14	[3]	0	**0**	**5**	LOSS	-
283P	31	[3]	0	-	-	-	-*
285K	139	[20]	0	**8**	**3**	LOSS	-*

The functional fingerprints will be determined for eight more missense mutations identified in the patient population for which there is no, or limited, functional data available (highlighted in red asterisks in Table 6). Functional status will be assessed by the *ADE2* color assay at various p53 expression levels. Mutations that are found to have an altered function will be further examined by the quantitative luciferase assay to determine how the specific mutation affects the "kinetics" of transactivation.

Given that many non hotspot p53 mutations associated with cancer retain function as indicated in Table 5, these results for the breast cancers in the neoadjuvant study at UNC are unexpected and may reflect a clinical feature of the breast cancers examined. Consistent with the findings of a shift in the nature of the p53 mutations seen in this population compared with the Carolina Breast Cancer Study, we may be seeing a trend for more deleterious p53 mutations among these highly-selected large, possibly more rapidly growing tumors. p53 mutations occurring in these tumors may be more deleterious and completely disable the transcriptional regulatory activity of p53.

Figure 6. Altered function p53 missense mutations associated with breast cancers reduce transactivationlevels *in vivo.* The quantitative luciferase reporter assay was used to determine the transactivation capacity of wt or mutant p53 from the p21 response element. Interestingly, the amount of p53 to initially induce transactivation is comparable for wt and mutant p53. Preliminary data suggests this initiation of transactivation correlates with the accumulation of protein above a basal level within the cell.



KEY RESEARCH ACCOMPLISHMENTS

- Optimized conditions for GeneChip assay using frozen breast cancer tissue
- Scaled down DNA requirements for GeneChip assay to require only 50ng DNA.
- Optimized method of concurrent RNA and DNA processing from core biopsies.
- Optimized large, single fragment PCR reaction to amplify entire coding region of p53 from large molecular weight DNA
- Optimized SSCP/sequencing methods for additional exons 2, 3, 9, 10, and 11 in addition to the existing protocol for exons 4-8.
- Identified positive control cell lines for SSCP that contain known p53 mutations in each of exons 2, 3, 9, 10, and 11.
- Adapted a system for in vivo site-directed mutagenesis by oligonucleotides to allow rapid construction in yeast of any p53 mutant.
- Developed seven isogenic p53 host yeast strains that can be used to generate any p53 mutation between amino acid 118 and 329
- Developed method to mate p53 host strain with p53 reporter strain allowing characterization of functional impact of chosen p53 mutation
- Collected clinical data, response to therapy data, and DNA from breast cancer tissue before, during, and after anthracycline- followed by taxane-based chemotherapy in an institutional pilot trial.
- Collected clinical data, response to therapy data, and DNA from breast cancer before, during, and after anthracycline-based chemotherapy in a multicenter trial that is nearing completion.
- p53 mutation status determined from the first 27 of 59 tumors from patients in the institutional trial, and 78 of 195 tumors from patients in the multicenter trial.
- Ongoing collaborations with investigators performing gene expression array (Perou) and aCGH (Gray) will allow examination of p53 mutation type with breast cancer subtype and gene copy number abnormalities.
- Identification of increased numbers of missense mutations among tumors in this study.
- Variable transactivation function identified in one p53 mutation from this study.

REPORTABLE OUTCOMES:

- Determination of optimal method to measure response to neoadjuvant chemotherapy in clinical / translational trials (Carey, Metzger et al. 2005)
- Correlation of p53 by immunohistochemistry and HER2 status with response to anthracycline- then taxane-based chemotherapy (Carey, Harden et al. 2004)
- In vivo site-directed mutagenesis system adapted to allow rapid construction of defined p53 mutants in yeast (Storici, Durham et al. 2003)
- Method of characterization of p53 alleles with altered transactivation function (Inga, Storici et al. 2003; Resnick and Inga 2003)

CONCLUSIONS

Ascertainment of tumor samples from patients undergoing neoadjuvant chemotherapy for locally advanced breast cancer is continuing. In addition to the UNC samples, the NCI-supported multiinstitutional study began in the fall 2002, has already accrued over 195 patients, and will close to accrual in late 2005. This confirms that the investigators have the tissue resources to perform the planned analysis of p53 as a predictive marker in breast cancer. Preliminary data from from the UNC instititutional pilot trial suggests that there is likely to be an 80% response rate to the neoadjuvant chemotherapy administered to patients in the UNC institutional trial and the CALGB 150007 multiinstitutional study. While technical obstacles have limited the gene expression array and array-CGH correlates to the p53 mutation analysis being performed under this award, these studies have been optimized for the small amount of tissue available and are ongoing.

The GeneChip method of p53 mutation analysis in human tumors has been optimized in Dr. Conway Dorsey's laboratory. Moreover, her laboratory has successfully reduced the required amount of DNA to 50 ng, and has performed multiplex PCR amplification upon both test and study core biopsies with good results. The screening method for GeneChip-negative samples using SSCP and sequencing has been optimized for the entire coding region of p53 from the large molecular weight DNA obtained from core biopsies, and have identified positive cell line controls for mutations in each exon. Preliminary analyses of the data from the multiinstitutional trial suggests that p53 mutations are frequent (42%) among the large tumors included in this trial, and that missense mutations are more frequent than expected based on previous studies in smaller tumors.

Once mutations are identified in the tumors from patients undergoing neoadjuvant chemotherapy, the specific mutations are provided to Dr. Resnick in order to correlate clinical response to therapy with functional evaluation of the effect of these mutations upon transactivation in a yeast-based transactivation assay. Dr. Resnick's laboratory has adapted their in vivo site-directed mutagenesis method to allow rapid construction of p53 mutations of choice. They have also demonstrated the ability to characterize p53 alleles with altered transactivation functions. Of 12 mutations from tumors in patients treated on the neoadjuvant trials, 11 were loss-of-function mutations. One exhibited variable transactivation function, with clinical significance to be determined in this study. The functional fingerprints remain to be determined for the remainder of the identified missense mutations.

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