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We are investigating cooperating genetic events in the genesis of breast cancer, using the mouse as a model system. We have shown cooperativity between a mutant allele of p53 and overexpressed ErbB2 in mammary tumorigenesis in transgenic mice. We are now performing a MMTV proviral mutagenesis screen to identify other events that can cooperate with these transgenes. We have put the mutant p53 allele onto the C3H background, and have generated mice that have both MMTV and mutant p53 transgene. These mice develop mammary tumor significantly faster than mice with MMTV alone (mice with mutant p53 alone do not develop tumors). These tumors are now being analyzed to identify the sites of proviral insertions. Other experiments, aimed at ErbB2 and MMTV were unsuccessful, but this may have been due to the mixed C3H x FVB strain background used in this cross.

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FOREWORD

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A S Perkins 3/13/00

PI - Signature Date

Annual Report for DAMD17-96-1-6242 for year ending July, 1999.

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Introduction

A central goal of current cancer research is the identification of the genes involved in tumorigenesis, and the definition of the precise role that these genes play in tumor development. Analysis of human breast carcinomas has implicated a number of genes in the genesis of these tumors, including *Erb*B2[43], *HST* and *INT2* [1], *p53* [15], *src* [39], *Rb* [26]. It is suggested by a number of studies that the development of breast cancer in humans requires changes in more than one of these genes, which may in part explain the long latency associated with this disease [15].

*Erb*B2 encodes a receptor tyrosine kinase related to the receptor for epidermal growth factor (EGFR or ErbB), and is amplified in nearly 30% of human cancers, particularly intraductal carcinomas[17, 44] . Numerous studies suggest that this amplification leads to increased mitogenic signaling in the cell. The importance of this amplification is supported by the finding that 70% of transgenic mice that overexpress rat *Erb*B2 in the mammary gland develop mammary carcinomas [11]. However, the latency of tumorigenesis is relatively long (over 200 d), suggesting that other oncogenic events are necessary. Analysis of these tumors revealed small in-frame deletions in the *Erb*B2 transgene in 65% of tumors analyzed [42]. These deletions resided in the extracellular domain adjacent to the transmembrane domain, and resulted in activation of ErbB2 tyrosine kinase activity. These findings indicate that activation of ErbB2 tyrosine kinase activity plays an important role in the development of these tumors. This is consistent with previous experiments, showing that mice carrying an MMTV-driven rat *Erb*B2 transgene with an activating mutation in the transmembrane domain develop multifocal mammary carcinomas with a significantly shorter latency [35].

In 30% of human breast carcinomas, expression of *ERB*B2 is associated with the presence of mutant *p53*, suggesting that activated tyrosine kinase receptors cooperate with mutant p53 in

the development of these tumors [15]. p53 is a multifunctional protein that is involved in the regulation of growth of nearly all cell types within mammalian organisms (reviewed in [23]. The wild type p53 protein can suppress tumor cell growth [10], and likely functions as a regulatory protein in two capacities: as a key component of apoptotis pathways within the cell [46]; and as a checkpoint protein to control G1 to S transition in the presence of genotoxic stress [24]. Structural domains of p53 include an amino-terminal transcriptional activation domain, a central DNA binding domain, and a carboxy-terminal domain important for oligomerization (reviewed in [23]). Genetic alterations at the *p53* locus are common in human cancers, and are primarily either missense mutations or allele loss [2, 14, 36]. While the majority of human tumors with altered *p53* have one allele bearing a missense mutation and one null allele, occasionally tumors are found to have one mutated allele and one normal allele [36]. These findings suggest a progression model in which the initial event is a missense mutation in one p53 allele, leading to a proliferative advantage, and then loss of the other allele, which confers a further selective advantage.

p53 point mutations are highly clustered into four regions that correspond to evolutionarily conserved domains of the protein that function in DNA binding. Some of the most commonly mutated amino acids are those that make direct contact with the DNA [4]. p53 proteins bearing these mutations have been found to have altered DNA binding and transactivation properties [20, 21]. Some mutant proteins fail to activate normal target genes, such as p21, but can activate atypical targets, such as *MDR1* [3]. Thus, certain mutations in p53 may lead to the acquisition of novel and dominant activities within the cell. It is evident from a number of studies that certain missense mutations in p53 function as dominant negative alleles that encode proteins that lack transcriptional activation potential, but retain the ability to oligomerize and thus can pull wildtype p53 into nonfunctional complexes [34]. An example of this is the 135V mutation, which

can accelerate tumor development in heterozygous but not nullizygous p53-deficient mice [12]. Other alleles, such as 143A, 175H, 248W, 248Q, 273H, and 281G act as dominant oncogenic alleles, since they can confer new malignant phenotypes upon gene transfer into cells that lack p53 [8, 16]. These phenotypes include the ability to grow in soft agar, and to form invasive tumors in nude mice. The molecular mechanisms that underlie the ability of mutant p53 alleles to induce these changes are unknown.

p53 alterations are common in human breast carcinomas [5, 38]. Missense mutations have been identified at many of the hotspot regions, including codons 175(R to H), and 248(R to Q). 175H represents approximately 8% of all p53 mutations in human breast cancers. These alleles are dominantly oncogenic in cell culture and nude mouse tumorigenicity assays [8, 16]. To obtain a more accurate picture of the effect that the 175H allele has on mammary cell growth, we used transgenic mice in which this allele was targeted to the mammary epithelium using the whey acidic protein (WAP) promoter. It was somewhat surprising to find that, despite high level expression in the mammary gland, mice carrying the WAP-driven p53-175H were not abnormally susceptible to mammary carcinomas – only one mouse developed a mammary carcinoma and this was with a latency of 11 months [27]. These data suggested that this allele is not dominantly oncogenic on its own in this setting, and requires other cooperating events. Indeed, these mice were much more susceptible than nontransgenic control mice to mammary tumors induced by carcinogens that are known to activate Ha-Ras [25, 28, 32]. This suggests that activated Ras is one molecule that can cooperate with p53-172H in this system.

It is known that ErbB2 can initiate a mitogenic signal within the cell, and that this signal utilizes the same pathway as activated ras. This suggested that if p53-172H can cooperate with activated Ras, it may also cooperate with ErbB2. In our previous studies, we demonstrated cooperativity between ErbB2 and p53-172H in the development of mammary carcinomas [29]. These bitransgenic

mice constitute a model system that closely mimics the genetic changes in human breast cancers, and that allows for further studies to uncover the mechanism of cooperativity between these two genes.

Body

Experimental Methods

Transgenic Mice The *p53-172H* transgenic mice, in which mutant *p53* transgene was preferentially overexpressed in the mammary epithelium by use of the whey acidic protein (WAP) promoter, were created and characterized as described [27]. p53 knockout mice were obtained from Tyler Jacks [18]. Unactivated *Erb*B2 transgenic mice (line N#202) which contain the wild type rat *Erb*B2 gene driven by MMTV have been described previously [11]. All three lines are on an FVB background. *p53/Erb*B2 bitransgenic mice were generated by crossing female and male offspring of line 8512 *WAP-p53-172H* transgenic mice to offspring of line N#202 of *MMTV-Erb*B2 transgenic mice. Mouse tail DNA from the offspring of this cross was isolated as described previously [29]. The WAP-172H and/or *MMTV-Erb*B2 transgenes, and the p53 knockout allele were identified by PCR as described [18, 29].

Histologic and immunohistologic analysis Mammary glands and mammary tumors were surgically removed, fixed in 10% neutral buffered formalin (ANATECH LTD, Battle Creek, MI) for 6 h, and placed in 70% ethanol until processed. These tissues were embedded in paraffin, and 5 _m sections were placed on regular slides and stained with hematoxalyn and eosin. Immunoperoxidase was performed on deparaffinized tissue sections using standard techniques. To detect vascular endothelial cells, an anti-CD34 antiserum was used (provided by D. Krause, Yale University). To detect phosphorylated neu protein, an antibody directed against a phosphopeptide within the cytoplasmic domain of activated Neu was used (courtesy of M. DiGiovanna).

Spectral Karyotyping. In situ hybridization of fluorescently labeled chromosome-specific probes was performed by Dr. Alan Coleman of the NIH, using techniques described by T. Reid and coworkers [41]

Results and Discussion

1. Identification of cooperating oncogenes in mammary tumorigenesis.

One of the goals of the studies in the past year has been to develop cohorts of mice that develop tumors due to the action of either a transgene alone or transgene plus MMTV, with the ultimate goal of then using DNAs from these tumors to isolate cooperating oncogenes.

Our work has focused on two genes that are known to play a role in human breast cancer: p53 and ErbB2. ErbB2 overexpression in the mammary glands of transgenic mice results in the development of mammary tumors with a latency of 200 to 280 days, depending on the strain of mouse. We have created transgenic mice in which the expression of a mutant p53 is directed to the mammary epithelium in transgenic mice through the use of a whey acidic protein (WAP) promoter. These mice do not exhibit a significant incidence of mammary tumors. Previous studies from our lab showed that when combined with the ErbB2 transgene, the WAPp53 causes a shortening of latency.

The long latency of tumorigenesis in the ErbB2 transgenic mice and the shortening of latency with combined expression of a mutant p53 transgene suggests that tumorigenesis is the result of the combined effect of several different genes. We wished to set up a screen to identify what other cooperating events may contribute to mammary tumorigenesis in the setting of either ErbB2 expression, mutant p53 expression, or both, in transgenic mice. One approach to this is to use retroviral proviral tagging to find cooperating oncogenes. Retroviruses can act as insertional mutagens in the somatic tissues of mice, and can either activate genes through promoter or enhancer insertion mechanism, or can knock out gene function through insertional disruption. The inserted provirus provides a molecular tag by which one can molecularly clone the locus into which the virus has inserted. For

mammary tumorigenesis, one can use mouse mammary tumor virus to achieve proviral insertional mutagenesis. This virus is transmitted horizontally through the milk from the dam to the pup in certain strains of mice, particularly C3H-He/N mice.

Group #	Median Age	mut p53	ErbB2	MMTV	# of mice with tumors	Total # mice	% with tumors
B1+	243	-	-	+	10	10	100
B1-	336	-	-	-	2	11	18
B2+	240	-	+	+	7	7	100
B2-	282	-	+	-	8	8	100
B3+	254	+	-	+	5	5	100
B4+	256	+	+	+	8	8	100
M1	245	+	-	+	26	26	100
M3	286	-	-	+	12	14	86

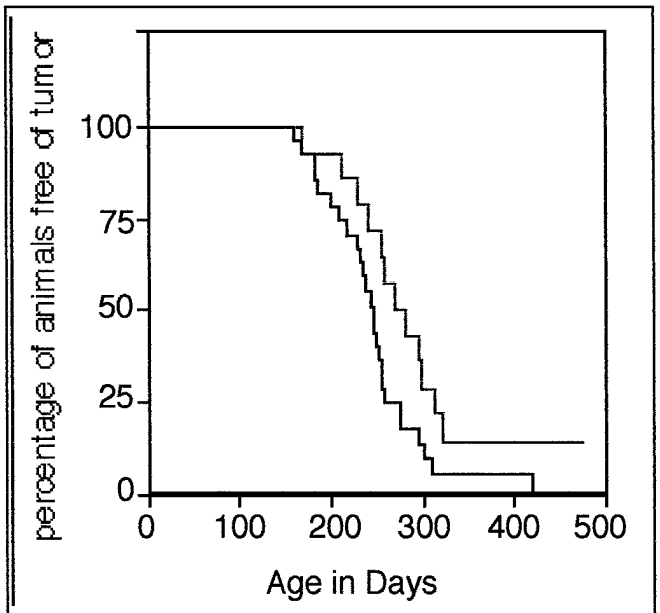

Figure 1. Kaplan-Meier plot showing the rate of tumor development in control C3H mice (MTV+, p53-; red line) and p53 transgenic C3H mice (MTV+, p53+; black line). The median age of death for control mice is 281 days, while that for transgenic mice is 245 days. The difference between the two groups is significant: $\chi^2 = 4.9$; $p<0.05$.

Two separate experiments are being carried out. In the first, (Table 1), the 172H transgene was put onto a C3H background by backcrossing for multiple generations, and then a transgenic male was crossed to a C3H MMTV+ female mouse, which passed MMTV onto the pups throught the milk. We aged a cohort of 26 mice with both MMTV and 172H (Group M1), and one of 14 mice with MMTV alone (Group M3). Group M1 all developed mammary adenocarcinomas, with lateny of tumor development ranging from 166 days to 421 days, and a median latency of 245 days (Figure 1).

Twelve of fourteen (86%) of Group M3 (MMTV alone and no mutant p53 transgene) developed mammary adenocarcinomas, with a median latency of 286 days. Figure 1 shows a Kaplan Meier plot of the latency of tumor development in the two groups. Performance of a log-rank test on these data indicate a statistically significant difference between the two groups (p,0.05).

Histologic analysis failed to reveal any consistent histopathoogical difference between tumors that developed in the M1 and those in the M3 group with the exception that in some M1 tumors, particularly the ones arising with longer latency, there was a striking degree of nuclear atypia and a very high mitotic rate. These preliminary data, if borne out with more thorough analysis, would suggest that additional tumorigenic "hits" are occurring in the longer latency tumors and this results in a more aggressive tumor phenotype. If these "hits" are MMTV insertions, then we can hope to identify them through the cloning of proviral insertion sites.

These tumors are now being analyzed to determine the sites of MMTV proviral insertion, which likely harbor growth regulatory genes that cooperate with p53 in the development of cancer. The identification of these genes will hopefully contribute signficantly to our understanding of mammary tumorigenesis.

In a second cross, we are creating 172H/neu bitransgenic mice with MMTV by mating bitransgenic male FVB mice with C3H MMTV+ female mice. This cross generates FVB x C3H F1 hybrid mice, with either one, neither, or both transgenes, all with MMTV. In a companion cross, we generated similar FVB x C3H F1 hybrid mice that lack MMTV (by using C3H males instead of females). The aging of these cohorts of mice resulted in the development in all of the mice that had either MMTV or ErbB2 transgene, but unfortunately, there was no difference in latency between mice harboring just MMTV compared with mice having both MMTV and the transgenes. Thus it appears that in this experiment, MMTV was the dominant force

driving tumorigenesis, and the presence of the transgenes did not significantly accelerate tumorigenesis relative to this. Thus, it appears unlikely that the analysis of proviral insertion sites in these tumors will reveal genes that have a particular propensity to cooperate with either mutant p53 or ErbB2. The reason why no acceleration of tumorigenesis was seen in mutant p53 + MMTV mice relative to MMTV mice alone (as was seen with Group M3 described above) is not clear. the most likely explanation is the difference in background strain in the mice: M3 is on a C3H background, while Group B3+ is on a mixed FVB x C3H F1 background.

2. Analysis of genomic instability.

In our bitransgenic model (mutant p53 plus ErbB2), we do not observe the emergence of tumors with kinetics that indicate direct and immediate malignant transformation by coexpression of 172H and *neu*: tumors arise following the second pregnancy rather than the first, and are unifocal, indicating the necessity for other events. This is thus distinct from the cell culture results described above [8], and is likely due to several things, including the lower transforming potential of native *neu* relative to Ras, the presence of endogenous p53 alleles in our transgenic mice, as well as other tumor control mechanisms that exist in the intact animal, such as tumor immunity, the inhibitory influence of surrounding tissue, and the requirement for tumor angiogenesis. Nonetheless, the 172H allele accelerates *neu*-induced tumorigenesis, albeit by an unknown mechanism. We present several possible mechanisms that our bitransgenic model will allow us to address. These models are based on the known or suggested functions of p53, which include an effect on apoptosis, on genome stability, and on transcriptional regulation of cell growth regulatory genes.

The data from cell culture experiments described above suggest a direct effect of 172H on tumor cell growth, and such an effect may indeed play an important role in our system. However,

other effects of this allele are also possible. One is that p53-172H increases the likelihood of additional mutational events in genes other than the *neu* transgene in the nonmalignant cells expressing MMTV-*neu*, and thus accelerates tumor formation. One type of genetic alteration known to contribute to mammary tumorigenesis is gene amplification. While an increased frequency of gene amplification is seen in p53 null cells, it is not observed in Li Fraumeni cells (mutated at 184 or 248) that retain one wild-type p53 gene [30] Since our 172H+*neu* bitransgenic tumors appear by Southern blot analysis to retain (a) wildtype copy or copies of p53 (data not shown), this mechanism may not apply to this model. We are currently assessing the frequency of other types of alterations — e.g., deletions, point mutations — in these bitransgenic tumors.

We are taking four approaches to assessing the contribution of the 172H transgene to mutation frequency and chromosomal instability.

1. Assessing aneuploidy over time in non malignant mammary glands from transgenic and nontransgenic mice.
2. Determination of mutation frequency in mammary tissue of transgenic and nontransgenic mice, using as a mutation sensor a lacI indicator gene residing in a lambda phage transgene.
3. Comaparative Genome Hybridization
4. Spectral karyotyping.

Approach One: We have harvested mammary glands from normal, 172H+, neu and 172H+neu+mice at various time points, and have processed these for histologic section. H+E slides have been prepared. We will stain these with Feulgen stain, and use image analysis to quantitate the amount of DNA per nucleus.

Approach Two. In collaboration with Dr. Peter Glazer of the Dept. of Therapeutic Radiology at Yale University, we are creating transgenic mice that contain a mutation sensor, which consists of

a lambda phage transgene that contains a lacI gene. Transgenic genomic DNA from these mice can be packaged using standard lambda packaging extracts to yield infectious lambda phage particles. When these are plated on an appropriate indicator strain of E. Coli, one can assess mutation in the lacI gene through blue-white color selection with X-gal. Through this approach, one can determine the mutation frequency in the DNA of any organ within the transgenic mice. We are crossing the lacI transgenic mice with mice containing the 172H and neu alleles to create bi- or tri-transgenic mice that contain lacI. We are now aging these, and will harvest mammary and liver (control) tissues at various time points before and after tumor formation. DNA will be extracted, and will be packaged and assayed for mutation frequency. This experiment will allow us to determine the mutation frequency in nonmalignant and malignant mammary glands in the different genetic backgrounds, which will allow us to assess the influence of the 172H and neu transgenes on mutation frequency.

Approach Three. To document large gains or losses of DNA in tumors arising in mice bearing neu and/or 172H, we are employing comparative genome hybridization (CGH). In this technique, tumor DNA is fluorescently labeled and hybridized to a metaphase spread of normal mouse chromosomes in the presence of normal DNA labeled with a different fluor. Digital photomicroscopy and image analysis are performed to quantitate the relative intensities of the normal and tumor DNA hybridization to the metaphase chromosomes. In this way, regions of DNA overrepresented (amplified) or underrepresented (deleted) in the tumor DNA can be identified. This project is being performed in collaboration with Dr. Alan Coleman at the NIH.

Approach Four. In collaboration with Dr. Alan Coleman at NIH we are also analyzing tumors for chromosomal abnormalities by spectral karyotyping. In this experiment, metaphase spreads are prepared from tumor cells, and these are hybridized with a fluorescent probe mix, which contains a probe for each mouse

chromosome labeled with a different Fluor. As a result, each mouse chromosome can be distinguished by the emission spectrum of the fluorescence. With this approach, we have documented a chromosomal translocation in a mammary tumor from a mouse containing 172H and neu on a p53+/- background. We are continuing this analysis to determine if the tumors arising in our mice have consistent nonrandom chromosomal abnormalities. Both the number of abnormalities and the identity of the chromosomal region involved will give insight into the degree of genomic instability in mice of different genetic background, and, potentially, the identity of cooperating oncogenic events.

3. Documentation of tumor angiogenesis. An alternative mechanism of *p53*-172H action in this model is that it may promote other aspects of tumor growth, such as tumor angiogenesis. The finding that mutant, but not wild-type, p53 can synergies with PKC to stimulate vascular endothelial growth factor (VEGF) **[22]**, suggests that the 172H allele could stimulate vascular ingrowth, which is known to be a rate-limiting step in tumorigenesis. To investigate this possibility, we are documenting the density of vessels in mammary tissue and tumors of the different genotypes (no transgene, neu alone, 172H alone, and 172H plus neu). This is being done by immunohistochemical staining for CD34, a cell surface protein present on mouse endothelial cells. This staining is highly specific, and allows for straightforward quantitation of microvessel density in mammary glands and tumors of the different genotypes.

4. Assessment of phosphorylation status of Neu in transgenic tissues, both nonmalignant and malignant. One key feature of neu-induced mammary tumorigenesis is the marked increase in Neu phosphorylation that appears to accompany tumor formation. One potential mechanism by which 172H could accelerate tumor formation is to hasten this increase in phosphorylated Neu. To document the timing of this increase in tumor progression, we are assessing the presence of phosphoNeu at different time points leading up to

tumor formation, using a anti-phosphoneu monoclonal antibody that has been well characterized by Dr. Michael DiGiovanna [6, 7], with whom we are collaborating. We have been able to obtain specific staining with low background, and are now embarking on the analysis of tissue sections from a panel of nonmalignant and malignant mammary tissue specimens obtained at different ages in our transgenic mice.

Conclusions. We have created a mouse model for human breast cancer closely mimics the genetic changes that occur in the human disease. Twenty-five to 30% of human breast cancers show amplification and overexpression of *Erb*B2 gene, and of these, many will have point mutations in p53 [15]. The 175H mutation is the most common p53 mutation in human breast cancers, and is often accompanied by loss of the other allele, arguing that it is not simply acting as a dominant negative [37]. Thus, we have created a useful model for the study of human breast cancer. We are now investigating the mechanism by which this acceleration occurs, taking a variety of approaches that address the role of genomic instability, tumor angiogenesis, and phosphorylation of Neu. Through these approaches we hope to contribute to the current understanding of mammary tumorigenesis as it occurs in the setting of a whole organism.

Relationship of Work Accomplished to SOW

Statement of Work

Concise restatement of the research proposal that outlines and establishes the PI/Contractor performance expectations for which the USAMRMC will provide support.

Task 1 The main goals of this grant are to examine cooperation between *p53* and genes encoding components of the class 1 receptor tyrosine kinases (RTKs). The first task is to set up crosses between mice bearing mutant *p53* alleles and the *NDF*, *TGFa*, and *ErbB2* transgenic mice. Also of interest is a general screen for genes involved in mammary tumorigenesis in mice that can cooperate with mutant *p53* alleles. This will be done by retroviral tagging with MMTV, and will be initiated by setting up crosses between *p53* mutants and C3H, to get the mutant alleles onto a C3H background.

(Years 1-3, with the bulk of the tumors being harvested in years two and three)

Task 2. Animals from the above crosses will be genotyped by DNA analysis and aged for tumors. Tumor incidence and latency will be documented. (Ongoing, through years 2-3)

Task 3. Tumors from the above crosses will be harvested and analyzed for histopathologic type, transgene expression at the RNA and protein level, and existence of metastases. In tumors with MMTV infection, DNA will be prepared for Southern blot analysis for retroviral insertions. (Years 2 and 3).

Task 4. Biochemical analyses of the tumors will be pursued by examining protein tyrosine phosphorylation of potential targets of the relevant class 1 RTK targets. This will be done in collaboration with Dr. David Stern's lab. (Year 3-4)

Task 5. Cloning of novel sites of MMTV insertion in the mice with mutant *p53* alleles. The genes at sites of insertion will be identified by lambda cloning of the preintegration locus, followed by exon trapping or cDNA hybrid selection. (Year 3-4).

Regarding Task I: We have completed the cross between the ErbB2 transgenic mice and the p53 mutant transgenic mice and this has been published (ref. 29). We have decided not to pursie the cross between the p53 mutant mice and the NDF transgenic mice, since in our hands, the phenotype observed in the NDF founder transgenics (salivary gland and Harderian gland adenomas and rare mammary adenocarcinomas) did not transmit to the subsequent generations. We have decided not to perform the cross between the p53 mutant mice and TGFalpha mice, since this is being pursued by another lab (that of Dr. J. Rosen). We have completed the cross between the p53 mutant transgenic mice and the MMTV-transmitting mice, and these tumors have been harvested. The histopathologic analysis of these tumors is almost complete.

Regarding Task 2: As described above, the genotyping has been completed. The aging of mice has been performed for the p53 mutant transgenics crossed to ErbB2 and for the p53 mutant transgenic mice crossed to MMTV-transmitting mice; or has been cancelled for the reasons listed in the preceeding paragraph.

Regarding Task 3: We have completed the harvesting and analysis of tumors from the p53 mutant transgenic mice crossed to ErbB2. For the tumors arising in the MMTV positive mice, the tumors have been harvested.. These will be analyzed shortly by Southern blot for the presence of the MMTV genome.

Regarding Task 4: We have completed the biochemical analysis for the tumors that arose in the p53 mutant transgenic/ErbB2 mice. For the other tumors, we have decided to substitute four analyses of genomic instability, as described in the text: 1. Assessing aneuploidy by Feulgen staining; 2. determination of mutation frequency using a lambda phage transgene; 3. comparative genome hybridization; 4. Spectral karyotyping. Given our current hypotheses about the function of mutant p53 in mammary tumorigenesis, we argue that these approaches will be more revealing.

Regarding Task 5: The cloning of novel sites of MMTV insertion in mice with mutant p53 allele has not been started. We will be analyzing over thirty tumors for MMTV hybridization by Southern blot within the next few weeks. Following Southern blot analysis, we will likely commence the cloning of the insertion sites. We have recently had good success with the cloning of Friend murine leukemia virus (F-MuLV) insertion sites using inverse PCR, a rapid and relatively facile cloning strategy.

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