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Mice Expressing a Mammary Gland–Specific R270H Mutation in the \( p53 \) Tumor Suppressor Gene Mimic Human Breast Cancer Development

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Abstract

The tumor suppressor gene \( p53 \) has an apparent role in breast tumor development in humans, as ~30% of sporadic tumors acquire \( p53 \) mutations and Li-Fraumeni syndrome patients carrying germ line \( p53 \) mutations frequently develop breast tumors at early age. In the present study, conditional expression of a targeted mutation is used to analyze the role of the human \( R273H \) tumor-associated hotspot mutation in \( p53 \) in mammary gland tumorigenesis. Heterozygous \( p53^{R270H/+} \) WAPCre mice (with mammary gland–specific expression of the \( p53 \) R270H mutation, equivalent to human \( R273H \), at physiological levels) develop mammary tumors at high frequency, indicating that the \( R270H \) mutation predisposes for mammary gland tumor development and acts in a dominant-negative manner in early stages of tumorigenesis. Spontaneous tumour development in these mice is further accelerated by 7,12-dimethylbenz(a)anthracene (DMBA) treatment at young age. The majority of spontaneous and DMBA-induced carcinomas and sarcomas from \( p53^{R270H/+} \) WAPCre mice is estrogen receptor \( \alpha \) positive, and expression profiles of genes also implicated in human breast cancer appear similarly altered. As such, \( p53^{R270H/+} \) WAPCre mice provide a well-suited model system to study the role of \( p53 \) in breast tumorigenesis and the responsiveness of mammary gland tumors to chemotherapeutics. (Cancer Res 2005; 65(18): 8166-73)

Introduction

Breast cancer is the most frequent tumor type among women worldwide with more than 1,000,000 new cases diagnosed every year (1). Human breast cancer is associated with different somatic alterations such as mutations in oncogenes and tumor suppressor genes, resulting in deregulation or loss of function of multiple, essential genes. The most frequently mutated gene in sporadic breast tumors is the tumor suppressor gene \( p53 \) (2). The frequency of acquired \( p53 \) mutations in primary breast carcinomas is ~30%, with the gene mutation often accompanied by loss of heterozygosity (LOH; ref. 3). Breast tumor progression seems to be associated with mutant \( p53 \), as illustrated by a higher frequency of \( p53 \) mutations in patients with advanced disease. Furthermore, the prevalence of \( p53 \) mutations is higher in recurrent tumors than in the primary ones (4). Finally, specific \( p53 \) mutations are associated with resistance to doxorubicin therapy in breast cancer patients (5). Taken together, these observations imply that acquiring \( p53 \) mutations in breast cancer predisposes to increased tumor malignancy. The role of \( p53 \) mutations early in breast cancer is further supported by the observation that Li-Fraumeni syndrome patients, carrying germ line \( p53 \) mutations, are predisposed to developing breast cancer at a relatively early age (6). Codons R175, R248, and R273 are the most common hotspots for mutations in both sporadic and hereditary \( p53 \) associated human breast cancer (7).

Given the apparent important role of \( p53 \) in preventing breast tumor development in humans, several attempts were made to generate mouse models with defects in \( p53 \) to study mammary gland tumor development. \( p53 \) knockout mice (\( p53^{-/-} \)) were generated and extensively studied (8). Although several important insights were obtained from these studies, \( p53 \) knockout mice did not fully recapitulate the spectrum of tumors found in Li-Fraumeni patients (9). In particular, the breast tumors associated with germ line mutation of \( p53 \) in humans did not arise in \( p53^{-/-} \) mice, and were only observed in heterozygous animals in a specific genetic background (10, 11). This could be due to the fact that in humans, null mutations are rarely found; rather, 50% of human tumors harbor a point mutation in the \( p53 \) gene. Mutated \( p53 \) might have a completely different effect on (breast) tumorigenesis than loss of the gene. In fact, several \textit{in vitro} studies suggest that mutant \( p53 \) has dominant-negative or gain of function properties distinct from \( p53 \) loss of function (12–15). Alternatively, early death due to lymphoma could be masking a phenotype in the mammary gland of \( p53^{-/-} \) mice. Indeed, mammary gland transplantation studies involving \( p53 \) knockout mice (10, 16) showed an increase in tumor burden and incidence after 7,12-dimethylbenz(a)anthracene (DMBA) treatment in combination with hormonal stimulation (16). In addition, epithelium-specific deletion of the \( p53 \) gene in recently developed conditional mouse models resulted in spontaneous mammary tumor development (17, 18). Recently, several strains of mice were reported that harbor targeted mutations of \( p53 \) in the endogenous gene locus (19–25). In particular, two different mouse models for Li-Fraumeni syndrome, with tumor-derived mutations in \( p53 \), were described (19, 20): \( p53^{R172H} \) and \( p53^{R270H} \). In both cases, expression of mutant \( p53 \) in the absence of wild-type \( p53 \) resulted in a shift in tumor spectrum compared with \( p53^{-/-} \) mice, indicative of an \textit{in vivo} gain of function property.
of mutant p53 (19). In addition, dominant-negative effects were observed in mouse embryonic fibroblasts and thymocytes, as was earlier found for heterozygous p53 R270H cells (26). In vivo expression of the mutation in all tissues was achieved using Protamine-Cre transgenic mice, resulting in a broad variety of tumors (19), but hardly any mammary tumors were found. Because one of the strengths of conditional mouse technology is the potential for tissue-specific analyses, we use the p53 R270H model in the present study to analyze the role of the p53 R270H mutation in spontaneous and carcinogen-induced mammary gland tumorogenesis. Expression of the mutation in mammary tissue was achieved by crossing p53 R270H mutant mice with mammary-specific Cre transgenic mice having Cre recombinase under the control of the hormone-inducible Whey Acidic Protein (WAPCre mice; ref. 27). We show that p53 R270H/+ WAPCre mice develop both spontaneous as well as carcinogen-induced mammary tumors at high frequency, indicating that the R270H mutation in p53 predisposes for mammary tumor development in mice.

Materials and Methods

Generation of p53 R270H WAPCre Mice

Conditional p53 R270H mice were generated by homologous recombination of mutant targeting vectors in J1 embryonic stem cells (19). Cloning of the p53 R270H mutant targeting vector and homologous recombination experiments in embryonic stem cells were described elsewhere (19, 26). The targeting vector with the stop cassette in intron 1 flanked by loxP sites in codon 270 of the p53 gene: 1156, 5'-TTGGGCTTAGGGACGTCTCTTATC-3' (intron 9).

The resulting PCR product (486 bp) was digested with NcoII. The p53 R270H mutation results in a new NcoII site in the PCR product resulting in two products of 269 and 217 bp, discriminating the mutant from the wild-type allele.

Mice expressing Cre recombinase under the control of a Whey Acidic Protein promoter, WAPCre mice, were used to induce Cre-mediated deletion of the floxed stop cassette specifically in the mammary gland (26). WAPCre mice [B6;129-Tg(N-WAPCre)11738Samm, in a mixed 129Sv/C57BL/6 background] were obtained from The Jackson Laboratory (Bar Harbor, ME). The presence of Cre recombinase was determined by PCR (product size, 676 bp) using the following primers: Cre 3, 5'-GCTGGGCGTGTTGCAATGTC-3; Cre 5, 5'-GTTACCGAGGACCGACGA-3'.

For all studies described, heterozygous p53 R270H mice and wild-type littermates in backcross generation F2-F3 (129Sv/J, C57BL/6) were crossed to WAPCre mice to generate respectively p53 R270H/+ WAPCre and p53 R270H WAPCre mice. Mice used in these experiments contained an estimated C57BL/6 allele contribution of 75% to 81.25%. Subsequent mating of female mice resulting in pregnancy and lactation of the litters was necessary for activation of the WAP gene promoter in the mammary gland of the dams, resulting in expression of Cre recombinase.

In Vivo Expression of the p53 R270H Mutant Allele

Female p53 R270H WAPCre mice (8-12 weeks old) were mated with p53 R270H+/+ mice. After either one or two pregnancies, followed by lactation of the litters, the mammary gland of the dams was isolated, snap-frozen in liquid nitrogen, and stored at -80°C. RNA isolation of tissues was done following the procedure of the manufacturer (Qiagen, Valencia, CA). The following primers were used for cDNA synthesis and the first PCR reaction (Titan One Tube RT-PCR system; Roche, Indianapolis, IN), amplifying exons 7 to 11 of the p53 gene: 1156, 5'-TTGCGCCACAGCGTGTTGACC-3'; 1157, 5'-AGAAGGGCAGCGGAATC-3'.

A second PCR reaction, amplifying exons 7 to 8, was done with nested primers: 1150: 5'-tgtaaaacgagccgcttagtattgtggaccca-3'; 1045, 5'-caggaaacagctatgacc-3'. The resulting PCR product (486 bp) was digested with NlaIII digestion to determine the presence of the p53 R270H allele specifically in the mammary gland, were exposed to the same protocol.

Expression of the R270H point mutation was determined in the obtained PCR product of 333 bp by digestion with NcoII. Digestion of wild-type p53 resulted in four products of 239, 67, 18, and 9 bp, whereas in the presence of the R270H mutation, five products are generated (157, 82, 67, 18, and 9 bp).

Sequencing. After purification of the second PCR product using the QiAquick PCR purification kit (Qiagen), DNA concentrations were determined. PCR product, 3 to 10 ng, was amplified in a subsequent sequence PCR reaction with the BigDye Terminator Reaction Kit (Applied Biosystems, Foster City, CA) and 3.2 pmol of primer 1150 or 1045 (described above) in the forward or reverse sequence reaction, respectively. Sequence analysis was done on a 3700 Genetic Analyzer (Applied Biosystems) using Sequencing Analysis 3.7 software.

Analysis of Spontaneous and 7,12-dimethylbenz(a)anthracene–induced Mammary Gland Tumor Development

Spontaneous mammary tumor development was determined in groups of female p53 R270H WAPCre mice and p53 R270H WAPCre littermates receiving 0.1 mL sunflower oil by gavage weekly for 6 weeks. Heterozygous p53 R270H WAPCre mice (17), crossed with WAPCre mice to delete exons 2 to 10 of one p53 allele specifically in the mammary gland, were exposed to the same protocol and used as control animals in this experiment.

In the DMBA exposure study, 4-week-old p53 R270H WAPCre mice and p53 R270H WAPCre littermates were treated once a week during 6 subsequent
weeks by gavage with 1 mg DMBA (Sigma, St. Louis, MO) dissolved in 0.1 mL sunflower oil. After 6 weeks of treatment, females were bred to activate the WAPCre gene and, consequently, the p53R270H mutation. All mice were weighed weekly and checked for the development of tumors until the age of 78 weeks. Moribund animals or those with visible tumors were killed as well as the surviving mice at the end of the experiment. Tumors and tissues were collected and processed for histopathology and DNA/RNA isolation following standard procedures.

**Histology and Immunohistochemistry**

Collected tissues were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde (10% neutral buffered formalin). The tissues were embedded in paraffin wax, sectioned at 5 μm, and stained with HE and for histopathologic evaluation. p53 protein accumulation was detected using the polyclonal CM5 antibody (1: 400; Santa Cruz Biotechnology, Santa Cruz, CA) using the same protocol as described for CM5. For antigen retrieval, polyclonal antibody MC-20 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently a streptavidin-peroxidase complex peroxidase Elite kit (Vector Laboratories, Burlingame, CA) were used. Immunohistochemical staining of p53 was done as described earlier (28) using a secondary goat anti-rabbit/biotin antibody (Vector Laboratories, Burlingame, CA), which recognizes several epitopes of both wild-type and mutant mouse p53 protein. CM5 immunostaining was done to control for hybridization efficiency.

**Molecular Analysis of Tumors and Tissues**

**Expression of the R270H point mutation.** Expression of the point mutation was determined as described above in mammary glands, tumors, and control tissues of both unexposed and DMBA-exposed p53R270H/+WAPCre females.

**Determination of loss of the wild-type p53 allele (loss of heterozygosity) in mammary tumors.** Both PCR (as described above) and Southern blot analysis were done to detect loss of the wild-type p53 allele. Genomic DNA was isolated from mammary tumors following standard procedures (Maniatis Laboratory Manual). After restriction with the Msel restriction enzyme and electrophoresis, DNA was transferred to nylon membranes (Hybond-N+, Amersham, Piscataway, NJ). A cDNA probe of 343 bp (SacII to Xpol fragment) spanning exons 7 to 10 of the p53 gene was randomly labeled with [32P]dCTP and used for hybridization (29). The size of the fragment was 2,976 and 1,496 bp for p53 wild-type allele. Genomic DNA was isolated from mammary tumors following standard procedures (Maniatis Laboratory Manual). After restriction with theSacI and KpnI restriction enzyme and electrophoresis, DNA was transferred to nylon membranes (Hybond-N+, Amersham, Piscataway, NJ). A cDNA probe of 343 bp (SacII to Xpol fragment) spanning exons 7 to 10 of the p53 gene was randomly labeled with [32P]dCTP and used for hybridization (29). The size of the fragment was 2,976 and 1,496 bp for p53 wild-type allele. Genomic DNA was isolated from mammary tumors following standard procedures (Maniatis Laboratory Manual). After restriction with the SacI and KpnI restriction enzyme and electrophoresis, DNA was transferred to nylon membranes (Hybond-N+, Amersham, Piscataway, NJ). A cDNA probe of 343 bp (SacII to Xpol fragment) spanning exons 7 to 10 of the p53 gene was randomly labeled with [32P]dCTP and used for hybridization (29). The size of the fragment was 2,976 and 1,496 bp for p53 wild-type allele. Genomic DNA was isolated from mammary tumors following standard procedures (Maniatis Laboratory Manual). After restriction with the SacI and KpnI restriction enzyme and electrophoresis, DNA was transferred to nylon membranes (Hybond-N+, Amersham, Piscataway, NJ). A cDNA probe of 343 bp (SacII to Xpol fragment) spanning exons 7 to 10 of the p53 gene was randomly labeled with [32P]dCTP and used for hybridization (29). The size of the fragment was 2,976 and 1,496 bp for p53 wild-type allele.

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**Analysis of Gene Expression Profiles in Mammary Glands and Mammary Tumors**

Macroarray analysis of gene expression profiles in normal mammary glands and mammary tumors was done using two different GEArray Q series kits (i.e., the Mouse Cell Cycle Gene Array and the Mouse p53 Signaling Pathway Gene Array kit (SuperArray Biosciences, Frederick, MD). For this analysis, three to four RNA samples of different tumors isolated from animals with the same genotype and treatment protocol were pooled.

Pooled total RNA (3.5 μg) was used as a template for [32P]dCTP DNA probe synthesis. Subsequently, the probe was hybridized overnight to membranes containing 96 gene-specific cDNA fragments supplemented with four housekeeping genes (GAPDH, cyclophilin A, RPLA13A, and actin) as positive controls, and pUC18 plasmid DNA and blank spots as negative controls. Analysis of the spots was done by scanning the membranes on a PhosphorImager/Storm 860 (Molecular Dynamics, Sunnyvale, CA) and quantifying the spots using the TotalLab program version 2.00 (Nonlinear Dynamics, Durham, NC).

**Statistical Analysis**

Statistical analyses of tumor-free survival curves included calculation of Kaplan-Meier distributions of survival of two different treatment groups and comparison by a two-sided log-rank test (SPSS version 11).
mammary tumor development was 8.5 ± 4.7 weeks and significantly shorter compared with untreated p53R270H/+ WAPCre mice (Kaplan-Meier, P = 0.0046). Evidently, exposure to DBA at an early age accelerates mammary tumor development in p53R270H/+ WAPCre mice. Other tumors that occurred in the DMBA-treated mice included lymphomas, ovary, lung, stomach, uterus, and skin tumors. A comparable DMBA-induced tumor spectrum was found in treated p53R270H/+ WAPCre mice (Fig. 3B), however, the latency time for (mammary) tumor development in DMBA-treated p53R270H/+ WAPCre mice was significantly decelerated compared with treated p53R270H/+ WAPCre mice (Kaplan-Meier, P = 0.047).

Table 1. Tumor spectrum in p53R270H/+ WAPCre mice

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Untreated</th>
<th>DMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of mice analyzed</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Tumor-bearing mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammary gland tumor</td>
<td>7 (64%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>Carcinoma*</td>
<td>3/7</td>
<td>2/4</td>
</tr>
<tr>
<td>Sarcoma/carcinosarcoma†</td>
<td>4/7</td>
<td>2/4</td>
</tr>
<tr>
<td>Intraepithelial neoplasia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mammary gland hyperplasia</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Ovary/uterus</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Other†</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Mean mammary gland tumor latency (wk)</td>
<td>19.7 ± 8.6</td>
<td>8.5 ± 4.7*</td>
</tr>
</tbody>
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*Carcinomas could be classified in adenocarcinomas, solid carcinomas, papillary carcinomas, and adenosquamous carcinomas (as classified in

Figure 3. Development of 7,12-dimethylbenz(a)anthracene–induced mammary tumors in p53R270H/+ WAPCre mice. Mammary tumors found in both untreated and DMBA-exposed p53R270H/+ WAPCre mice were heterogeneous, including the frequently found adenocarcinomas, solid carcinomas, papillary carcinomas, and adenosquamous carcinomas (as classified in

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*Carcinomas could be classified in adenocarcinomas, solid carcinomas, papillary carcinomas, and adenosquamous carcinomas.† Sarcomas found consisted of carcinosarcomas and fibrosarcomas.† Other tumors were found in lung (bronchioalveolar carcinoma), abdominal cavity (sarcoma), stomach (squamous cell papilloma and carcinoma), and skin (sebaceous cell adenoma, squamous cell papilloma, and carcinoma).

† Mean latency time is depicted as the number of weeks after expression of the p53R270H point mutation (t = 0 at birth of the litters) was statistically significantly reduced when compared with untreated mice (Kaplan-Meier analysis, P = 0.0046).
ref. 30). Carcinosarcomas and sarcomas of the mammary gland were also found (Fig. 4A–F). In the majority of mammary tumors, the number of p53 positive cells was greatly increased compared with normal mammary glands of young p53 mutant mice (Fig. 4G–I), suggesting these tumors originated from a clonally expanded p53R270H mutant epithelial cell. These results were confirmed by molecular analysis of mammary tumors using reverse transcription-PCR (RT-PCR). All, except one, mammary tumors were found to express the point mutant p53 allele (data not shown), indicating that WAPCre-induced expression of the R270H mutant protein is linked to mammary tumor development.

Estrogen is thought to be play an important role in the pathogenesis of human breast tumors, with the estrogen receptor α acting as mediator of estrogen responsiveness in human breast cancer (31). Unfortunately, most mouse models of breast cancer exclusively develop estrogen receptor α–negative tumors (32). However, in our study, 67% (10 of 15) of the mammary tumors obtained from untreated as well as DMBA-treated p53R270H/+ WAPCre mice appeared estrogen receptor α positive to varying degrees (Fig. 4f–I). The group of estrogen receptor α–positive tumors consisted of both carcinomas as well as sarcomas, and the grade of estrogen receptor α positivity seemed to be not associated with a specific mammary tumor or cell type.

Molecular analysis of spontaneous and 7,12-dimethylbenz(a)anthracene–treated tumors. In human breast cancer, p53 mutations are frequently accompanied by LOH (3). Here, the majority (6 of 7, 86%) of mammary tumors from p53R270H/+ WAPCre mice showed evidence of LOH at the p53 locus (Fig. 5A), ranging from 8% (lane 3) to 46% loss (lane 8). Samples with only a partial loss of the wild-type allele may result either from LOH in a subset of tumor cells or from infiltration of the tumor with wild-type stromal cells. Only tumor #3 almost completely retained the wild-type fragment (8% loss). These observations indicate a selective advantage for p53 LOH (late) in mammary tumor development in this model.

In humans, various breast tumor subtypes harbor distinct profiles of gene expression that are likely to reflect basic differences in the cell biology of the tumors (33, 34). Here, differences in gene expression profiles between normal mammary gland versus mammary tumors and spontaneous versus DMBA-induced mammary gland tumors were analyzed by macroarray analyses, focusing on cell cycle and p53-signaling genes. A selection of the analyzed genes is shown in Fig. 5B. Clearly, gene expression profiles change during mammary tumor development in both DMBA-treated as well as untreated animals, with a high number of genes down-regulated in mammary tumors. For instance, cyclin G, p57Kip2, and cyclin E levels are significantly lower in tumors of both untreated as well as DMBA-exposed p53R270H/+ WAPCre mice compared with normal mammary glands. Up-regulation in tumors is detected for the cell proliferative marker Ki67, and cyclin D1, a breast tumor-related oncogene, abnormally accumulated in up to 35% of human breast cancer cases (35). For these and other genes like GADD45, cyclin D3, and Noxa, differences in gene expression are dependent on DMBA treatment (Fig. 5B), indicating that differences exist in gene expression profiles of mammary gland tumorigenesis in untreated and DMBA-treated p53R270H/+ WAPCre mice.

Discussion

In the present study, we analyzed development of mammary tumors in a conditional mouse model with the p53R270H missense mutation, equivalent to human R273H, targeted into the genome of the mouse. Importantly, the wild-type and mutant p53 proteins are

![Figure 4. Histologic characterization of mammary tumors. Primary mammary tumors from p53R270H/+ WAPCre mice show various histopathologic features, including acinar carcinoma (A), adenocarcinoma (B), adenosquamous carcinoma (C), papillary carcinoma (D), carcinosarcoma (E), and sarcoma (F). Carcinosarcomas were characterized by both malignant epithelial components and poorly defined malignant stromal components. The sarcomas found were poorly differentiated tumors of mesenchymal origin. Molecular phenotypes of mammary tumors were determined using CM5 (p53) and MC-20 (estrogen receptor α) stainings. Examples of p53– and estrogen receptor α–positive tumors are a papillary carcinoma showing positive epithelial lining (G and I), carcinosarcoma with positive epithelial and mesenchymal staining (H and K), and sarcoma (L and I) with positive mesenchymal cells. Objective used for all pictures is 20×. Insets, stained cells (arrows) at a higher magnification (40×).](cancerres.aacrjournals.org/.../fig4.jpg)
Mammary Tumor Development in p53.R270H/WAPCre Mice

Figure 5. Molecular analysis of spontaneous and DMBA-induced mammary tumors. A, LOH analysis by Southern blotting in spontaneous mammary tumors (1, 2, 5, 7, 8, 10, and 11) of p53R270H/WAPCre mice. Msil-restricted tumor DNA was hybridized with a p53 cDNA probe. Kidney DNA of the same mouse was used as control DNA (2, 4, 6, 9, and 12). LOH was quantified by calculating the percent loss of the wild-type allele in a mammary tumor by using kidney as an internal control for hybridization efficiency (mean loss in kidney, 34.7%). Percent LOH in mammary tumors is 40% (lane 1), 8% (lane 3), 40% (lane 5), 46% (lane 7), 43% (lane 9), 23% (lane 10), and 41% (lane 11). B, RNA expression levels of cell cycle– and p53 signaling–related genes were determined in mammary glands and mammary tumors from untreated and DMBA-treated mice. Changes in the relative expression level of the different genes were determined in tumors and normalized to the expression level in normal mammary glands. A selection of down-regulated and up-regulated genes is depicted in bars for both spontaneous (striped bars) and DMBA-induced tumors (filled bars).

expressed at physiologic levels (19). Heterozygous p53R270H/WAPCre mice developed a high frequency of spontaneous mammary tumors after tissue-specific expression of the p53.R270H mutation using WAPCre mice. The mean latency time for tumor development in p53R270H/WAPCre mice was strongly reduced when compared with previous studies with tissue-specific transplantation models (16), even though the BALB/c mouse strain used in the earlier studies is much more sensitive for mammary tumor development than C57BL/6 / 129 mice (11, 36), the genetic background of mice used here.

We here show that heterozygous loss of p53 in p53R270H/WAPCre mice does not predispose for spontaneous mammary tumors up to 48 weeks after Cre expression, whereas, at this time point, 64% of p53R270H/WAPCre mice had developed one or more mammary gland tumors (Fig. 3A). These observations clearly show that the p53.R270H mutation may have dominant-negative properties. In addition, comparison of mammary tumor development in p53R270H/WAPCre mice with p53R270H/F2–/– and p53R270H/F2–/– mice reveals that latency times are not significantly different between the three genotypes (P = 0.86). These results point towards a lack of gain of function mechanisms of the p53.R270H mutant in the mammary gland. Interestingly, because in a previous study Olive et al. (19) did show gain of function properties of this p53 mutant allele mainly in the lung using the same mouse model, gain of function properties of p53 apparently are tissue specific. In summary, mammary tumor development in p53R270H/WAPCre mice is evidently not simply caused by functional inactivation of one p53 allele, but rather shows functional inactivation of both alleles through dominant-negative action of mutant p53, as also described previously for these mice (19). Further indication for this was provided by LOH analysis of spontaneous p53R270H/WAPCre tumors. Although we observed the majority of mammary tumors (partly) losing the wild-type p53 allele, the wild-type fragment could still be detected, indicating that a proportion of tumor cells retained the wild-type p53 allele. We therefore hypothesize that the p53.R270H mutation has a dominant-negative effect in early stages of mammary tumorigenesis, resulting in overall genome instability, followed by LOH later in tumor development. However, to address this, further studies are needed in analyzing LOH in preneoplastic stages of mammary gland development in p53R270H/WAPCre mice.

Tumor types found were adenocarcinomas and (carcino)sarcomas, the former also frequently found in human breast cancer patients, the latter uncommon in mammary glands of mice and man. In general, sarcomas are often found in p53+/– mice (8) and Li-Fraumeni syndrome patients (9), but no sarcomas of the mammary gland were reported thus far. However, (carcino)sarcomas of the mammary gland were found recently in conditional p53F2/–/– WAPCre mice, with homozygous p53 deletion of exons 2 to 10 in epithelia, pointing towards a role for defective p53 in mammary sarcoma development.

Apart from mammary gland tumors, some untreated p53.R270H mutant mice developed additional tumors. Expression of the p53.R270H mutation seemed to be not exclusively restricted to the mammary gland, but was also visible in some tumors and kidneys of older mice. This is most likely caused by some Cre expression in other tissues than the mammary gland, as was also described by others (18, 27). Another possibility is minor leakiness of the stop cassette, resulting in low levels of transcription of the mutant p53 allele in some tissues when mice age.

Steroid status is one of the main differentiating characteristics of human breast cancer. About 70% of human breast tumors are estrogen receptor α positive and estrogen receptor dependent; however, mouse models rarely produce estrogen receptor α–positive mammary tumors (32). Therefore, it would be highly valuable to have mouse models developing estrogen receptor α–positive as well as estrogen receptor α–negative mammary tumors to study the factors that control estrogen receptor α expression and the effect of therapeutics. In the present study, mammary tumors of both estrogen receptor types were found in p53R270H/WAPCre mice, with the pattern of estrogen receptor α expression (groups of contiguous cells) similar to that found in human breast carcinomas (37). These results resemble those reported recently (18), where homozygous conditional deletion of mouse p53 in mammary epithelial cells by WAPCre also led to estrogen receptor α–positive mammary tumors in mice. Apparently, inactivation of p53 induced by WAPCre expression, either through complete loss of both alleles or expression of mutant variants, directs estrogen receptor α–positive tumor development.

It is generally agreed that environmental factors and somatic genetic events are the predominant contributors to the development of sporadic cancer. Whether exposure to environmental compounds also has a significant effect on cancer development in the presence of an inherited, dominant mutant p53 allele was examined by exposing p53R270H/WAPCre mice to the mammary carcinogen DMBA. Others have shown cooperation of DMBA with

5 W.P. Wijnhoven et al., preliminary results.

6 Jos Jonkers, personal communication.
induced mammary tumors, with estrogen receptor differences were observed between spontaneous and DMBA-treated mice (data not shown), indicating that age at the time of exposure is a significant factor in mammary tumor development. No histologic differences were observed between spontaneous and DMBA-induced mammary tumors, with estrogen receptor α–positive and –negative tumors found in all groups. A small difference was found in the grade of estrogen receptor positivity: DMBA-induced tumors stained slightly more positive (data not shown).

Expression profiles of specific genes seemed to change during mammary tumor development in both DMBA-treated as well as untreated mice. Interestingly, the breast tumor–related oncogene cyclin D1 is specifically induced in DMBA-treated tumors, in line with previous results obtained with DMBA-treated rats (38, 39). In contrast, other cyclins are down-regulated, which was unexpected because expression levels of these genes are frequently induced in mouse mammary tumors (40). The clearest example for this is cyclin G. Presumably, this reflects the p53 dependence of this gene because we showed before that p53.R270H embryonic stem cells display diminished activation of cyclin G after γ-irradiation compared with wild-type cells (26). As a result, in mammary gland tumors solely consisting of p53 mutant cells, levels of cyclin G will be much lower compared with noncancerous mammary glands also containing cells that do not express the R270H mutant protein. Indeed, comparing expression profiles of p53-mutant mammary glands with those of wild-type littermates reveals cyclin G and other genes differentially expressed, underscoring the p53 dependency of these genes and the disturbance of RNA expression levels in R270H mutant mammary glands.

Differences exist in gene expression profiles in tumors obtained from untreated and DMBA-treated p53.R270H/+WAPcre mice, limited to a few selected genes like GADD45, cyclin D1 and cyclin D3, K67, and Noxa. The levels of GADD45, a well-known DNA damage and p53 responsive gene, were slightly induced in untreated tumors, whereas in DMBA-induced tumors levels were decreased. This difference may well be explained by the role GADD45 is playing in nucleotide excision repair, a repair system recognizing DMBA-induced adducts (41). Levels of GADD45 in normal mammary glands might be higher in DMBA-treated than in untreated mice to facilitate DNA repair in response to the introduction of DNA damage. Indeed, expression levels of GADD45 are twice higher in mammary glands of DMBA-treated mice compared with those of untreated p53.R270H/+WAPcre mice (data not shown), explaining the relative difference of GADD45 levels in tumors. Another explanation could be the selective proliferation of cells with low repair capacity in preneoplastic lesions. Clearly, these primary expression analyses reveal unique signatures for p53 mutant and carcinogen-induced mammary tumors. In addition, we found some human relevant breast cancer genes up-regulated in mammary tumors of p53.R270H/+WAPcre mice (i.e., cyclin D1, a breast tumor related oncosine found up-regulated in 35% of human breast cancers, and Ki67, frequently coexpressed with estrogen receptor α in estrogen receptor α–positive human tumors). These results, although the number of genes analyzed is low, are interesting, in that they show that molecular events underlying mammary gland tumor development in the p53.R270H/+WAPcre mouse model may be, at least to some extent, similar to those occurring in human breast cancer development. However, to identify the cooperating oncogenic events in the development of mammary tumors in p53.R270H/+WAPcre mice, a genome-wide analysis of both spontaneous and DMBA-induced tumors needs to be done.

In conclusion, conditional knock-in mouse models (as the p53.R270H mutant), with mutations equivalent to those found in humans targeted into the mouse genome, show tumor responses and tumor types highly comparable to human cancer. As such, these models are very suitable to establish precise genotype–phenotype relationships between p53 hotspot mutations found in humans and tumorigenesis in specific tissues like the breast. Ultimately, these highly relevant mouse models can be used to study the effectiveness of novel cancer therapies.

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