ABSTRACT

Preneoplastic lesions in the mammary gland represent a population of cells at increased risk of progression to tumors. Because p53 is the most commonly mutated gene in human breast cancer, we sought to determine whether mutations in p53 were present in preneoplastic lesions or were acquired during progression to overt tumors. In the mouse mammary gland, hyperplastic alveolar nodules (HAN) are the most common preneoplastic lesion. Analysis of the TM series of transplantable murine HAN outgrowths and tumors allowed the status of p53 to be determined at distinct stages of mammary tumorigenesis. Alterations in the p53 gene or in the pattern of p53 expression were observed in all five HAN outgrowth lines examined. Altered expression of p53 protein was detected in 3 of 5 TM HAN outgrowth lines as determined by immunohistochemistry. Overexpression of nuclear p53 was detected in only a fraction of the cells (10-50%) in TM3 and TM4 HAN outgrowths, whereas in tumors that arose from TM4 HAN outgrowths, the proportion of cells overexpressing p53 protein approached ~100%. Despite overexpression of p53 in TM3 HAN outgrowths, no tumors have developed in this line. The TM9 outgrowth line exhibited a different pattern of p53 expression by immunohistochemistry: p53 protein was overexpressed in the cytoplasm of virtually all cells in the HAN outgrowths but was localized to the nuclei of TM9 tumor cells. Direct sequencing of p53 transcripts from tumors and cell lines revealed various genetic changes: point mutations in exons 4 and 5 (TM2H, nonsense; TM4, missense); a deletion in exon 5 (TM4); and an insertion in exon 7 (TM3). Although p53 protein was overexpressed in TM9 tumors, it was shown to be wild-type both by immunoprecipitation and direct sequencing of the entire coding region of the cDNA. TM4 cells were homogeneous with respect to mutant p53 genotype and uniformly expressed p53 by immunohistochemical staining in vitro, but transplantation of TM4 cells to fat pads of BALB/c hosts resulted in HAN outgrowths in situ in which ~95% of the cells expressed the mutant p53 at detectable levels. In summary, mutation of the p53 gene and overexpression of p53 protein can occur in preneoplastic mammary epithelial cells, and those mutations are maintained in tumors that arise from the HAN. It appears that mutation of p53 conferred a biologically relevant growth advantage to cells in vivo. Overexpression of p53 in one outgrowth line in the absence of mutations in the coding sequence suggests the presence of cellular factors that can enhance accumulation of wild-type p53 protein. Conversely, expression of mutant p53 was decreased when cells were grown in vivo, implicating the presence of cellular factors that can suppress p53 expression in vivo. These observations demonstrate that the p53 pathway may be a common target for mutation in murine mammary tumorigenesis.

INTRODUCTION

It is necessary to identify the initiating events in breast cancer to design effective strategies for prevention and treatment. The lesions associated with proliferative breast disease represent the earliest identifiable intermediates in breast tumor development (1, 2). These lesions are preneoplastic in that their presence increases a woman’s risk of developing subsequent breast cancer; however, these lesions are not irreversibly committed to a pathway of tumorigenesis because only a small proportion actually progress to tumors. The occurrence of proliferative breast disease may, in part, be genetically determined (3). Although the importance of preneoplastic lesions in human breast cancer has long been recognized (4), their involvement in the development of breast cancer has not been fully characterized.

The association of preneoplastic lesions with the development of breast cancer has been most clearly documented in mice (5). Development of mammary tumors in mice is presaged by a prevalence of hyperplastic lesions in susceptible strains as compared to resistant strains, suggesting a genetic component (6-8). These lesions also increase in number as mice age. The most common preneoplastic intermediates have been termed HAN. The biological features of HAN that distinguish them from normal mammary epithelium include: (a) extensive alveolar development in virgin mice; (b) immortal life span, in that they can be serially transplanted to syngeneic hosts for indefinite periods; and (c) increased risk of tumor formation (9). Because HAN can be propagated in vivo by serial transplantation, the transplantable HAN outgrowths provide a reproducible source of preneoplastic tissue for analysis of genetic changes. These attributes make the murine mammary gland amenable to analysis of the genetic basis for neoplasia.

Insights into the genetic basis of breast cancer have been afforded by the identification of frequent losses of heterozygosity from human chromosome 17p (10-12) and the identification of p53, located on chromosome 17p, as the most commonly mutated cellular gene in human breast cancers (13, 14). In general, overexpression of p53 protein is an indication that it has suffered mutations in evolutionarily conserved regions of the polypeptide (15, 16). Mutant p53 proteins may have longer half-lives than the wild-type protein (17), resulting in increased cellular concentrations of p53 that become detectable by IHC (18, 19). Missense mutations within conserved regions II through V (15) cause the p53 protein to lose its wild-type conformation, detected by the PAb246 antibody, and acquire a mutant-specific epitope recognized by the PAb240 antibody (20, 21). In breast carcinomas that react positively for p53 by IHC, point mutations that result in amino acid substitutions are most common (11, 14, 18, 19). Deletions and truncations of the p53 protein are observed less frequently (22, 23).

The possibility that mutation of p53 can serve as an initiating event in breast cancer is suggested by the presence of germ-line mutations of p53 in cancer-prone Li-Fraumeni families in whom breast cancer is prevalent (24, 25) and by susceptibility to many types of tumors in mice rendered nullizygous for p53 by homologous recombination (26). Overexpression of p53, however, is a common feature of high-grade breast tumors and appears to be an indicator of poor prognosis (27-31). Therefore, it is unclear whether mutation of p53 coincides with establishment of preneoplastic breast lesions or whether p53 becomes mutated during progression to aggressive tumors.

Transplantable murine HAN outgrowth lines offer an opportunity to monitor sequential changes in p53 by analyzing both preneoplastic lesions and tumors that develop from them. A series of transplantable HAN outgrowth lines was developed recently (32). These HAN out-
growth lines, designated TM2H through TM10, vary in tumor incidence. In addition, mammary epithelial cell lines have been established in vitro from these in vivo HAN outgrowths. The cell lines are preneoplastic in that they form characteristic HAN outgrowths when transplanted into fat pads of BALB/c hosts. Establishment of the HAN outgrowth lines from primary cultures of mammary epithelial cells has been reported previously (32). Briefly, mammary glands were removed from 10-12-week-old virgin BALB/c female mice, the glands were enzymatically dissociated, and the cells were grown in collagen for 2-6 weeks. The cells were removed from collagen and were plated into plastic flasks in medium containing Dulbecco’s modified Eagle’s medium/F12 (1:1), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 μg/ml insulin/meal, 5 ng of epidermal growth factor/ml, and 2% adult bovine serum and were designated FSK1 through FSK12 (Table 1). Cells were passaged until sufficient cells were available for injection into mammary fat pads of BALB/c mice that had been cleared of host epithelium. The resulting alveolar outgrowths, designated TM2H through TM10, were maintained in vivo by serial transplantation of 1-mm³ pieces of HAN outgrowth. Tissues from two separate donors were transplanted into the inguinal fat pads of female BALB/c mice (10 recipient fat pads for each donor) every 8-10 weeks. Some animals bearing outgrowths were not used as donors but were maintained for 12 months or until tumors developed. These latter tissues were used for biochemical and immunohistochemical analyses. Cell lines were established from the TM HAN outgrowths as described previously (32). The origin of each TM HAN outgrowth line and tissue culture cell line is detailed in Table 1.

IHC. Tissues were excised, placed in 10% neutral buffered formalin for 6 h, rinsed with water, and placed in 70% ethanol until processed. The tissues were embedded in paraffin, and 5-μm sections were placed on Probe-On Plus slides (Fisher Scientific, Houston, TX). The sections were processed for immunohistochemical staining using the capillary gap method (33). The sections were incubated for 15 min with CMS antisera diluted 1:100. This antiserum was produced essentially as described by Midgley et al. (34), except that the antigen was murine p53 expressed in bacteria. Specific immune complexes were visualized with 4-chloronaphthol as the substrate for horseradish peroxidase.

Sequencing of RNA-PCR Products. Total RNA was isolated from tissues using RNeasy (QIAGEN). RNA extracts were treated with DNase to ensure the absence of DNA contamination. Total RNA (0.5 μg) was reverse transcribed and then used as template for amplification by PCR with specific primers. The PCR products were electrophoresed on 0.8% agarose gels. The bands were excised and purified using the QIAquick Gel Extraction Kit (QIAGEN). DNA sequencing was performed by the Nucleic Acids Core Facility in the Institute for Molecular Genetics at Baylor College of Medicine using fluorescently labeled primers (44). For direct sequencing, the complete p53 cDNA was amplified with either primers 34A and 27B (961-base pair product) or 21B and 30B (918-base pair product). The 5’ ends of the coding-strand primers (34A and 21A) were biotinylated (Institute for Molecular Genetics) to allow capture of single-stranded DNA for sequencing as described by Gibbs et al. (45). Briefly, 8 μl of Dynabeads M-280 Streptavidin (Dynal Inc., Great Neck, NY) were used to capture the coding strand in a 100-μl reaction volume. The complementary strand was eluted after denaturation with 225 μl of 0.15 N NaOH. The dideoxy chain termination method of sequencing (46) was carried out using Sequenase Version 2.0 (United States Biochemicals, Cleveland, OH) with the following primers at 0.5 pmol/reaction: 30B; 21B; 27B; 2b; 29B. The reaction products were separated on 7% sequencing gels run at 65 W constant power in 90 mm Tris-86 mm H₂BO₃-2.5 mm Na₂EDTA, pH 8.5 (47). The gels were fixed and then exposed to XAR5 film. The nucleotide sequences were compared to wild-type p53 cDNA (48) using software provided by the Molecular Biology Computational Resource (49).

RESULTS

Expression of p53 Protein. TM2H outgrowths completely filled the fat pads with uniform hyperplasias and frequently formed tumors (62.5% tumor incidence; Table 3). All of the TM2H HAN outgrowths and tumors were negative for p53 expression when analyzed by IHC. Neither was overexpression detected when tumors were analyzed by immunoprecipitation-immunoblotting (0 of 8 tumors analyzed; Table

<table>
<thead>
<tr>
<th>Progenitor cell line</th>
<th>Treatment of progenitor cells</th>
<th>FSK cell passage at origin of HAN line</th>
<th>HAN outgrowth line</th>
<th>HAN transplant generation at origin of TM cell line</th>
<th>Cell line derived from HAN outgrowth</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSK2</td>
<td>DMBA</td>
<td>2</td>
<td>TM2H</td>
<td>9</td>
<td>TM2H</td>
</tr>
<tr>
<td>FSK3</td>
<td>None</td>
<td>11</td>
<td>TM3</td>
<td>3</td>
<td>TM3</td>
</tr>
<tr>
<td>FSK4</td>
<td>DMBA</td>
<td>8</td>
<td>TM4</td>
<td>2</td>
<td>TM4</td>
</tr>
<tr>
<td>FSK9c</td>
<td>None</td>
<td>1</td>
<td>TM9</td>
<td>10</td>
<td>TM9</td>
</tr>
<tr>
<td>FSK10d</td>
<td>DMBA</td>
<td>2</td>
<td>TM10</td>
<td>12</td>
<td>TM10</td>
</tr>
</tbody>
</table>

 Behm et al. (32).

DMBA, dimethylbenz(a)anthracene. Because mutations in p53 were detected in both untreated and DMBA-treated cells, the expression of progenitor cells to DMBA appears to be of no significance (32, 75).
Table 2 Primers used for RNA-PCR and sequence analysis of murine p53 transcripts

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Coding/noncoding strand</th>
<th>Location relative to coding strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>34A</td>
<td>biotin-TCA</td>
<td>coding: exon 1</td>
<td>nt* 43-62</td>
</tr>
<tr>
<td>21A</td>
<td>biotin-CAG</td>
<td>coding: exon 4</td>
<td>nt 491-510</td>
</tr>
<tr>
<td>29B</td>
<td>TCCTGGT</td>
<td>noncoding: exon 4</td>
<td>nt 381-364</td>
</tr>
<tr>
<td>25B</td>
<td>CTCCCA</td>
<td>noncoding: exon 5</td>
<td>nt 612-595</td>
</tr>
<tr>
<td>2B</td>
<td>CTGTCT</td>
<td>noncoding: exon 6</td>
<td>nt 774-751</td>
</tr>
<tr>
<td>27B</td>
<td>CTCTTG</td>
<td>noncoding: exon 8</td>
<td>nt 1004-985</td>
</tr>
<tr>
<td>21T</td>
<td>CATCGAA</td>
<td>noncoding: exon 10</td>
<td>nt 1177-1157</td>
</tr>
<tr>
<td>30B</td>
<td>AGG</td>
<td>noncoding: exon 11</td>
<td>nt 1409-1390</td>
</tr>
<tr>
<td>33A</td>
<td>AGTA</td>
<td>coding: exon 4</td>
<td>nt 491-510</td>
</tr>
</tbody>
</table>

* nt, nucleotide.

3). These results are compatible with the presence of wild-type levels of p53, although mutation of p53 may cause loss of expression or result in a truncated protein that fails to accumulate to detectable levels.

The TM3 outgrowth line also formed alveolar hyperplasias but differed in that the outgrowths were less extensive and did not produce mammary tumors. Although TM3 HAN outgrowths represent nonmutagenic hyperplasias, overexpression of nuclear p53 was detected by IHC in 3 of 5 samples (Fig. 1B). The staining was localized in the nuclei of positive cells, but only a fraction of the cells was positive (range, 10 to 40%).

Overexpression of nuclear p53 also was observed in the TM4 HAN outgrowth line (Fig. 1C), another tumor-producing line (61.9% tumor incidence; Table 3). In the TM4 HAN outgrowths, only a fraction of the cells stained positively (50%). Nuclear p53 immunoreactivity in TM4 tumors was similar to that observed in TM4 HAN outgrowths, except that nearly all of the cells were positive (Fig. 1C). The p53 protein overexpressed in the tumors was predominantly in the mutant conformation (PAb240 reactive) as determined by immunoprecipitation-immunoblotting (data not shown).

Intermediate tumor incidence was characteristic of the TM9 HAN outgrowth line (38.4% tumor incidence; Table 3). Overexpression of p53 was observed in TM9 HAN outgrowths. In contrast to the other outgrowth lines, p53 reactivity in TM9 HAN outgrowths was localized to the cytoplasm and virtually all of the cells were positive (Fig. 1D). In the TM9 tumors, all of the cells remained positive for p53, but the specific immunoreactivity was now localized to the nucleus (Fig. 1E). These observations were consistent over at least four transplant generations (transplant generations 13–16). Immunoprecipitation-immunoblot analysis of two tumors revealed PAb246-reactive p53 but no PAb240-reactive p53 (Fig. 2A). This suggested that the p53 present in the TM9 outgrowth line was either wild-type or "pseudo-wild-type" (18).

Another HAN outgrowth line (TM10) produced tumors at an intermediate frequency (27.3% tumor incidence; Table 3). In general, TM10 HAN outgrowths did not express p53 at levels detectable by IHC (Table 3). However, all of the tumors overexpressed p53 in the nucleus as determined by IHC (data not shown). The level of p53 expression was sufficient for detection by immunoprecipitation-immunoblotting and was in the mutant conformation (Fig. 2B; Table 3).

Sequence Analysis of p53. It was postulated that if a mutant allele of p53 preexisted in the preneoplastic cells of the HAN outgrowths, then all the tumors that originate from that outgrowth line should bear identical mutations. However, if mutations in p53 accumulate during progression, then the p53 mutations in the tumors would be expected to be dispersed throughout the coding region. The p53 transcripts in three to five tumors from each of the HAN outgrowth lines, TM2H, TM4, TM9, and in the preneoplastic cell lines were sequenced. Because no tumors have developed from the TM3 HAN outgrowth line, nucleotide sequence information was derived from only the cultured cells (Table 4). The nucleotide sequence of p53 in TM10 tumors was not determined because no cell line has been established from TM10 HAN for comparison.

In TM2H cells, a C→T transition at nucleotide 491 introduced a translational stop signal at codon 112 (Table 4). Although this sequence predicts a 15K peptide, no immunoreactive form of p53 was observed in the TM2H cell line by metabolic labeling and immunoprecipitation with a variety of monoclonal antibodies (43). There was no evidence of the wild-type sequence at nucleotide 491 in the TM2H cells and one tumor. However, two tumors had both wild-type and mutant sequences at nucleotide 491 in approximately equal abundance. The wild-type allele may be present in some of the tumors, suggesting heterogeneity in the population of cells in TM2H HAN outgrowths. Alternatively, the wild-type sequence may have been contributed by surrounding normal tissue.

TM3 cells also expressed mutant p53 transcripts. Insertion of 3 nucleotides in exon 7 created an additional serine in conserved region IV (Table 4; Ref. 43). Although the TM3 cell line expressed only mutant p53 transcripts, as detected by direct sequencing, only a fraction of the cells in TM3 HAN outgrowths overexpressed p53 protein by IHC (Fig. 1B).

Table 3 Summary of p53 status in murine mammary HAN outgrowth lines and derived tumors

<table>
<thead>
<tr>
<th>Designation of outgrowth line</th>
<th>HAN outgrowths in vivo</th>
<th>Tumors</th>
<th>Tumorigenicity of HAN outgrowth lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM2H</td>
<td>0 of 2</td>
<td>0 of 1</td>
<td>62.5</td>
</tr>
<tr>
<td>TM3</td>
<td>3 of 5 (+)</td>
<td>NA*</td>
<td>38.4</td>
</tr>
<tr>
<td>TM4</td>
<td>2 of 2 (++)</td>
<td>4 of 4</td>
<td>61.0</td>
</tr>
<tr>
<td>TM9</td>
<td>5 of 4*</td>
<td>2 of 2</td>
<td>50.0</td>
</tr>
<tr>
<td>TM10</td>
<td>1 of 6 (+)</td>
<td>2 of 3</td>
<td>27.3</td>
</tr>
</tbody>
</table>

* Number of HAN outgrowths or tumors containing >5% positive cells/number tested. Numbers in parentheses, the relative intensity of IHC staining with the CM5 polyclonal antibody, rated from weak to strong on an arbitrary scale of 1+ to 4+. The staining was nuclear except in TM9 HAN outgrowths.

† NA, not applicable.

‡ Cytoplasmic staining.

§ The positive tumors contained only PAb246-reactive p53 (see Fig. 2B). Exceptions are noted.
Two mutations were identified in the TM4 HAN-derived cell line and tumors (Table 4). A point mutation at nucleotide 571 (C→G transversion) resulted in a nonconservative amino acid substitution of tryptophan for cysteine at codon 138. A deletion of 21 nucleotides (524 to 544) also was present, resulting in a protein lacking codons 123–129 (Fig. 3). Because the deletion encompassed the first 21 nucleotides of exon 5, it was presumed that a point mutation in intron 4 caused aberrant splicing of the p53 transcript. To determine if the two mutations were in separate alleles of p53, the region containing both mutations was amplified as a single fragment by RNA-PCR and then cloned into a plasmid vector and individual clones were sequenced. Each mutation was found in a separate clone and in equal frequency. No wild-type clones were recovered, so presumably all cells express both mutant alleles. Both mutations were present in all of the tumors analyzed (4 of 4), as well as in the HAN-derived cell line. The 123–129 deletion and Trp→Cys138 mutations were both located in conserved region II of the p53 protein. Despite the presence of only mutant alleles of p53 in TM4 tumors and cell line, only a fraction of the cells in the TM4 HAN outgrowths overexpressed p53 protein by IHC (Fig. 1C).

Analysis of exons 5–9 of p53 transcripts in the TM9 tumors yielded only wild-type sequences. This was unexpected, since overexpression of p53 was observed by IHC (Fig. 1, D and E) and by immunoprecipitation-immunoblotting (Fig. 2A). To search exhaustively for mutations, sequence analysis was extended to the entire coding region of p53 mRNA from five TM9 tumors. No p53 mutations were found in the tumors analyzed. However, it was determined that the TM9 cell line is heterozygous at nucleotide 665 (Fig. 4) resulting in a substitution of methionine for valine at codon 170 in conserved region III. The presence of both wild-type and mutant proteins in the TM9 cell line has been confirmed by immunoprecipitation with PAb246 and PAb240 (data not shown). As the mutation is present only in the cultured cells, we believe this mutation was acquired in vitro and is not present in the TM9 HAN outgrowth line. Together, the sequence data (Fig. 4) and the immunoprecipitation-immunoblotting results (Fig. 2A) substantiate that wild-type p53 was overexpressed in the TM9 HAN outgrowths and tumors.

In summary, mutations in p53 were observed in the preneoplastic TM cell lines derived from HAN outgrowths (Table 1). The mutations in the p53 gene differed among TM2H, TM3, and TM4 preneoplastic cell lines. There was a notable lack of any wild-type p53 sequence in the cell lines, suggesting that mutation of p53 and loss of the remaining wild-type allele may be coincident. Similarly, TM2H and TM4 tumors contained mutations in p53 that were identical to those detected in the preneoplastic TM cell lines (Table 4). Because both the TM tumors and cell lines were derived from HAN outgrowths in vivo, it would appear that the mutations in p53 were present in the preneoplastic HAN and were not acquired during progression.

Expression of p53 Protein in TM4 Cells in Vitro and in Situ. Only a fraction of the cells in the TM3 and TM4 HAN outgrowths in situ overexpressed p53 by IHC. However, TM3 and TM4 HAN cell lines expressed only mutant alleles of p53 and uniformly overexpressed p53 in all cells in vitro (Fig. 5A). These observations could be explained either by heterogeneity of p53 genotypes in the HAN outgrowths or by environmental factors in situ that, in some manner, serve to limit expression or accumulation of the mutant p53 protein.
The TM9 cell line expresses both a wild-type (G) and a mutant (A) allele of p53 at codon 524. In the TM4 tumor shown, p53 protein was expressed at detectable levels and was immunoprecipitated with the pan-specific and mutant-specific antibodies but not with the wild-type-specific antibody.

To distinguish between these alternatives, approximately $5 \times 10^5$ cells from the TM4 cell line were injected into cleared mammary fat pads of BALB/c hosts. The HAN outgrowths, designated 125TM4, were excised 8 weeks later and the pattern of p53 expression was analyzed by IHC. Overexpression of p53 in the 125TM4 HAN outgrowths in situ was clearly evident (Fig. 5B), but less than 50% of the cells stained positively. Similar results were obtained with TM3 cells in vitro and cells grown as transplants in situ (data not shown). Therefore, it appears that growth in situ can alter expression or accumulation of mutant p53 proteins.

### TABLE 4: Sequence analysis of p53

<table>
<thead>
<tr>
<th>Outgrowth line</th>
<th>Mutation; nucleotide position</th>
<th>Codon affected; mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM2H cell line</td>
<td>C $\rightarrow$ T; 491</td>
<td>Exon 4; stop$^{112}$</td>
</tr>
<tr>
<td>TM4H tumors</td>
<td>C $\rightarrow$ T; 491</td>
<td>Exon 4; stop$^{112}$</td>
</tr>
<tr>
<td>TM3 cell line</td>
<td>3 nucleotide insertion$^a$</td>
<td>Exon 7; Ser233-234$^a$</td>
</tr>
<tr>
<td>TM3 tumors</td>
<td>NA$^b$</td>
<td>NA$^b$</td>
</tr>
<tr>
<td>TM4 cell line</td>
<td>Deletion; 524-544/C $\rightarrow$ G; 571</td>
<td>Exon 5; 123-129 deletion/exon 5; Trp138</td>
</tr>
<tr>
<td>TM4 tumors</td>
<td>Deletion; 524-544/C $\rightarrow$ G; 571</td>
<td>Exon 5; 123-129 deletion/exon 5; Trp138</td>
</tr>
<tr>
<td>TM9 cell line</td>
<td>G $\rightarrow$ A; 665$^c$</td>
<td>Exon 5; Met170</td>
</tr>
<tr>
<td>TM9 tumors</td>
<td>Wild-type</td>
<td>Not analyzed</td>
</tr>
<tr>
<td>TM10</td>
<td>Not analyzed</td>
<td>Not analyzed</td>
</tr>
</tbody>
</table>

$^a$ See Osbun et al. (43) for sequence. Insertion could begin at either nucleotide 856 (TCG) or 857 (CGT).

$^b$ NA, not available because no tumors have arisen.

$^c$ The TM9 cell line expresses both a wild-type (G) and a mutant (A) allele of p53 at nucleotide 665.
Mutation of p53 in preneoplastic mammary lesions

Fig. 3. TM4 cell line and tumors express p53 transcripts bearing mutations in exon 5. Direct sequencing of p53 mRNAs was performed as described in “Materials and Methods” using primer 2B to obtain the nucleotide sequences shown. The wild-type nucleotide sequence is shown for comparison and was derived from a TM2H tumor. Two mutations were identified in the TM4 cell line and tumors. Both G (wild-type) and C (mutant) nucleotides were present at position 571 (bottom arrow). The wild-type and mutant sequences, beginning at nucleotide 524 (top arrows), resulted from deletion of 21 nucleotides (Δ21) from some of the p53 transcripts. Right, the nucleotide sequences of the wild-type and Δ21 transcripts are compared. The order of the lanes for each set of sequencing reactions is A, C, G, T from left to right. The deletion spans the first 21 nucleotides of exon 5 (bracketed by dotted lines). These two mutations were shown to be in separate alleles of p53 by cloning and sequencing each.

Mutation of p53 in TM9 cell line but not tumors derived from TM9 HAN outgrowths. The nucleotide sequence of p53 transcripts from the TM9 cell line is compared with the sequence from two TM9 tumors. Arrow, the heterozygous G to A transition in TM9 cells. Right, the wild-type nucleotide sequence. The order of the lanes for each set of sequencing reactions is A, C, G, T from left to right. Sequence of noncoding strand shown.

Fig. 4. Mutation of p53 in TM9 cell line but not tumors derived from TM9 HAN outgrowths. The nucleotide sequence of p53 transcripts from the TM9 cell line is compared with the sequence from two TM9 tumors. Arrow, the heterozygous G to A transition in TM9 cells. Right, the wild-type nucleotide sequence. The order of the lanes for each set of sequencing reactions is A, C, G, T from left to right. Sequence of noncoding strand shown.

during serial transplantation in vivo. Although the p53 mutations apparently conferred some growth advantage in vivo, the cells remained preneoplastic and, in one instance (TM3), have never formed a tumor.

Mutations in p53 have been observed in association with many human cancers. The stage at which p53 is mutated appears to depend on the tissue and type of cancer. In the mouse skin carcinogenesis system, wild-type p53 was expressed in premalignant papillomas but was found to be mutated during progression to carcinomas (58, 59). In colon cancer, mutation of p53 also has been characterized as a late event because mutations were observed in 64% of the adenocarcinomas (52). However, p53 was found to be mutated in a significant proportion (24%) of adenomatous polyps (52) and appeared to be an early event in familial polyposis coli (60). Mutations in p53 also have been documented in preinvasive lesions of the bronchus (61), preneoplastic lesions in the lung (62), and benign breast disease (54).

Mutation of p53 has been suggested to be a late event in breast cancer owing to the rarity of overexpression of p53 in ducal carcinoma in situ and its association with some malignant breast carcinomas. However, the absence of p53 mutations in early lesions has not been demonstrated directly but rather inferred by the lack of accumulation of p53 protein. This assumption overlooks the possibility that p53 may not be expressed due to truncation or gene loss. Mutations in a significant portion of the coding sequence have been shown recently to not result in overexpression of p53 (63). Furthermore, our data suggest that overexpression of p53 can be mitigated by the influence of the tissue environment in vivo. Both TM3 and TM4 cells grown in vitro overexpress p53 uniformly (Fig. 5A; Ref. 43). However, <50% of the cells accumulated levels of p53 that were detectable by IHC when grown as transplants in situ (Fig. 5B). Differential expression of mutant p53 in vitro and in vivo has also been observed in a thyroid tumor cell line (57). Therefore, assessment of the incidence of p53 mutations in early breast lesions by IHC may dramatically underestimate the true incidence of mutations at this stage. More importantly, these data imply that the growth environment in the intact host provides factors that may serve to diminish overexpression of mutant p53 proteins. This regulation appears to be lost or overwhelmed when preneoplastic cells progress to overt tumors, since the tumors appear to overexpress p53 uniformly (Fig. 1C).

TM9 HAN outgrowths and tumors present the converse situation in which wild-type p53 is overexpressed. The p53 protein in the TM9 tumors was shown to be wild-type by immunoprecipitation with PAb246 and by sequencing the entire coding region of p53 mRNA. Overexpression of wild-type p53 has been reported in human breast cancers as well (64). In the HAN outgrowths, the p53 immunoreactivity was localized primarily to the cytoplasm, whereas in the TM9 tumors specific reactivity was localized in the nucleus. Cytoplasmic localization of p53 in human breast carcinomas has been reported by several investigators (14, 23, 65, 66). Sequence analysis of the p53 expressed in tumors exhibiting cytoplasmic staining has shown them to be uniformly wild-type (23, 65). Although nuclear localization is thought to be essential for the transforming activity of mutant p53 (67), the significance of cytoplasmic localization of p53 has been demonstrated by analysis of the Val135 temperature-sensitive mutant. At 37°C, this mutant p53 was localized in the cytoplasm and was able to cooperate with ras in transformation assays (68). Overexpression of wild-type p53 also has been observed in the nuclei of cells transformed by a combination of myc plus ras oncogenes (69).

The biochemical basis for overexpression of wild-type p53 remains speculative, but it is clearly not unique to the TM9 HAN outgrowths or tumors. It is possible that the "p53 pathway," as well as p53 itself, is commonly altered in breast carcinomas. This would include mutations in nuclear proteins that interact with p53 (70), mutations in...
proteins required for intracellular translocation of p53 (68, 71), as well as mutation of the coding sequence of p53. Indeed, whether the frequent losses of heterozygosity at 17p actually include the p53 gene is the subject of debate. Losses of genes on 17p in addition to p53 have been suggested to be significant in breast tumorigenesis (72-74).

This study demonstrates certain similarities between mammary tumorigenesis in humans and mice. As reported for human breast carcinomas, mutation of the p53 gene or altered expression of p53 protein was observed frequently in murine mammary tumors derived from TM HAN outgrowths. The mutations were present in preneoplastic lesions as well as in tumors, suggesting that this can be an early event in mammary tumorigenesis. However, tumor formation was not an invariant consequence of p53 mutations. The studies reported here demonstrate novel, and significant, features of p53 regulation. Partial suppression of mutant p53 overexpression in TM4 HAN outgrowths and the apparent loss of that suppression in TM4 tumors suggest an important role for signals that are capable of inhibiting accumulation of mutant p53 protein. Overexpression of wild-type p53 was observed in the TM9 HAN outgrowth line and its derived tumors. These data suggest that the p53 pathway may be a remarkably common target for mutation during mammary tumor development and may include alterations in genes in addition to p53. The HAN cell lines should provide valuable tools with which to probe this pathway and to identify essential collaborating proteins.

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REFERENCES


Fig. 5. Comparison of overexpression of p53 in TM4 cells in vitro and in situ. In A, TM4 cells were seeded onto coverslips and stained with CMS antiserum as described previously (43) (X 200). In B, TM4 cells were transplanted into cleared mammary fat pads of BALB/c hosts. The outgrowths (designated 12TM4 to distinguish from the TM4 HAN outgrowth line) were excised after 8 weeks and processed for IHC as described in "Materials and Methods." Uniform overexpression of p53 was observed in TM4 cells grown in vitro, whereas in the in situ outgrowth, only a fraction of cells expressed levels of p53 that were sufficient to be detected by IHC (X 200). Solid arrowheads, positive HAN cells; open arrowheads, negative HAN cells.


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