p53 Mutations Selected in Vivo When Mouse Mammary Epithelial Cells Form Hyperplastic Outgrowths Are Not Necessary for Establishment of Mammary Cell Lines in Vitro

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ABSTRACT

Breast cancer is a consequence of multiple alterations occurring over a long period of time. Genetic changes in early stages of tumorigenesis have not been defined. A recently developed murine system permits the study of mammary preneoplastic cells in vivo and in vitro (F. S. Kittrell et al., Cancer Res., 52: 1924–1932, 1992). To assess the potential role of p53 mutations in early stages of breast cancer, the status of p53 was determined in a series of mouse mammary epithelial cell lines which give rise to preneoplastic outgrowths (hyperplastic alveolar nodules) when transplanted into cleared mammary fat pads of syngeneic mice. Protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were also analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatography; p53 alterations and the effects on protein stability and conformation were analyzed using immunoprecipitations and immunochromatin
Selection of p53 Mutations in Preneoplasias

**Table 1** PCR primers used in cloning and sequencing p53 from MMECL

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Exon</th>
<th>Sense or antisense</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>34A</td>
<td>5′-biotin TCA GTT CAT TGG GAC CAT CC-3′</td>
<td>1</td>
<td>Sense</td>
<td>43-62</td>
</tr>
<tr>
<td>21A</td>
<td>5′-biotin CAG TCT GGG ACA GCC AAG TC-3′</td>
<td>4</td>
<td>Sense</td>
<td>491-510</td>
</tr>
<tr>
<td>33A</td>
<td>5′-GTA GGA ATT CAG TCT GGG ACA GCC AAG TC-3′</td>
<td>4</td>
<td>Sense</td>
<td>491-510</td>
</tr>
<tr>
<td>25B</td>
<td>5′-CCT CCC AGC TGG AGG AGT G-3′</td>
<td>5</td>
<td>Antisense</td>
<td>595-612</td>
</tr>
<tr>
<td>2B</td>
<td>5′-CTG TCT TCC AGA TAC TCG GGA TAC-3′</td>
<td>6</td>
<td>Antisense</td>
<td>751-774</td>
</tr>
<tr>
<td>27B</td>
<td>5′-CTT CTG TAC GCC GGT TCC TCT-3′</td>
<td>8</td>
<td>Antisense</td>
<td>985-1004</td>
</tr>
<tr>
<td>21B</td>
<td>5′-CAT CGA ATT CTC CCG GAA CAT CTC GAA GC-3′</td>
<td>10</td>
<td>Antisense</td>
<td>1157-1177</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bold-face type indicates nucleotides added to form EcoRl restriction sites in the final PCR products.

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**RESULTS**

Analyses of p53 Protein Stability and Conformation Expressed in MMECL. To determine the forms of p53 expressed in each of the cell lines, subconfluent cells were metabolically labeled with Trn<sup>35S</sup> and extracted from the clones pooled from individual clonings of separate RT-PCR reactions were analyzed by automated fluorescence sequencing of single-stranded DNA (Institute for Molecular Genetics, Baylor College of Medicine; Applied Biosystems, Inc., Foster City, CA). p53 sequences from the FSK-2 cell line were analyzed by a direct sequencing strategy<sup>4</sup> (35). All of the mutations detected from the cloned p53 fragments were verified using the direct sequencing method as well. The sequences obtained were compared to that published for WT murine p53 (36) using software provided by the Molecular Biology Computational Resource at Baylor College of Medicine. All restriction enzyme digests were performed according to the manufacturer’s recommendations.

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<sup>4</sup> D. J. Jerry, M. A. Ozben, F. S. Kittrell, D. P. Lane, D. Medina, and J. S. Butel. Mutations in p53 are frequent in the preneoplastic stage of mouse mammary tumor development, submitted for publication.
Fig. 2. Analysis of p53 protein conformations expressed in mammary epithelial cell lines. FSK-7, FSK-3 (A), TM-3, TM-4 (B), and TM-2H (C) cells were metabolically labeled with TracS for 2 h. Cell extracts were immunoprecipitated with an irrelevant monoclonal antibody (M73; negative control) or with anti-murine p53 monoclonal antibodies PAb240 (240; p53 MT specific) or PAb246 (246; p53 WT specific). TM-2H cell extracts were also immunoprecipitated with PAb248, a monoclonal antibody directed to the NH2-terminus of p53. As a positive control for p53, labeled TM-4 cell extracts were immunoprecipitated with PAb248 (248). The samples in A and B were analyzed by 10% SDS-PAGE and those in C by 12% SDS-PAGE. Left ordinate, molecular weight standards in kilodaltons; arrows, migration of p53.

To estimate the relative stability (i.e., half-life) of p53 in the FSK cell lines, subconfluent cultures of cells were starved in methionine-free media without insulin and epidermal growth factor for 2 h. The cells were then pulse-labeled for 30 min with 35S-containing MFEM (without insulin and epidermal growth factor); the 35S-containing medium was removed and replaced with normal cell culture medium containing unlabeled methionine plus insulin and epidermal growth factor. Cells were extracted at various time points (T in minutes) to determine the rate of p53 protein turnover (Fig. 3). Immunoprecipitation assays and these labeling conditions confirmed that the FSK cells expressed only the WT form of p53 that displayed no reactivity with the MT-specific PAb240 antibody (Fig. 3, Lanes 240). The WT form of p53 was completely degraded by 90 min of chase in unlabeled media (Fig. 3, Lanes T = 90 min). These data revealed that primary cultures of mammary epithelial cells and cells from FSK lines 2, 3, 4, and 7 express only WT p53 protein with a relative half-life of ~30 min.

Cell lines FSK-3 (P14, P16), FSK-7 (P17–25), and TM-2H (P4, P7) failed to stain for p53 protein by ICC (Table 2; Fig. 4). However, TM-3 (P14–19) and TM-4 (P18, P19) cells were readily stained for p53 expression (>75% of cells reacted; Fig. 4). Although the population of FSK-4 (P13) showed no signs of the MT conformation or stabilized forms of the p53 protein, when examined at P15 and P19 a fraction (~25%) of the FSK-4 cells were positive by ICC staining. These data are in agreement with the premise that detection of p53 expression by immunohistochemical staining is indicative of overexpression and presumably mutation of p53 (12, 13). The FSK cell lines, with the exception of higher passages of FSK-4, express only the WT

Table 2 Summary of p53 Mutations in MMECL

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>PAb246 (WT)</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-ND</td>
</tr>
<tr>
<td>PAb240 (MT)</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-ND</td>
</tr>
<tr>
<td>Half-life</td>
<td>&lt;30 min/ND*</td>
<td>&lt;30 min/ND*</td>
<td>&lt;30 min/ND*</td>
<td>&lt;30 min/ND*</td>
</tr>
<tr>
<td>ICC</td>
<td>ND0</td>
<td>0/4+</td>
<td>2+/4+</td>
<td>0/0*</td>
</tr>
<tr>
<td>Amino acid sequence</td>
<td>WT/MT*</td>
<td>WT/MT*</td>
<td>WT/MT*</td>
<td>WT/ND</td>
</tr>
</tbody>
</table>

* ND, not determined.
1 Scale used: 0, negative; 1+, 1–25% of cells positive; 2+, 25–50% positive; 3+, 50–75% positive; 4+, 75–100% positive.
2 Investigated only in outgrowth lines passaged in mammary fat pads in vivo.
3 WT indicates the sequences were wild type compared with that in the TM cell lines; the p53 sequence in FSK-3, -4, and -7 was analyzed over the entire region between PCR primers 33A and 21B.
4 One allele, stop112.
5 One allele, insertion Ser233-234.
6 One allele, deletion of amino acids 123–129; one allele, Cys → Trp138.
In the TM-2H cell line, a C to T transversion at nucleotide 491 (in exon 4, just 5' to conserved region II) results in a nonsense codon at residue 112. This mutation was homogeneous by sequence analysis, suggesting that the second p53 allele in the cells is converted, lost, or transcriptionally repressed. In any event, the predicted result would be loss of expression of the full-length p53 gene product, consistent with the p53 protein analyses. The product of a specific truncation at codon 112 would be Mr ~15,000 in size. Monoclonal antibody PAB248, which recognizes an epitope between amino acids 14 and 69, should detect the putative MT protein in immunoprecipitation assays, if it were expressed, but no such truncated p53 protein was identified (Fig. 2C, Lane 248). Polyclonal antiserum CM5, able to react with multiple epitopes, failed to detect p53 in TM-2H cells by ICC, showing that no forms of p53 accumulated.

The TM-3 cell line was found to express p53 transcripts having a 3-nucleotide insertion that causes a serine residue to be inserted between codons 233 and 234 (exon 7, conserved region IV); no differing allele was detected by direct sequencing (Fig. 5). Thus, this single MT allele apparently gave rise to both WT (PAB246+) and MT (PAB246+) conformations of the p53 protein (Fig. 2B).

Two MT p53 alleles were characterized in the TM-4 cell line, both of which lies within conserved region II in exon 5. One p53 allele contains a C to G transversion at nucleotide 571 (Cys→Trp138), and the second allele has a 21-nucleotide deletion from 524–544 (deletes seven amino acid residues: 123–129). Although the protein analyses indicated both a WT (PAB246+) and MT (PAB246+) conformations of p53, it is currently unknown which p53 allele contributes to which protein conformation.

Analysis of the FSK cell lines revealed only WT sequences at the sites of the p53 mutations found in their corresponding TM cell lines (Table 2). The sequence data were consistent with the protein data, which showed only WT conformation and WT stability of p53 protein in the FSK cells.

**DISCUSSION**

A number of genetic alterations are associated with cancer of the breast (for a review see Ref. 41), but changes in p53 are the most frequently identified (12, 13, 42–48). Mutations in p53 are generally believed to be somatic, although inherited mutations have been demonstrated in patients from cancer-prone families, including those with Li-Fraumeni syndrome (2, 49, 50). Females in these families tend to have a high incidence of breast cancer. Overexpression of the p53 gene is documented in as many as 53% of breast tumors studied (11–13, 47, 51–55). It has been shown that overexpression in breast cancer often arises from mutations of p53 (11–13, 47) occurring within highly conserved domains of the protein (38). Although overexpression of p53 has been noted in benign breast disease (56), recent investigations suggest that detection of p53 overexpression, used in conjunction with other established factors, may be a valuable prognostic indicator (15, 53–55, 57, 58). Technical difficulties associated with analysis of small early lesions, as well as the impracticability of studying human preneoplasia in vivo, have precluded efforts to determine at which stages in mammary tumorigenesis p53 mutations occur.

We have examined the expression and sequence status of p53 in mammary preneoplasia using a group of established MMECL. The ability of the established parental cell lines (FSK lines) to give rise to preneoplastic outgrowths (HAN) in vivo is a unique aspect of the system (27). Reestablishment of these outgrowths in vitro (TM cell lines) permits investigation of the mutational events and selective
SELECTION FOR p53 MUTATIONS IN PRENEOPLASIAS

forces that may be important in the early stages of mammary tumorigenesis in vivo. Our findings revealed that there is a strong selection during the preneoplastic mammary outgrowth in vivo for cells that express mutant p53.

We first asked whether the TM cell lines that were established from preneoplasias in vivo expressed WT or MT p53 gene products. Of the two outgrowth cell lines (TM-3 and TM-4) that were found to overexpress p53 by immunochemical staining, both synthesized WT as well as MT conformations of p53 protein. However, sequence analysis of p53 cDNA revealed only MT p53 transcripts in both cell lines. The TM-3 cell line expressed a single mutant p53 allele, whereas the TM-4 cell line synthesized two distinct mutant p53 alleles. The fact that both WT (PAb246-') and MT (PAb240-') forms of the p53 protein were detected in both TM-3 and TM-4 cell lines is probably explained by the "pseudo-wild-type" p53 phenomenon described by Bátek et al. (12). These investigators found that MT p53 transcripts may give rise to a variable fraction of proteins that have the WT p53 (PAb246-') conformation. Alternatively, the amino acid change induced by one of the p53 mutations expressed by TM-4 may not be sufficient to alter the WT (PAb246-') protein conformation. The TM-2H cell line expressed no p53 protein detectable by either ICC or immunoprecipitation. This loss of expression is explained by a mutation creating a stop codon in the NH2-terminal one-third of the p53 coding sequences.

We next determined whether the p53 mutations discovered in the TM cell lines were expressed in the parental FSK cell lines prior to transplantation in vivo. Protein analyses of FSK cells demonstrated that all p53 gene products expressed were in the WT conformation and exhibited short half-lives. Nevertheless, ICC staining indicated that a minority of cells overexpressed p53 in the FSK-4 cell line. Sequence analysis of p53 cDNA from the FSK cell lines did not reveal any of the mutations which were documented in their respective TM outgrowth cell lines. It should be emphasized, however, that neither the methods used for p53 sequence analysis nor those used for protein analysis may be sensitive enough to detect small amounts of mutant p53 expression in a background that is largely WT p53.

Several important conclusions can be drawn from these data. First, establishment of MMECL in vitro does not require mutation of the tumor suppressor gene p53. This is in contrast to the high frequency of mutations in p53 detected in spontaneously immortalized murine fibroblasts (18, 59). The difference in p53 status may be due to variation in cell types (fibroblastic versus epithelial) or may reflect dissimilar procedures used in establishing the cell lines (59). Second, alterations in p53 are frequent in preneoplasias. All three of the preneoplastic outgrowth cell lines studied expressed mutant p53 genes. It is noteworthy that the same mutations are retained in mammary tumors which arise from the preneoplasias. However, those changes are not necessary for in vivo immortalization, inasmuch as four independent EL lines carried by fat pad transfers are immortal but do not express MT p53 by ICC (60). Thus, the acquisition of MT p53 appears to be coincident with alveolar hyperplasia and increased tumorigenic potential. Although we are unable to conclude from these data that mutations in p53 are sufficient to produce preneoplasia in vivo, there does appear to be a strong selection for those cells, likely a clonal population, which have sustained p53 mutations.

We propose two alternative explanations for the origin of p53 mutations that are selected in the preneoplastic outgrowths. One possibility is that after the FSK cells are transplanted in vivo, mutation of p53 may occur in a rare cell which is then selected during outgrowth.
Conversely, during culturing in vitro, a cell may sustain a p53 mutation which confers a growth advantage following in vivo transplantation. The methods used in this study lack sufficient sensitivity to unequivocally determine which sequence of events has ensued in each cell line. Although it was not possible to ascertain whether a minor population of cells expressing mutant p53 might be a constituent of a larger population of cells that synthesize only WT p53 in all the FSK cell lines, ICC data suggest that is the case in the FSK-4 cell line. Regardless of when the mutations occur, we conclude that these specific p53 mutations are capable of conferring a selective growth advantage upon the cells which express them in the HAN outgrowth. Consequently, the predominant population of cells constituting the preneoplastic outgrowth in vivo expresses the mutation(s). In addition to the selection for cells with a p53 mutation in HAN outgrowths, there seems to be a concomitant selection against the expression of the WT p53 gene product. This was evidenced by the loss (or repression or conversion) of the remaining WT p53 allele in the TM-2H and TM-3 cell lines.

The growth advantage provided by mutant p53 to mammary epithelial cells in vivo in the fat pad might be expected to cause an enrichment for those mutant p53-expressing cells in vitro as well. However, in this epithelial cell system the selective forces appear to be much stronger in vivo than they are in vitro. After 15–19 passages in culture few cells in the FSK-4 line overexpress p53 protein, whereas the TM-4 outgrowth cells appear homogeneous for overexpression of mutant p53 after passage through an animal. Studies are in progress to determine whether the p53 mutations expressed in the TM cell lines preexist in small subpopulations of the FSK cells prior to transplantation in vivo and, if they are preexisting, whether they provide any selective growth advantage during passage in vitro.

ACKNOWLEDGMENTS

We thank Daniel Lane for providing the CMV antisera.

REFERENCES


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