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Accelerated paper

p53 Mutations in human immortalized epithelial cell lines

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Although rodent cells have been immortalized following transfection with a mutant p53 gene, the role of p53 in the immortalization of human cells is unknown. Therefore, human epithelial cell lines were examined for p53 mutations in exons 4–9 which include the evolutionarily conserved regions. A spontaneously immortalized skin keratinocyte cell line, HaCat, and three ras-transfected clones, have a p53 mutational spectrum that is typical of ultraviolet light induced mutations. A normal finite lifespan cell strain (184) and two benzo[*a*]pyrene immortalized mammary epithelial cell lines derived from 184 (184A1 and 184B5) contain wild type p53 sequences in exons 4–9, although elevated levels of nuclear p53 indicate an alteration in the stability of the normally transient protein. Wild type p53 was found in human bronchial, esophageal and hepatic epithelial cells immortalized by SV40 T antigen gene and human renal epithelial cells immortalized by adenovirus 5. BEAS-2B, an SV40 T antigen immortalized bronchial epithelial cell line and two subclones, have a germline polymorphism at codon 47. Inactivation of p53 by mechanisms such as mutation or complexing with proteins of DNA tumor viruses appears to be important in the immortalization of human epithelial cells.

Introduction

The involvement of p53 in controlling cell proliferation was initially suggested by the observation of binding of p53 protein to oncoproteins of DNA tumor viruses. Wild type cellular p53 protein has been demonstrated to form *in vivo* complexes with SV40 large T antigen (1–3), adenovirus E1b (4–6), and *in vitro* complexes with HPV16 and HPV18 E6 'high cancer risk' proteins (7–9).

p53 was originally considered to be an oncogene that was able to cooperate with ras serving 'myc-like' function in *in vitro* transformation studies of rodent cells (10–12). However, it has since been shown that the murine p53 used in all of these studies was mutated (13,14). Recently, it has been shown that: (i) p53 gene constructs with many different point mutations, deletions and insertions are able to complement ras in these rodent cell assays (13–15); (ii) transfected wild type p53 can inhibit the growth and/or tumorigenicity of neoplastic rodent and human cells (16–21); and (iii) certain transfected mutant p53 can increase the *in vitro* plating efficiency of primary rat cells and cause their immortalization (11,13). Thus, certain mutant p53 genes may play a role in immortalization of rodent cells. However, unlike rodent cells, immortalization of human cells is a rare event (22,23).

Tainsky and coworkers (24) have shown that fibroblasts with germline p53 mutations from patients with Li–Fraumeni cancer syndrome develop changes in culture not present in normal fibroblast controls. Some of these alterations are in morphology, anchorage independent growth, aneuploidy and extended life span in culture. These fibroblasts were non-tumorigenic when injected into athymic nude mice (24). We have reported that mutant p53 (143^{ala}) causes neoplastic transformation of human bronchial epithelial cells immortalized with SV40 T antigen (BEAS-2B cells) (25). Thus, certain p53 mutants can act as an oncoprotein in human epithelial cells.

We have selected human epithelial cells of several tissue origins (bronchus, liver, kidney, breast and skin) which have been immortalized by a variety of mechanisms in order to investigate the involvement of p53 with immortalization. The mechanisms of immortalization of cells studied in this paper include introduction of transforming DNA viral genes (SV40 T antigen or adenovirus 5), spontaneous immortalization, and immortalization associated with exposure to benzo[*a*]pyrene.

Materials and methods

Cell lines

All the human cell lines and strains were expanded in culture for immunohistochemical staining and to generate DNA for PCR and sequencing analysis. BEAS-2B (26), BEAS-2B/S6 and BEAS-2B/R1 (27), BES-1A1 (26) and HB56B/5T (28) are SV40 T antigen containing human bronchial epithelial cell lines. These cells were cultured in LHC-9 medium (29). THLE-2 and THLE-3 (30) are SV40 T antigen containing human liver cultures which were grown in LCM medium (31). HET-1A (32) is an SV40 T antigen transformed human espohageal cell line which was grown in KGM medium (Clonetics). Spontaneously immortalized human skin keratinocyte cell line HaCaT (33), clones derived from HaCaT (34) and 293 fetal kidney adenovirus transformed cells (35) were cultured in RMPI 1640 + 10% fetal bovine serum (FBS*). Normal finite lifespan human mammary epithelial cell strains 184, 48R and 161 and two derivative benzo[*a*]pyrene exposed immortalized mammary epithelial cell lines 184A1 and 184B5 (36) were cultured in MEGM (Clonetics), and breast fibroblast strain 184 was cultured in DMEM + 10% FBS. Cell line A549 (adenocarcinoma of the lung, American Type Culture Collection) was cultured in RPMI 1640 + 10% FBS.

DNA amplification and dideoxy sequencing

Genomic DNA (500 ng) was amplified by PCR [37] using p53 specific primer pairs in the intron regions surrounding exons 4–9, [primer sequences in reference (38). PCR conditions: 0.42 mM each dNTP, 40 pmol of each primer, 5 U AmpliTaq (Cetus, Emeryville, CA), 50 mM Tris, pH 9.0, 3 mM $MgCl_2$. PCR program: denature at 100°C for 5 min, 85°C for 3 min (add Amplitaq); cycles 1–35: 94°C for 30 s, 60°C for 1 min, 78°C for 30 s. Each DNA sample was subjected to at least two separate PCR reactions for DNA sequencing. Purified DNA was sequenced directly by a modification of the dideoxy chain-termination method of Sanger *et al.* (39). Template DNA (1–2μg) was denatured at 98°C for 3 min, anneled with 3 pmol sequencing primer and sequenced with the Sequenase kit reagents (US Biochemical). Radioactive label incorporation was achieved by incubation with [α-^{35}S]-deoxynucleotide (New England Nuclear) corresponding to the first base of the nascent chain (40). Samples were run on 8% gels (Gel-Mix 8, BRL) for 2–5 h. Dried gels were placed against Kodak X-AR 5 film at room temperature for 1–2 days.

*Abbreviations: FBS, fetal bovine serum; PBS, phosphate buffered saline.

Cloning and sequencing

Genomic DNA from the HaCaT cell line was amplified using an intron 4 primer containing an EcoR I site (ACGTGAATTCTGAGGACCTGGTCCTCTGAC) and an intron 9 primer containing a BamHI site (TGCACCTAGGAGTGTTAG-ACTGGAAACTTT). The 1.85 kb fragment was isolated from a 0.8% low melting point agarose gel, extracted, precipitated and digested with EcoRI and BamHI. This fragment was cloned into Bluescript SK(+) (Stratagene) which was digested with EcoRI and BamHI. DH5α bacteria were transformed and 10 colonies were isolated. Plasmid DNA from each colony was sequenced using both the T3 primer (Stratagene) and a sequencing primer in intron 8 (AGGCATAACTGC-ACCCTTGG).

Immunohistochemical analysis

Cells were seeded onto glass multiwell chamber slides (LAB-TEK No.177402, Nunc, Naperville, IL) and subsequently fixed in acetone at −20°C for 10 min and stored at −20°C. Endogenous peroxidase activity was quenched for 20 min at room temperature with a 0.3% hydrogen peroxide solution in phosphate-buffered saline (PBS). After copious washing in PBS, antigenic cross-reactivity was blocked with a 1:50 dilution of normal horse serum for 30 min at room temperature. All sera, monoclonal antibodies, polyclonal antibodies and the ABC reagents were diluted in PBS with 2% crystallized bovine albumin (#81−001, Pentex, miles, Inc., Kankakee, IL). Saturating concentrations of murine monoclonal primary antibodies were incubated overnight at 4°C and subsequently detected by a biotinylated secondary antibody and an avidin-biotin peroxidase system according to the manufacturer's protocol (Vecta-stain Elite Kit, Vector Laboratories Inc., Burlingame, CA). The chromogen was diaminobenzidine (final conc. 0.05 mg/ml) osmicated with nickel chloride (final conc. 0.03%; there was no counterstain. A proliferation marker, Ki-67, demonstrated the viability of the cells under analysis (working dilution 1:1000, M722, Dakopatts, Glostrup, Denmark). A monoclonal antibody to SV40 large T antigen served as an isotype matched negative control (working dilution 1:500, PAb 416, AB-2, Oncogene Science Inc., Manhasset, NY).

p53 protein expression was demonstrated by concordant staining with two monoclonal antibodies. An epitope near the amino terminus was recognized by PAb 1801 (working dilution 1:1000, AB-2, Oncogene Science Inc., Manhasset, NY), and an epitope near the carboxyl terminus was recognized by PAb 122 (working dilution 1:3000, 14091A, Pharmingen Inc., San Diego, CA). Intense extra-nucleolar, nuclear staining was the criterion for a positive reaction for p53 and SV40 large T antigen protein accumulation. Punctuate nuclear staining is characteristic for the Ki67 proliferation marker.

Results

Sixteen human immortalized epithelial cell lines have been examined for mutations in exons 4−9 of the p53 gene. The cell lines, tissue of origin, mode of immortalization and p53 status are listed in Table I. Of these cell lines, ten were completely wild type in the areas examined, or contained only the codon 72 germline polymorphism (CGC to CCC) which alters an arg residue to a pro residue (41). The human bronchial epithelial cell lines BES-1A1 and HB56B/5T (codon 72 polymorphism present) were p53 wild type, as were THLE-2 and THLE-3 (both have codon 72 polymorphism) human liver epithelial cell lines, and HET-1A, a human esophageal cell line. All of these cell lines were immortalized with SV40 T antigen. Human kidney cell line 293 immortalized by adenovirus 5 was wild type for p53. Human mammary epithelial cell strain 184 and immortalized cell lines 184A1 and 184B5 derived from 184 by benzo[*a*]pyrene exposure were also wild type for p53. The treatment of these cells with benzo[*a*]pyrene did not cause mutations in the p53 gene, although benzo[*a*]pyrene is a potent mutagen.

Examination of the 'spontaneously' immortalized skin keratinocyte line HaCaT (33) and individual clones derived after c-Ha-ras oncogene transfection derived from HaCaT (34) revealed a heterozygous mutation in codon 179 of exon 5 and consecutive heterozygous CC → TT mutations in codons 281 and 282 of exon 8. The DNA samples which were sequenced initially had been maintained in culture for 37 passages, so DNA was obtained from the earliest possible passages (passages 4 and 5) for a second analysis. These early passage HaCaT DNAs showed identical mutations, which reduced the possibility that the p53 mutations arose as a result of continuous culturing. In addition, DNA was obtained from the malignant melanoma which was excised ~1.5 cm from the location of the normal tissue which developed into the HaCaT cell line. p53 sequence of the DNA from this tissue revealed wild type sequence at codons 179, 281 and 282, which elimates the possibility that these are germline mutations.

In order to determine if all three of the p53 mutations were carried on the same allele, genomic HaCaT DNA as amplified using intron primers carrying restriction sites. A 1.85 Kb region of the p53 gene between exons 5 and 9 was produced. The resultant PCR product was digested with the restriction enzymes and cloned into Bluescript SK(+) (Stratagene) for DNA sequencing. Analysis of 10 clones revealed three clones mutated at codon 179 and wild type at codons 281 and 282 and seven clones wild type at codon 179 and mutated at codons 281 and 282 (Figure 1). The location and amino acid changes caused by these mutations, with respect to the evolutionarily conserved domains of p53, are also shown in Figure 1. Thus, both alleles of the HaCaT cell line are mutated in the p53 gene.

Sequence analysis of the SV40 T antigen immortalized BEAS-2B cell line, and two subclones BEAS-2B/S6 and BEAS-2B/R1, revealed presence of a codon 47 missense mutation (CCG → TCG) which causes a pro to ser substitution, as well as the codon 72 polymorphism.

Esophageal tissue from the donor was available for p53 sequence analysis (HEA437). The exon 4 PCR product of HEA437 was digested and cloned into Bluescript SK(+) (Stratagene) for sequencing of individual clones as described above. Of 12 clones, 11 clones contained the mutant codon 47 sequence, and 1 clone contained the wild type codon 47 sequence (Figure 2). Thus the codon 47 mutation is germline, and both a wild type and mutant p53 gene are present.

Cell lines which did not contain either SV40 T or adenovirus oncoproteins were analyzed by p53 immunohistochemical staining. HaCaT cell lines were positive for p53 (Figure 3a) and Ki67 (proliferation antigen) in all cells, while SV40 T antigen staining was negative for all these cells (Figure 3b). The HaCaT cells have also been shown to be negative for human papilloma virus by Southern blot analysis (33) (de Villiers, personal communication). These positive staining results are consistent with an extended p53 protein half-life in cells containing mutant p53.

All of the mammary epithelial cells examined were positive for p53 by immunohistochemical staining (Figure 3c−e), while staining for SV40 T antigen was negative (Figure 3f). However, normal fibroblasts from the donor patient 184 were obtained for immunohistochemical staining, and these cells were mostly negative for nuclear p53 expression (Figure 3g). Two other normal mammary epithelial cell strains (48R and 162) were analyzed for p53 protein by immunohistochemical staining, and high levels of nuclear p53 were detected in the 48R cell strain (Figure 3h) as well as in the 161 cell strain (data not shown). The mammary epithelial cell strains 184, 184A1 and 184B5 were also examined by Southern blot analysis for mdm2 gene amplification, and found to be negative (data not shown).

Discussion

The phenomenon of cellular senescence (42) appears to be characteristic of all normal human cells in culture (reviewed in (22)). SV40 T antigen transformed human cells typically have extended *in vitro* lifespans but frequently reach a 'crisis' period (43) at which net proliferation is reduced completely or in part,

Table I. Origin and p53 status in immortalized human cell lines

Cell line	Tissue origin	Mode of immortalization	p53 status	Reference
BEAS-2B	Bronchus	SV40 T antigen	G.polymor. c. 47, 72	26
BEAS-2B/S6	Bronchus	BEAS-2B subclone	G. polymer. c. 47, 72	27
BEAS-2B/R1	Bronchus	BEAS-2B subclone	G.polymor. c. 47, 72	27
HB56B/5T	Bronchus	SV40 T antigen	G.polymor. c. 72	28
BES-1A1	Bronchus	SV40 T antigen	Wild type	26
HET-1A	Esophagus	SV40 T antigen	Wild type	32
THLE-2	Liver	SV40 T antigen	G.polymor. c. 72	31
THLE-3	Liver	SV40 T antigen	G.polymore. c 72	31
293	Fetal kidney	Adenovirus 5	Wild type	35
HaCaT	Skin	Spontaneous	M. c. 179, 281–282	33
HaCaT-ras A-4	Skin	HaCaT + c-Ha-ras	M. c. 179, 281–282	34
HaCaT-ras I-7	Skin	HaCaT + c-Ha-ras	M. c. 179, 281–282	34
HaCaT-ras II-4	Skin	HaCaT + c-Ha-ras	M. c. 179, 281–282	34
184	Breast	Not applicable	Wild type	36
184A1	Breast	184 + B[*a*]P	Wild type	36
184B5	Breast	184 + B[*a*]P	Wild type	36

G, Germline; Polymor, Polymorphism; M, Mutant; c, codon.

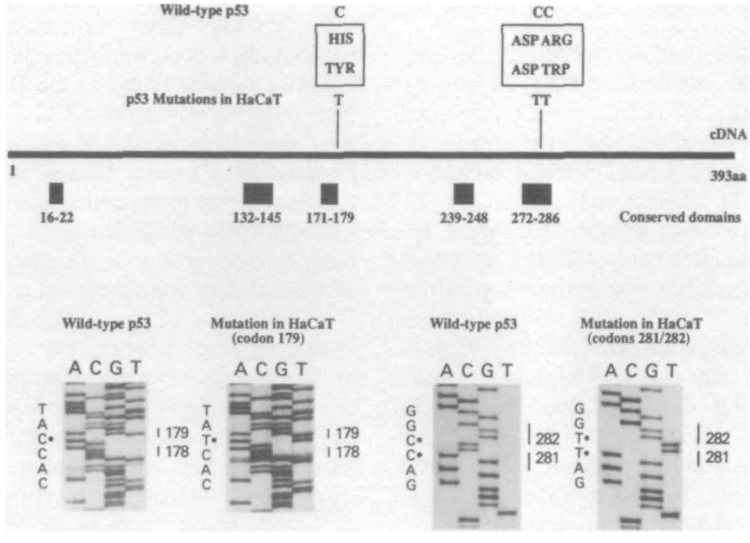

Fig. 1. p53 sequencing of HaCaT clones in codon 179 and 281–282 regions in relationship to the p53 conserved domains.

and the surviving cells have aberrant morphology. Furthermore, the development of a continuously growing cell line from normal human cells expressing SV40 T antigen is a rare event, on the order of 1×10^{-7} (44,45). The immortalized cells are associated with aneuploidy and clonally-derived marker chromosomes (26,46,47). Since p53 mutations have been shown to immortalize rodent cells (11,48), and human fibroblasts with germline p53 mutations from Li–Fraumeni patients 'spontaneously' immortalized [24], we tested the hypothesis that p53 mutations and loss of the wild type allele may be a method by which human cells escape from 'crisis' and expand into an established cell line.

DNA sequence analysis of SV40 T antigen immortalized human bronchial cell lines (BES-1A1 and HB56B/5T), immortalized liver cell lines (THLE-2 and THLE-3), and an esophageal cell line (HET-1A), revealed the presence of wild type p53. Likewise, the immortalized fetal kidney cell line, 293, containing adenovirus 5 sequence is also wild type for the regions of p53 examined. Similar data were observed by several groups (49–51) in cervical cancer samples. Mutations in the p53 gene were observed only in cells which were negative for human

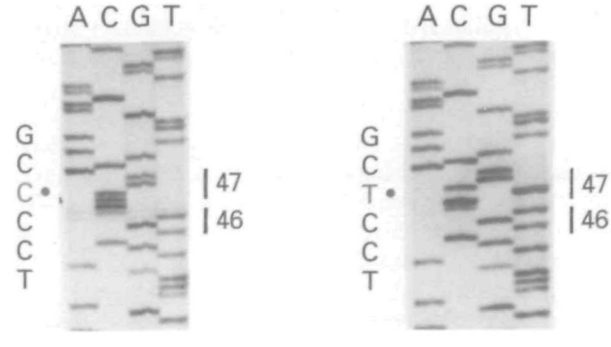

Fig. 2. p53 sequencing of BEAS-2B donor espohageal tissue (HEA437) clones in codon 47 region.

papilloma virus. It was shown previously that cells which harbored HPV 16 or 18 accomplish inactivation of wild type p53 by ubiquitin-dependent degradation (8,9). These results suggest that inactivation of p53 may be essential, and that this inactivation

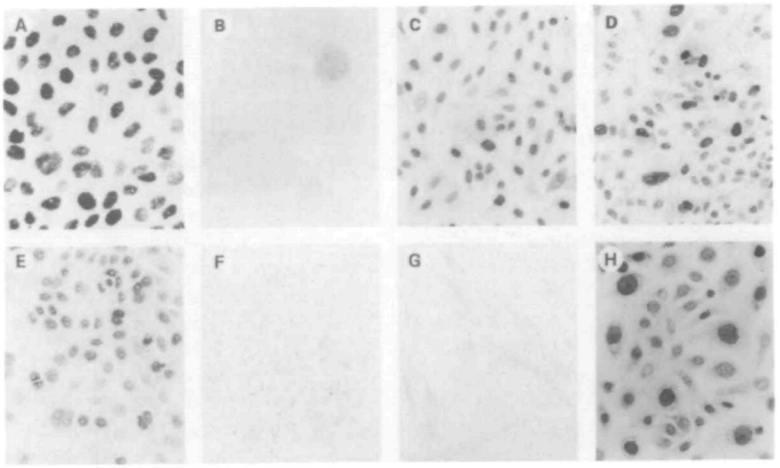

Fig. 3. Immunohistochemical analysis of p53 expression in HaCaT (**a**) normal mammary cell strain 184 (**c**) immortalized mammary cell strain 184A1 (**d**) and 184B5 (**e**), normal mammary fibroblast cell strain 184 (**g**) and normal mammary epithelial cell strain 48R (**h**). Immunohistochemical analysis of SV40 T antigen in HaCaT cell line (**b**) and in mammary epithelial cell strain 184 (**f**).

could be achieved by either viral protein complexing p53, or by p53 gene mutation.

The SV40 T antigen immortalized cell line BEAS-2B (26) and its two subclones (BEAS-2B/S6 and BEAS-2B/R1) (27) were found to have a mutation at codon 47 (CCG → TCG) causing an amino acid change from pro to ser. Analysis of cloned DNA from normal esophageal tissue of the donor from which the cell lines were derived revealed 11 clones with the codon 47 alteration, as well as one wild type codon 47 clone (see Figure 2). Thus, the BEAS-2B codon 47 mutation is most likely a germline polymorphism. Previous immunoprecipitation analysis has shown that p53 in BEAS-2B cells binds to the SV40 T antigen protein but does not bind to the heat shock 70 protein (25). When a plasmid containing p53 with the codon 47 mutation was transfected into a human lung carcinoma cell line, cell proliferation was inhibited (79). These results indicate that the p53 in BEAS-2B cells has normal wild type properties. Therefore, the presence of the viral oncoprotein SV40 T antigen complexed with p53 may be necessary for immortalization of this cell line.

Mutations in the *N*-terminal region of the p53 gene are infrequent, and only one case of a codon 47 mutation has been reported to date (52). The non-small cell lung cancer line H1373 has a CCG → CTG mutation at codon 47 causing a change from pro to leu. While the mutation caused weak p53 immunocytostaining using antibody Pab 1801, no staining was observed with Pab 421 (53).

The p53 mutations in the early and late passage HaCaT cells are C → T and CC → TT transitions which were found at dipyrimidine sites in codons 178 to 179 (CAC CAT) and 281 to 282 (GAC CGG). The 179 mutation causes a his residue to be altered to a tyr residue, while the 282 mutation changes an arg to a trp. Cloning analysis revealed that the 179 mutation was present on one allele, while the 281 and 282 mutations were on the other allele. Although codon 282 contains a frequently mutated CpG site and many investigators have reported mutations from CGG to TGG in this position (54–60), the codon 281 mutation observed in this study has not been previously described. Furthermore, the 281 mutation is silent (GAC and GAT both code for asp), so presence of this mutation would not confer a selective growth advantage to the cells which harbor it, unless differences in codon utilization are a factor. These consecutive mutations carried on the same allele are therefore almost certainly not two independent events, but the result of a single event such as pyrimidine dimer formation. Since both p53 alleles are mutated, there is no wild type p53 protein in the HaCaT cell lines.

The mutations found in the HaCaT cell line and its clones, especially the consecutive CC → TT mutations, are consistent with sunlight induced UV damage. Brash and coworkers [40] have recently reported three 'sunlight signature' consecutive CC → TT mutations and 5 C → T transition mutations at CC sites in the p53 gene of invasive carcinomas of the skin. Another study reported a G → A transition in a squamous cell carcinoma which was opposite a potential pyrimidine dimer site (CC) (61). Over expression of p53 was observed by histochemical methods in 83% of basal cell carcinomas of the head and neck (62) and in 50% of basal cell carcinomas (63). Although Loeb and coworkers have also described C → T transitions induced by oxy-radicals generated by copper exposure (64), the rarity of CC → TT in cancers arising in internal organs and the characteristic mutations at dipyrimidines implicate ultraviolet light as the mutagenic agent in the HaCaT cells.

DNA sequencing of the melanoma from the HaCaT donor revealed that p53 codons 179, 281 and 282 were wild type. Not only does this result eliminate the possibility that the mutations in the HaCaT cell lines are germline, but it suggests a mutagenic mechanism by which the skin keratinocyte cells spontaneously immortalized may involve p53, and that a separate mechanism was involved in the development of the malignant melanoma in the patient.

Although accumulation of high levels of p53 protein frequently correlates with missense mutation, the breast epithelial cell strain 184 and its immortalized derivative lines 184A1 and 184B5 probably represent exceptions to this rule since they contain normal DNA sequence in exons 4–9 of the p53 gene. Similar results were reported in several lung carcinoma lines in which the p53 coding region was entirely sequenced (38), colorectal cell lines (65), and during certain phases of the cell cycle in normal lymphocytes (66). Lane and coworkers recently demonstrated abnormal overexpression of wild type p53 protein in normal cells of a cancer family patient (67). Levine and coworkers found that some breast cancers which have wild type p53 can inactivate the protein by sequestering it in the cytoplasm, removed from the nucleus where it presumably exerts its tumor-suppressive functin (68).

Several possibilities exist which may account for this presence of high levels of p53 in cell lines which contain wild type p53 sequence. First, these cells may produce p53 with an abnormally long half-life. Band and coworkers have recently found the half-life of p53 in normal human mammary epithelial cell strains to be ~3 h (69). One of several normal mammary epithelial cell strains which stained positively for p53 was completely sequenced in the p53 coding region (codons 1–393) and found to contain the wild type p53 gene (V.Band, personal communication). The mechanism by which the half-life of p53 is altered in these cells is unknown.

Human p53 from other cell types may have longer half-lives than rodent p53 which is commonly cited at ~20 min (70,71). For example, the half-life of p53 in GM47.23 human glioblastoma cell line was recently demonstrated by Ullrich and coworkers to be 1.9 h (72). The half-life of newly synthesized p53 in human keratinocytes is ~4 h (73), while the half-life of the p53 protein in human T-lymphocytes is 65 min (74). The understanding of p53 involvement in cancer may be more complete when the factors which are involved in extending the half-life of the protein are elucidated.

Mutations in the human p53 gene outside of the highly conserved regions can occur, although it is uncommon in human cancers (75). However, if mutations occurred outside of the regions which were sequenced, this may be responsible for the staining results. Alternatively, a protein which is involved in p53 degradation may be mutated or a p53 'stabilizing' protein could be present. Although the mdm2 protein is a candidate for stabilizing both mutant and wild type p53 protein (76,77), the gene is not amplified in these human mammary epithelial cell strains. Finally, post-translational modifications such as phosphorylation could alter the biological activity and physical conformation of p53 [reviewed in (78)].

In conclusion, we have demonstrated that human epithelial cells of various tissue organs which have been immortalized by oncogenic DNA viruses do not require mutations in the p53 gene. Spontaneously immortalized HaCaT cells acquired p53 mutations consistent with UV damage, and chemically immortalized breast epithelial cell lines, although wild type for p53 in the evolutionarily conserved domains, accumulate high amounts of p53 protein due to unknown mechanisms. Inactivation of p53 by mechanisms such as mutation or complexing with proteins of DNA tumor viruses appears to be important in the immortalization of human epithelial cells.

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