Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5523–5527, July 1991
Medical Sciences

The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines

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Communicated by Robert A. Weinberg, March 18, 1991

ABSTRACT **Human cervical carcinoma cell lines that were either positive or negative for human papillomavirus (HPV) DNA sequences were analyzed for evidence of mutation of the p53 and retinoblastoma genes. Each of five HPV-positive cervical cancer cell lines expressed normal pRB and low levels of wild-type p53 proteins, which are presumed to be altered in function as a consequence of association with HPV E7 and E6 oncoproteins, respectively. In contrast, mutations were identified in the p53 and *RB* genes expressed in the C-33A and HT-3 cervical cancer cell lines, which lack HPV DNA sequences. Mutations in the p53 genes mapped to codon 273 and codon 245 in the C33-A and HT-3 cell lines, respectively, located in the highly conserved regions of p53, where mutations appear in a variety of human cancers. Mutations in *RB* occurred at splice junctions, resulting in in-frame deletions, affecting exons 13 and 20 in the HT-3 and C-33A cell lines, respectively. These mutations resulted in aberrant proteins that were not phosphorylated and were unable to complex with the adenovirus E1A oncoprotein. These results support the hypothesis that the inactivation of the normal functions of the tumor-suppressor proteins pRB and p53 are important steps in human cervical carcinogenesis, either by mutation or from complex formation with the HPV E6 and E7 oncoproteins.**

Cervical cancer is one of the leading causes of female death from cancer worldwide with ≈500,000 deaths per year. Epidemiologic studies have implicated a sexually transmitted agent in the etiology of cervical cancer, and laboratory studies over the past decade have established a strong association between certain human papillomaviruses (HPVs) and cervical cancer and several other anogenital carcinomas (for review, see ref. 1). Over 65 different HPVs have now been described, and ≈20 of these have been associated with anogenital lesions (2). A subgroup of these viruses, including HPV types 16, 18, 31, 33, and 39, have been etiologically implicated in cervical carcinogenesis because they are found in a high percentage of the cancers and because the benign lesions with which these viruses are associated are precursors for malignant progression.

Additional evidence that HPVs have an etiologic role in cervical neoplasia derives from the analysis of the properties of the viral gene products expressed in these cancers. The viral *E6* and *E7* genes are regularly expressed in the HPV-positive tumors and cervical carcinoma cell lines (3–6), and both genes have transforming properties. *E7* alone can transform established rodent cells, such as NIH 3T3 cells (7–12), and can cooperate with an activated *ras* oncogene to transform primary rat cells (8, 13). The transforming potential of *E6* was revealed by studies showing that efficient immortalization of primary human keratinocytes or human fibroblasts required the combination of *E6* with *E7* (14–16).

Insight into the mechanisms by which DNA tumor viruses transform cells has come from the recognition that the virus-encoded oncoproteins interact specifically with important cell regulatory proteins. The E7 protein of the genital tract HPVs, similar to the adenovirus E1A proteins (17) and the large tumor antigens of the polyomaviruses (18, 19), can complex with the product of the retinoblastoma tumor-suppressor gene pRB (20, 21). The E7 proteins of the "high risk" HPVs, such as HPV-16 and HPV-18, bind pRB with ≈10-fold higher affinity than do the E7 proteins of the "low risk" HPV types 6 and 11, and this difference in binding affinity correlates with the transforming potential of the different E7 proteins (21). Like simian virus 40 (SV40) large tumor antigen and adenovirus 5 E1B (22–24), the E6 protein of the "high risk" HPVs can complex with the p53 protein (25), which is now also recognized as having tumor-suppressor properties (26, 27). Because of the tumor-suppressor properties of pRB and p53, the oncogenic effects of these viruses are believed to result, at least in part, from these specific interactions.

In this study we have examined the status of the pRB- and p53-encoding genes in a series of human cervical carcinoma cell lines previously analyzed for HPV DNA. In each of the two HPV-negative cell lines, elevated levels of p53 protein were found. Because mutations in the gene encoding p53 can cause accumulation of ostensibly inactive p53 aggregates, the p53-encoding genes were sequenced and found to be mutated. In contrast, the levels of p53 protein in five HPV-positive cell lines were low, and sequence analysis of the p53 cDNAs revealed no mutations. pRB appeared normal in the HPV-positive cell lines, in that normal-sized phosphorylated as well as hypophosphorylated forms of the protein were detected by immunoblot analysis. In the HPV DNA-negative cell lines, however, mutations in the pRB-encoding gene were found that affected the capacity of the encoded proteins to be phosphorylated and complexed with adenovirus E1A, characteristics of pRB inactivation seen in a variety of other tumors. These results support the hypothesis that the normal functions of pRB and p53 proteins are abrogated in human cervical cancer, either by mutation of the genes themselves or as a consequence of specific interaction of these proteins with the E6 and E7 oncoproteins.

MATERIALS AND METHODS

Cell Lines. The following human cervical carcinoma cell lines were obtained from the American Type Culture Collection: C-33A, HT-3, ME-180, SiHa, C-4II, HeLa, and CaSki (Table 1). The Saos-2 cell line was originally derived from a human osteosarcoma and was obtained from Stephen Friend (Harvard Medical School). Nontransformed primary and secondary human foreskin keratinocytes (HFKs) were prepared and maintained as described (29). The SV40-immortalized HFK cell line (HFK/SV40) was obtained from Richard Schlegel (30). The HFK/1321 cell line was immortalized by the HPV-16 *E6/E7* genes expressed from the

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Abbreviations: SV40, simian virus 40; nt, nucleotide(s); HFK, human foreskin keratinocyte; HPV, human papillomavirus.

Table 1. Human cervical carcinoma cell lines

Cell line	HPV DNA	HPV RNA	Ref.
HeLa	HPV-18	Yes	3
C-4II	HPV-18	Yes	3
SiHa	HPV-16	Yes	4, 28
CaSki	HPV-16	Yes	4, 28
ME-180	HPV*	Yes*	28
C-33A	Negative	No	28
HT-3	Negative	No	28

*The ME-180 cell line was reported as containing HPV DNA detected with a HPV-18 DNA probe (28). Further analysis of this cell line has revealed transcriptionally active HPV sequences that are more closely related to HPV-39 than to HPV-18 but for which the HPV type number is yet unassigned (Elizabeth Schwarz, personal communication).

human β-actin promoter (14), the HFK/1319 cell line was immortalized by a plasmid (p1319) containing the HPV-16 early region expressed from the human β-actin promoter (K.M., unpublished work), and the HFK/698 (29) and HFK/769 cell lines were immortalized by cloned HPV-16 DNA.

Immunologic Procedures. For immunoblotting, cellular protein lysates were prepared from 80% confluent cells in lysis buffer (1% Nonidet P-40/100 mM NaCl/2 mM EDTA/20 mM Tris, pH 8.0) containing phenylmethylsulfonyl fluoride (0.01%)/aprotinin (1 μg/ml)/leupeptin (1 μg/ml)/NaF (5 mM), and sodium orthovanadate (1 mM) at 0°C for 30 min. Lysates were cleared by centrifugation at 15,000 × *g* for 15 min and stored at −80°C. Protein concentrations were determined by the Bio-Rad protein assay. Samples (100 μg) were analyzed by SDS/PAGE followed by immunoblotting (31). The mouse monoclonal antibodies Mh-Rb-02 (PharMingen, San Diego) and PAb1801 (32) (marketed as AB2; Oncogene Sciences, Mineola, NY) were used to detect pRB and p53, respectively. An ^{125}I-labeled sheep anti-mouse antibody (Amersham) was used for detection.

PCR Analysis, Cloning, and DNA Sequencing. Cytoplasmic RNA, prepared by using standard procedures (33), served as a template for cDNA synthesis. Reverse transcription was followed by PCR amplification, according to the suggestions of the manufacturer (Cetus). The primer for reverse transcription of p53 mRNA extended from nucleotide (nt) 1015 to nt 996 with a *Hin*dIII site at the 5′ end for subsequent cloning, where nt 1 is the adenine of the ATG initiation codon. The opposing primer used for PCR amplification extended from nt 296 to nt 315 and contained a *Sal* I site at its 5′ end. The RB sequences for the RB primers used in this study use the nucleotide numbering system of Friend *et al.* (34) and are as follows: exon 12 (sense), nt 1171–1190; exon 13 (sense and antisense), nt 1300–1321; exon 16 (antisense), nt 1470–1494; exon 16/17 (sense), nt 1489–1508; exon 18/19 (sense and antisense), nt 1807–1831; exon 20 (sense and antisense), nt 2005–2026; exon 21 (antisense), nt 2123–2145; and exon 22/23 (antisense), nt 2321–2339. For genomic analysis additional primers derived from RB intron sequences (35) were used: CACAGTATCCTCGACATTGATTTCTG (intron 12, sense), CGAACTGGAAAGATGCTGC (intron 13, antisense), CTCTGGGGGAAAGAAAAGAGTGG (intron 19, sense). All RB primers contained guanine- or cytosine-rich regions at their 5′ ends and either *Sal* I (sense primers) or *Bam*HI (antisense primers) cloning sites. For PCR analysis of genomic DNA, 250 ng to 1 μg of cellular DNA was used as a template under the conditions suggested by the manufacturer (Cetus). PCR products were cloned into pUC19 and pGEM-1 vectors (Promega), and sequence analysis was carried out on several clones from independent PCR reactions by using modified T7 DNA polymerase (Sequenase; United States Biochemical).

RESULTS

The cervical carcinoma cell lines examined in this study and their status with respect to the presence and the expression of HPV DNA are summarized in Table 1. To verify that the C-33A and HT-3 cell lines were indeed HPV negative, DNA from each cell line was further examined by filter hybridization under nonstringent conditions (36) and by PCR with consensus primers. Southern blot hybridization at a melting temperature (t_m) of −45°C with mixed HPV DNA probes, and PCR analysis with consensus HPV primers capable of detecting a wide spectrum of genital-tract HPV types (37) also failed to reveal any HPV DNA in C-33A or HT-3 cell lines (data not shown).

Analysis of pRB in Human Cervical Carcinoma Cell Lines. At least part of the transforming capacity of the E7 protein has been proposed to be a consequence of its ability to interact with pRB protein, thus abrogating the function of pRB as a negative regulator of cell proliferation. This hypothesis leads to the prediction that mutations in *RB* would not, therefore, be of selective advantage in HPV-positive cancer cell lines. Furthermore, if *RB* were an essential target in human cervical cancer, one might expect *RB* inactivation in the HPV-negative cervical carcinoma cell lines to be achieved by other means, such as mutation of the *RB* gene. An immunoblot analysis of pRB was, therefore, done on the human cervical carcinoma cell lines (Fig. 1). In each HPV-positive cervical cell line normal pRB was detected with evidence of both hypophosphorylated and hyperphosphorylated forms of the protein (indicated as pRB and ppRB in Fig. 1). In contrast, a protein with an altered mobility was detected in each of the HPV-negative cell lines C-33A and HT-3. A small amount of normal forms of the RB proteins could also be detected in the HT-3 cells, possibly from admixture of some cells with normal RB proteins (see below). The faster migrating forms of pRB seen in each of these two HPV-negative cell lines appear as single bands, indicating the presence of only the hypophosphorylated form of pRB. Complex formation with adenovirus E1A *in vitro*, an attribute of the wild-type protein, could not be detected, providing further evidence that pRB protein was abnormal in each of these cell lines (data not shown).

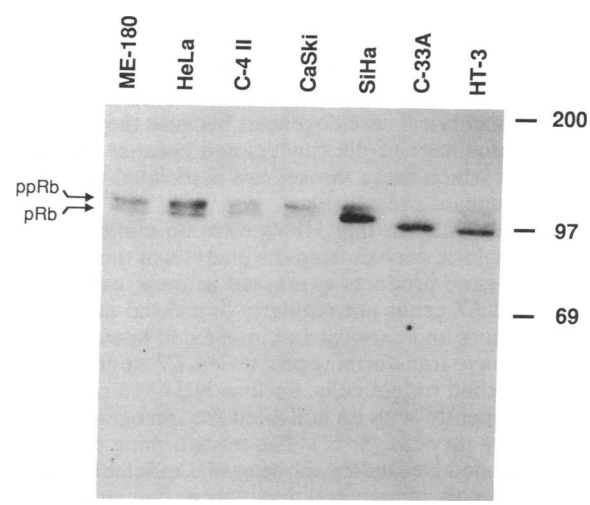

FIG. 1. Immunoblot analysis of pRB in cervical carcinoma cell lines. One hundred micrograms of protein extract of each indicated cell line was separated on SDS/7.5% polyacrylamide gel and electroblotted to a nitrocellulose membrane. The RB protein was detected with the monoclonal mouse antibody Mh-Rb-02. Positions of the pRB protein in hypophosphorylated (indicated here as pRB) or hyperphosphorylated (ppRB) forms.

Structure of RB mRNA in C-33A and HT-3 Cell Lines. To verify that C-33A and HT-3 cell lines express mutant pRB, cDNA representing the pRB mRNA was examined by PCR analysis. Altered forms of pRB protein defective in their ability to be phosphorylated and to complex adenovirus E1A have been demonstrated in a variety of human cancers. Because mutations that affect these properties of pRB have been mapped to genomic sequences encoding exons 13–22 (38–40), PCR primers were designed to examine these exons in the cDNAs. cDNA from HeLa cells, which contain normal pRB, was used as control.

Analysis of PCR products for the C-33A cell line revealed a small deletion, evidenced by the shorter PCR product seen with the E18/19 primer and either the E21 or E20 primer. Sequence analysis of the PCR product revealed a 12-base deletion at the 5′ end of exon 20, resulting in an in-frame deletion of four amino acids (Fig. 2). A similar analysis of the HT-3 RB cDNA revealed that exon 13 was entirely deleted from the cDNA (Fig. 2).

Determination of the Genomic Mutation in *RB* in C-33A and HT-3 Cell Lines. To determine the basis for each of the altered cDNAs in these two cell lines, we examined the genomic sequences surrounding the junctions for the exon 20 splice-acceptor site in C-33A and for the exon 13 splice-donor and -acceptor sites in HT-3; exon skipping is commonly associated with splice-junction mutations (41). A single G → A mutation was found in the exon 20 splice acceptor in C-33A cells, and an A → G mutation was found in the exon 13 splice donor in HT-3 cells (Fig. 3). These mutations were found in multiple independent clones of PCR-amplified segments of genomic DNA from these cell lines, indicating that the mutations did not represent PCR-generated artifacts. One of five clones from the HT-3 cells contained a wild-type RB sequence, supporting the observation that the cell line is probably not clonal and contains some cells expressing normal pRB, seen faintly in Fig. 1. No mutation was found at the exon 13 splice acceptor in HT-3 cells. Thus, in each of the HPV-positive lines, pRB was normal but apparently complexed with E7, whereas in the HPV-negative cell lines examined, pRB was present in a mutant form.

Analysis of p53 in Cervical Carcinoma Cell Lines. Levels of p53 protein were examined in these cell lines by immunoblot analysis with mouse monoclonal antibody 1801 to human p53

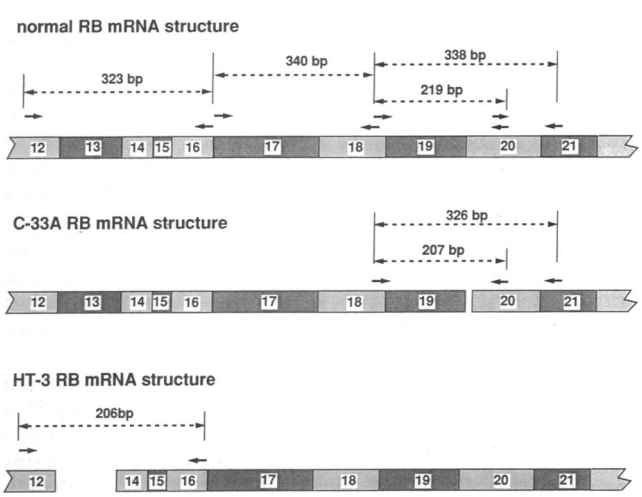

FIG. 2. Schematic representation of mutations in RB mRNA determined from PCR analyses and DNA sequence analysis. The structure of normal RB mRNA is shown at top. The RB mRNA from C33-A cells has a deletion of 12 base pairs (bp) at the beginning of exon 20, resulting in in-frame deletion of four amino acids. The RB mRNA from HT-3 cells contains a precise deletion of exon 13, resulting in in-frame deletion of 39 amino acids.

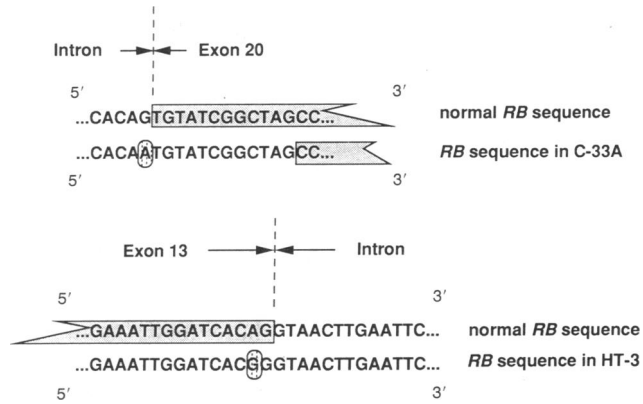

FIG. 3. Genomic mutations in the *RB* gene in C-33A (*Upper*) and HT-3 (*Lower*) cell lines. In the C-33A cell line a G → A mutation occurs at the intron/exon 20 splice junction. A new cryptic splice acceptor is used 12 bases downstream. The HT-3 cell line contains an A → G mutation at the −2 position of the 5′ splice junction, thus skipping exon 13.

(32). A specific band of p53 was detected in HFKs and in each of the cervical carcinoma cell lines (Fig. 4*A*). Saos-2 cells were included in this analysis as a negative control because they do not contain or express p53 (42). p53 levels in the

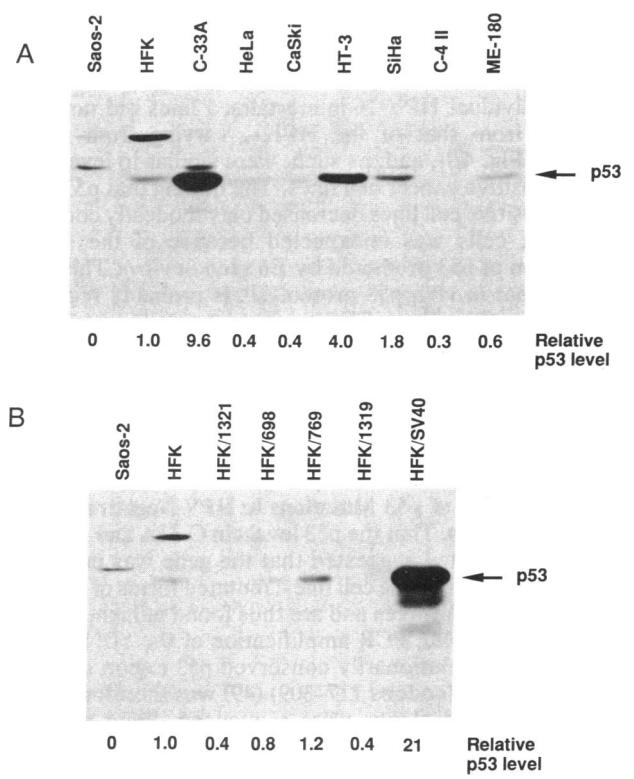

FIG. 4. Immunoblot analysis of p53 in human cervical carcinoma cell lines and in HFKs. Equal amounts of protein (100 μg) extracted from several human cervical carcinoma cell lines (*A*) or HFK cell lines immortalized by cloned viral plasmids (*B*) were separated through SDS/10% polyacrylamide gel before electroblot transfer to nitrocellulose filters. In these experiments, the human osteosarcoma cell line (Saos-2), which does not contain p53 (42), and secondary cultures of HFKs, which express very low levels of p53, served as controls. The p53 protein was detected using mouse monoclonal antibody PAb 1801 (32), as described. An AMBIS scanner (AMBIS Systems, San Diego) was used for measurement, and p53 levels are expressed relative to the level in nonimmortalized HFKs. In subsequent cultures of HT-3 cells, the p53 level was <4.0 in relative level, seen in the experiment of *A* with a relative level of 2.0.

carcinoma cell lines were measured and are expressed relative to the p53 level detected in HFK cells, which is extremely low, as it is in most primary cells. A previous analysis of p53 in HeLa cells reported no detectable protein despite the presence of translatable mRNA (43); however, very low p53 levels could be demonstrated in HeLa cells in this analysis. This result was similar to that seen with the four other HPV-positive cell lines examined (Fig. 4*A*), in which p53 levels detected ranged from 0.3- to 1.8-times that found in secondary cultures of HFKs. Thus, in nontransformed HFKs as well as in HPV-positive cervical carcinoma cell lines, the levels of p53 are very low. The low p53 levels in these cell lines contrasted with those found in the two HPV-negative cervical carcinoma cell lines studied. In the experiment depicted in Fig. 4*A*, p53 levels in C-33A and HT-3 cell lines were 9.6- and 4.0-times that of HFKs.

Analysis of p53 and pRB in SV40 and HPV-Immortalized Keratinocytes. These results with the cervical carcinoma cell lines prompted examination of p53 levels in a series of human keratinocyte lines immortalized by HPV-16 and by SV40. The E6 oncoprotein of the HPV types associated with cervical cancer can exist in a complex with p53 in *in vitro* assays (25) and can promote its degradation *in vitro* (44). In contrast, SV40 large tumor antigen, which also complexes p53, increases the half-life and steady-state levels of p53 in transformed cells (45). The levels of p53 were therefore measured in a series of four independent HFK cell lines immortalized by different plasmids expressing the full HPV-16 genome or portions of the HPV-16 early region containing *E6* and *E7*. The levels of p53 were measured directly from the immunoblot and compared with the level in nontransformed HFKs. The levels observed for the individual HPV-16-immortalized lines did not differ markedly from that of the HFKs, varying from 0.4- to 1.2-times (Fig. 4*B*), and, as such, were similar to levels seen in HPV-positive cancer cell lines. The finding that p53 levels in HPV-positive cell lines decreased only modestly compared with HFK cells was unexpected because of the striking degradation of p53 promoted by E6 seen *in vitro*. This result suggests that *in vivo* p53 proteolysis is probably regulated, and perhaps the effect of E6 on this process is restricted to certain times in the cell cycle. As anticipated, the p53 level in the SV40-immortalized HFKs was markedly elevated over that of the nonimmortalized HFKs (45). Immunoblot analysis of pRB in these immortalized cell lines revealed normal levels of phosphorylated and hypophosphorylated forms of pRB (data not shown).

Determination of p53 Mutations in HPV-Negative Cervical Cancer Cell Lines. That the p53 levels in C-33A and HT-3 cell lines were elevated suggested that the gene was potentially mutated in each of these cell lines: mutated forms of p53 often have extended half-lives and are thus found at higher steady-state levels (46–48). PCR amplification of the cDNA region spanning the evolutionarily conserved p53 region often mutated in cancers (codons 117–309) (49) was therefore carried out, and multiple clones were sequenced. Point mutations resulting in amino acid substitutions were found in the p53 cDNAs in C-33A and HT-3 cell lines within this region, affecting codons 273 and 245, respectively. A CGT → TGT at codon 273 in C-33A cells resulting in an amino acid change of Arg → Cys was found, and a GGC → GTC at codon 245 resulting in a Gly → Val substitution was found in HT-3 cells. Multiple independent clones verified these mutations. These p53 mutations have also been independently noted in each of these two cell lines (T. Crook and K. Vousden, personal communication).

Despite the low levels of p53, the p53 genes might also be mutated in the HPV-positive cell lines. The same conserved region of p53 analyzed above was therefore amplified and sequenced from the cDNA of each of the five HPV-positive cell lines. No p53 gene mutations were found.

DISCUSSION

Approximately 85% of human cervical cancers harbor HPV DNA sequences (1, 50), and the viral E6 and E7 oncoproteins are generally expressed within these tumors (3–6). The tumor-suppressor proteins pRB and p53, which can be complexed by the E7 and E6 oncoproteins, respectively (20, 21, 25), may be relevant targets of the HPVs. Indeed, mutations that inactivate or alter the functions of each of these genes characterize many different human cancers. Mutations in *RB* that eliminate expression of the gene or result in a truncated or functionally altered product have been demonstrated in a variety of human cancers other than retinoblastomas, including sarcomas, small cell carcinomas of the lung, and breast cancers (51). Mutations in the p53 gene have been similarly detected in a high percentage of colon, breast, lung, brain, and esophageal human cancers (49).

The availability of a series of HPV-positive and HPV-negative human cervical carcinoma cell lines provided the opportunity to evaluate whether or not genetic events that altered pRB and p53 might play a role in this cancer. The results were consistent with the hypothesis that pRB and p53 regulatory functions are commonly annulled in human cervical cancers, either by mutation in the HPV-negative cases or as a consequence of their complex formation with the HPV E6 and E7 oncoproteins.

The binding of viral oncoproteins to pRB is thought to functionally inactivate its tumor-suppressor activity. The active form of pRB appears to be the hypophosphorylated form of the protein (52–54), and it is this form that is preferentially found in complex with SV40 large tumor antigen (55) and HPV E7 (K.M., unpublished observation). By this model, one assumes that the functional form of pRB is bound in an inactive complex, no longer inhibiting cellular proliferation.

In human retinoblastomas and other sporadic cancers, mutations in *RB* have been compiled and found to map to regions of the cellular protein involved in complexing with the viral oncoproteins (38, 39). The mutated forms of pRB found in cancer cells can no longer complex with viral oncoproteins, suggesting that the former proteins may also be deficient in their ability to associate with the normal cellular targets of pRB (38, 39, 56). In addition, these mutated forms of pRB are impaired in their ability to be phosphorylated (38, 39, 56). The mutated forms of pRB in C-33A and HT-3 cells have these same characteristics in that they were not phosphorylated and could not complex with adenovirus E1A. The mutations in each of the cell lines mapped to splice junctions affecting exons 13 and 20, respectively, and fall within the domains of pRB necessary for complexing the viral oncoproteins. The splice-acceptor mutation in C-33A cells leads to the in-frame deletion of four amino acids through the use of an alternate acceptor site 12 nt downstream. The splice-donor mutation in HT-3 cells (AGGT → GGGT) results in the precise deletion of exon 13 from the mRNA. Mutations in the 5′ splice junctions that result in exon skipping have been previously described at the −1, +1, and +2 positions, but to our knowledge this is the only example of a naturally occurring mutation with this effect at the −2 position (41).

The complex formation between the viral oncoproteins and p53 is also thought to inactivate the normal function of p53 in regulating cell proliferation. In SV40 and adenovirus 5-transformed cells, association of the virus-encoded oncoproteins and p53 increased half-life and steady-state levels of p53 (45). The association of HPV-16 or HPV-18 E6 in complex with p53 has been demonstrated *in vitro* (25). Because of this association *in vitro*, p53 is targeted for degradation through the ubiquitin-dependent proteolysis system (44). As anticipated, the p53 level in SV40-immortalized keratinocytes was very high, and the levels in the several lines of HPV-immortalized keratinocytes examined were quite low, al-

though still detectable. Low levels of p53 were also found in HPV-positive cervical carcinoma cell lines, indicating that the E6 association with p53 does not cause an increase in its steady level *in vivo*. Relative to nonimmortalized HFKs, the steady-state level of p53 measured in Fig. 4 was lower in four of five HPV-positive cervical carcinoma cell lines examined and in three of four independent HPV-immortalized HFK lines. Assuming that the E6-promoted degradation of p53 seen *in vitro* is of physiological significance, these data indicate that not all cellular p53 is targeted by E6. This discrepancy between the marked *in vitro* degradation of p53 promoted by E6 and the modestly decreased levels of p53 seen *in vivo* is yet to be understood.

The elevated levels of p53 seen in the C-33A and HT-3 cell lines suggested that this gene might be mutated in each of these two HPV-negative cell lines; this possibility was confirmed by direct sequence analysis of cDNA from each line. The mutations affected codons 245 and 273 and, as such, map to an evolutionarily conserved domain in which many mutations have been detected in a variety of human cancers (49).

This study provides evidence that p53 and pRB are relevant targets in cervical carcinogenesis. Inactivation of these two cellular tumor-suppressor proteins through their interaction with E6 and E7 may be the functional equivalent of specific mutations in the p53 and *RB* genes. Some mutations in p53 may actually result in a gain of function (57), something that may not be achieved by the E6/p53 interaction. Furthermore, such activating p53 mutations could even be associated with neoplastic progression in some HPV-positive cancers if the mutated p53 were not able to complex with E6 and were therefore not targeted for degradation.

We are grateful to Drs. Jon Huibregtse and Scott Vande Pol for a critical reading of this manuscript. We are grateful to Carol Comlish for her editorial assistance in preparing this manuscript. M.S. was supported by the Deutsche Forschungsgemeinschaft, and K.M. was supported by an advanced training grant from the Swiss National Science Foundation.

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