American Journal of Pathology, Vol. 143, No. 5, November 1993

p53 Gene Mutations and MDM2 Amplification Are Uncommon in Primary Carcinomas of the Uterine Cervix

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The p53 gene is the most frequently altered gene known thus far in a wide variety of human cancers. Inactivation of p53, either through mutation or through interaction with the human papillomavirus (HPV) E6 oncoprotein, is a characteristic feature of all cervical carcinoma cell lines that have been studied. These findings suggest that p53 inactivation is required for cervical carcinoma development and that HPV infection and p53 mutation may be mutually exclusive. We have studied the p53 gene in 35 primary cervical carcinomas. DNA sequence and single strand conformational polymorphism analyses were used to evaluate p53 in 27 squamous carcinomas (25 HPV-positive) and eight adenocarcinomas (four HPV-positive). A missense mutation of p53 was observed in one HPV 16-positive squamous carcinoma, demonstrating that p53 mutations can occur in combination with HPV infection. The HPV-negative tumors all lacked p53 gene mutations. The absence of p53 mutations in HPV-negative cases prompted an assessment of tumors for MDM2 gene amplification. The MDM2 gene encodes a p53 binding protein and has been found to be amplified in some human tumors lacking p53 mutations. MDM2 amplification was not identified in any of the tumors we examined, including four HPV-negative cases. Our findings show that HPV infection and p53 gene mutation are not mutually exclusive and suggest that many HPV-negative carcinomas may arise via a pathway independent of p53 inactivation. (Am J Pathol 1993, 143:1398–1405)

Human papillomaviruses (HPV) have been implicated in the development of a wide variety of benign and malignant lesions of the lower genital tract, upper respiratory region, and skin. Over 60 distinct viral types have been identified to date. These viruses can be grouped as either cutaneous or mucosal and further subdivided as high- or low-risk types with respect to their association with malignant neoplasia. In the uterine cervix, the majority of high-grade squamous intraepithelial lesions and invasive carcinomas harbor high-risk HPV types (most notably HPV 16 and HPV 18), whereas low-risk types are more frequently associated with low-grade squamous intraepithelial lesions and benign condylomas.[1] Although the vast majority of primary invasive cervical carcinomas and high-grade squamous intraepithelial lesions contain HPV, viral infection is undetectable in a small percentage of cases examined.[2]

The viral genome present in primary cervical carcinomas and tumor-derived cell lines is usually found in an integrated state, often disrupting the E1 and E2 genes and allowing active expression of the E6 and E7 transforming genes.[3–5] The E6 and E7 genes have been found to immortalize primary cells *in vitro*, but a fully transformed phenotype is rarely achieved, suggesting the requirement of additional events.[6,7] One interesting feature of the E6-transforming proteins expressed by oncogenic HPV types is their ability to bind the cellular tumor-suppressor protein p53.[8] Previous studies have shown that high-risk HPV E6 pro-

Supported by Council for Tobacco Research grant #3223 and NIH grant #AI16959.

Accepted for publication May 17, 1993.

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teins translated *in vitro* are able to bind wtp53 protein and mediate its degradation through a ubiquitin-mediated mechanism.[9,10] This E6-wtp53 interaction suggests a mechanism through which HPVs might exert their oncogenic potential in HPV-infected cells. In tumors that do not harbor HPVs, p53 may be inactivated by mutation or through alteration of proteins responsible for mediating p53 function. Little is known about these normal cellular mediators, but one potential mediator is the MDM2 gene, which encodes a p53 binding protein. Amplification of this gene has recently been found in a subset of human sarcomas lacking p53 mutations.[11,12]

Two previous studies have analyzed the state of p53 in cervical carcinoma-derived cell lines.[13,14] Mutations of p53 were absent in each of six HPV-positive cell lines, whereas the two HPV-negative cell lines tested were both found to contain missense mutations. These findings suggest that 1) E6/wtp53 interactions may be functionally equivalent to p53 mutations and 2) wtp53 is an important target of inactivation during cervical tumorigenesis, both in HPV-infected and uninfected cells. To test the validity of these hypotheses in primary tumors, we examined 35 cervical carcinomas for the presence of HPV sequences and p53 gene mutations. Tumors for which sufficient quantities of DNA were available were also analyzed for losses of heterozygosity (LOHs) of chromosome 17p, which contains the p53 gene. Three polymorphic markers in close proximity to the p53 gene were used for this analysis. In addition, this same group of tumors was analyzed for MDM2 gene amplification.

Materials and Methods

Tissue and Processing

A group of 16 primary cervical tumors (eight squamous carcinomas and eight adenocarcinomas), and corresponding normal tissue obtained for each case, were acquired through the Johns Hopkins Surgical Pathology Tumor Bank. An additional group of 19 invasive squamous carcinomas of the cervix were obtained from a study conducted in Spain and Colombia.[15,16] All tissues were stored frozen at −80 C until processed. Genomic DNA was isolated from cryostat sections of frozen primary tumors essentially as described by Fearon et al.[17] Hematoxylin and eosin-stained sections were examined at regular intervals and used as guides to dissect away portions of tissue heavily contaminated by normal or necrotic tissue. Only regions containing greater than 75% tumor cells were retained and used to prepare genomic DNA for polymerase chain reaction (PCR) and Southern blot analyses.

HPV Detection and Typing

A previously described set of consensus primers capable of amplifying a region of the L1 capsid gene conserved among HPV types was utilized for PCR detection of HPV.[18] Primers flanking a 265-bp region of the human β-globin gene were included in each reaction as an internal control for PCR amplification of target DNA. Genomic DNA from the cell lines SiHa (HPV 16-positive) and HeLa (HPV 18-positive), representing approximately 1,000 copies of viral DNA template per reaction, were included in each test to monitor the sensitivity of the assay. Reactions in which the DNA template was replaced by PCR buffer alone were interspersed between test specimens as negative controls. PCR reactions were carried out in a Coy thermal cycler set to the following parameters: 35 cycles consisting of 30 seconds at 95 C, 30 seconds at 61 C, and 1 minute at 72 C. Reactions (100 μl) contained a mixture of 1× PCR buffer (Cetus, Emmeryville, CA), 100 μmol/L of each dNTP, 0.25 μmol/L of each primer, 0.1 μg template DNA and 2.5 U of Taq polymerase (Cetus). PCR products were electrophoretically separated on 2% agarose gels, transferred to nylon filters (Zeta Probe GT, Bio-Rad Laboratories, Richmond, CA) by standard capillary techniques[19] and probed with HPV 6, 11, 16, and 18 type specific [^{32}P]ATP end-labeled oligonucleotides.[18]

Southern blot analysis was performed to confirm the PCR results in cases where adequate amounts of tissue were available for isolation of genomic DNA. Ten μg of genomic DNA from each sample were digested with the restriction enzyme *Pst*I, electrophoresed on 1.0% agarose gels, and transferred to nylon filters. Filters were hybridized successively at high (10 C below the T_m) and low (37 C below the T_m) stringency with HPV type 6, 16, 18, and 31 full-length viral genome probes. All probes were labeled with [^{32}P]dCTP by the random primer method.[20]

For cases in which ethidium bromide staining of PCR products and/or low stringency Southern hybridization detected HPV sequences but failed to allow assignment of HPV type, HPV typing was performed by sequencing the HPV L1 PCR product essentially as described by Casanova et al.[21] The consensus primers used to generate the PCR product were employed as forward and reverse sequencing primers.

E6 Gene Sequencing

The E6 gene from several HPV 16-positive cases was PCR-amplified utilizing a previously characterized set of primers[22] then cloned into the Blue Script/SK vector (Stratagene, La Jolla, CA). Pooled clones were then sequenced en masse as described by Nigro et al.[23]

RFLP Analysis of Chromosome 17p

Allelic loss of the chromosomal arm harboring p53 was evaluated using the probes p144D6, pYNZ22.1, and pYNH37.3, which detect anonymous loci located telomeric to the p53 gene.[24–26] Genomic DNA (10 μg) from primary tumors and corresponding normal tissue was digested with the appropriate restriction enzyme (*Msp*I for probes p144D6 and pYNH37.3; *Hin*fI for pYNZ22.1), electrophoresed on 1.2% agarose gels, transferred to nylon, and probed at high stringency essentially as outlined under HPV detection.

Single Strand Conformational Polymorphism (SSCP) Analysis of the p53 Gene

Separate PCR amplifications of exons 5, 6, 7, and 8 of the p53 gene were performed as outlined above under HPV detection, except that reactions were reduced to a volume of 50 μl and included 0.1 μCi of [^{32}P]dCTP. This region of the p53 gene was examined because the majority of mutations identified to date occur within conserved domains located in these exons.[27] The following intron-derived primers were used for PCR amplifications: exon 5, 295-bp product, (sense) 5′gactttcaactctgtctcc3′ and (antisense) 5′gagcaatcagtgaggaatc3′; exon 6, 190-bp product, (sense) 5′tccccaggcctctgattcc3′ and (antisense) 5′tgacaaccacccttaaccc3′; exon 7, 192-bp product, (sense) 5′caaggcgcactggcctcatc3′ and (anti-sense) 5′cacagcaggccagtgtgcag3′; exon 8, 243-bp product, (sense) 5′gatttccttactgcctcttgc3′ and (anti-sense) 5′gtgaatctgaggcataactgc3′. At the completion of the PCR, 3 μl of each reaction was removed and added to 100 μl of a solution containing 0.05% sodium dodecyl sulfate, 5 mmol/L ethylenediaminetetraacetic acid, 50% formamide, 10 mmol/L NaOH, 0.025% bromophenol blue, and 0.025% xylene cyanol. Samples were heated to 95 C for 5 minutes, iced, and 3 μl was loaded onto a 6% polyacrylamide/5% (w/w) glycerol gel. Following electrophoresis for 15 hours at 8 watts, gels were placed on Whattman 3MM paper, dried, and exposed to XAR film (Kodak) for 16 to 72 hours.

p53 Gene Sequencing

A single 1.8-kb genomic fragment containing exons 5 through 8 of the p53 gene was generated by PCR utilizing the exon 5 sense primer and exon 8 antisense primer described above. Primers included *Bam*HI and *Eco*RI restriction enzyme recognition sequences to facilitate directional subcloning of the amplified products into the Blue Script/SK vector (Stratagene). Pools of recombinant plasmids, containing no less than 50 individual clones, were then sequenced as previously described.[28,29] Cases that displayed nucleotide alterations were subjected to verification by a second independent extraction of genomic DNA, followed by PCR amplification, cloning, and sequencing as described above.

MDM2 Gene Amplification

Tumors were evaluated for MDM2 gene amplification by Southern blot hybridization. Genomic DNA (10 g) from paired tumor and normal tissues was digested with restriction enzymes *Hin*fI or *Pst*I, electrophoresed on agarose gels, transferred to nylon, and hybridized to a 1.7-kb MDM2 complementary DNA probe (generously provided by Dr. Bert Vogelstein). Autoradiographs were scored by comparing the MDM2 signal in each tumor with that in its corresponding normal DNA. In keeping with previous studies,[11,12] tumors with fivefold or greater MDM2 signal were considered to have amplified the gene. Single copy reference probes (pYNZ22.1, p144D6, and pYNH37.3) were used to control for variations in sample loading.

Results

HPV Detection

HPV DNA was detected in 29 of 35 tumors following PCR amplification with the L1 consensus primers and assessment of the electrophoresed products on ethidium-stained agarose gels. Transfer of the PCR products to nylon filters and successive hybridization with HPV type-specific oligonucleotide probes (HPV 6, 11, 16, and 18) revealed the following tumor and HPV type distribution: 20 HPV 16-positive (19 squamous carcinomas and one adenocarcinoma), four HPV 18-positive (one squamous

carcinoma and three adenocarcinomas), and 11 HPV-negative (seven squamous carcinomas and four adenocarcinomas). The L1 PCR products from five cases that were HPV-negative by hybridization, but HPV-positive on ethidium bromide-stained gel, were then directly sequenced to determine the HPV type present. Of these cases, five HPV types with significant homology to HPV 16 (two cases), HPV 18 (two cases), and HPV 51 (one case) were detected (Table 1).

When adequate amounts of tissue were available for extraction of high molecular weight genomic DNA, high and low stringency Southern blot hybridizations were performed. The results obtained by Southern hybridization were in agreement with those obtained by PCR in that each case positive by Southern was also PCR-positive. The cases in which HPV type was determined by DNA sequence analysis demonstrated varying degrees of cross reactivity with HPV 16 and 18 probes following low stringency Southern hybridization (Table 1).

Analysis of 17p LOHs by RFLP Analysis

Because the p53 gene itself contains few known frequent polymorphisms, allelic losses of 17p were evaluated using the probes p144D6, pYNZ22.1, and pYNH37.3, which detect highly polymorphic loci located telomeric to the p53 gene. Cases were classified as informative when two alleles were detected at a given locus in the normal tissue of an individual. Chromosome 17p LOHs were not detected in any of 18 cases informative with at least one marker (Table 1, Figure 1).

Detection of p53 Mutations by SSCP and DNA Sequence Analyses

SSCP analysis takes advantage of the unique secondary structure assumed by each strand of a DNA duplex upon electrophoretic separation under nondenaturing conditions.[30] Melting of the duplex under denaturing conditions followed by polyacrylamide gel electrophoresis under nondenaturing conditions results in the resolvable separation of the coding and noncoding DNA strands. Single base pair substitutions, which alter the secondary structure and mobility of individual DNA strands, can be detected when compared to DNA with wild type sequence (Figure 2). The p53 genes in all 35 tumors were evaluated by SSCP. Initially the p53 gene was cloned and sequenced from 16 tumors. In every case, the SSCP and sequencing results were in total agreement (Table 1), and subsequent cases showing no SSCP band shifts were not subjected to additional sequence analysis.

Variations in exon 6 of the p53 gene were detected by SSCP analysis in two tumors (Table 1). DNA sequence analysis of exon 6 revealed an A to G nucleotide substitution at codon 213 in one HPV 18-positive adenocarcinoma (Table 1, case C-29). This change was also detectable in the normal tissue of this individual and represents a silent (no amino acid change) sequence polymorphism

Table 1. *Tumor Type, HPV Type Detected, and p53 Gene Status in 35 Primary Cervical Carcinoma*

No.	Type	HPV type detected		p53 Gene status†	17p LOH	MDM2 amplification
		PCR	Southern			
C-1 thru C-4	Squamous	16	16	wt	(–)	(–)
C-5	Squamous	16	16	214 T → G‡	(–)	ND
C-6 thru C-18	Squamous	16	ND	wt	ND	ND
C-19 thru C-20	Squamous	16§	16§	wt	ND	ND
C-21	Squamous	16	16	wt	(–)	(–)
C-22	Squamous	18	18	wt	(–)	(–)
C-23	Squamous	18§	18§	wt	(–)	(–)
C-24	Squamous	18§	18§	wt	(–)	(–)
C-25	Squamous	51§	16/18§	wt	(–)	ND
C-26	Squamous	(–)	ND	wt	ND	ND
C-27	Squamous	(–)	ND	wt	ND	ND
C-28	Adeno‖	16	16	wt	(–)	(–)
C-29	Adeno	18	18	213 A → G¶	(–)	ND
C-30	Adeno	18	18	wt	(–)	(–)
C-31	Adeno	18	18	wt	(–)	(–)
C-32 thru C-35	Adeno	(–)	(–)	wt	(–)	(–)

* Southern blot hybridization was performed to confirm PCR results when adequate tissue was available.
† p53 gene status as determined by SSCP or DNA sequence analysis.
‡ T to G missense mutation at codon 214 (His to Gln) in exon 6 of the p53 gene.
§ HPV 16, 18, or 51 related type as determined by low stringency Southern hybridization and confirmed by sequencing of the HPV L1 PCR product. Specific HPV types were not assignable.
‖ Group of adenocarcinomas includes one clear cell carcinoma.
¶ Silent A to G polymorphism at codon 213 in exon 6 of the p53 gene.

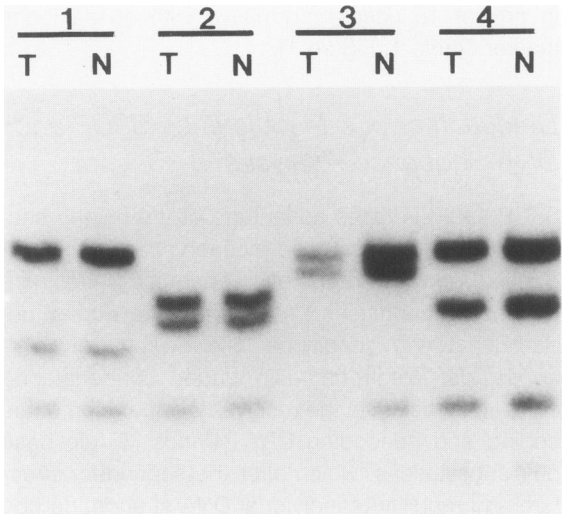

Figure 1. *An example of RFLP analysis performed to detect LOH on chromosome 17p in the vicinity of the p53 locus. Four primary cervical tumors and normal tissue from the same individuals hybridized with the probe pYNZ22.1 are shown. Comparison between the number of alleles present in the tumor and normal tissue reveals that case 1 is not informative at this locus. Cases 2 through 4 are informative and show no allelic losses.*

present in approximately 5% of the population.[31] A missense mutation (T to G) affecting codon 214 and resulting in an amino acid substitution (histidine to glutamine) was detected in one HPV 16-positive squamous carcinoma (Table 1, case C-5 and Figure 3). This mutation seems to affect only one allele of the p53 gene because both the wild type nucleotide (T) and mutant nucleotide (G) are detectable at the same position in the sequencing gel. Alternatively, the wild type nucleotide present at this position may represent PCR amplification of wtp53 from normal cells contaminating this tumor sample. This possibility is unlikely because this sample was highly enriched for tumor (approximately 95%).

Because the interaction between E6 and wtp53 seems to be an important consequence of HPV infection, the unexpected finding of a p53 mutation in an HPV 16-positive tumor led us to examine the E6 gene present in this case. DNA sequence analysis detected an A to G transition at codon 10 (arginine to glycine) and a T to G transversion at codon 83 (leucine to valine). Whereas these sequence alterations probably reflect minor variations in highly related viral types, the codon 10 alteration is of particular interest because this codon is in a region of E6 found to be critical for mediating p53 degradation.[9]

MDM2 Gene Amplification

The absence of p53 gene mutations in our HPV-negative tumors prompted us to look for alterations

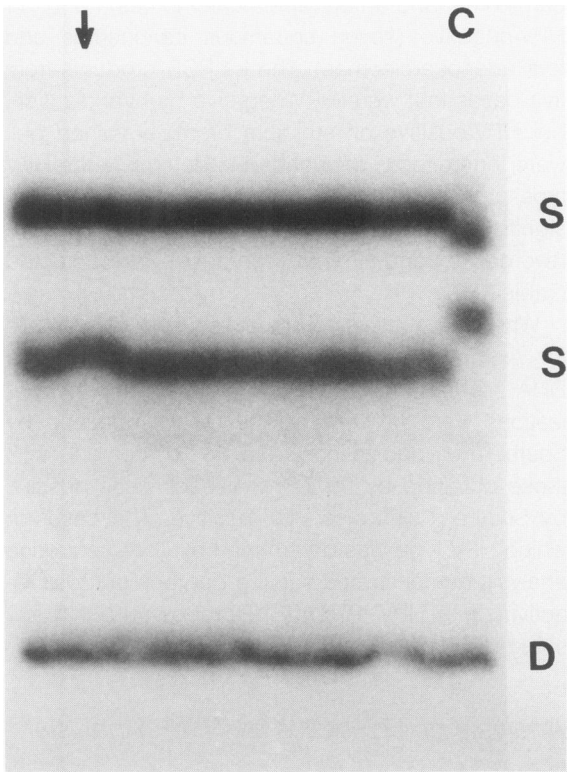

Figure 2. *SSCP analysis of the p53 gene. The three bands resolved in each gel represent the coding strand of the DNA duplex (S), the non-coding strand (S), and reannealed double stranded DNA (D). Shifts in mobility represent single base-pair deviations from the wild type sequence. A change in the nucleotide sequence was detected in exon 6 of the p53 gene in one HPV 16-positive squamous carcinoma (case C-5 marked by an arrow). The colorectal carcinoma cell line SW837, which also contains an exon 6 mutation, was included as a positive control (C).*

of the MDM2 gene in this set of primary cervical carcinomas. Southern blot hybridization failed to identify amplification or rearrangement of MDM2 in any of 15 cases for which adequate quantities of high quality genomic DNA were available (Table 1). A summary of tumor type, 17p LOHs, p53 gene mutations, and MDM2 amplification in our HPV-positive *versus* HPV-negative tumors is presented in Table 2.

Discussion

The cellular tumor suppressor gene p53 is the most frequently mutated gene identified to date in a wide spectrum of human cancers.[27] Until recently, little was known about the function of wtp53 within the cell. Kastan et al have recently shown that wtp53 plays a central role in the temporary inhibition of replicative DNA synthesis observed in normal cells following DNA damage.[32,33] Normal mammalian cells exhibit G_1 cell cycle arrest and inhibition of replicative DNA synthesis following DNA damage.

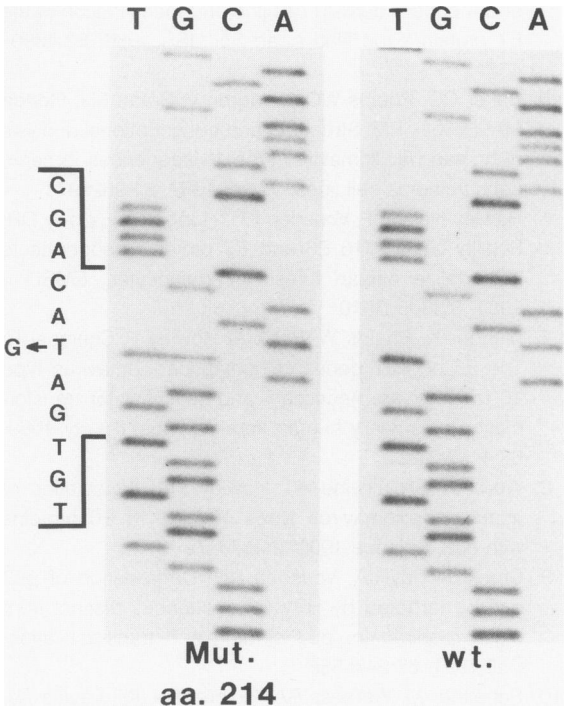

Figure 3. *DNA sequence analysis of exon 6 of the p53 gene.* (**Left**) *The HPV 16-positive squamous cell carcinoma, case C-5. A missense mutation (T to G transversion) affecting codon 214, which results in a change in amino acid sequence from histidine to glutamine, was detected.* (**Right**) *Wild type p53 sequence shown for comparison.*

Table 2. *Summary of Molecular Characterization of Primary Cervical Tumors*

	HPV-positive*		HPV-negative†	
	No. tested	No. positive*	No. tested	No. positive*
17p LOH	14	0	4	0
p53 mutation	29	1	6	0
MDM2 amplification	11	0	4	0

* Twenty-five of 27 squamous carcinomas and four of eight adenocarcinomas were HPV-positive.

† Two of 27 squamous carcinomas and four of eight adenocarcinomas were HPV-negative.

This mechanism, mediated by transient increases in wtp53, may be a protective physiological response that preserves the integrity of the DNA template by allowing cells to undertake DNA repair and avert the fixation of mutations in daughter cells. Cells lacking wtp53 or expressing mutant p53 fail to arrest in response to DNA damage. The inhibition of DNA synthesis associated with the normal cellular response to DNA damage can also be disrupted by expression of E6 proteins encoded by high-risk HPV types.[34] This observation suggests a plausible mechanism through which HPV may predispose infected cells to the accumulation of genetic alterations necessary for cervical tumor progression. Such a scenario is consistent with our current understanding of the multistep nature of tumorigenesis.[35]

The interaction of viral oncoproteins such as simian virus 40 T-Ag, adenovirus E1b, and HPV E6, with p53 protein provides a mechanism to inactivate p53 functionally and may bypass the need for p53 gene mutations. Alteration of genes encoding cellular mediators of p53 function, such as MDM2, may also bypass this need. The detection of p53 mutations in HPV-negative cervical carcinoma cell lines and absence of p53 mutations in HPV-positive cell lines further support the notion that p53 is an important cellular target within the HPV-infected cell.[13,14] Two previous studies[36,37] found p53 mutations in HPV-negative primary carcinomas, suggesting that the observations in cervical cancer cell lines could be extended to primary tumors and that inactivation of p53 is an obligatory event in cervical tumorigenesis. However, together these studies examined only nine HPV-negative cases.

Our results suggest that mutation of the p53 gene is not always required in HPV-negative tumors and may occasionally occur in HPV-positive tumors. In a similar study,[38] Fujita et al identified mutations of p53 in two HPV-positive carcinomas and no mutations in seven HPV-negative cases. Allelic losses of p53 were not found in any of 10 informative cases. In combination with our study, 13 HPV-negative cervical carcinomas were found to lack p53 mutations in the most frequently mutated portions of the gene. Many HPV-negative cervical tumors may therefore arise through pathways entirely independent of p53. The absence of MDM2 gene amplification in these tumors further supports this notion. Clearly, other cellular mediators of p53 function must be evaluated as they are identified. For instance, the radiation inducible gene GADD45 has been demonstrated to be a target of transcriptional activation by wtp53 and thus may serve as such a target for inactivation during tumor development.[39]

The detection of a p53 mutation in an HPV-positive cervical carcinoma led us to examine the E6 gene expressed in this case. We speculated that this p53 mutation may have arisen through selective pressure brought on by a functionally inactive E6 variant. Examination of E6 in this tumor revealed a missense nucleotide substitution in one domain of the protein implicated in mediating degradation of p53. However, ongoing functional studies have not yet demonstrated that this E6 variant is defective. Furthermore, this E6 sequence variation is not unique to this case nor limited to tumors with mutant p53 genes (Slebos et al, unpublished data). The occurrence of p53 mutations in HPV-positive cervical

carcinomas may confer a growth advantage to the affected cells of these tumors, in addition to that gained by the interaction of E6 with wtp53. Characterization of p53 mutations occurring in HPV-positive cases may yield insight into the functional domains of p53 that are required for the interaction of p53 with E6 or other cellular proteins.

Numerous studies have documented frequent mutations in the p53 gene in a wide variety of human tumors. The majority of cervical carcinomas contain integrated HPV and express E6 oncoproteins. Accumulating evidence suggests that the E6-p53 interaction inactivates wtp53 in HPV-infected cervical epithelium and therefore provides the functional equivalent of p53 gene mutations in these cells. Our study supports this hypothesis, as the great majority of HPV-positive squamous carcinomas were found to lack mutant p53 genes. In contrast to some previous studies, the HPV-negative cervical carcinomas in our series also lacked p53 gene mutations. This finding, in addition to absence of MDM2 amplification, suggests that the development of many HPV-negative carcinomas does not require inactivation of the p53 gene and may therefore arise through p53-independent pathways.

Acknowledgments

The authors wish to thank Dr. Robert J. Kurman for helpful discussions pertaining to this work and for critical review of the manuscript. TDK is a recipient of the Frederik B. Bang Research Award. RJS is supported by an award from the Dutch Cancer Society (KWF). KRC is a recipient of a Sandoz Clinician Scientist Award. LH is a Howard Hughes Medical Institute Physician Scientist Research Fellow. This project was supported by funds from the Richard W. Telinde Endowment, The Council for Tobacco Research (#3223), and the NIH (AI16959).

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