Expression of p16, Rb, and p53 Proteins in Squamous Cell Carcinomas of the Anorectal Region Harboring Human Papillomavirus DNA

Danielle W. Lu, M.D., Samir K. El-Mofty, D.M.D., Ph.D., Hanlin L. Wang, M.D., Ph.D.

Lauren V. Ackerman Laboratory of Surgical Pathology, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri

Human papillomavirus (HPV) has been implicated as an etiologic agent for the development of squamous cell carcinoma of the anorectal region. It has been shown that the HPV E6 and E7 oncoproteins are able to inactivate the tumor suppressor functions of p53 and Rb. In cervical and head and neck cancers, HPV infection is also associated with an overexpression of p16, a cyclin-dependent kinase inhibitor. The expression of these cell cycle regulators in squamous cell carcinomas of the anorectal region has not been well studied. In the current study, 29 cases of squamous cell carcinoma of the anorectal region were immunohistochemically examined for the expression of p16, Rb, and p53 proteins. Tumor cell DNA was also extracted from paraffin blocks and subjected to broad-spectrum HPV DNA testing and typing. The results show that the tumor cells exhibited a strong and diffuse nuclear stain (with some cytoplasmic positivity) for p16 in all 29 cases (100%). The adjacent nonneoplastic squamous epithelium or colonic mucosa, in contrast, was completely negative. Loss of Rb nuclear staining in tumor cells was observed in 20 cases (69%). The p53 protein was essentially undetectable, with only 6 cases containing <10% positive cells. HPV DNA was detected in every case (100%), with 25 cases (86%) harboring Type 16. In addition, almost identical results were obtained in 12 HPV-positive squamous cell carcinomas of the upper aerodigestive tract. This was in marked contrast to those of HPV-negative tumors, where positive p16 staining and loss of Rb expression were seen in only 2/21 (10%) and 1/21 (5%) cases, respectively. These

observations indicate that overexpression of p16 and loss of Rb nuclear staining are commonly associated with high-risk HPV infection, which may serve as useful surrogate biomarkers for identifying squamous cell carcinomas harboring HPV DNA.

KEY WORDS: Anorectal, Human papillomavirus, p16, p53, Rb, Squamous cell carcinoma.

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Epidemiological and laboratory investigations have established a strong association of human papillomaviruses (HPV) with human cancers, particularly squamous cell carcinomas of the uterine cervix and anal canal, and some carcinomas of the upper aerodigestive tract (1-3). The carcinogenic effect of these "high-risk" HPVs, as defined by their documented association with invasive carcinomas (4), has been largely attributed to the products of two viral genes, E6 and E7, which are consistently expressed in cells infected by the viruses (5-7). It has been shown that the transforming activity of these two viral oncoproteins is mediated at least in part through inactivating the functions of two important cellular tumor suppressors p53 and Rb, which serve critical roles in regulating cell cycle control. The E6 oncoprotein interacts physically with p53 to promote its degradation via the ubiquitin-proteasome pathway (8-10). On the other hand, the oncoprotein E7 forms a complex with Rb protein, leading to its functional inactivation as well as accelerated degradation (11, 12). These synergistic effects result in a deregulated cell cycle control and are thus believed to be critically important in HPV-induced carcinogenesis (1-3, 5, 6, 13-17). More recent studies have also demonstrated that the protein level of another cell cycle regulator, p16 (also known as p16^{INK4A}, MTS1, and CDKN2), is overexpressed in high-risk-HPV-related cervical cancers (18-22) and head and neck cancers (23). This is interesting because the expression of p16 is regulated by Rb, presumably through a negative feedback mecha-

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Address reprint requests to: Hanlin L. Wang, M.D., Ph.D., Lauren V. Ackerman Laboratory of Surgical Pathology, Department of Pathology and Immunology, Campus Box 8118, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110-1093; fax: 314-747-2040; e-mail: hwang@path.wustl.edu.

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nism (24, 25), and because p16 itself also functions as a tumor suppressor by inhibiting cyclin-dependent kinases 4 and 6 that phosphorylate Rb and thus decelerating the cell cycle progression (26).

Squamous cell carcinoma of the anal canal is uncommon, but its incidence has increased in recent years (27). Like in cervical cancers, high-risk types of HPV have been implicated in the pathogenesis in the majority of the cases (27–29). However, there are only a few studies that have examined the expression of p53 protein (30–33) and only one study that has examined the expression of Rb protein (32) in HPV-positive anal carcinomas, which have generated inconclusive results. The expression status of p16 protein in anal carcinomas has not yet been investigated. In the studies described in this report, the expression of p16, Rb and p53 proteins in 29 cases of squamous cell carcinoma of the anorectal region was examined immunohistochemically, and the HPV status in these tumors was analyzed by sensitive PCR-based assays. Our data demonstrate that high-risk HPV infection correlates with overexpression of p16 and loss of Rb nuclear staining.

MATERIALS AND METHODS

Clinical Specimens

Twenty-nine cases of squamous cell carcinoma of the anorectal region were retrieved from the 1989–2002 surgical pathology archives at Washington University Medical Center, including 25 biopsies and 4 resections. Clinical data were reviewed to ensure that they were indeed anorectal primaries. Formalin-fixed, paraffin-embedded tissue blocks were available from each case. Hematoxylin and eosin (H&E)-stained slides were reexamined to confirm the original diagnosis. In addition, 12 HPV-positive and 21 HPV-negative squamous cell carcinomas from the upper aerodigestive tract, which have been characterized previously (34), were included for comparison.

DNA Extraction and HPV DNA Detection and Typing

Tumor tissue from paraffin blocks was dissected using a sterile blade, followed by deparaffinization twice in a 1.5-mL tube with 1 mL xylene and washing twice with 1 mL of 100% ethanol. The tissue samples were digested with proteinase K (1 mg/mL) in a volume of 0.1–0.3 mL at 56° C overnight. The proteinase K was then inactivated by boiling for 10 minutes, and 2 μ L of DNA aliquot was directly used for polymerase chain reaction (PCR). In these experiments, broad-spectrum HPV DNA amplifica-

tion was performed employing the short PCR fragment (SPF10) primer set provided by Delft Diagnostic Laboratory (the Netherlands), exactly following the manufacturer's instructions. Specifically, the SPF10 primers amplify a 65-bp DNA fragment from the L1 region of the HPV genome (35, 36), with the primer sequences described previously (35). The PCR products were subjected to electrophoresis on a 4% low melting agarose gel, and the 65-bp band was visualized by ethidium bromide staining. In every experiment, an HPV-positive and an HPV-negative control were included. All cases were analyzed at least twice using different DNA concentrations. In addition, PCR amplification of the β -globin gene was performed using the primers described by Saiki *et al.* (37) to ensure adequate DNA quality.

Samples positive for HPV DNA were further genotyped using the INNO-LiPA (line probe assay) HPV genotyping research prototype kit provided by Delft Diagnostic Laboratory. This assay detects 25 different HPV genotypes (high-risk HPVs: 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70; and low-risk HPVs: 6, 11, 34, 40, 42, 43, 44, 54, 74) simultaneously in a single analysis (36). Briefly,

FIGURE 1. Squamous cell carcinoma of the anorectal region showing basaloid features without overt keratinization (H&E; original magnification, 200 $\times$).

biotin-labeled PCR products were reverse hybridized to specific probes on the LiPA strips under highly stringent conditions. Alkaline phosphatase-labeled streptavidin was then added, and the hybridized complex was visualized by incubation with chromogens nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The results of hybrid-

ization were analyzed by comparing to the standard grid provided by the manufacturer.

Immunohistochemistry

Immunohistochemical studies were performed on 4- μ m tissue sections using commercially avail-

FIGURE 2. Identification of HPV genotypes in anorectal squamous cell carcinomas using line probe assay (LiPA). Note that HPV 58 (Case 11) was reactive with three probes, 31/40/58, c56, and 58; HPV 18 (Case 15), with two probes, 18 and c68; and HPV 33 (Cases 22 and 25), with two probes, c31 and 33. Two HPV types (16 and 35) were detected in Case 27. These experiments have been repeated once, and those results were identical.

FIGURE 3. Immunohistochemical staining for p16 showing a strong and diffuse immunoreactivity in anorectal squamous cell carcinomas, with a predominantly nuclear staining and some cytoplasmic positivity (A; original magnification, 400 $\times$). The adjacent nonneoplastic squamous epithelium (B; right) and colonic mucosa (C; right lower) were essentially negative for nuclear staining (original magnification, 100 $\times$).

able monoclonal antibodies. These included mouse IgG2b against p16 (clone 6H12) obtained from Novocastra Laboratories Ltd. (United Kingdom) and mouse IgG1 against Rb (clone IF8) and mouse IgG2a against p53 (clone DO-1) obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immunohistochemical staining was conducted employing the DAKO LSAB2 horseradish peroxidase system (DAKO Corp., Carpinteria, CA), following the manufacturer's instructions with slight modifi-

cations (38). Briefly, deparaffinized tissue sections were first treated with 3% H₂O₂ for 15 minutes to inhibit endogenous peroxidase followed by antigen retrieval using microwave heating in 10 mM citrate buffer (pH6.0) for 10 minutes. After incubation with blocking serum for 20 minutes, sections were incubated with primary antibodies described above for 1 hour at room temperature, with an antibody dilution of 1:40 for anti-p16 and 1:100 for anti-Rb and anti-p53. After further incubation with biotinylated link antibody and peroxidase-labeled streptavidin, the staining was developed by reaction with 3,3'-diaminobenzidine substrate-chromogen solution, followed by counterstaining with hematoxylin. In each experiment, a negative control in which the primary antibodies were replaced by preimmune mouse IgG and a positive control were included.

Nuclear staining was considered positive for p16, Rb, and p53. A tumor was recorded positive if >10% of the tumor cells showed immunoreactivity. The staining characteristics were compared with adjacent nonneoplastic squamous epithelium and/or colonic mucosa.

RESULTS

Clinicopathologic Features of Anorectal Squamous Cell Carcinomas

The patients with anorectal squamous cell carcinomas included in this study ranged in age from 38 to 85 years (mean age, 61.5 y), with a male to female ratio of 1:2.2. Clinical presentation was either an anal mass (16 cases) or a rectal mass (13 cases). Twenty-five patients were managed with radiation and chemotherapy after the diagnosis was established by biopsies, without further surgical intervention. The remaining 4 patients underwent surgical resections, with all tumors being Stage I. Histologically, the majority of the cases (83%) exhibited basaloid features without overt keratinization (Fig. 1). Only 5 cases showed focal keratinization.

HPV DNA Detection and Typing

Using the SPF10 PCR primer set, HPV DNA was amplified in all 29 anorectal squamous cell carcinomas (100%), irrespective of the presence or absence of keratinization in tumor cells (data not shown). Further HPV genotyping employing INNO-LiPA demonstrated that all the cases were associated with high-risk types, with HPV16 being the most prevalent one detected in 25 cases (86%). The other HPV types included 18 (1 case), 33 (2 cases), and 58 (1 case). One case also showed coinfection with two viral types (16 and 35). These results are presented in Figure 2.

FIGURE 4. Immunostaining for Rb showing a uniform nuclear positivity in nonneoplastic squamous epithelium (A) and colonic mucosa (B) adjacent to anorectal squamous cell carcinoma (left; original magnification, 200×). Higher power view highlighting the loss of Rb nuclear staining observed in two thirds of the anorectal squamous cell carcinomas (C; original magnification, 400×). Note that intratumoral inflammatory cells were positive, which served as “built-in” controls. The nuclear immunoreactivity for Rb retained in the remaining cases (D; original magnification, 400×).

Immunohistochemical Findings

Strong and diffuse nuclear staining (with some cytoplasmic positivity) for p16 was observed in all 29 cases (100%) of anorectal squamous cell carcinoma (Fig. 3). The adjacent nonneoplastic squamous epithelium or colonic mucosa present in some of the cases was either completely nonimmunoreactive or exhibited weak membranous or focal cytoplasmic positivity. No nuclear staining was evident in nonneoplastic epithelial cells in any of the cases.

Nuclear positivity for Rb was universally present in nonneoplastic cells (Fig. 4). However, nuclear staining for Rb was observed in only 9 cases of anorectal squamous cell carcinomas. Twenty cases (69%) showed a loss of Rb nuclear staining in tumor cells with only weak cytoplasmic immunoreactivity detected (Fig. 4). The presence or absence of keratinization in tumor cells did not appear to influence the Rb immunostaining characteristics. The p53 protein was essentially undetectable in both tumor and nonneoplastic cells in 23 cases. The remaining 6 cases (21%) showed positive staining for p53 in scattered tumor cells. Because the positive tumor cells accounted for <10% of the population in these cases, they were also considered to be negative (Fig. 5).

FIGURE 5. Immunostaining for p53 showing a completely negative reactivity in the majority of the anorectal squamous cell carcinomas (A). Only six cases exhibited focal positivity seen in <10% of the tumor cells (B; original magnification, 400×).

Because all anorectal squamous cell carcinomas included in this study harbored HPV DNA, control cases from upper aerodigestive tract were similarly studied immunohistochemically for comparison. These included 12 cases of HPV-positive and 21 cases of HPV-negative squamous cell carcinomas. The results of these studies and a comparison with the findings from anorectal cancers are presented in Table 1.

DISCUSSION

The etiopathogenetic importance of high-risk HPV infection in several human cancers has been well established by numerous studies (1–3). In keeping with this notion, we demonstrate that all 29 cases of squamous cell carcinoma of the anorectal region studied in this report are associated with high-risk HPV types, particularly Type 16. In addition, our results show aberrant expression patterns of several important cell cycle regulators in these HPV-related anorectal cancers, further supporting the hypothesis that disruption of the normal cell cycle regulation by the E6 and E7 viral oncoproteins serves critical roles in mediating HPV-induced carcinogenesis (1–3, 15).

One of the major functions of the viral protein E6 is to inactivate p53, a well-studied cellular tumor suppressor that, under the normal physiologic conditions, promotes cell cycle arrest at the late G1 phase and prevents the propagation of genetically damaged cells (39). It is interesting to note, however, that the mechanism by which HPV E6 oncoprotein deregulates p53 is via a direct protein–protein interaction to induce degradation of the p53 protein (8–10). This is different than that occurring in many other types of human cancers, where mutations of the *p53* gene are the targeting events (39). In this regard, an early study by Crook *et al.* (40) demonstrated no *p53* gene mutations in a limited number of HPV-positive anal squamous cell carcinomas, suggesting that *p53* mutations and HPV infection may be mutually exclusive. However, the results of a few immunohistochemical studies, an indirect but useful method to detect *p53* mutations (41), are somewhat conflicting. The reported fre-

quency of coexistence of p53 protein overexpression and HPV infection in anal squamous cell carcinomas in these studies varied from 10.3% to 64.3% (30–33). Although our data presented in this report are broadly in agreement with these published findings in that 6 of 29 cases (21%) showed p53 nuclear overexpression in some of the tumor cells, it is important to emphasize that the positivity was focal and only seen in a minority of the tumor cells. It is thus reasonable to speculate that mutations of the *p53* gene may not serve a major role in HPV-induced carcinogenesis in the anorectal region. This notion is in line with the fact that HPV E6 oncoprotein facilitates p53 protein degradation (8–10).

Another important tumor suppressor that is also targeted by HPV is Rb, a nuclear phosphoprotein that plays a key role in regulating the cell cycle (26). The HPV E7 oncoprotein preferentially interacts with the hypophosphorylated Rb, the active form, resulting in a functional inactivation as well as an acceleration of protein degradation (11, 12). Similar to what has been reported for the *p53* gene, no gross rearrangement or loss of the *Rb* locus was observed in squamous cell carcinomas of the anus in one early study by Southern blotting analysis (40). In the current study, we demonstrate that loss of Rb nuclear staining by immunohistochemistry is frequently associated with HPV infection in anorectal squamous cell carcinomas, which occurred in 69% of the cases. Although these findings differ somewhat from those reported by Tanum and Holm (32), who showed a heterogeneous Rb nuclear staining in 95% of the cases they studied, our results support the concept that Rb protein is deregulated through two different mechanisms in HPV-related anorectal cancers. That is, in some of the tumors, Rb is inactivated by the HPV E7 oncoprotein at the functional level, and thus the nuclear Rb protein remains detectable immunohistochemically. In other tumors, however, Rb deregulation is achieved through accelerated protein degradation, as evidenced by a negative nuclear staining. This concept is further indirectly supported by the results of p16 immunostaining, as discussed below.

The p16 protein is also a key cell cycle regulator that functions as a tumor suppressor and is a com-

TABLE 1. Comparison of p16, Rb, and p53 Expression between Squamous Cell Carcinomas of the Anorectal Region and the Upper Aerodigestive Tract with and without HPV Infection

Staining Characteristics	No. of Cases (%)		
	Anorectal Region	Upper Aerodigestive Tract	
	HPV-Positive	HPV-Positive	HPV-Negative
Positive p16 nuclear staining	29 (100)	12 (100)	2 (10)
Loss of Rb nuclear staining	20 (69)	7 (58)	1 (5)
Positive p53 nuclear staining	0	0	6 (29)
Total cases	29	12	21

mon target of inactivation in human cancers (42). It has been recently reported, however, that in HPV-related cervical and head and neck cancers, p16 protein is overexpressed as detected by immunohistochemistry (18–23). These paradoxical findings are thought to result from a negative feedback control mechanism secondary to Rb deregulation (22, 24, 25). It has been well established that there exists an inverse relationship between the expression of p16 and the presence of functional Rb in many cell systems and that Rb represses the transcription of the *p16* gene (24, 25). However, once the inhibitory effect of Rb is abolished by either functional inactivation or protein degradation, as seen in HPV-infected cells, the transcriptional activity and the protein expression level of p16 are expected to increase. Similar to those reported in cervical and head and neck cancers (18–23), the results presented in this report show that p16 is overexpressed in 100% of the cases of anorectal squamous cell carcinomas that harbor HPV DNA. The p16 immunoreactivity seen in tumor cells is strong, highly reproducible, and detected in nearly every tumor cell in all cases. These observations further support the hypothesis that in HPV-related anorectal squamous cell carcinomas, the tumor suppressor function of the Rb protein is nullified even if it remains detectable immunohistochemically in a subset of the tumors.

A number of studies have attempted to investigate whether HPV infection bears any prognostic significance in squamous cell carcinomas of the cervix (43–48), penis (49), lung (50), and head and neck region (51, 52). Although the accumulated data are inconclusive and controversial, it seems to be useful to separate tumors that are HPV related from those that are not because different etiopathogenetic pathways are involved. In general, molecular detection of HPV DNA in tumor cells requires sophisticated laboratory facilities and experienced personnel and is costly. It therefore may not be feasible to be implemented into daily practice in many surgical pathology laboratories. From this perspective, the expression patterns of cell cycle regulators, as detected by immunohistochemical staining, may serve as good surrogate markers (20, 53). This view is substantiated by our findings showing a strong correlation between p16 overexpression and high-risk type HPV infection in anorectal squamous cell carcinomas. Because all the cases we studied harbor HPV DNA, we have also included HPV-positive and HPV-negative tumors from the upper aerodigestive tract for comparison. The results of these studies further indicate that p16 overexpression is fairly specific for HPV-related tumors. Our data also show that loss of Rb nuclear staining is another relatively specific marker for high-risk type HPV infection, which is seen in about two thirds of HPV-related tumors but only rarely

occurs in HPV-negative cases. Furthermore, immunostaining for p53 does not appear to be useful in the distinction between HPV-positive and HPV-negative cancers.

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