

Human Papillomavirus Genotyping and p16 Expression As Prognostic Factors for Patients With American Joint Committee on Cancer Stages I to III Carcinoma of the Anal Canal

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Terms in [blue](#) are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose

Carcinomas of the anal canal are strongly associated with the human papillomavirus (HPV). Expression of p16 is used as a surrogate marker of HPV infection. In a retrospective study, we evaluated HPV genotyping and p16 expression as prognostic markers of overall survival (OS) and disease-specific survival (DSS) in patients diagnosed with American Joint Committee on Cancer (AJCC) stages I to III carcinoma of the anal canal.

Patients and Methods

HPV genotyping polymerase chain reaction (high-risk subtypes 16, 18, 31, 33, 45, 52, and 58) and immunohistochemical expression of p16 were analyzed by using paraffin-embedded tumor biopsies from 143 anal carcinomas. The patients were treated with combined chemoradiotherapy or radiotherapy alone.

Results

HPV16 was detected in 81.0% of the tumors, followed by HPV33 (5.1%), HPV18 (2.2%), and HPV58 (0.7%). p16 positivity was found in 92.9% of the tumors. In univariable survival analysis, HPV positivity was significantly correlated with improved OS (74% v 52%; $P = .036$) and DSS (84% v 52%; $P = .002$), and p16 positivity was significantly correlated with improved OS (76% v 30%; $P < .001$) and DSS (85% v 30%; $P < .001$). In multivariable COX analysis that included HPV status, p16 status, sex, T stage, N stage, and treatment, p16 positivity remained an independent prognostic factor for OS (hazard ratio [HR], 0.07; 95% CI, 0.01 to 0.61; $P = .016$) and DSS (HR, 0.07; 95% CI, 0.01 to 0.53; $P = .011$).

Conclusion

p16 positivity is an independent prognostic factor for OS and DSS in patients with AJCC stages I to III carcinoma of the anal canal.

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INTRODUCTION

Anal carcinoma is a rare disease with an incidence ranging from 1.0 to 2.5 per 100,000 people per year in many western countries. For both sexes, risk factors are human papillomavirus (HPV) infection,^{1,2} HIV infection,³ immune suppression in recipients of transplantation,⁴ smoking,^{1,5} receptive anal intercourse,¹ lifetime number of sexual partners,¹ age,⁶ and in women, previous in situ or invasive cervical, vulva, or vaginal cancer.^{2,5,7} Two prospective randomized phase III trials demonstrated improved locoregional control and reduced the need for colostomy by using chemoradiotherapy compared with radiotherapy alone.^{8,9} Both randomized and retrospective se-

ries have reported the T stage and N stage as independent prognostic factors.⁹⁻¹³

Among more than 130 different HPV subtypes, HPV16 is the most prevalent in anal carcinoma and is present in up to 89% of all patients with anal carcinoma.^{2,14,15} HPV-associated cancers often have a viral sequence integrated into the genome of the cancer cells. Two of the HPV early structural genes, E6 and E7, are known as oncogenes promoting tumor growth and malignant transformation. The E6 and E7 proteins contribute to the genetic instability through their inactivation of p53 and the retinoblastoma protein (pRb). pRb is a negative regulator of the cyclin-dependent kinase inhibitor p16, and inactivation of pRb leads to upregulation of p16. p16 is often used as a [surrogate](#) marker of HPV infection.

Studies of head and neck squamous cell carcinoma (HNSCC) have demonstrated high concordance between expression of p16 by immunohistochemistry (IHC) and HPV positivity by in situ hybridization or polymerase chain reaction (PCR).¹⁶⁻¹⁸ In HNSCC, HPV and p16 status have been evaluated as prognostic factors with positive HPV status or increased p16 expression being associated with improved prognosis.^{19,20} One study of cervical cancer found that increased p16 expression was associated with a better prognosis.²¹ Few studies have evaluated HPV or p16 status as prognostic factors in patients with anal carcinoma.²²⁻²⁵ The aim of our retrospective study was to evaluate HPV status and p16 expression as prognostic markers for overall survival (OS) and disease-specific survival (DSS) in a large single-institution cohort of patients with American Joint Committee on Cancer (AJCC) stage I to III carcinoma of the anal canal.

PATIENTS AND METHODS

Patients

Between January 2000 and January 2010, patients with newly diagnosed AJCC stages I to III anal canal cancer, either squamous cell carcinoma (SCC) or verrucous carcinoma, referred to the Department of Oncology at Herlev Hospital (Herlev, Denmark) were enrolled onto the study. Patients with anal margin tumors, AJCC stage IV disease, recurrent anal SCC, adenocarcinoma, patients treated with primary surgery or palliative intent, and patients who did not have paraffin-embedded tumor biopsy tissue were excluded. The patients were staged by using palpation of inguinal lymph nodes, digital anal examination, anoscopy, endoanal and inguinal ultrasound, and computed tomography scan of the thorax and pelvis eventually combined with [¹⁸F]fluorodeoxyglucose-positron emission tomography to identify regional or distant metastasis.

Tumors overlapping the anal canal and anal margin were classified as anal canal tumors. Patient characteristics, treatment, and treatment outcome were collected from patients' medical records. Data collection was approved by the Danish Data Protection Agency (Reference No. 750.24-311), and the ethical aspects of the study were approved by the Regional Research Ethics Committee (Reference No. H-3-2010-068).

Real-Time PCR HPV16, -18, -31, -33, -45, -52, and -58 Genotyping

DNA was extracted from five, 3- μ m-thick, formalin-fixed and paraffin-embedded tissue slides. DNA extraction was carried out with a QIAGEN QIAcube by using the DNA mini kit. DNA concentration was measured with an ultraviolet-visible spectrometer and diluted to 3 ng/ μ L. Genotyping was performed with an in-house assay based on published studies by Lindh et al²⁶ for subtypes 16 and 18. The TaqMan assay consisted of TaqMan Singleplex real-time PCR targeting the E6/E7 region of the HPV genome. Probes and corresponding primers were chosen, with specificity for HPV genotype 16, HPV18, and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase as positive DNA control. Subsequently, all samples without HPV16 and HPV18 infections were reanalyzed with an in-house multiplex assay for detection of HPV subtypes 31, 33, 45, 52, and 58 that was based on the method designed by Schmitz et al.²⁷ Each reaction included 0.5 μ mol/L of primers and 0.2 μ mol/L of probe in a 25- μ L volume. In addition, 12.5 μ L TaqMan universal mastermix with uracil deoxyribonucleic acid glycosylase and 5 μ L sample template was added per well. Real-time PCR was performed on the Roche LC480 thermocycler with the following cycling parameters: 50°C for 2 minutes and 94°C for 10 minutes followed by 45 cycles of 94°C for 15 seconds, 50°C for 20 seconds, and 60°C for 40 seconds. A cycle threshold less than 35 was used in the interpretation of the PCR results according to the original assay design.

p16 IHC Staining

The primary formalin-fixed paraffin-embedded biopsy tissues were cut into 4- μ m thick sections and deparaffinized. p16 expression was visualized by using a purified mouse immunoglobulin G_{2a} antihuman p16 (JC8) monoclo-

nal antibody (Sc-56330, dilution 1:200, Santa Cruz Biotechnology, Dallas, TX). The EnVision FLEX system (DAKO, Glostrup, Denmark) was used for detection according to the manufacturer's recommendations for the antibody. A multiblock with endomyometrium and cervical SCC as positive controls and skin as negative control was used as control for specific staining. The p16 IHC score was derived by using a method described by Naggar and Westra^{28,29} that included a 70% tumor cell positivity cut point. The p16 score was evaluated in a blinded manner for HPV PCR status and clinical data.

Statistical Analysis

The primary end points were 5-year OS and DSS. The OS was calculated from the date of biopsy to the date of death from any cause or date of last follow-up. DSS was calculated from the date of biopsy to the date of death as a result of anal carcinoma, death as a result of causes other than anal carcinoma, or date of last follow-up. The comparisons of clinical variables between the HPV-positive and the HPV-negative group and between the p16-positive and the p16-negative group were made by using Fisher's exact test. The OS and DSS were estimated by the Kaplan-Meier method. The Cox proportional analysis was used to evaluate the significance of risk factors for the two end points. The significant variables in univariable analysis were included in the multivariable Cox model. For all analyses, a two-sided *P* value of less than .05 was considered statistically significant. SPSS software, version 20 (IBM, Armonk, NY) was used for all statistical analyzes, and the Kaplan-Meier curves were generated in R version 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patient Population

A total of 201 patients were identified; 58 patients were excluded because of anal margin tumors (*n* = 16), AJCC stage IV disease (*n* = 12), recurrent cancer (*n* = 2), primary surgery (*n* = 13), being treated with palliative intent (*n* = 9), adenocarcinoma (*n* = 1), or unavailability of paraffin-embedded tumor tissues from the time of diagnosis (*n* = 5). Thus, 143 patients were included in the study.

Patient Characteristics

Of the study patients, 142 had SCC, and one patient had verrucous carcinoma. The mean age was 63 years (range, 36 to 97 years). Patient characteristics are listed in Table 1. Two patients were known to be HIV positive and both were HPV positive and p16 positive.

Treatment

Radiotherapy was delivered as external beam radiotherapy (*n* = 131) or a combination of external beam radiotherapy followed by brachytherapy (*n* = 12). Sixty-eight patients (48%) received combined chemoradiotherapy and 75 patients (52%) received radiotherapy alone. The prescribed radiotherapy dose was usually 60 Gy with chemotherapy and 64 Gy with radiotherapy alone. Fifty-three (37%) of 143 patients received neoadjuvant and concomitant chemotherapy, five (3%) neoadjuvant chemotherapy, and 10 (7%) concomitant chemotherapy. The chemotherapeutic drugs predominantly used were cisplatin and fluorouracil; only a few patients received other drugs such as mitomycin. Three patients received radiotherapy with dose delays of up to 12 days because of treatment breaks related to adverse effects. Their HPV and p16 status were as follows: HPV negative/p16 negative, HPV positive/p16 positive, and HPV positive/p16 positive. All the patients received the prescribed dose. Thirteen patients were hospitalized during treatment because of adverse effects; only one of these patients was HPV negative/p16 negative.

Table 1. Demographic and Patient Characteristics According to HPV and p16 Status

Characteristic	Total (n = 143)		HPV Positive (n = 120)		HPV Negative (n = 17)		P	p16 Positive (n = 131)		p16 Negative (n = 10)		P
	No.	%	No.	%	No.	%		No.	%	No.	%	
Sex							.013					.015
Male	37	26	25	21	9	53		29	22	6	60	
Female	106	74	95	79	8	47		102	78	4	40	
Age at diagnosis, years							.443					.197
< 63	74	52	63	53	7	41		70	53	3	30	
≥ 63	69	48	57	47	10	59		61	47	7	70	
T stage							1.000					1.000
1-2	85	59	72	60	10	59		78	60	6	60	
3-4	58	41	48	40	7	41		53	40	4	40	
N stage							1.000					.518
Negative	87	61	73	61	10	59		80	61	5	50	
Positive	56	39	47	39	7	41		51	39	5	50	
Primary treatment							.796					.748
Chemoradiotherapy	68	48	56	47	9	53		63	48	4	40	
Radiotherapy	75	52	64	53	8	47		68	52	6	60	

Abbreviation: HPV, human papillomavirus.

Clinical Follow-Up

Median follow-up after completion of treatment was 51.2 months (range, 0.4 to 144.4 months). After treatment, clinical follow-up including a digital anal examination, anoscopy, and a palpation of the inguinal lymph nodes was performed once per month for 3 months, every 3 months for the first and second year, and every 6 months during years 3 to 5. At the time of analysis, 104 patients (73%) were still alive, and 39 patients (27%) had died as a result of anal carcinoma (n = 25), treatment complications (n = 3), and causes other than anal carcinoma (n = 12). Thirty-three patients (23%) developed recurrent disease during follow-up, 25 (76%) within the first 2 years.

HPV Genotyping and p16 IHC Staining

There was a significantly larger proportion of males among the HPV-negative ($P = .013$) and p16-negative ($P = .015$) patients (Table 1). The HPV DNA subtype status was obtainable in 137 patients (96%) and is presented in Table 2. Overall, 87.6% of the tumors were HPV positive. In six patients (4%), HPV genotyping was not performed because of the small amount of DNA.

Table 2. HPV DNA Genotype Status in 137 Patients

HPV Genotype	No. of Patients	%
16	109	79.6
18	1	0.7
16/18	2	1.5
31	0	0.0
33	7	5.1
45	0	0.0
52	0	0.0
58	1	0.7
No genotype	17	12.4

Abbreviation: HPV, human papillomavirus.

p16 status was evaluable in 141 patients. Two samples were not evaluable because of lack of carcinoma in the residual tissue material. One hundred thirty-one tumors (92.9%) were positive, and 10 (7.1%) were scored as negative. Both HPV and p16 status were available in 135 tumors (94.4%). Table 3 provides details regarding the concordance between HPV DNA status and p16 expression status. The concordance analysis between HPV and p16 showed a sensitivity of 0.95 and a specificity of 0.90.

Primary End Points and Prognostic Factors

Actuarial OS for all patients was 72% (95% CI, 68% to 76%), and DSS for all patients was 80% (95% CI, 77% to 83%). The HPV-positive group had a significantly better survival compared with the HPV-negative group (OS: 74% v 52%, $P = .036$; DSS: 84% v 52%, $P = .002$; Fig 1A-B). In the p16-positive group, both the OS and DSS were significantly longer compared with that in the p16-negative group (OS: 76% v 30%, $P < .001$; DSS: 85% v 30%, $P < .001$; Figs 1C and 1D). The results of the univariable and multivariable COX analyzes for OS and DSS are presented in Table 4.

In univariable analysis for OS, the following variables were related to improved survival: HPV positivity ($P = .036$), p16 positivity ($P < .002$), and female sex ($P = .037$). The following variables were significant for better DSS: HPV positivity ($P = .002$), p16 positivity ($P < .001$), and N-stage negativity ($P = .020$). The multivariable analyzes revealed that p16 status ($P = .016$) was an independent

Table 3. Concordance Between HPV and p16 Status

HPV Status	No.	%	p16 Positive		p16 Negative	
			No.	%	No.	%
All patients	135		125	92.6	10	7.4
HPV positive	120	88.9	119	99.2	1	0.8
HPV negative	15	11.1	6	40.0	9	60.0

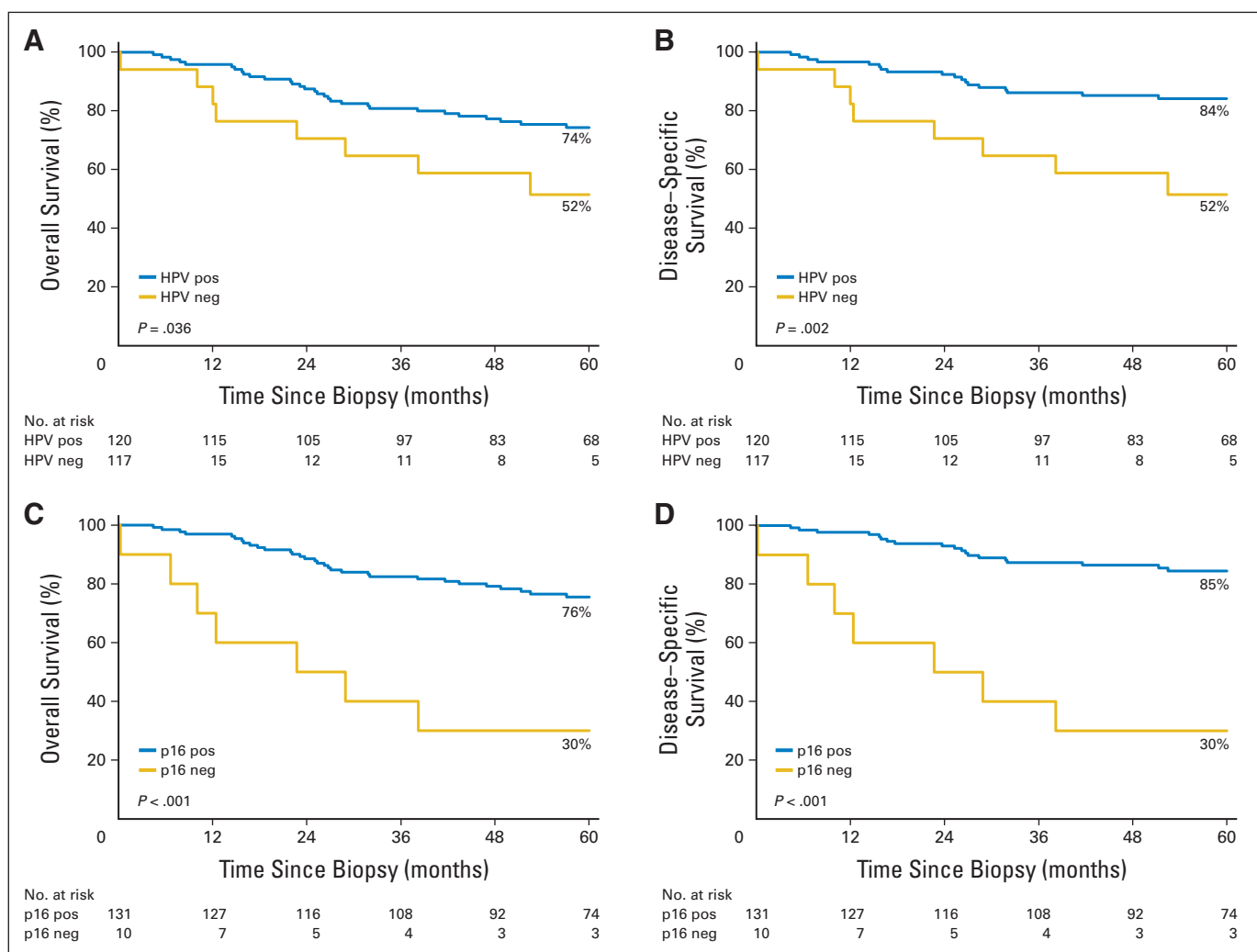


Fig 1. Kaplan-Meier survival curves according to human papillomavirus (HPV) and p16 status. (A) Overall survival according to HPV status, (B) disease-specific survival according to HPV status, (C) overall survival according to p16 status, and (D) disease-specific survival according to p16 status. neg, negative; pos, positive.

prognostic factors for OS and p16 status ($P = .011$) and N stage ($P = .011$) were independent prognostic factors for DSS.

Data regarding smoking was available for 129 patients (90%), with 67 patients (52%) reported as smokers or former smokers and 62 patients (48%) as nonsmokers. In the p16-negative group, there were three smokers and seven nonsmokers. There was no significant difference in OS and DSS between the smokers and nonsmokers (data not shown).

DISCUSSION

In our study, the most frequently detected HPV genotype was HPV16. Less than 10% of the patients were HPV18, HPV33, or HPV58 positive. These results are in agreement with other studies.^{14,15} HPV status as a prognostic factor is not well documented in anal carcinoma. Williams et al²⁴ found no difference in disease-free survival between HPV in situ hybridization-positive and -negative patients, and Yhim et al²⁵ found significantly better 4-year progression-free survival and OS in PCR HPV16-positive patients. The PCR method detects whether the HPV genome is integrated into tumor DNA but it cannot

distinguish transcriptional active HPV from inactive HPV because it is a DNA-based method. Increased p16 expression is a well-established surrogate marker for tumors with transcriptional active HPV. p16 has been investigated primarily in HNSCC and cervical cancer as a prognostic factor. These studies concluded that increased p16 expression was associated with improved prognosis.¹⁹⁻²¹ In our study, we found that p16 positivity was a strong independent prognostic factor for improved OS and DSS in patients with anal carcinoma, in both univariable and multivariable analyzes. To unify the assessment of p16 IHC in SCC, we used a scoring system similar to the validated system used in HNSCC that includes the 70% tumor cell cut point. Yhim et al²⁵ used an 80% tumor cell cut point and found significantly improved 4-year progression-free survival but not statistically different OS in patients with p16-positive tumors compared with patients with p16-negative tumors. Two smaller studies, one by Ajani et al²² and one by Bruland et al,²³ evaluated p16 as a prognostic factor and found no association. The discordant results may be explained by smaller sample sizes and/or different scoring systems; Ajani et al used the median percentage of tumor cells stained and Bruland et al used a

Table 4. Prognostic Value of Risk Factors in Univariable and Multivariable Analyses for OS and DSS

Risk Factor	OS						DSS							
	Univariable Analysis			Multivariable Analysis			Univariable Analysis			Multivariable Analysis				
	OS (%)	HR	95% CI	P	HR	95% CI	P	DSS (%)	HR	95% CI	P	HR	95% CI	P
HPV status				.036			.228				.002			.458
Positive	74	0.43	0.20 to 0.95		3.71	0.44 to 31.17		84	0.27	0.12 to 0.62		2.20	0.27 to 17.64	
Negative	52	1			1			52	1			1		
p16 status				< .001			.016				< .001			.011
Positive	76	0.21	0.09 to 0.47		0.07	0.01 to 0.62		85	0.13	0.06 to 0.31		0.07	0.01 to 0.53	
Negative	30	1			1			30	1			1		
Sex				.037			.171				.510			
Female	77	0.50	0.26 to 0.96		0.61	0.31 to 1.24		81	0.76	0.33 to 1.73				
Male	58	1			1			78	1					
Age, years				.645							.723			
≥ 63	70	1.16	0.62 to 2.17					81	0.87	0.41 to 1.86				
< 63	74	1						79	1					
T stage				.295							.150			
1-2	75	0.71	0.38 to 1.34					84	0.57	0.27 to 1.22				
3-4	69	1						75	1					
N stage				.115							.020			.011
Negative	77	0.60	0.32 to 1.13					87	0.40	0.19 to 0.87		0.34	0.15 to 0.781	
Positive	65	1						70	1			1		
Treatment				.928							.916			
Chemoradiotherapy	73	0.97	0.52 to 1.82					80	1.04	0.49 to 2.22				
Radiotherapy	72	1						81	1					

Abbreviations: DSS, disease-specific survival; HR, hazard ratio; HPV, human papilloma virus; OS, overall survival.

combination of staining intensity and number of positive cells when evaluating the p16 IHC.

Regarding conformity between HPV and p16, we found good agreement, with a sensitivity of 0.95 and a specificity of 0.90. The patients with discrepancy between HPV and p16 may be the result of the presence of HPV genotypes other than 16, 18, 31, 33, 45, 52, and 58 or inactive HPV.

Only two patients were known to be HIV positive; however, it is not a standard at our institution to check for HIV in patients with newly diagnosed anal cancer. Thus, the number of HIV-positive patients might be higher.

In this study, only 10 patients had a negative p16 status. These patients had a significantly shorter OS and DSS compared with the p16-positive patients, although all of the patients were treated with curative intent. The shorter OS and disease-free survival of the p16-negative patients could not be explained by differences in treatment delivery; only one p16-negative patient had a break during the course of treatment.

p16-negative tumors are likely dependent on oncogenic pathways other than HPV-induced pathogenesis and thus represent a different tumor biology. In future studies in anal carcinoma, stratifying according to p16 status should be considered. This might select candidates for a more rigorous follow-up and facilitate initiation of studies with alternative treatment regimens for this prognostically unfavorable group. Extrapolating from other SCCs (eg, HNSCC), hyperfractionated radiotherapy, addition of an epidermal growth factor receptor inhibitor or a hypoxic modifier could be alternative treatment regimens to pursue. Because anal cancer is a rare disease, participating in multicenter clinical trials is recommended.

In conclusion, we found that p16 expression is an independent prognostic factor for OS and DSS in patients with carcinoma of the anal canal. Further larger studies are warranted to confirm these results.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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GLOSSARY TERMS

disease-specific survival rate: the percentage of people in a study who have not died from a specific disease since diagnosis or treatment. Patients who died as a result of some other cause are not counted.

immunohistochemistry: the application of antigen-antibody interactions to histochemical techniques. Typically, a tissue section is mounted on a slide and incubated with antibodies (polyclonal or monoclonal) specific to the antigen (primary reaction). The antigen-antibody signal is then amplified using a second antibody conjugated to a complex of peroxidase-antiperoxidase, avidin-biotin-peroxidase, or avidin-biotin alkaline phosphatase. In the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding. Immunofluorescence is an alternate approach to visualize antigens. In this technique, the primary antigen-antibody signal is amplified using a second antibody conjugated to a fluorochrome. On ultraviolet light absorption, the fluorochrome emits its own light at a longer wavelength (fluorescence), thus allowing localization of antibody-antigen complexes.

overall survival: the duration between random assignment and death.

p16: molecule that binds to cyclin-dependent kinase 4 and 6, thereby preventing their interaction with cyclin D. p16 (also known as p16^{INK4}) behaves as a negative regulator of proliferation and arrests cells in the G0/G1 phase of the cell cycle.

polymerase chain reaction (PCR): a method that allows logarithmic amplification of short DNA sequences within a longer DNA molecule.

prognostic factor: a measurable patient characteristic that is associated with the subsequent course of disease (whether or not therapy is administered). The identification of a prognostic factor does not necessarily suggest a cause-and-effect relationship. However, within a suitable outcome model, the measurement of a prognostic factor contributes to an estimate of an outcome probability (eg, the probability of disease-free survival within a given time interval).

surrogate: a biologic marker evaluated in place of the actual marker of interest. For example, studying a marker for drug effect in blood instead of tumor. The relationship between the marker under study and the marker of interest needs to be established before using the term surrogate.