ONCOLOGY LETTERS 13: 1891-1898, 2017

Characterization of topoisomerase II α and minichromosome maintenance protein 2 expression in anal carcinoma

CRISTOVAM SCAPULATEMPO-NETO[1], CARLOS VEO[2], JOSÉ HUMBERTO T.G. FREGNANI[1,3], ADRIANA LORENZI[3], ALLINI MAFRA[3], ARMANDO G.F. MELANI[2], EDGAR ANTONIO ALEMÁN LOAIZA[2], LUCIANA ALBINA REIS ROSA[4], CRISTINA MENDES DE OLIVEIRA[4], JOSÉ EDUARDO LEVI[4] and ADHEMAR LONGATTO-FILHO[3,5-7]

Departments of [1]Pathology (CSN) and [2]Surgery; [3]Molecular Oncology Research Center, Barretos Cancer Hospital, Pio XII Foundation, Barretos, São Paulo, SP 14784-400; [4]Laboratory of Virology, Institute of Tropical Medicine São Paulo, University of São Paulo, São Paulo, SP 05403-000; [5]Laboratory of Medical Investigation 14, Faculty of Medicine, University of São Paulo, São Paulo, SP 01246-903, Brazil; [6]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga; [7]ICVS/3B's - PT Government Associate Laboratory, 4806-909 Caldas das Taipas/Guimarães, Portugal

Received November 30, 2015; Accepted August 25, 2016

DOI: 10.3892/ol.2017.5650

Abstract. The present study aimed to ascertain the significance of topoisomerase II α (TOP2A) and minichromosome maintenance protein (MCM) 2 expression in anal carcinoma. A total of 75 anal lesions were retrieved from the files of the Department of Pathology of Barretos Cancer Hospital (Barretos, Brazil) in order to verify the human papillomavirus (HPV) statuses of these lesions and characterize the immunohistochemical expression levels of TOP2A and MCM2 in anal carcinoma, as these are important markers for cervical HPV-induced lesions; their expression was also compared with respect to p16 and Ki-67. The vast majority of the cases tested positive for HPV16 (84%); 1 case tested positive for both HPV16 and HPV18. Positive HPV16 status was more frequent in early stages than in advanced stages (P=0.008). Positive immunohistochemical reactivity for MCM2 and TOP2A protein was observed in 71.6 and 100% of cases, respectively. Positive reactivity for p16 was significantly associated (P=0.001) with histological grade, and was more commonly expressed in squamous cell carcinoma than adenocarcinomas. HPV16 was strongly associated with positive p16 protein expression (76.6%). However, the high expression of Ki-67 combined with the high expression of p16 was predominantly observed in Stage III-IV cases. MCM2, TOP2A, p16 and Ki-67 exhibited intense positive staining in the anal lesions, indicating that these markers were significantly and constantly expressed in anal carcinoma.

Correspondence to: Dr Adhemar Longatto-Filho, Laboratory of Medical Investigation 14, Faculty of Medicine, University of São Paulo, 455 Dr. Arnaldo Avenue, São Paulo, SP 01246-903, Brazil
E-mail: longatto16@hotmail.com

Key words: anal carcinoma, p16, Ki-67, minichromosome maintenance protein 2, topoisomerase II α, human papillomavirus

Introduction

Human papillomavirus (HPV) is thought to be the carcinogenic agent responsible for all cases of cervical cancer, and for carcinomas of other anatomical sites, including anal carcinomas. Currently, >85% of anal carcinomas are thought to be associated with oncogenic HPV, and, among all high-risk HPV types, type 16 is recognized as the most common, with prevalence rates estimated at ~70% of all cases (1). In contrast to cervical carcinoma, the incidence of anal carcinoma is gradually increasing, accounting for ~2.2% of all gastrointestinal tract malignancies in the United States, with 6,230 cases newly diagnosed each year (2). Similar data have been also documented in other countries, such as Denmark (3). Populations at increased risk include women with cervical HPV-related neoplasia, immunosuppressed transplant patients and human immunodeficiency virus (HIV)-positive individuals. Risk factors for anal carcinoma acquisition also comprise history of smoking, history of condylomata (due to HPV exposition), and history of anal intercourse, indicating HPV infection in the anal canal (4). Notably, anal carcinoma is most frequent in men who have sex with men (MSM), who are ~20 times more likely than heterosexual men to develop the disease. Furthermore, MSM with HIV are at increased risk for anal cancer development (5). The estimated rate of anal carcinoma among HIV-positive persons is 174/100,000, as compared to an incidence of 2/100,000 among the HIV-negative population. MSM represent ~75% of the population at risk for the development of anal carcinoma (6).

Experimental studies have revealed that the E7 gene of HPV16 is a major participant in anal carcinogenesis (7). Thus, HPV vaccination is expected to be useful for MSM and the HIV-positive population to avoid anal cancer development (6). Alternatively, screening for anal cancer in the HIV-positive population has been proposed; however, at present, the efficacy of available markers has not been satisfactorily verified (8). Despite this, the use of markers to improve diagnosis and prognosis in anal cancer screening is of interest as brush sampling of the anus represents a realistic, minimally invasive option for this proposal (9).

In recent years, a number of different molecular markers have been tested with regard to optimization of the diagnosis of high-grade anal lesions (10). The assay most frequently investigated for this goal is immunohistochemical analysis of p16, which is widely used and can be used to successfully ascertain high-grade lesions in cytologically doubtful cervical HPV-induced lesions (11). The sensitivity and specificity of p16 for the detection of high-grade lesions may be markedly improved with the addition of Ki-67, a cell cycle marker, in the immunostaining panel (12). Considering the histological and etiological similarities between cervical and anal carcinomas, it is unsurprising that these two markers yielded promising results when added to anal carcinoma analyses (13). Notably, Ki-67 was demonstrated to detect anal HPV-related alterations with a high sensitivity and specificity, and p16 positive reactivity was found to be strongly associated with anal high-grade lesions (14). In cervical lesions, the expression of p16 and Ki-67 increases in parallel according to the degree of severity of the lesions (15). Additionally, combined p16 and Ki-67 immunostaining reduces the diagnostic variability among professionals and improves the detection of anal high-grade lesions (16).

Other adjunct markers have been added in different panels to improve anal lesions diagnosis. Among them is the minichromosome maintenance proteins (MCMs), a protein family with six major isoforms (MCM2-7) that are critical in restricting DNA synthesis to once per cell cycle, and also regulating DNA elongation (17). Dysregulated expression of MCM family proteins has been observed in a plethora of solid tumors, including anal cancer, and the results encourage its use to discriminate high-grade lesions of the anus (17). In cervical carcinoma, the usefulness of MCMs (MCM2 and MCM7) has also been documented, and their performances in the dectection of high-grade lesions were comparable to that of p16 and Ki-67 expression (18).

Similarly, topoisomerase II α (TOP2A) is frequently overexpressed in cervical neoplasia. This nuclear enzyme acts to relax the supercoiled DNA during cell replication and is involved in chromosome condensation, and it is critical for the normal segregation of daughter chromosomes at the end of cell division (18). Notably, TOP2A overexpression is associated with the progression from cervical intraepithelial neoplasia grade 2 to a more advanced cervical lesion (18). Currently, an immunohistochemical biomarker named BD ProEx C (BD Diagnostics, Burlington, NC, USA), combining MCM2 and TOP2A antibodies, is available and has been used to identify cervical high-grade lesions (18). Despite the importance of MCM2 and TOP2A in the investigation of cervical carcinoma, to the best of our knowledge, these markers have not been studied in anal carcinoma. Accordingly, the present study investigated the expression of MCM2 and TOP2A in 75 cases of anal carcinoma in order to characterize the expression of these markers with regard to the diagnostic and prognostic evaluation of anal cancer in routine practice, and compared their expression with that of p16 and Ki-67.

Materials and methods

Study design. A total of 75 consecutive cases of anal high-grade lesions were retrieved from the files of the Department of Pathology of Barretos Cancer Hospital (Barretos, Brazil) between January 2000 and December 2010. Sociodemographic and clinicopathological data were recovered from the patients' records. Histopathological revision of the slides was performed by one qualified pathologist in the group (C.S.N.). New sections from the paraffin-blocks of the tumors were cut for immunohistochemical analysis and HPV DNA test.

Ethics. The study protocol was approved by the local Ethics Committee of Barretos Cancer Hospital, and the authors declare no conflict of interest.

DNA extraction. DNA was extracted from fresh material using a QIAamp DNA Mini Kit (Qiagen Biotecnologia Brasil, Ltda., São Paulo, Brazil), according to the manufacturer's instructions, and from formalin-fixed paraffin-embedded tissue using a QIAamp DNA Micro Kit (Qiagen Biotecnologia Brasil, Ltda.), also according to the manufacturer's instructions.

HPV identification followed protocols published previously (19). The main steps are described below.

E7 HPV16 type-specific quantitative polymerase chain reation (qPCR). Type-specific TaqMan-based qPCR targeting HPV16 E7 used HPV16 E7 type-specific (20) oligonucleotide primers (forward, 5'-GATGAAATAGATGGTCCAGC-3', and reverse, 5'-GCTTTGTACGCACAACCGAAGC-3') and a probe (5'-FAM-CAAGCAGAACCGGACAG-MGB-NFQ), in a final reaction volume of 25 μl. Each qPCR mixture contained 1X TaqMan Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA), 400 nM of each forward and reverse primer, 200 nM of fluorogenic TaqMan probe and 5 μl of extracted DNA. The amplification conditions were as follows: 50˚C for 2 min; 95˚C for 10 min; and 40 cycles of 95˚C for 15 sec, 55˚C for 1 min and 60˚C for 1 min. qPCR was performed in an ABI 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each qPCR run included the following controls: i) SiHa cell line DNA (harboring 1-2 copies/cell of HPV16), and ii) water as a negative control. All samples and controls were run in duplicate.

E7 HPV-18 type-specific qPCR. Type-specific TaqMan-based qPCR targeting HPV18 E7 was performed using HPV18 E7 type-specific (20) oligonucleotide primers (forward, 5'-AAGAAAACGATGAAATAGATGGA-3', and reverse, 5' GGCTTCCACCTTACAACACA-3') primers and probe (5'-VIC-AATCATCAACATTTACCAGCC-MGB-NFQ-3'), in a final reaction volume of 25 μl. Each qPCR mixture contained 1X TaqMan Master Mix, 400 nM of each forward and reverse primer, 400 nM of fluorogenic TaqMan probe and 5 μl of extracted DNA. The amplification conditions were as

Table I. Characterization of the study population according to demographic information, clinical and histopathological findings.

Variable	n	%
Age, years (n=75)		
<40	9	12.0
40-59	41	54.7
≥60	25	33.3
Gender (n=75)		
Female	49	65.3
Male	26	34.7
Clinical stage (n=70)		
Stage 0	7	10.0
Stage I	3	4.3
Stage II	33	47.1
Stage III	19	27.1
Stage IV	8	11.4
Histopathological type (n=74)		
Squamous (invasive and *in situ*)	55	74.3
Adenocarcinoma	19	25.7
Histological grade (n=40)		
Grade 1	9	20.0
Grade 2	18	40.0
Grade 3	18	40.0
Regional recurrence (n=75)		
No	71	94.7
Yes	4	5.3
Distant metastasis (n=75)		
No	62	82.7
Yes	13	17.3
Status (n=75)		
Alive without disease	39	52.0
Alive with disease	7	9.3
Deceased (from cancer)	25	33.3
Deceased (from other causes)	4	5.3

Table II. Characterization of the study population according to immunohistochemical information (immunostaining of p16, Ki-67, MCM and TOP2A) and HPV infection.

Variable	n	%
p16 immunoexpression (n=72)		
Negative	17	23.6
Positive	55	76.4
Ki-67 immunoexpression (n=73)		
1+	3	4.1
2+	2	2.7
3+	9	12.3
4+	59	80.8
MCM immunoexpression (n=74)		
Negative	21	28.4
+1	16	21.6
+2	13	17.6
+3	24	32.4
TOP2A immunoexpression (n=67)		
1+	8	11.9
2+	42	62.7
3+	17	25.4
HPV16 detection (n=75)		
Negative	12	16.0
Positive	63	84.0
HPV18 detection (n=75)		
Negative	74	98.7
Positive[a]	1	1.3

[a]The case with HPV18 had co-infection with HPV16. MCM, minichromosome maintenance protein; TOP2A, topoisomerase II α; HPV, human papillomavirus.

follows: 50˚C for 2 min; 95˚C for 10 min; and 40 cycles of 95˚C for 15 sec, 50˚C for 1 min and 60˚C for 1 min. qPCR was performed in an ABI 7300 Real-Time PCR System and included the following controls: i) HeLa cell line DNA (harboring 20 copies/cell of HPV18), and ii) water as negative control. All samples and controls were run in duplicate.

Human β-globin PCR. Samples that were determined to be negative for HPV16 and HPV18 were submitted to a PCR analysis capable of detecting a 110-bp fragment of the human β-globin gene, in order to assess the DNA quality and integrity. β-globin PCR was conducted using 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 200 μM dNTP mix (all from Invitrogen; Thermo Fisher Scientific, Inc.), 200 nM forward primer PCO3 (5'-ACACAACTGTGTTCACTAGC-3'), 200 nM reverse primer PCO4 (5'-CAACTTCATCCACGTTCACC-3') (21), 1.25 U Platinum® Taq DNA Polymerase (Invitrogen; Thermo Fisher Scientific, Inc.), 5.7% glycerol, 0.25 μg/μl of Cresol Red, 5 μl of extracted DNA, and water, to a final volume 25 μl. The amplification conditions were as follows: 94˚C for 5 min; followed by 40 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min; and a final extension phase of 72˚C for 10 min. PCR was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany).

Immunohistochemical reactions. Histological sections of 4 μm thickness were deparaffinized in an oven at 80˚C for 30 min. Immunostaining was conducted in an Ultra Benchmark Autostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) using the following antibodies under specific conditions: Ki-67 (cat. no. M3060; Spring Bioscience Corporation, Pleasanton, CA, USA; dilution, 1:600), with 60 min antigen retrieval using CC1 (Ventana Medical Systems, Inc.) followed by incubation for 32 min; p16 (CINtec®; Ventana Medical Systems, Inc.; prediluted), with 30 min antigen retrieval using CC1 followed by incubation for 32 min; TOP2A (cat. no. ab52934; Abcam, Cambridge, MA, USA; dilution, 1:200), with 60 min antigen retrieval using CC1 (Ventana Medical Systems, Inc.) and incubation for 32 min; MCM2 (cat. no. ab6153; Abcam; dilution,

Table III. Five-year overall survival probabilities according to the study variables.

Variable	n	5-year overall survival rate (%)	P-value
Age, years (n=75)			0.052
<40	9	63.5	
40-59	41	74.8	
≥60	25	48.0	
Gender (n=75)			0.556
Female	49	66.9	
Male	26	60.1	
Clinical stage (n=70)			0.012
Stage 0-II	43	73.4	
Stage III-IV	27	47.4	
Histopathological type (n=74)			<0.001
Squamous (invasive and *in situ*)	55	74.0	
Adenocarcinoma	19	35.1	
Treatment (n=75)			0.003
Chemotherapy + radiotherapy	40	74.8	
Chemotherapy + radiotherapy + surgery	16	73.4	
Other	19	34.7	
Oncological response, QT/RT (n=69)			<0.001
Complete	36	91.2	
Incomplete/progression	33	28.1	
p16 immunoexpression (n=72)			0.200
Negative	17	52.3	
Positive	55	68.6	
Ki-67 immunoexpression (n=73)			0.648
1+/2+	5	80.0	
3+/4+	68	64.2	
MCM immunoexpression (n=74)			0.114
0/1+	37	55.1	
1+/2+	37	72.7	
TOP2A immunoexpression (n=67)			0.548
1+	8	72.9	
2+/3+	59	58.9	
HPV16 detection (n=75)			0.137
Negative	12	48.6	
Positive	63	67.6	

The survival analysis for HPV18 variable could not be performed as there was only 1 case. Data were compared using Student's t-tests. MCM, minichromosome maintenance protein; TOP2A, topoisomerase II α; HPV, human papillomavirus.

1:50), with 60 min antigen retrieval using CC1 (Ventana Medical Systems, Inc.) and incubation for 60 min.

Immunohistochemical quantification. The p16 reaction was categorized as positive if nuclear and/or cytoplasmic immunostaining was detected. Ki-67, MCM2 and TOP2A were considered positive only if brown nuclear staining was clearly observed. The scoring of positive reactions followed that of a previous report (22), with slight modifications: Negative (0); faintly positive (+), 1-10% positively stained cells; sporadic (++), 11-50% positively stained cells; intermediate (+++), 51-75% positively stained cells; and diffuse (++++), >75% positively stained cells.

For statistical calculations, variables were clustered as follows: p16 and MCM2 were scored as positive or negative reactions; Ki-67 and TOP2A expression levels were scored as 1+/2+/3+/4+ and 1+/2+/3+, respectively (TOP2A positive reactions did not reach 4+ in any of the cases tested). The tumoral stages were dichotomously classified as 0, I and II and III and IV; histological grades were divided into grades 1-2, and grade 3; and the presence of distant metastasis was designated as no or yes.

Table IV. Distribution of patient data according to histological type.

Variable	Squamous/HSIL, n (%)	Adenocarcinoma, n (%)	P-value
Age, years (n=74)			0.008
<40	5 (9.1)	4 (21.1)	
40-59	36 (65.5)	5 (26.3)	
≥60	14 (25.5)	10 (52.6)	
Gender (n=74)			0.579
Female	37 (67.3)	11 (57.9)	
Male	18 (32.7)	8 (42.1)	
Clinical stage (n=69)			0.150
Stage 0-I	34 (66.7)	8 (44.4)	
Stage II-IV	17 (33.3)	10 (55.6)	
p16 (n=71)			<0.001
Negative	6 (11.3)	10 (55.6)	
Positive	47 (88.7)	8 (44.4)	
Ki-67 (n=73)			0.591
1+/2+	3 (5.5)	2 (11.1)	
3+/4+	52 (94.5)	16 (88.9)	
<4+	9 (16.4)	5 (27.8)	0.312
≥4+	46 (83.6)	13 (72.2)	
MCM (n=74)			<0.001
0/1+	20 (36.4)	17 (89.5)	
2+/3+	35 (63.6)	2 (10.5)	
TOP2A (n=66)			0.664
1+	6 (12.5)	1 (5.6)	
2+/3+	42 (87.5)	17 (94.4)	
HPV16 detection (n=74)			0.276
Negative	7 (12.7)	5 (26.3)	
Positive	48 (87.3)	14 (73.7)	

It was not possible to analyze the association between histological type and HPV18 status as there was only 1 case that waspositive for HPV18. P-values were calculated by Fisher's exact test. HSIL, high-grade squamous intraepithelial lesion; MCM, minichromosome maintenance protein; TOP2A, topoisomerase II α; HPV, human papillomavirus.

Statistical analysis. The statistical data were analyzed with SPSS for Windows® version 20.0 (IBM SPSS, Armonk, NY, USA). Data were compared using Student's t-tests or Fisher's exact test. Prevalence rates were compared by means of a 'z' approximation in the SPSS package. Confidence intervals were also calculated. The significance level was set at 5%.

Results

General data. The patients comprised 49 females (65.3%) and 26 males (34.7%); 63 were Caucasians (84.0%) and 12 patients were of African descent (16.0%). The mean age [± standard deviation (SD)] was 55.9±13.56 years. The follow-up periods among the patients varied from 1 to 123 months (mean, 39.93; median, 33.50).

The majority of the patients (n=38, 52.1%) declared that they were married (with a long/stable relationship of several years), 18 (24.7%) were single, 8 (11.0%) were divorced and 9 (12.3%) were widows/widowers. The vast majority of the patients (n=53) had only basic school education, and 12 (16.9%) declared that they were illiterate.

Clinicopathological data. In the present study, a history of tobacco use was not significantly associated with anal cancer: 33 (47.1%) had no history of tobacco smoking, 30 (40.0%) were current frequent tobacco smokers, and 7 (10.0%) reported that they had smoked tobacco in the past. Familial history of cancer was noted for 20 (27.0%) patients. The characterization of the study population according to the demographic information, and clinical and histopathological findings, is depicted in Table I. Table II shows the frequencies of immunohistochemical reactivity for p16, Ki-67, MCM and TOP2a, and HPV infection; notably, HPV18 was simultaneously positive with HPV16 in only 1 case. Table III shows the correlation of 5-year overall survival rates according to the other variables of the study. As expected, patients in stages 0-II had a greater 5-year overall survival rate compared with stages III-IV (P=0.012); the group with a complete oncological response also had a greater

Table V. Distribution of the variables according to the clinical stage.

Variable	Stage 0-II, n (%)	Stage III-IV, n (%)	P-value
Age, years (n=66)			0.733
<40	5 (11.6)	4 (14.8)	
40-59	23 (58.1)	13 (48.1)	
≥60	13 (30.2)	10 (37.0)	
Gender (n=66)			0.306
Female	30 (69.8)	15 (55.6)	
Male	13 (30.2)	12 (44.4)	
p16 (n=63)			0.251
Negative	8 (19.0)	8 (32.0)	
Positive	34 (81.0)	17 (68.0)	
Ki-67 (n=64)			0.642
1+/2+	4 (9.5)	1 (3.8)	
3+/4+	38 (90.5)	25 (96.2)	
<4+	11 (26.2)	3 (11.5)	0.219
≥4+	31 (73.8)	23 (88.5)	
MCM (n=65)			0.805
0/1+	21 (50.0)	15 (55.6)	
2+/3+	21 (50.0)	12 (44.4)	
TOP2A (n=60)			0.125
1+	7 (18.9)	1 (3.8)	
2+/3+	30 (81.1)	25 (96.2)	
HPV16 detection (n=66)			0.008
Negative	3 (7.0)	9 (33.3)	
Positive	40 (93.0)	18 (66.7)	

It was not possible to analyze the association between histological type and HPV18 status as there was only 1 case positive for HPV18. P-values were calculated by Fisher's exact test. MCM, minichromosome maintenance protein; TOP2A, topoisomerase II α; HPV, human papillomavirus.

survival rate compared with those showing an incomplete response/progression (P=0.003). The expression levels of none of the proteins studied correlated significantly with overall survival rates.

Analysis of the histological types of anal carcinomas among the included cases revealed that the majority of cases were squamous cell carcinomas (P=0.008) among patients of all ages (Table IV). In addition, squamous cell carcinoma was the histological type that was most frequently associated with p16 and MCM2 positive immunoreactivity (both P<0.001). The histological grade was significantly associated with negative p16 expression (P=0.001; data not shown), as p16 was not expressed in grade 3 tumors. Notably, TOP2A expression was associated with the absence of metastasis (P=0.008; data not shown).

The distribution of the variables according to the clinical stage is depicted in Table V. HPV detection was positive in the vast majority of cases, but was proportionally more prevalent (P=0.008) in cases of stages 0-II (40 cases, 93.0%) than in stages III-IV (18 cases, 66.7%).

Discussion

The current study presents notable findings regarding the regulation of HPV-induced tumors of the anus. Firstly, HPV was detected in squamous cell carcinomas, adenocarcinomas and high-grade squamous intraepithelial lesions (HSIL). The present study investigated only the two major types of high-risk HPV that are associated with anal carcinoma: HPV16 and HPV18. Only 1 case tested positive for HPV18, which limited any discussion regarding the importance of HPV18 in anal lesions in comparison with HPV16.

HPV-induced tumors are frequently associated with p16 overexpression, which was confirmed in the present series of patients. However, the aggressiveness of these malignancies did not differ significantly among the tumor stages or in tumors with or without metastasis. Notably, negative p16 expression was not observed in grade 3 tumors. In previous studies, p16 expression was observed in both the less aggressive alterations and the more aggressive anal lesions (10,13). Evidence on p16 expression is controversial in the literature. The widespread positive immunoreactivity of p16 in anal cancer tumors of varying histological grades and clinical stages, and in tumors with or without distant metastasis, was suggested to demonstrate the limited usefulness of p16 as a prognostic marker (23). Longacre *et al* (23) suggested that p16 was more likely to be useful in distinguishing hyperplastic and atypical metaplastic squamous epithelium from dysplastic conditions. However, Serup-Hansen *et al* (24) demonstrated recently that

p16 positivity is an independent prognostic factor for overall survival and disease-specific survival in anal carcinoma of stages I-III.

In the present study, Ki-67 was highly expressed (score +4) in the majority of cases, which indicated that these anal tumors were highly proliferative lesions; however, tumors with high proliferation rates did not differ from less proliferative tumors in terms of clinical behavior, as Ki-67 was expressed in tumors of different stages and all histological grades. The association between Ki-67 and p16 positive expression was previously reported, revealing a parallel increase in expression of the two markers according to the aggressiveness of cervical lesions (15). The anal lesions assessed in the current study were all of a high-grade, which may explain the augmented indexes of proliferation demonstrated by Ki-67 immunoreactivity. Previously, Ki-67 was reported to be increased in anal lesions that were positive for HPV, as compared with normal healthy anal tissues, although tissue samples from sites of anal inflammation with reactive changes also exhibited increased Ki-67 values. However, p16 has been recognized as a specific biomarker for the presence of HPV in anal tissue, and has been associated with high-grade lesions (25).

The primary goal of the current study was to characterize and identify whether TOP2A and MCM2 may be useful as prognostic parameters in anal cancers, as they are for cervical carcinomas, particularly when assessed in association with the analysis of the p16 expression (26). In addition, the combined assessment of TOP2A and MCM2 expression provides a lower false positive rate in the diagnosis of malignant intraepithelial lesions, as compared with other currently used biological markers (27). In the present study, TOP2A was demonstrated to have an important role in anal carcinoma, since it exhibited moderate-to-strong positive reactivity in all cases; however, no statistical association was observed with regard to tumor aggressiveness. TOP2A in anal carcinoma was partially confirmed as all cases studied exhibited moderate to strong positive reactivity for this marker; however, no statistical association was observed with regard to tumor aggressiveness. Positive MCM2 reactivity was observed in almost 80% of the cases, which suggests that MCM2 is important in the progression of anal carcinoma. However, as for TOP2A, no correlation was observed with the clinicopathological parameters due to the persistent distribution of this marker.

Notably, the results herein have demonstrated that all the immunohistochemical markers were highly expressed in ~80% of the cases, which indicates that anal carcinoma has a significant potential for proliferation. Additionally, these markers appear to have some association with HPV status, since 84% of cases tested positive for HPV16. HPV16 is highly prevalent in anal cancer, reaching 90% in some studies, and is significantly associated with high-risk anal lesions and cancer progression (28).

The present series failed to establish any associations with respect to the HIV status in these patients; as a retrospective study, data regarding HIV statuses were unavailable for many patients. Currently, HIV testing is mandatory in routine clinical practice at our center, but was not in the past. For this reason, this parameter was not assessed. HIV patients, predominantly MSM, have an increased risk for anal cancer development, and the synergism with HPV is assumed to be a causative factor for anal carcinoma pathogenesis (29). Further studies considering HIV status may reveal differences among the markers tested and HPV status, with respect to prognostic impact.

In conclusion, the present study demonstrated that the evaluated biomarkers were significantly and constantly expressed in anal carcinoma and may be used in combination to evaluate the prognosis of anal cancer.

References

1. Wong AK, Chan RC, Aggarwal N, Singh MK, Nichols WS and Bose S: Human papillomavirus genotypes in anal intraepithelial neoplasia and anal carcinoma as detected in tissue biopsies. Mod Pathol 23: 144-150, 2010.
2. Zandberg DP, Bhargava R, Badin S and Cullen KJ: The role of human papillomavirus in nongenital cancers. CA Cancer J Clin 63: 57-81, 2013.
3. Nielsen A, Munk C and Kjaer SK: Trends in incidence of anal cancer and high-grade anal intraepithelial neoplasia in Denmark, 1978-2008. Int J Cancer 130: 1168-1173, 2012.
4. Palefsky JM and Rubin M: The epidemiology of anal human papillomavirus and related neoplasia. Obstet Gynecol Clin North Am 36: 187-200, 2009.
5. Machalek DA, Poynten M, Jin F, Fairley CK, Farnsworth A, Garland SM, Hillman RJ, Petoumenos K, Roberts J, Tabrizi SN, *et al*: Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: A systematic review and meta-analysis. Lancet Oncol 13: 487-500, 2012.
6. Firnhaber C and Wilkin T: Human papillomavirus vaccines: Where do they fit in HIV-infected individuals? Curr HIV/AIDS Rep 9: 278-286, 2012.
7. Thomas MK, Pitot HC, Liem A and Lambert PF: Dominant role of HPV16 E7 in anal carcinogenesis. Virology 421: 114-118, 2011.
8. Salit IE, Tinmouth J, Chong S, Raboud J, Diong C, Su D, Sano M, Lytwyn A, Chapman W and Mahony J: Screening for HIV-associated anal cancer: Correlation of HPV genotypes, p16, and E6 transcripts with anal pathology. Cancer Epidemiol Biomarkers Prev 18: 1986-1992, 2009.
9. Valari O, Koliopoulos G, Karakitsos P, Valasoulis G, Founta C, Godevenos D, Dova L, Paschopoulos M, Loufopoulos A and Paraskevaidis E: Human papillomavirus DNA and mRNA positivity of the anal canal in women with lower genital tract HPV lesions: Predictors and clinical implications. Gynecol Oncol 122: 505-508, 2011.
10. Roldán Urgoiti GB, Gustafson K, Klimowicz AC, Petrillo SK, Magliocco AM and Doll CM: The prognostic value of HPV status and p16 expression in patients with carcinoma of the anal canal. PLoS One 9: e108790, 2014.
11. Roelens J, Reuschenbach M, von Knebel Doeberitz M, Wentzensen N, Bergeron C and Arbyn M: p16INK4a immunocytochemistry versus human papillomavirus testing for triage of women with minor cytologic abnormalities: A systematic review and meta-analysis. Cancer Cytopathol 120: 294-307, 2012.
12. Wentzensen N, Schwartz L, Zuna RE, Smith K, Mathews C, Gold MA, Allen RA, Zhang R, Dunn ST, Walker JL and Schiffman M: Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. Clin Cancer Res 18: 4154-4162, 2012.
13. Bean SM, Eltoum I, Horton DK, Whitlow L and Chhieng DC: Immunohistochemical expression of p16 and Ki-67 correlates with degree of anal intraepithelial neoplasia. Am J Surg Pathol 31: 555-561, 2007.
14. Pirog EC, Quint KD and Yantiss RK: P16/CDKN2A and Ki-67 enhance the detection of anal intraepithelial neoplasia and condyloma and correlate with human papillomavirus detection by polymerase chain reaction. Am J Surg Pathol 34: 1449-1455, 2010.
15. Longatto Filho A, Utagawa ML, Shirata NK, Pereira SM, Namiyama GM, Kanamura CT, Santos Gda C, de Oliveira MA, Wakamatsu A, Nonogaki S, *et al*: Immunocytochemical expression of p16INK4A and Ki-67 in cytologically negative and equivocal pap smears positive for oncogenic human papillomavirus. Int J Gynecol Pathol 24: 118-124, 2005.
16. Walts AE, Lechago J, Hu B, Shwayder M, Sandweiss L and Bose S: P16 and Ki67 immunostains decrease intra- and interobserver variability in the diagnosis and grading of anal intraepithelial neoplasia (AIN). Clin Med Pathol 1: 7-13, 2008.

17. Kreuter A, Jesse M, Potthoff A, Brockmeyer NH, Gambichler T, Stücker M, Bechara FG, Pfister H and Wieland U: Expression of proliferative biomarkers in anal intraepithelial neoplasia of HIV-positive men. J Am Acad Dermatol 63: 490-498, 2010.
18. Brown CA, Bogers J, Sahebali S, Depuydt CE, De Prins F and Malinowski DP: Role of protein biomarkers in the detection of high-grade disease in cervical cancer screening programs. J Oncol 2012: 289315, 2012.
19. Veo CA, Saad SS, Fregnani JH, Scapulatempo-Neto C, Tsunoda AT, Resende JC, Lorenzi AT, Mafra A, Cinti C, Cotrim ID, *et al*: Clinical characteristics of women diagnosed with carcinoma who tested positive for cervical and anal high-risk human papillomavirus DNA and E6 RNA. Tumour Biol 36: 5399-5405, 2015.
20. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ and Muñoz N: Human Papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189: 12-19, 1999.
21. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA and Arnheim N: Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354, 1985.
22. Longatto Filho A, Utagawa ML, Shirata NK, Pereira SM, Namiyama GM, Kanamura CT, Santos Gda C, de Oliveira MA, Wakamatsu A, Nonogaki S, *et al*: Immunocytochemical expression of p16INK4A and Ki-67 in cytologically negative and equivocal pap smears positive for oncogenic human papillomavirus. Int J Gynecol Pathol 24: 118-124, 2005.
23. Longacre TA, Kong CS and Welton ML: Diagnostic problems in anal pathology. Adv Anat Pathol 15: 263-278, 2008.
24. Serup-Hansen E, Linnemann D, Skovrider-Ruminski W, Høgdall E, Geertsen PF and Havsteen H: 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. J Clin Oncol 32: 1812-1817, 2014.
25. Shi J, Liu H, Wilkerson M, Huang Y, Meschter S, Dupree W, Schuerch C and Lin F: Evaluation of p16INK4a, minichromosome maintenance protein 2, DNA topoisomerase IIalpha, ProEX C and p16INK4a/ProEX C in cervical squamous intraepithelial lesions. Hum Pathol 38: 1335-1344, 2007.
26. Bala R, Pinsky BA, Beck AH, Kong CS, Welton ML and Longacre TA: p16 is superior to ProEx C in identifying high-grade squamous intraepithelial lesions (HSIL) of the anal canal. Am J Surg Pathol 37: 659-668, 2013.
27. Pinto AP, Schlecht NF, Woo TY, Crum CP and Cibas ES: Biomarker (ProEx C, p16(INK4A), and MiB-1) distinction of high-grade squamous intraepithelial lesion from its mimics. Mod Pathol 21: 1067-1074, 2008.
28. Grulich AE, Poynten IM, Machalek DA, Jin F, Templeton DJ and Hillman RJ: The epidemiology of anal cancer. Sex Health 9: 504-508, 2012.
29. Legarth R, Helleberg M, Kronborg G, Larsen CS, Pedersen G, Pedersen C, Jensen J, Nielsen LN, Gerstoft J and Obel N: Anal carcinoma in HIV-infected patients in the period 1995-2009: A Danish nationwide cohort study. Scand J Infect Dis 45: 453-459, 2013.