HPV status and immunohistochemical analysis of p16, p53 and PD-L1 expression as prognostic biomarkers in patients with squamous cell anal cancer receiving definitive radiotherapy/chemoradiotherapy

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Abstract. Anal squamous cell carcinoma (SCC) treated with definitive radiotherapy (RT)/chemoradiotherapy (CRT) has shown high success rates, yet challenges such as treatment resistance and recurrence persist. The present study aimed to investigate the associations between immunohistochemical (IHC) evaluation, treatment response and prognosis in anal SCC. A retrospective cohort analysis included 42 patients with anal SCC treated at a single institution between 2006 and 2022. Human papillomavirus (HPV) status was determined, and the IHC analysis of p16, p53 and PD-L1 expression was conducted using formalin-fixed, paraffin-embedded biopsies. A complete response to RT/CRT was observed in 71.4% of patients. Recurrence occurred in 38.1% of cases, of which 7.1% had local-regional recurrence (LRR), 14.3% had distant recurrence (DR), and 16.7% had both LRR and DR. HPV positivity (71.4%) was significantly associated with p16 positivity. Lack of complete response was associated with HPV-negative status, p16-negative status, increased recurrence and DR. In addition, recurrence was significantly associated with p53-positive status, and p53 positivity was significantly associated with increased LRR. PD-L1 positivity, defined as a combined positive score (CPS) ≥1% was found in 73.8% of the patients, and exhibited significant associations with HPV positivity and p16 positivity. PD-L1 CPS ≥ 1% was also associated with an increased LRR. Univariate analysis revealed that age <65 years, a complete response and HPV positivity were associated with increased 5-year overall survival (OS), while a complete response, HPV positivity and p53-negative status were associated with increased 5-year disease-free survival (DFS). Multivariate analysis identified that age <65 years and HPV positivity are independent prognostic factors for 5-year OS, and a complete response and p53-negative status are independent prognostic factors for 5-year DFS. In conclusion, these findings suggust that the identification of HPV status and poor prognostic biomarkers at diagnosis may be used to guide personalized treatment strategies, with the combination of immunotherapy with standard CRT potentially providing improved outcomes.

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Abbreviations: CPS, combined positive score; CRT, chemoradiotherapy; CTV, clinical target volume; DFS, disease-free survival; DR, distant recurrence; FFPE, formalin-fixed, paraffin-embedded; HIV, human immunodeficiency virus; HPV, human papillomavirus; IHC, immunohistochemical; LRR, local-regional recurrence; MMC, mitomycin-C; OS, overall survival; RT, radiotherapy; SCC, squamous cell carcinoma; TPS, tumor proportion score; 5-FU, 5-fluorouracil

Key words: anal cancer, radiotherapy, chemoradiotherapy, human papillomavirus, p16, p53, PD-L1

Introduction

Anal squamous cell carcinoma (SCC) presents a unique challenge in cancer care, with most cases diagnosed as local-regional disease (1). While treatment approaches have undergone limited changes over the past 40 years and favorable outcomes have been observed, a challenging subgroup with a poor prognosis necessitates careful treatment evaluation (2-4). The European Society for Medical Oncology, European Society of Surgical Oncology, and European Society for Therapeutic Radiology and Oncology guidelines prioritize only clinical digital rectal examinations until 26 weeks after the start of treatment (5). Currently, no test or marker apart from clinical examination is able to predict the response to treatment. Incomplete responses adversely affect survival (6-8), and limited options in such cases create issues

for clinicians and patients. This underscores the imperative for the early recognition and tailored treatment of this challenging subgroup.

In head and neck cancers, human papilloma virüs (HPV) positivity is associated with improved treatment responses and survival outcomes (9,10). The positive association between HPV status and p16 expression, particularly in head and neck tumors, has been thoroughly investigated. As a result, p16 expression is now integrated into the staging process for head and neck cancers as a reliable indicator of HPV infection (11,12). Further investigations into the relationship between HPV status and p16 expression have led to their inclusion as prognostic factors in the National Comprehensive Cancer Network Guidelines for anal SCC (13,14). However, their clinical utility and role in treatment require further clarification. The implications of p53 status in anal cancer are also unclear (15). The different p53 mutations and functions in HPV-positive and -negative tumors are further complicating factors (16). In anal SCC, the expression of p53, which is often described as a negative prognostic factor in numerous types of cancer (17), has not been fully evaluated.

Immunotherapy has emerged as a promising means of treating local-regional and metastatic anal cancer, as it is for numerous other types of cancer. Programmed death ligand 1 (PD-L1) is a key component of the immune checkpoint pathway during the late effector phase of the immune response; therefore, the investigation of its expression is of interest. Previous data have indicated uncertainty regarding the associations between PD-1 expression on tumor-infiltrating lymphocytes and PD-L1/2 expression on tumor cells with poor prognosis (18), and discrepancies in these associations also exist for anal cancer (19-21).

The influence of HPV status and p16, p53 and PD-L1 expression on treatment response, recurrence patterns and overall survival (OS) in anal SCC were investigated in the current study with the aim of gaining prognostic insights. Another aim was to elucidate the immunological landscape of anal SCC, facilitating the exploration of targeted therapies involving immune checkpoint inhibitors.

Materials and methods

Patients. A retrospective evaluation was conducted on 42 patients diagnosed with anal SCC and treated with definitive radiotherapy (RT)/chemoradiotherapy (CRT) at the Department of Radiation Oncology of Ege University (Izmir, Türkiye) between January 2006 and January 2022. Exclusion criteria comprised age <18 years, metastatic disease, a history of other cancers within the last 3 years, anal carcinoma *in situ* or anal intraepithelial neoplasm, prior RT, human immunodeficiency virus (HIV) positivity, unavailable current status data, and lack of diagnostic biopsy material. The study was approved by the Medical Research Ethics Committee at Ege University (Izmir, Türkiye; reference: 21-3.1T/63). Written informed consent was obtained from all patients.

Treatment. The staging elements of tumor (T), node (N) and metastasis (M) were evaluated according to version 9 of the American Joint Committee on Cancer staging system before treatment (22).

The sequential boost technique (23) was used for the treatment of the high- and low-risk clinical target volumes (CTVs). After delivering the initial dose to the low-risk CTV, treatment focuses on a smaller, high-risk target volume. This involves administering an additional, intensified dose of radiation, commonly known as the 'boost'. High-risk CTV included the gross disease CTV, mesorectum, presacral nodes, and bilateral internal and external iliac nodes below the sacroiliac joint. Additional inguinal nodes were included in the high-risk CTV if gross inguinal nodal involvement was present. Low-risk CTV encompassed the high-risk CTV, and presacral, bilateral internal and external iliac nodes above the sacroiliac joint to the L5/S1 vertebral body junction. Bilateral inguinal nodes were included in the low-risk CTV if there was no evident involvement of these nodes. A dose comprising 45 Gy in 1.8-Gy fractions was applied to the low-risk CTV followed by a 5.4-9-Gy boost in 1.8-Gy fractions to the high-risk CTV. Use of the simultaneous integrated boost technique (24) delivers stage-based doses to different target volumes with the same number of fractions, simplifying planning but reducing the biological dose to elective nodal areas. The single CTV included the gross disease CTV, bilateral inguinal nodes, mesorectum, presacral nodes, and bilateral internal and external iliac nodes above the sacroiliac joint to the L5/S1 vertebral body junction. A dose of 45-54 Gy was targeted at the single CTV, with a specific dose within of 54-59.4 Gy for the gross disease CTV.

Chemotherapy received by the patients included mitomycin-C (MMC; 10 mg/m^2) administered intravenously on days 1 and 29, together with 5-fluorouracil (5-FU; 1,000 mg/m^2) administered by continuous infusion for 24 h on days 1-4 and 29-32. No cases received induction chemotherapy.

Post-treatment evaluation. At 3 months after the completion of treatment, patients underwent an initial evaluation of local response using standard anoscopy and digital rectal examination to identify indications of lesion progression. At 6 months post-treatment, a comprehensive examination was conducted, including digital rectal examination, endoscopic examination, computed tomography and pelvic magnetic resonance imaging. Fluorodeoxyglucose positron emission tomography was also performed when clinically indicated, encompassing the assessment of suspicious pelvic and inguinal nodes, as well as distant metastasis. If suspicion arose regarding a residual lesion, a biopsy was performed for histopathological confirmation. Those patients with confirmed residual lesions based on biopsy findings were referred for surgical intervention. The response status was assessed following the Response Evaluation Criteria in Solid Tumors (RECIST 1.1) (25).

A complete response was defined as the absence of disease in the primary tumor site and regional lymph nodes within 6 months after the completion of RT/CRT. A non-complete response was categorized as stable disease, partial response or progression.

Recurrence definitions. Local-regional recurrence (LRR) refers to progression independent of time after definitive RT/RCT, such that it was not possible to perform salvage surgery, or the reappearance of cancer in the primary tumor and pelvic area ≥6 months post-treatment in patients with a complete response. Distant recurrence (DR) indicates the presence of

metastasis outside the pelvic or inguinal lymph node areas, regardless of local-regional status.

Follow-up and monitoring. Patients underwent regular follow-up after their treatment, which involved digital rectal examinations every 3 months for the first 2 years, biannually from year 3 to 5, and yearly thereafter. Females underwent gynecological examinations, and imaging was conducted as necessary. The assessment included the detection of LRR and DR, with time intervals measured from the end of RT.

HPV-DNA analysis and typing. First, tissue microarrays were prepared from Formalin-fixed paraffin-embedded (FFPE) blocks for each patient with a thickness of 2 μm. From these, two FFPE sections were cut, each labeled with biopsy number, block name, and date, and barcode. FFPE sections were processed with 300 μl deparaffinization solution (Qiagen, Inc.) in 1.5- or 2-ml microcentrifuge tubes, followed by 10 sec of vigorous vortexing at room temperature. After centrifugation at 15,000 x g for 10 sec at 25˚C to bring the sample to the bottom of the tube, the samples were incubated at 56˚C for 3 min and then cooled to room temperature. HPV DNA amplification was performed using the QIASCREEN HPV PCR kit (Qiagen, Inc.). Deparaffinized samples were treated with 25 μl Buffer FTB, 55 μl RNase-free water, and 20 μl proteinase K, with controlled temperature incubations. Following removal of the upper phase, the aqueous lysate was subjected to RNase A treatment and a final incubation with proteinase K. DNA was then extracted using Buffer AL and ethanol, with purification using QIAamp UCP MinElute columns during centrifugation at 15,000 x g for 1 min at 25˚C, followed by elution using Buffer ATE. This produced high-quality DNA for downstream molecular analyses. Quality control was performed using spectrophotometry and gel electrophoresis, with optional enhancements for small elution volumes. Small elution volumes are the final liquid volumes used to collect DNA from purification columns. They are employed when working with limited sample sizes or to achieve higher DNA concentrations, using less elution buffer to concentrate the DNA. The sequences of the primers in the PCR kit are proprietary and were not revealed by the company.

The study targeted 15 high-risk HPV types, namely HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67 and 68, which were grouped into HPV 16, HPV 18 and other high-risk types. In the analysis phase, patients were categorized as HPV positive or HPV negative.

Expression analysis and scoring of p16, p53 and PD-L1. First, tissue microarrays were prepared from FFPE blocks for each patient with a thickness of 2 μm. From these, five FFPE sections were cut, each labeled with biopsy number, block name, date and barcode. The sections were incubated at 60˚C in an oven for at least 2 h for deparaffinization. Following this initial preparation step, the process continued with the p16, p53 and PD-L1 immunohistochemical (IHC) staining using the Benchmark XT device (Roche Tissue Diagnostics). Treatment steps included antigen retrieval, reagent application, and the use of CINtec P16 Histology, CONFIRM anti-p53 (DO-7) Primary Antibody, and VENTANA® PD-L1 (SP263) Assay, all from Roche Tissue Diagnostics.

Viable tumor tissue without necrosis was examined. Positive p16 expression was defined as intense cytoplasmic and nuclear staining in >5% of tumor cells (26,27). For p53 expression, positivity was defined as intense cytoplasmic and nuclear staining in >5% of tumor cells (26,27). The tumor proportion score (TPS) was calculated as the percentage of PD-L1-positive tumor cells among all viable tumor cells: TPS (%)=[(PD-L1-positive tumor cells/total viable cells) x100]. The combined positive score (CPS) was determined by dividing the sum of PD-L1-positive tumor cells, lymphocytes and macrophages by the total number of viable tumor cells: CPS (%)=[(PD-L1-positive tumor cells + lymphocytes + macrophages/total viable cells) x100] (28). The analysis was conducted blindly.

Statistical analysis. The associations among HPV-DNA status, p16, p53, PD-L1 expression, complete response and clinical factors were assessed using Pearson's Chi-square or Fisher's exact tests. Univariate and multivariate Cox regression analyses were performed to identify independent prognostic factors for OS and disease-free survival (DFS), including factors achieving $P<0.01$ from the univariate analysis in the multivariate analysis. Survival analysis was performed by the generation of Kaplan-Meier curves, and the log-rank test was used for this analysis. IBM SPSS Statistics version 25 (IBM Corp.) was utilized for statistical analysis. $P<0.05$ was considered to indicate a statistically significant result.

Results

Patient characteristics. A total of 42 patients with anal SCC participated in the study, with a median age of 61 years (age range, 35-86 years). Among them, 25 (59.5%) were female and 17 (40.5%) were male. The distribution of stages was as follows: 2 (4.8%) cases in stage I, 6 (14.3%) in stage IIA, 7 (16.7%) in stage IIB, 4 (9.5%) in stage IIIA, 3 (7.1%) in stage IIIB and 20 (47.6%) in stage IIIC. Concurrent chemotherapy (5-FU/MMC) with RT was administered in 36 cases (85.7%). This treatment approach took into account the patients' comorbidities, including severe hypersensitivity or allergy, myelosuppression, renal or hepatic impairment, and cardiopulmonary disease. The characteristics of the patients and the therapies received are presented in Table I.

In terms of treatment response, 30 cases (71.4%) achieved a complete response, 5 (11.9%) had a partial response, 4 (9.5%) had a stable response and 3 (7.2%) experienced progression. Among the 12 patients without a complete response, 8 (19%) underwent abdominoperineal resection surgery, 1 (2.4%) underwent wide local excision, 2 (4.8%) were referred for systemic treatment due to the detection of distant metastasis and 1 (2.4%) experienced progression, leading to early death due to acute abdominal perforation. Of the six patients who did not receive chemotherapy, only one did not exhibit a complete response.

During the follow-up period (median length, 64 months), the 24 surviving cases comprised 20 (47.6%) patients who remained disease-free and 4 (9.5%) who had ongoing disease. Recurrence was observed in 16 cases (38.1%), with 3 (7.1%) having only LRR, 6 (14.3%) having only DR and 7 (16.7%) experiencing both LRR and DR. The median recurrence time

Table I. Patient and clinical characteristics.

Characteristic	Value
Sex, n (%)	
Female	25 (59.5)
Male	17 (40.5)
Median age (range), years	61 (35-86)
T stage, n (%)	
T1	2 (4.8)
T2	11 (26.2)
T3	18 (42.8)
T4	11 (26.2)
N stage, n (%)	
N0	18 (42.8)
N1a	16 (38.1)
N1b	1 (2.4)
N1c	7 (16.7)
Stage, n (%)	
I	2 (4.8)
IIA	6 (14.3)
IIB	7 (16.7)
IIIA	4 (9.5)
IIIB	3 (7.1)
IIIC	20 (47.6)
Median total radiotherpay dose (range), Gy	59.4 (50.4-59.4)
Concurrent chemotherapy, n (%)	
Yes	36 (85.7)
No	6 (14.3)

T stage, primary tumor stage; N stage, regional lymph node stage.

was 10 months (range, 1-72 months). The median local recurrence-free time was 16.5 months (range, 3-72 months) and the median DR-free time was 11 months (range, 1-96 months). The most common metastatic sites were the lungs, followed by the liver. In addition, no statistical difference was observed in recurrence (P=0.510), LRR (P=0.614) and DR (P=0.433) between RT with or without chemotherapy.

HPV status and p16 expression. The 42 patients comprised 30 (71.4%) cases who were HPV positive and 12 (28.6%) who were HPV negative. Within the HPV-positive subgroup, 22 (73.3%) were solely positive for HPV 16, which was the most prevalent HPV type. Additionally, 5 (16.6%) cases were positive for other high-risk HPV types, 2 (6.7%) had a combination of HPV 16 with other HPV types, and 1 (3.4%) had a combination of HPV 16, HPV 18 and other HPV types. The total study cohort included 31 (73.8%) p16-positive cases and 11 (26.2%) p16-negative cases. A robust association was observed between HPV-positive and p16-positive tumors (P<0.001).

HPV-negative and p16-negative tumors were found to be associated with male sex (P=0.001 and P<0.001, respectively). No significant associations were found with other patient or clinical factors for both HPV status and p16 expression.

Recurrence was observed in 9 (30%) of 30 cases with HPV-positive status and in 7 (58.3%) of 12 cases with HPV-negative status, although this difference lacked statistical significance (P=0.088). Also without reaching statistical significance, HPV-negative tumors exhibited a trend for higher LRR (16.7% in HPV positive vs. 41.7% in HPV negative; P=0.086) and DR (26.7% in HPV positive vs. 41.7% in HPV negative; P=0.277). Patients with HPV-positive tumors demonstrated a statistically significant improved outcome (P=0.008); 21 (70%) patients with HPV-positive tumors survived, compared with only 43 (25%) patients with HPV-negative tumors. The clinical, recurrence and IHC characteristics of HPV-positive and -negative tumors are presented in Table II.

p53 expression. The 42 cases included 15 (35.7%) patients who were p53 positive. No significant associations were identified between p53 expression and p16 expression (P=0.333), HPV status (P=0.193) or other patient characteristics (Table III).

Recurrence was observed in 10 (66.7%) patients with p53-positive tumors, but only 6 (22.2%) patients with p53-negative tumors; a significant association was established between recurrence and p53-positive tumors (P=0.006). Similarly, p53 positivity was associated with an increased occurrence of LRR, with 7 of the 10 cases of LRR being p53 positive (P=0.014; Table III).

In the 30 cases with HPV-positive tumors, 5 (55.6%) of the 9 patients with p53-positive tumors and 4 (19%) of the 21 patients with p53-negative tumors exhibited recurrence. p53 expression was found to be associated with increased recurrence in the HPV-positive subgroup (P=0.046). However, a similar predictive effect was not observed for survival status (alive vs. ex) within the HPV-positive subgroup.

In the 12 cases with HPV-negative tumors, an equal distribution of p53-positive and -negative patients was noted (6 of each). The analysis did not reveal a significant impact of p53 expression on the recurrence and survival status (alive vs. deceased) in HPV-negative cases (P=0.079 and P=0.505, respectively).

PD-L1 expression. The median TPS was 1% (range, 0-100%), with a mean value of 8%, while the median CPS was 3% (range, 0-100%), with a mean value of 10%. PD-L1 expression was higher in tumor cells compared with immune cells.

Exploring the number of cases with TPS <1 or ≥1%, as well as CPS <1% and ≥1%, revealed that 19 cases (45.2%) had a TPS <1% and 23 cases (54.8%) had a TPS ≥1%. In addition, 11 cases (26.2%) had a CPS <1% and 31 cases (73.8%) had a CPS ≥1%. The distribution of TPS and CPS cases exhibited a statistically significant difference (P<0.001).

A significant association of female sex with TPS ≥1%-positive (P=0.038) and CPS ≥1%-positive (P=0.011) tumors was observed. Statistical significance was established for the associations of CPS positivity with HPV-positive (P=0.026) and p16-positive tumors (P=0.013). Although no statistically significant association was noted in terms of recurrence (P=0.109), out of the 16 cases with recurrence, 14 were found to have a CPS ≥1%. In addition, all 10 cases with LRR were positive for PD-L1 (P=0.031) (Table IV).

Table II. Clinical and immunohistochemical characteristics of HPV-positive and -negative anal squamous cell carcinoma.

Characteristics	HPV-positive, n (%)	HPV-negative, n (%)	P-value
Sex			0.001
Female	23 (76.7)	2 (16.7)	
Male	7 (23.3)	10 (83.3)	
Age, years			0.406
<65	17 (56.7)	8 (66.7)	
≥65	13 (43.3)	4 (33.3)	
T stage			0.446
T1-T2	10 (33.3)	3 (25)	
T3-T4	20 (66.7)	9 (75)	
N status			0.600
N-	13 (43.3)	5 (41.7)	
N+	17 (53.7)	7 (58.3)	
Clinical stage			0.292
I-II	2 (40)	3 (25)	
III	18 (60)	9 (75)	
Recurrence			0.088
Yes	9 (30)	7 (58.3)	
No	21 (70)	5 (41.7)	
Locoregional recurrence			0.086
Yes	5 (16.7)	5 (41.7)	
No	25 (83.3)	7 (58.3)	
Distant recurrence			0.277
Yes	8 (26.7)	5 (41.7)	
No	22 (73.3)	7 (58.3)	
Survival status			0.008
Alive	21 (70)	3 (25)	
Deceased	9 (30)	9 (75)	
p16 expression			<0.001
Positive	29 (96.7)	2 (16.7)	
Negative	1 (3.3)	10 (83.3)	
p53 expression			0.222
Positive	9 (30)	6 (50)	
Negative	21 (70)	6 (50)	

HPV, human papillomavirus; T stage, primary tumor stage; N stage, regional lymph node stage.

Table III. Clinical and immunohistochemical characteristics of p53-positive and -negative anal squamous cell carcinoma.

Characteristics	p53-positive, n (%)	p53-negative, n (%)	P-value
Sex			0.613
Female	9 (60)	16 (59.3)	
Male	6 (40)	11 (40.7)	
Age, years			0.055
<65	6 (40)	19 (70.4)	
≥65	9 (60)	8 (29.6)	
T stage			0.534
T1-T2	5 (33.3)	8 (29.6)	
T3-T4	10 (66.7)	19 (70.4)	
N status			0.094
N-	9 (60)	9 (33.3)	
N+	6 (40)	18 (66.7)	
Clinical stage			0.220
I-II	7 (46.7)	8 (29.6)	
III	8 (53.3)	19 (70.4)	
Recurrence			0.006
Yes	10 (66.7)	6 (22.2)	
No	5 (33.3)	21 (77.8)	
Locoregional recurrence			0.014
Yes	7 (46.7)	3 (11.1)	
No	8 (53.3)	24 (88.9)	
Distant recurrence			0.101
Yes	7 (46.7)	6 (22.2)	
No	8 (53.3)	21 (77.8)	
Survival status			0.307
Alive	7 (46.7)	17 (63)	
Deceased	8 (53.3)	10 (37)	
HPV status			0.193
Positive	9 (60)	21 (77.8)	
Negative	6 (40)	6 (22.2)	
p16 expression			0.333
Positive	10 (66.7)	21 (77.8)	
Negative	5 (33.3)	6 (22.2)	

HPV, human papillomavirus; T stage, primary tumor stage; N stage, regional lymph node stage.

The distribution of TPS and CPS values for PD-L1 expression in cases according to HPV status, p16 and p53 expression is shown in Table V.

Factors and outcomes associated with treatment response. Both HPV-negative and p16-negative tumors were found to be strongly associated with the absence of a complete response to definitive RT/CRT (both P<0.001). The presence of HPV and p16 positivity was identified as a significant predictor for achieving a complete response. The absence of a complete response was also associated with an increased recurrence rate (P=0.016), elevated DR rate (P=0.015) and unfavorable survival status (alive vs. deceased; P=0.049) (Table VI).

Survival outcomes. The 3-year and 5-year OS rates of the study cohort were 78.4 and 66.7%, respectively, while the corresponding DFS rates were 72 and 65.5%.

In univariate analyses, age (<65 vs. ≥65 years) was found to have a significant association with improved 5-year OS (P=0.049; Fig. 1A). No significant associations were found

Table IV. Clinical and immunohistochemical characteristics associated with TPS (<1 vs. ≥1%) and CPS (<1 vs. ≥1%).

Characteristics	TPS <1%, n (%)	TPS ≥1%, n (%)	P-value	CPS <1%, n (%)	CPS ≥1%, n (%)	P-value
Sex			0.038			0.011
Female	8 (42.1)	17 (73.9)		3 (27.3)	22 (71)	
Male	11 (57.9)	6 (26.1)		8 (72.7)	9 (29)	
Age, years			0.453			0.299
<65	12 (63.2)	13 (56.5)		8 (72.7)	17 (54.8)	
≥65	7 (36.8)	10 (43.5)		3 (27.3)	14 (45.2)	
T stage			0.401			0.759
T1-T2	5 (26.3)	8 (34.8)		3 (27.3)	10 (32.3)	
T3-T4	14 (73.7)	15 (65.2)		8 (72.7)	21 (67.7)	
N status			0.344			0.224
N-	7 (36.8)	11 (47.8)		3 (27.3)	15 (48.4)	
N+	12 (63.2)	12 (52.2)		8 (72.7)	16 (51.6)	
Clinical stage			0.428			0.496
I-II	6 (31.6)	9 (39.1)		3 (27.3)	12 (38.7)	
III	13 (68.4)	14 (60.9)		8 (72.7)	19 (61.3)	
Complete response			0.371			0.149
Yes	4 (21.1)	7 (30.4)		6 (54.5)	24 (77.5)	
No	15 (78.9)	16 (69.6)		5 (45.5)	7 (22.6)	
Recurrence			0.320			0.109
Yes	6 (31.6)	10 (43.5)		2 (18.2)	14 (45.2)	
No	13 (68.4)	13 (56.5)		9 (81.8)	17 (54.8)	
Locoregional recurrence			0.230			0.031
Yes	3 (15.8)	7 (30.4)		0 (0)	10 (32.3)	
No	16 (84.2)	16 (69.6)		11 (100)	21 (67.7)	
Distant recurrence			0.599			0.286
Yes	6 (31.6)	7 (30.4)		2 (18.2)	11 (35.5)	
No	13 (68.4)	16 (69.6)		9 (81.8)	20 (64.5)	
Survival status			0.474			0.695
Alive	12 (63.2)	12 (52.2)		6 (54.5)	18 (58.1)	
Deceased	7 (36.8)	11 (47.8)		5 (45.5)	13 (41.9)	
HPV status			0.078			0.026
Positive	11 (57.9)	19 (82.6)		5 (45.5)	25 (80.6)	
Negative	8 (42.1)	4 (17.4)		6 (54.5)	6 (19.4)	
p16 expression			0.141			0.013
Positive	12 (63.2)	19 (82.6)		5 (45.5)	26 (83.9)	
Negative	7 (36.8)	4 (17.4)		6 (54.5)	5 (16.1)	
p53 expression			0.203			0.158
Positive	5 (26.3)	10 (43.5)		2 (18.2)	13 (41.9)	
Negative	14 (73.7)	13 (56.5)		9 (81.8)	18 (58.1)	

TPS, tumor proportion score; CPS, combined positive score; T stage, primary tumor stage; N stage, regional lymph node stage; HPV, human papillomavirus.

for other clinical factors (T stage, N status and clinical stage) or patient characteristics with survival. HPV-positive tumors exhibited higher rates for 5-year OS (77.3 vs. 41.7%; P=0.010; Fig. 1B) and DFS (73.4 vs. 42.9%; P=0.025; Fig. 1D). Although p53 expression was not found to have a significant association with 5-year OS (94.6 vs. 72.2%; P=0.642), it was strongly associated with a decreased 5-year DFS (50.9 vs. 74.2%; P=0.010; Fig. 1E). A complete response was also significantly associated with an improved 5-year OS (77.8 vs. 40%; P=0.015; Fig. 1C) and DFS (76.5 vs. 38.1%; P=0.001; Fig. 1F). Conversely, no

Table V. Distribution of TPS and CPS values.

A, TPS, n (%)

Characteristics	<1%	≥1 to <5%	≥5 to <10%	≥10 to <25%	≥25 to <50%	≥50%
HPV status						
Positive	11 (26.2)	8 (19)	4 (9.5)	2 (4.8)	4 (9.5)	1 (2.4)
Negative	8 (19)	2 (4.8)	0 (0)	1 (2.4)	0 (0)	1 (2.4)
p16 expression						
Positive	12 (28.6)	8 (19)	4 (9.5)	2 (4.8)	4 (9.5)	1 (2.4)
Negative	7 (16.7)	2 (4.8)	0 (0)	1 (2.4)	0 (0)	1 (2.4)
p53 expression						
Positive	5 (11.9)	4 (9.5)	1 (2.4)	2 (4.8)	3 (7.1)	0 (0)
Negative	14 (33.3)	6 (14.3)	3 (7.1)	1 (2.1)	1 (2.4)	2 (4.8)
Total	19 (45.2)	10 (23.8)	4 (9.5)	3 (7.1)	4 (9.5)	2 (4.8)

B, CPS, n (%)

Characteristics	<1%	≥1 to <5%	≥5 to <10%	≥10 to <25%	≥25 to <50%	≥50%
HPV status						
Positive	5 (11.9)	11 (26.2)	7 (16.7)	2 (4.8)	4 (9.5)	1 (2.4)
Negative	6 (14.3)	4 (9.5)	0 (0)	1 (2.4)	0 (0)	1 (2.4)
p16 expression						
Positive	5 (11.9)	12 (28.6)	7 (16.7)	2 (4.8)	4 (9.5)	1 (2.4)
Negative	6 (14.3)	3 (7.1)	0 (0)	1 (2.4)	0 (0)	1 (2.4)
p53 expression						
Positive	2 (4.8)	7 (16.7)	1 (2.4)	2 (4.8)	3 (7.1)	0 (0)
Negative	9 (21.4)	8 (19)	6 (14.3)	1 (2.4)	1 (2.4)	2 (4.8)
Total	11 (26.2)	15 (35.7)	7 (16.7)	3 (7.1)	4 (9.5)	2 (4.8)

TPS, tumor proportion score; CPS, combined positive score; HPV, human papillomavirus.

significant impacts of p16 expression on 5-year OS (P=0.183) and DFS (P=0.160), or of PD-L1 expression on 5-year OS (P=0.963) and DFS (P=0.179) were observed.

In the multivariate analysis, age (<65 vs. ≥65 years; P=0.010) and HPV positivity (P=0.002) emerged as independent prognostic factors for 5-year OS, while a complete response (P=0.007) and p53 expression (P=0.038) were identified as independent prognostic factors for 5-year DFS.

Discussion

In early studies of concurrent CRT, a complete response rate of 80-90% was reported (2,29,30), while Ajani *et al* (31) observed a 73% complete response rate. In the present study, the complete response rate was 71.4%, which is lower compared with that in previous studies. However, the present study did not identify any factors influencing the treatment-associated complete response rate. A complete response was found to be associated with recurrence and survival as an independent prognostic factor in the present series, aligning with previous reports (6-8). It is important to highlight that the factors found to be influencing a complete response in the previous studies were limited to clinical data and treatment strategy, and sufficient information for a complete response evaluation regarding the identification of guiding HPV status and molecular characteristics were lacking. The inclusion of these factors is an notable aspect of the present study. In that context, HPV negativity was found to be strongly associated with resistance to definitive CRT, consistent with the findings of Soares *et al* (32). The lower complete response rate in the present study compared with previous studies may be explained by there being a higher proportion of HPV-negative tumors (28.6%) in the present study (33-35). The robust association of HPV positivity with a complete response and the significant impact of a complete response on DR in the present data indirectly suggest that HPV status should be considered when assessing the risk of DR. Indeed, the present study was consistent with previous studies (36,37) in finding significantly increased 5-year OS and DFS rates in HPV-positive patients. HPV status was identified to be a stage-independent prognostic factor.

Several studies have emphasized that p16 expression alone serves as a favorable prognostic factor for anal SCC and is linked to a higher likelihood of achieving a complete

Table VI. Clinical and immunohistochemical characteristics associated with response status.

Characteristics	Complete response (+), n (%)	Complete response (-), n (%)	P-value
Sex			0.127
Female	20 (66.7)	5 (58.3)	
Male	10 (33.3)	7 (41.7)	
Age, years			0.921
<65	18 (60)	7 (41.7)	
≥65	12 (40)	5 (58.3)	
T stage			0.554
T1-T2	9 (30)	4 (33.3)	
T3-T4	21 (70)	8 (66.7)	
N status			0.400
N-	12 (40)	6 (50)	
N+	18 (60)	6 (50)	
Clinical stage			0.566
I-II	11 (36.7)	4 (33.3)	
III	19 (63.3)	8 (66.7)	
Recurrence			0.016
Yes	8 (26.7)	8 (66.7)	
No	22 (73.3)	4 (33.3)	
Locoregional recurrence			0.296
Yes	6 (20)	4 (33.3)	
No	24 (80)	8 (66.7)	
Distant recurrence			0.015
Yes	6 (20)	7 (41.7)	
No	24 (80)	5 (58.3)	
Survival status			0.049
Alive	20 (66.7)	4 (33.3)	
Deceased	10 (33.3)	8 (66.7)	
HPV status			<0.001
Positive	27 (90)	3 (25)	
Negative	3 (10)	9 (75)	
p16 expression			<0.001
Positive	27 (90)	4 (33.3)	
Negative	3 (10)	8 (66.7)	
p53 expression			0.193
Positive	9 (30)	6 (50)	
Negative	21 (70)	6 (50)	
PD-L1 expression, TPS %			0.371
<1	4 (21.1)	15 (78.9)	
≥1	7 (30.4)	16 (69.6)	
PD-L1 expression, CPS %			0.149
<1	6 (54.5)	5 (45.5)	
≥1	24 (77.5)	7 (22.6)	

T stage, primary tumor stage; N stage, regional lymph node stage HPV, human papillomavirus; PD-L1, programmed cell death ligand 1.

response (26,38,39), while the study by Ajani *et al* did not find this association (31). In the present study cohort, p16 expression demonstrated a significant association with an increased complete response rate. Regarding survival, the association of p16 positivity with OS is currently uncertain, but it may be linked with increased DFS (13,26,37). In the present study cohort, despite the strong association between HPV positivity and p16 expression, the favorable survival outcomes observed

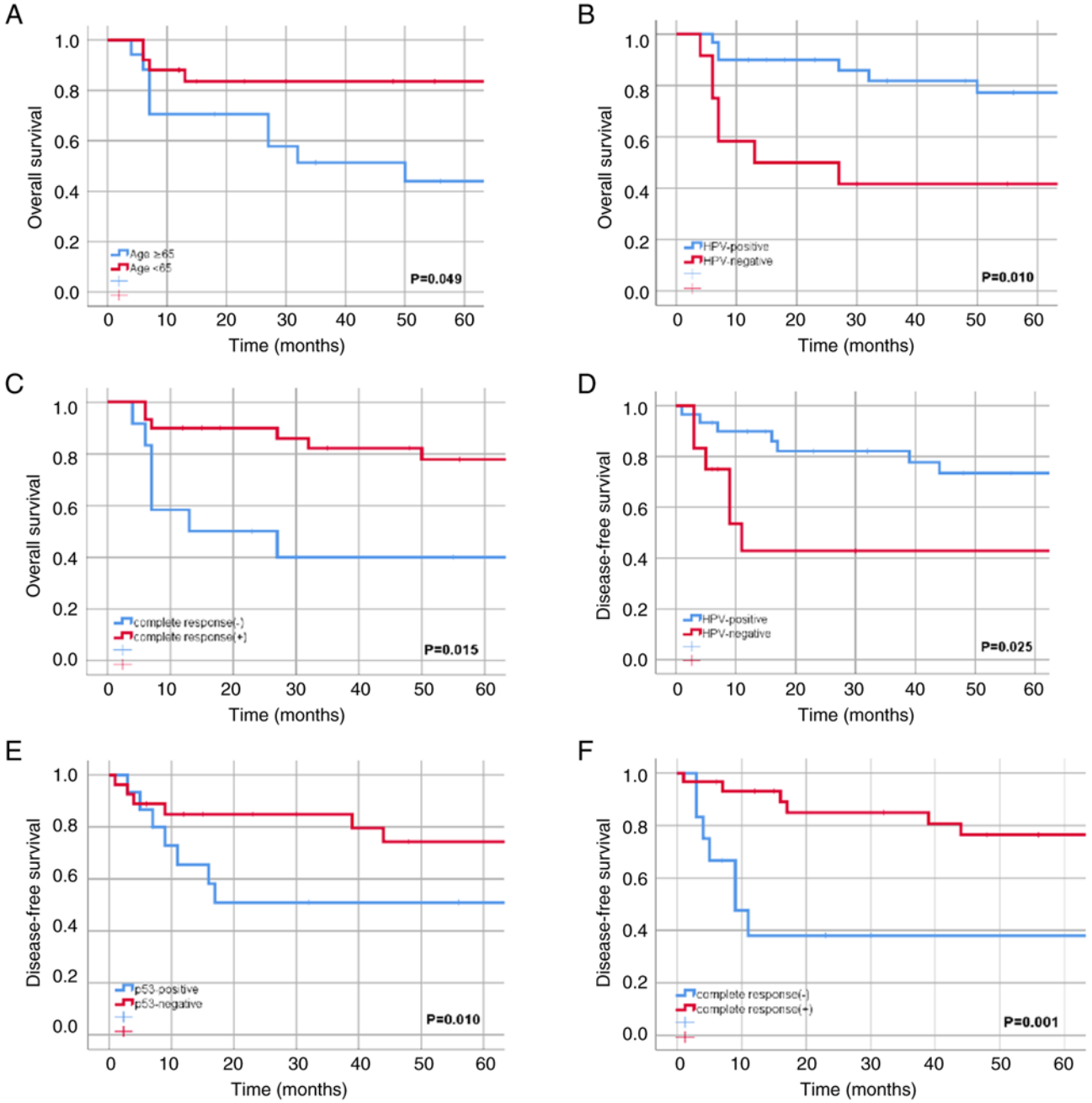

Figure 1. Kaplan-Meier curves of overall survival and disease-free survival in patients with anal squamous cell carcinoma. Kaplan-Meier curves of overall survival according to (A) age <65 vs. ≥65 years, (B) HPV status and (C) complete response status, and disease-free survival according to (D) HPV status, (E) p53 expression status and (F) complete response status. HPV, human papillomavirus.

in the HPV-positive subgroup were not mirrored in p16 expression. Given the discordant survival outcomes, prioritizing the use of HPV in prognostic evaluations for anal SCC cases appears consistent and rational.

In HPV-assocated cancers, the association between p53 mutations and prognosis is not conclusive due to differences in mutation and function. p53 mutations can be examined using IHC and mutational analyses, including sequencing or PCR-single-strand conformation polymorphism. IHC expression analysis of p53 using a p53 antibody, which was also employed in the current study for the detection of p53 mutations, is frequently used due to its simplicity and accessibility compared with mutational analysis (26). The rationale for using IHC analysis in the detection of p53 is the rapid degradation of the non-mutant wild-type p53 under normal cellular conditions, which makes it challenging to detect. By contrast, mutant p53 has a longer half-life, leading to its accumulation within cells and increased IHC staining (40). HPV-negative cancers generally exhibit more mutations and functional losses in the p53 gene compared with HPV-positive cancers (41). Consequently, in HPV-positive tumors, low p53 expression is expected immunohistochemically. However, the HPV-induced degradation of p53 may mask increased p53 expression immunohistochemically in HPV-positive tumors with p53 mutations, causing potential confusion in the analysis. In SCCs of the head and neck, the correspondence between both tests has been

investigated, and IHC and mutation analysis were reported to be consistent with each other for the detection of p53 mutations; it is noteworthy that the majority of the cases were HPV negative (64%) (42). By contrast, Meulendijks *et al* (43) noted a low concordance between p53 gene mutation and abnormal p53 expression (>70 and 0%, respectively) in their analysis of anal SCC cases, of which 87% were HPV positive.

A variable range of 34-100% has been reported for p53 mutations in anal SCC (15). In the present study, p53 mutations were detected in 35.7% of the patients. Previous studies have suggested that HPV-negative tumors may be more resistant to definitive CRT due to an association between the p53 mutation and HPV-negative status (43-46). Soares *et al* (32) detected p53 gene mutations in all HPV-negative cases and identified a significant association between poor treatment response at 6 months and the p53 mutation. Contrary to this, the present data did not reveal any association of p53 expression with either HPV-negative status or a complete response.

Meulendijks *et al* (43) noted that the presence of p53 mutations was associated with HPV- and p16-negative tumors, but did not significantly affect survival or serve as a prognostic factor in the HPV-negative subgroup. Similarly, in the present study, p53 expression exhibited no impact on recurrence or survival in the HPV-negative subgroup. However, a notable finding was the first-time identification of a significant association between p53 expression and increased recurrence in the HPV-positive subgroup. Consistent with the present study, Gilbert *et al* (26) found that high p53 expression (>5%) was associated with increased recurrence and, similarly, Bruyere *et al* (47) found that abnormal p53 expression (0 or >50%) had an association with increased recurrence. Meulendijks *et al* (43) found that abnormal P53 expression and the presence of a P53 gene mutation exhibited an independent association with poor local-regional control. The present study demonstrated that LRR was associated with >5% p53 expression. In terms of survival outcomes linked to p53 expression, previous studies have presented ambiguous results (31,32,48-50). However, the present study is consistent with the aforementioned research, with the exception of the study of Zhu *et al* (49), in indicating a reduction in DFS.

In non-metastatic anal SCC, PD-L1 expression rates have been reported in previous studies by Armstrong *et al* (50), who found >5% PD-L1 expression in 40.5% of cases; Iseas *et al* (51), who observed a CPS >1% in 57% of cases; and Chan *et al* (52), who found a TPS >1% in 71.4% of cases. In the present study, the prevalance of PD-L1 expression with TPS ≥1 was 54.8%, and with CPS ≥1 was 73.8%. Consistent with the findings of Iseas *et al* (51), higher PD-L1 expression was observed in tumor cells than in immune cells in the present study, which has an impact on treatment decisions. Therefore, the use of PD-1 or PD-L1 inhibitors may be effective for tumor regression. The quantity and quality of lymphocytes in the tumor microenvironment, regulated by various types of T cells, are highly important. It has been shown that the number, location and quality of $CD8^+$ T cells positively correlate with the prognosis of a number of malignant tumors. However, regulatory T cells can inhibit the immune response against tumor cells, which is related to the failure of immunotherapy (53). Ongoing studies are aiming to enhance the immune response, and include the use of adaptive T-cell therapies such as CAR-T cells and tumor-infiltrating lymphocytes. RT is a significant stimulator and enhancer of lymphocytes, which counters the immunosuppressive effect of PD-L1 (53).

The existing evidence on the relationships between HPV status, p16, p53 expression and PD-L1 in non-metastatic cases of anal SCC is contradictory (19,52). However, PD-L1 positivity has been shown to be associated with HPV negativity in anogenital tumors (54). In the present study, ≥1% PD-L1 positivity was significantly associated with HPV and p16 positivity, highlighting their potential for guiding immunotherapy decisions in patients with anal SCC.

Iseas *et al* (51) reported higher complete response rates and improved OS in PD-L1-positive cases with CPS >1% after definitive CRT. In addition, Chan *et al* (52) found an improved 10-year OS in cases with ≥5% PD-L1 positivity, and Wessely *et al* (19) reported an improved OS for patients who were PD-L1 positive with TPS >1%. By contrast, Zhao *et al* (21) observed a tendency for PD-L1 positivity to be associated with worse DFS and OS. In the present study, PD-L1 positivity (CPS >1%) was identified as a factor increasing the LRR, although no statistically significant association was found between PD-L1 (CPS >1%) status and complete response, 5-year OS or DFS. The present study presents a well-suited population for the low-incidence anal SCC, as the exclusion of HIV-positive patients ensured a homogeneous group with a consistent treatment approach. Despite being retrospective, all data were obtained and cases with missing information were excluded. Treatment response, HPV status and IHC analysis were meticulously evaluated by an experienced team. However, the limited number of patients (14.3%) who did not receive chemotherapy in the present study could be considered a limitation, despite no statistical significance being observed between the patients who received chemotherapy and those who did not in terms of treatment response and survival. As a useful suggestion, p53 mutation analysis alongside p53 expression should be evaluated in future studies.

The present study also highlights the crucial role of HPV vaccination. Anal cancer prevention strategies mirror those for cervical cancer prevention, focusing on both primary and secondary prevention methods. Providing the 9-valent HPV vaccine to girls and boys before the onset of sexual activity could effectively prevent nearly all anal cancers. There is evidence to suggest that vaccination may also reduce the risk of recurrent precancerous lesions and potentially prevent the progression to anal cancer, particularly in high-risk individuals (55). However, addressing the needs of individuals with persistent HPV infection requires a different approach. Therapeutic vaccination aims to stimulate cellular immunity against existing HPV infections and lesions, potentially preventing cancer progression. Multiple therapeutic vaccines are currently in clinical development, utilizing various platforms (56).

Despite the high efficacy of HPV vaccines in preventing infection, several challenges persist. Guidelines advocate for the cancer screening of vaccinated individuals, underscoring the ongoing importance of preventive measures. Disparities in global HPV vaccination rates highlight that targeted interventions are necessary to ensure equitable access to vaccination. In addition, there is a concerning lack of awareness among adolescents, including medical students, regarding HPV and

its vaccines. Greater efforts, potentially including mandatory measures, are required to increase awareness, particularly among males and young people (57). Accordingly, a risk-adaptive approach is necessary for patients with anal SCC. Further randomized controlled studies comparing patients with and without HPV based on risk stratification are essential. The findings of the present study suggest that additional agents tailored to the treatment for both subgroups are necessary.

Concerning the HPV-positive subgroup, a strong association with complete response and significantly improved overall and DFS were observed. Although p16 expression was found to be associated with an improved treatment response, survival outcomes were more consistently associated with HPV positivity. A number of recommendations for the HPV-positive subgroup may be made. Firstly, in the entire cohort, p53 exhibited an association with increased recurrence and LRR. However, it may be beneficial to assess the impact of p53 mutation within HPV-positive and -negative subgroups separately due to the p53 status in HPV-negative tumors having no significant effect on survival or prognosis, contrasting with HPV-positive tumors, where p53 expression or mutation exhibited an association with heightened recurrence. Addressing mutated p53 in HPV-positive tumors may involve directly targeting the aberrant protein to restore the wild-type conformation and transcriptional activity. Agents for targeting p53 include COTI-2 and Ad-p53, which are being studied in head and neck cancers (58). Furthermore, treatments targeting HPV-positive cancer cells, even without mutated p53, could focus on viral enzymes E6/E7, responsible for p53 degradation. Notable examples are Ad-E6/E7-As and bortezomib (58). Further studies are warranted to uncover p53 reactivators that are more specific, safe and efficient, in order to provide an enhanced treatment of anal SCC. Secondly, in the present study PD-L1 expression was found to be associated with HPV and p16 positivity, suggesting a potential role in guiding immunotherapy decisions for this subgroup of patients. The integration of HPV status with p16, p53 and PD-L1 expression analysis may provide a comprehensive understanding of the immunological landscape, and aid in risk stratification and personalized treatment approaches for HPV-positive anal SCC. Thirdly, additional genetic alterations may influence the survival in patients with HPV-positive tumors, including somatic PIK3CA exon 9/20 and KMT2C pathogenic variants. These mutations may play a key role in tumor biology and the response to treatment, further highlighting the complexity of the molecular mechanisms involved in HPV-positive tumors (59). Finally, while efforts to further increase survival outcomes in the HPV-positive subgroup are necessary, the reduction of potential side effects is also important. For example, the potential for dose de-escalation and the substitution of chemotherapy with alternative therapeutic agents may be considered.

The HPV-negative subgroup is currently overlooked due to its relatively lower incidence, and there is currently no randomized controlled trial focusing on this subgroup. Based on the key insights gained from the present study, further research focusing on the HPV-negative subgroup of patients with anal SCC would be beneficial. Recommendations for the HPV-negative subgroup include investigating the hypothesis that RT dose escalation based on HPV status could be successful for the HPV-negative resistant subgroup. In addition, mutational profiles that notably differ between HPV-positive and HPV-negative patients may be explored, as they could suggest multiple avenues for the investigation of targeted therapies in anal SCC (59). Also, hyperthermia could be considered as a potential means of increasing the sensitivity of cancer cells to therapeutic agents, inducing direct cytotoxicity, triggering anticancer immune responses and improving drug delivery, as supported by previous studies (60,61). Hypoxia-sensitizing methods may also be explored, particularly in radioresistant HPV-negative or p16-negative subgroups, as in the DAHANCA 5 trials (46,62). Furthermore, the potential of metformin in the prevention of multidrug resistance and the resensitization of cancer cells to standard chemotherapeutic agents, as well as enhancing cancer cell sensitivity to RT, may be considered (63). Notable PD-L1 positivity in the HPV-negative subgroup suggests that immunotherapy may be considered as an aggressive option, including concurrent RT. While the expression of p53 in HPV-negative tumors appears to have no significant impact on survival or prognosis, additional larger population-based studies are required to confirm these findings within this subgroup. These approaches all have the potential to be further optimized with nanoparticle-based treatments, highlighting the advancement of radiosensitizer nanoparticles. However, despite progress, challenges remain in the translation of nanoparticle-enhanced RT from the laboratory to clinical practice, including concerns about biosafety, nanoparticle clearance and the optimization of nanoparticle properties for effective interaction with radiation and biological systems (64).

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BBT conceived and designed the analysis, collected data, contributed data or analysis tools, performed analyses, wrote the paper and supervised the study. FS, MSe and MSo collected data and contributed data or analysis tools. DY contributed data or analysis tools and performed analyses. SO conceived and designed the analysis, collected data and supervised the study. BBT, FS, DY and SO confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Research Ethics Committee at Ege University (Izmir, Türkiye; reference:

21-3.1T/63). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. National Cancer Institute: SEER cancer statistics factsheets: Anal Cancer. Available from: http://seer.cancer.gov/statfacts/html/anus.html. Accessed at Jun 7, 2021).
2. Bartelink H, Roelofsen F, Eschwege F, Rougier P, Bosset JF, Gonzalez DG, Peiffert D, van Glabbeke M and Pierart M: Concomitant radiotherapy and chemotherapy is superior to radiotherapy alone in the treatment of locally advanced anal cancer: Results of a phase III randomized trial of the European organization for research and treatment of cancer radiotherapy and gastrointestinal cooperative groups. J Clin Oncol 15: 2040-2049, 1997.
3. Oliveira SC, Moniz CM, Riechelmann R, Alex AK, Braghirolli MI, Bariani G, Nahas C and Hoff PM: Phase II study of capecitabine in substitution of 5-FU in the chemoradiotherapy regimen for patients with localized squamous cell carcinoma of the anal canal. J Gastrointest Cancer 47: 75-81, 2016.
4. Peiffert D, Tournier-Rangeard L, Gérard JP, Lemanski C, François E, Giovannini M, Cvitkovic F, Mirabel X, Bouché O, Luporsi E, *et al*: Induction chemotherapy and dose intensification of the radiation boost in locally advanced anal canal carcinoma: Final analysis of the randomized UNICANCER ACCORD 03 trial. J Clin Oncol 30: 1941-1948, 2012.
5. Rao S, Guren MG, Khan K, Brown G, Renehan AG, Steigen SE, Deutsch E, Martinelli E and Arnold D; ESMO Guidelines Committee. Electronic address: clinicalguidelines@esmo.org: Anal cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up*. Ann Oncol 32: 1087-1100, 2021.
6. Glynne-Jones R, Sebag-Montefiore D, Meadows HM, Cunningham D, Begum R, Adab F, Benstead K, Harte RJ, Stewart J, Beare S, *et al*: Best time to assess complete clinical response after chemoradiotherapy in squamous cell carcinoma of the anus (ACT II): A post-hoc analysis of randomised controlled phase 3 trial. Lancet Oncol 18: 347-356, 2017.
7. Chapet O, Gerard JP, Riche B, Alessio A, Mornex F and Romestaing P: Prognostic value of tumor regression evaluated after first course of radiotherapy for anal canal cancer. Int J Radiat Oncol Biol Phys 63: 1316-1324, 2005.
8. Gerard JP, Ayzac L, Hun D, Romestaing P, Coquard R, Ardiet JM and Mornex F: Treatment of anal canal carcinoma with high dose radiation therapy and concomitant fluorouracil-cisplatinum. Long-term results in 95 patients. Radiother Oncol 46: 249-256, 1998.
9. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, Westra WH, Chung CH, Jordan RC, Lu C, *et al*: Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 363: 24-35, 2010.
10. Ragin CC and Taioli E: Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: Review and meta-analysis. Int J Cancer 121: 1813-1820, 2007.
11. Rischin D, Young RJ, Fisher R, Fox SB, Le QT, Peters LJ, Solomon B, Choi J, O'Sullivan B, Kenny LM and McArthur GA: Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol 28: 4142-4148, 2010.
12. Lewis JS Jr: p16 Immunohistochemistry as a standalone test for risk stratification in oropharyngeal squamous cell carcinoma. Head Neck Pathol 6 (Suppl 1): S75-S82, 2012.
13. 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.
14. NCCN: NCCN Guidelines for Anal Cancer Version 1.2024. https://www.nccn.org/professionals/physician_gls/pdf/anal_blocks.pdf. Accessed March 4, 2024.
15. Lampejo T, Kavanagh D, Clark J, Goldin R, Osborn M, Ziprin P and Cleator S: Prognostic biomarkers in squamous cell carcinoma of the anus: A systematic review. Br J Cancer 103: 1858-1869, 2010.
16. Gillison ML, Chaturvedi AK, Anderson WF and Fakhry C: Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. J Clin Oncol 33: 3235-3242, 2015.
17. Balachandran S and Narendran A: The developmental origins of cancer: A review of the genes expressed in embryonic cells with implications for tumorigenesis. Genes (Basel) 14: 604, 2023.
18. Doroshow DB, Bhalla S, Beasley MB, Sholl LM, Kerr KM, Gnjatic S, Wistuba II, Rimm DL, Tsao MS and Hirsch FR: PD-L1 as a biomarker of response to immune-checkpoint inhibitors. Nat Rev Clin Oncol 18: 345-362, 2021.
19. Wessely A, Heppt MV, Kammerbauer C, Steeb T, Kirchner T, Flaig MJ, French LE, Berking C, Schmoeckel E and Reinholz M: Evaluation of PD-L1 expression and HPV genotyping in anal squamous cell carcinoma. Cancers (Basel) 12: 2516, 2020.
20. Balermpas P, Martin D, Wieland U, Rave-Fränk M, Strebhardt K, Rödel C, Fokas E and Rödel F: Human papilloma virus load and PD-1/PD-L1, CD8+ and FOXP3 in anal cancer patients treated with chemoradiotherapy: Rationale for immunotherapy. Oncoimmunology 6: e1288331, 2017.
21. Zhao YJ, Sun WP, Peng JH, Deng YX, Fang YJ, Huang J, Zhang HZ, Wan DS, Lin JZ and Pan ZZ: Programmed death-ligand 1 expression correlates with diminished CD8+ T cell infiltration and predicts poor prognosis in anal squamous cell carcinoma patients. Cancer Manag Res 10: 1-11, 2017.
22. Goodman KA, Gollub M, Eng C, Brierley J, Palefsky J, Gress D, Williams A and Goldberg R: Anus: AJCC Cancer Staging Manual. 9th edition. Washington MK (ed). American College of Surgeons, 2022.
23. Glynne-Jones R, Nilsson PJ, Aschele C, Goh V, Peiffert D, Cervantes A and Arnold D; ESMO; ESSO; ESTRO: Anal cancer: ESMO-ESSO-ESTRO clinical practice guidelines for diagnosis, treatment and follow-up. Radiother Oncol 111: 330-339, 2014.
24. Kachnic LA, Winter K, Myerson RJ, Goodyear MD, Willins J, Esthappan J, Haddock MG, Rotman M, Parikh PJ, Safran H and Willett CG: RTOG 0529: A phase 2 evaluation of dose-painted intensity modulated radiation therapy in combination with 5-fluorouracil and mitomycin-C for the reduction of acute morbidity in carcinoma of the anal canal. Int J Radiat Oncol Biol Phys 86: 27-33, 2013.
25. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, *et al*: New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer 45: 228-247, 2009.
26. Gilbert DC, Williams A, Allan K, Stokoe J, Jackson T, Linsdall S, Bailey CM and Summers J: p16INK4A, p53, EGFR expression and KRAS mutation status in squamous cell cancers of the anus: Correlation with outcomes following chemo-radiotherapy. Radiother Oncol 109: 146-151, 2013.
27. Yang Y, Forslund A, Remotti H, Lönnroth C, Andersson M, Brevinge H, Svanberg E, Lindnér P, Hafström L, Naredi P and Lundholm K: P53 mutations in primary tumors and subsequent liver metastases are related to survival in patients with colorectal carcinoma who undergo liver resection. Cancer 91: 727-736, 2001.
28. de Ruiter EJ, Mulder FJ, Koomen BM, Speel EJ, van den Hout MFCM, de Roest RH, Bloemena E, Devriese LA and Willems SM: Comparison of three PD-L1 immunohistochemical assays in head and neck squamous cell carcinoma (HNSCC). Mod Pathol 34: 1125-1132, 2021.
29. Northover J, Glynne-Jones R, Sebag-Montefiore D, James R, Meadows H, Wan S, Jitlal M and Ledermann J: Chemoradiation for the treatment of epidermoid anal cancer: 13-year follow-up of the first randomised UKCCCR anal cancer trial (ACT I). Br J Cancer 102: 1123-1128, 2010.
30. James RD, Glynne-Jones R, Meadows HM, Cunningham D, Myint AS, Saunders MP, Maughan T, McDonald A, Essapen S, Leslie M, *et al*: Mitomycin or cisplatin chemoradiation with or without maintenance chemotherapy for treatment of squamous-cell carcinoma of the anus (ACT II): A randomised, phase 3, open-label, 2x2 factorial trial. Lancet Oncol 14: 516-524, 2013.
31. Ajani JA, Wang X, Izzo JG, Crane CH, Eng C, Skibber JM, Das P and Rashid A: Molecular biomarkers correlate with disease-free survival in patients with anal canal carcinoma treated with chemoradiation. Dig Dis Sci 55: 1098-1105, 2010.

32. Soares PC, Abdelhay ES, Thuler LCS, Soares BM, Demachki S, Ferro GVR, Assumpção PP, Lamarão LM, Pinto LF and Burbano RMR: HPV positive, wild type TP53, and p16 overexpression correlate with the absence of residual tumors after chemoradiotherapy in anal squamous cell carcinoma. BMC Gastroenterol 18: 30, 2018.
33. Lu DW, El-Mofty SK and Wang HL: Expression of p16, Rb, and p53 proteins in squamous cell carcinomas of the anorectal region harboring human papillomavirus DNA. Mod Pathol 16: 692-699, 2003.
34. Mai S, Welzel G, Ottstadt M, Lohr F, Severa S, Prigge ES, Wentzensen N, Trunk MJ, Wenz F, von Knebel-Doeberitz M and Reuschenbach M: Prognostic relevance of HPV ınfection and p16 overexpression in squamous cell anal cancer. Int J Radiat Oncol Biol Phys 93: 819-827, 2015.
35. Foster CC, Lee AY, Furtado LV, Hart J, Alpert L, Xiao SY, Hyman NH, Sharma MR and Liauw SL: Treatment outcomes and HPV characteristics for an institutional cohort of patients with anal cancer receiving concurrent chemotherapy and intensity-modulated radiation therapy. PLoS One 13: e0194234, 2018.
36. Baricevic I, He X, Chakrabarty B, Oliver AW, Bailey C, Summers J, Hampson L, Hampson I, Gilbert DC and Renehan AG: High-sensitivity human papilloma virus genotyping reveals near universal positivity in anal squamous cell carcinoma: Different implications for vaccine prevention and prognosis. Eur J Cancer 51: 776-785, 2015.
37. Yhim HY, Lee NR, Song EK, Kwak JY, Lee ST, Kim JH, Kim JS, Park HS, Chung IJ, Shim HJ, *et al*: The prognostic significance of tumor human papillomavirus status for patients with anal squamous cell carcinoma treated with combined chemoradiotherapy. Int J Cancer 129: 1752-1760, 2011.
38. Rödel F, Wieland U, Fraunholz I, Kitz J, Rave-Fränk M, Wolff HA, Weiss C, Wirtz R, Balermpas P, Fokas E and Rödel C: Human papillomavirus DNA load and p16INK4a expression predict for local control in patients with anal squamous cell carcinoma treated with chemoradiotherapy. Int J Cancer 136: 278-288, 2015.
39. 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.
40. Neal CP, Garcea G, Doucas H, Manson MM, Sutton CD, Dennison AR and Berry DP: Molecular prognostic markers in resectable colorectal liver metastases: A systematic review. Eur J Cancer 42: 1728-1743, 2006.
41. Sun Y, Wang Z, Qiu S and Wang R: Therapeutic strategies of different HPV status in head and neck squamous cell carcinoma. Int J Biol Sci 17: 1104-1118, 2021.
42. Ganly I, Soutar DS, Brown R and Kaye SB: p53 alterations in recurrent squamous cell cancer of the head and neck refractory to radiotherapy. Br J Cancer 82: 392-398, 2000.
43. Meulendijks D, Tomasoa NB, Dewit L, Smits PH, Bakker R, van Velthuysen ML, Rosenberg EH, Beijnen JH, Schellens JH and Cats A: HPV-negative squamous cell carcinoma of the anal canal is unresponsive to standard treatment and frequently carries disruptive mutations in TP53. Br J Cancer 112: 1358-1366, 2015.
44. Cacheux W, Rouleau E, Briaux A, Tsantoulis P, Mariani P, Richard-Molard M, Buecher B, Dangles-Marie V, Richon S, Lazartigues J, *et al*: Mutational analysis of anal cancers demonstrates frequent PIK3CA mutations associated with poor outcome after salvage abdominoperineal resection. Br J Cancer 114: 1387-1394, 2016.
45. Mireștean CC, Iancu RI and Iancu DPT: p53 modulates radiosensitivity in head and neck cancers-from classic to future horizons. Diagnostics (Basel) 12: 3052, 2022.
46. Liu C, Mann D, Sinha UK and Kokot NC: The molecular mechanisms of increased radiosensitivity of HPV-positive oropharyngeal squamous cell carcinoma (OPSCC): An extensive review. J Otolaryngol Head Neck Surg 47: 59, 2018.
47. Bruyere D, Monnien F, Colpart P, Roncarati P, Vuitton L, Hendrick E, Lepinoy A, Luquain A, Pilard C, Lerho T, *et al*: Treatment algorithm and prognostic factors for patients with stage I-III carcinoma of the anal canal: A 20-year multicenter study. Mod Pathol 34: 116-130, 2021.
48. Allal AS, Waelchli L and Bründler MA: Prognostic value of apoptosis-regulating protein expression in anal squamous cell carcinoma. Clin Cancer Res 9: 6489-6496, 2003.
49. Zhu X, Jamshed S, Zou J, Azar A, Meng X, Bathini V, Dresser K, Strock C, Yalamarti B, Yang M, *et al*: Molecular and immunophenotypic characterization of anal squamous cell carcinoma reveals distinct clinicopathologic groups associated with HPV and TP53 mutation status. Mod Pathol 34: 1017-1030, 2021.
50. Armstrong SA, Malley R, Wang H, Lenz HJ, Arguello D, El-Deiry WS, Xiu J, Gatalica Z, Hwang JJ, Philip PA, *et al*: Molecular characterization of squamous cell carcinoma of the anal canal. J Gastrointest Oncol 12: 2423-2437, 2021.
51. Iseas S, Golubicki M, Robbio J, Ruiz G, Guerra F, Mariani J, Salanova R, Cabanne A, Eleta M, Gonzalez JV, *et al*: A clinical and molecular portrait of non-metastatic anal squamous cell carcinoma. Transl Oncol 14: 101084, 2021.
52. Chan AM, Urgoiti GR, Jiang W, Lee S, Kornaga E, Mathen P, Yeung R, Enwere EK, Box A, Konno M, *et al*: The prognostic impact of PD-L1 and CD8 expression in anal cancer patients treated with chemoradiotherapy. Front Oncol 12: 1000263, 2022.
53. Chen C, Liu Y and Cui B: Effect of radiotherapy on T cell and PD-1/PD-L1 blocking therapy in tumor microenvironment. Hum Vaccin Immunother 17: 1555-1567, 2021.
54. Qin Y, Luan J, Zhou X and Li Y: PD-L1 expression in anogenital and oropharyngeal squamous cell carcinomas associated with different clinicopathological features, HPV status and prognosis: A meta-analysis. Biosci Rep 41: BSR20203669, 2021.
55. Stier EA, Chigurupati NL and Fung L: Prophylactic HPV vaccination and anal cancer. Hum Vaccin Immunother 12: 1348-1351, 2016.
56. Skolnik JM and Morrow MP: Vaccines for HPV-associated diseases. Mol Aspects Med 94: 101224, 2023.
57. Garolla A, Graziani A, Grande G, Ortolani C and Ferlin A: HPV-related diseases in male patients: An underestimated conundrum. J Endocrinol Invest 47: 261-274, 2024.
58. de Bakker T, Journe F, Descamps G, Saussez S, Dragan T, Ghanem G, Krayem M and Van Gestel D: Restoring p53 function in head and neck squamous cell carcinoma to ımprove treatments. Front Oncol 11: 799993, 2022.
59. Hamza A, Masliah-Planchon J, Neuzillet C, Lefèvre JH, Svrcek M, Vacher S, Bourneix C, Delaye M, Goéré D, Dartigues P, *et al*: Pathogenic alterations in PIK3CA and KMT2C are frequent and independent prognostic factors in anal squamous cell carcinoma treated with salvage abdominoperineal resection. Int J Cancer 154: 504-515, 2024.
60. Mao W, Li W and Hu X: Tumor hyperthermia research progress and application prospect in tumoroids (Review). Mol Clin Oncol 20: 31, 2024.
61. Ott OJ, Schmidt M, Semrau S, Strnad V, Matzel KE, Schneider I, Raptis D, Uter W, Grützmann R and Fietkau R: Chemoradiotherapy with and without deep regional hyperthermia for squamous cell carcinoma of the anus. Strahlenther Onkol 195: 607-614, 2019.
62. Telarovic I, Wenger RH and Pruschy M: Interfering with tumor hypoxia for radiotherapy optimization. J Exp Clin Cancer Res 40: 197, 2021.
63. Ugwueze CV, Ogamba OJ, Young EE, Onyenekwe BM and Ezeokpo BC: Metformin: A possible option in cancer chemotherapy. Anal Cell Pathol (Amst) 27: 7180923, 2020.
64. Liu J, Wu J, Chen T, Yang B, Liu X, Xi J, Zhang Z, Gao Y and Li Z: Enhancing X-Ray sensitization with multifunctional nanoparticles. Small 26: e2400954, 2024.