

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) $\geq 1\%$ was found in 73.8% of the patients, and exhibited significant associations with HPV positivity and p16 positivity. PD-L1 CPS $\geq 1\%$ 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 suggest 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 virus (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/m²) administered intravenously on days 1 and 29, together with 5-fluorouracil (5-FU; 1,000 mg/m²) 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 μ m. From these, two FFPE sections were cut, each labeled with biopsy number, block name, and date, and barcode. FFPE sections were processed with 300 μ l 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 μ l Buffer FTB, 55 μ l RNase-free water, and 20 μ l 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 μ m. 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: $TPS (\%) = [(PD-L1\text{-positive tumor cells} / \text{total viable cells}) \times 100]$. 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: $CPS (\%) = [(PD-L1\text{-positive tumor cells} + \text{lymphocytes} + \text{macrophages} / \text{total viable cells}) \times 100]$ (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 radiotherapy 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 $\geq 1\%$, as well as CPS $<1\%$ and $\geq 1\%$, revealed that 19 cases (45.2%) had a TPS $<1\%$ and 23 cases (54.8%) had a TPS $\geq 1\%$. In addition, 11 cases (26.2%) had a CPS $<1\%$ and 31 cases (73.8%) had a CPS $\geq 1\%$. The distribution of TPS and CPS cases exhibited a statistically significant difference ($P<0.001$).

A significant association of female sex with TPS $\geq 1\%$ -positive ($P=0.038$) and CPS $\geq 1\%$ -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 $\geq 1\%$. 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

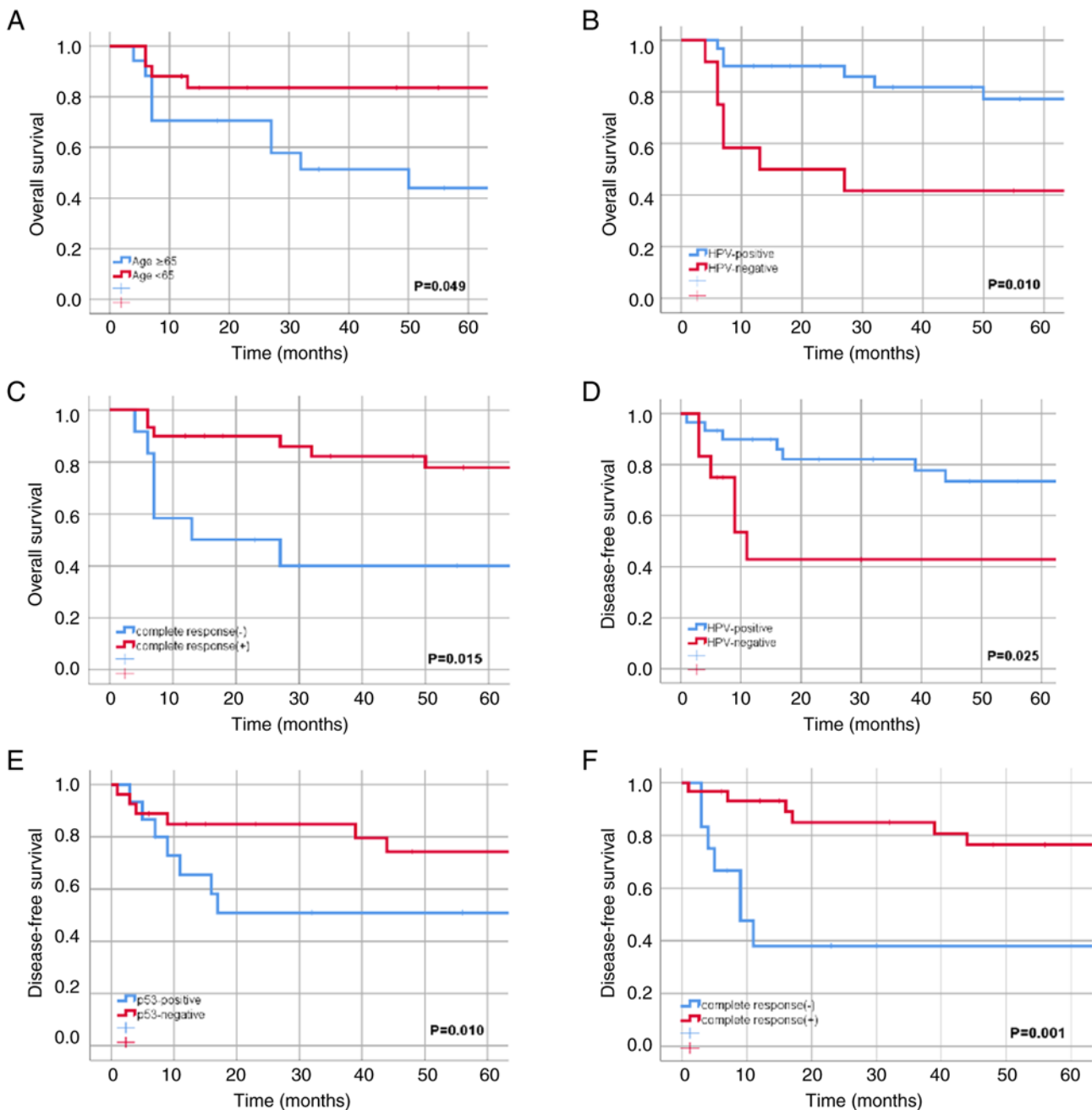


Figure 1. Kaplan-Meier curves of overall survival and disease-free survival in patients with anal squamous cell carcinoma. Kaplan-Meier curves of overall survival according to (A) age < 65 vs. ≥ 65 years, (B) HPV status and (C) complete response status, and disease-free survival according to (D) HPV 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-associated 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 prevalence 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, $\geq 1\%$ 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 $\geq 5\%$ 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.

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