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RESEARCH

Protease-Activated Receptor 1 (PAR1) Expression Contributes to HPV-Associated Oropharyngeal Cancer Prognosis

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Abstract

Background Human papillomavirus (HPV)-associated oropharyngeal cancer occasionally has a poor prognosis, making prognostic risk stratification crucial. Protease-activated receptor-1 (PAR1) is involved in carcinogenesis, and its expression is regulated by alpha-arrestin domain-containing protein 3 (ARRDC3). It is also involved in the tumor microenvironment. We sought to evaluate the predictive ability of PAR1, ARRDC3, and tumor-infiltrating lymphocyte (TIL) scores in patients with oropharyngeal, hypopharyngeal, and uterine cervical cancers, serving as comparators for HPV-associated oropharyngeal cancer.

Methods Immunohistochemical analysis of p16, ARRDC3, and PAR1 expression was performed on 79 oropharyngeal, 44 hypopharyngeal, and 42 uterine cervical cancer samples. The TIL scores were assessed and classified into the following groups based on invasion: low: 0–10%, medium: 20–40%, and high: > 50%. For prognostic analysis, the three groups were evaluated by dividing them into low, medium, and high categories, or alternatively into two groups using the median value as the cutoff.

Results p16 was expressed in 44 (56%) oropharyngeal, 8 (18%) hypopharyngeal, and all uterine cervical cancer samples. ARRDC3 was detected in 39 (49%) oropharyngeal, 25 (57%) hypopharyngeal, and 23 (55%) uterine cervical cancer samples. PAR1 was expressed in 45 (57%) oropharyngeal, 22 (50%) hypopharyngeal, and 22 (50%) uterine cervical cancer samples. Patients diagnosed with p16-positive oropharyngeal cancer had a substantially improved prognosis compared to those diagnosed with p16-negative cancer. The PAR1-negative cases had a considerably improved prognosis compared to the positive cases (disease-specific survival [DSS] and -negative cases (disease-free survival [DFS]). Multivariate analysis revealed that ARRDC3-positive cases had an appreciably better DSS prognosis than patients with p16-negative oropharyngeal cancers. PAR1-positive patients among patients with p16-positive oropharyngeal cancer had a poor prognosis. With respect to DFS, patients with PAR1-positive and p16-negative oropharyngeal cancer had a 35-fold higher recurrence rate than those with PAR1-negative and p16-negative oropharyngeal cancer.

Conclusion Our results suggest that PAR1 expression affects the prognosis and recurrence rate of HPV-associated oropharyngeal cancer.

Keywords Arrestin domain-containing 3 · Oropharyngeal cancer · Protease-activated receptor 1 · Tumor-infiltrating lymphocyte

Introduction

The number of cases of oropharyngeal cancers caused by human papillomavirus (HPV) infection has increased in recent years [1]. In the 2000s, HPV was detected in approximately 50% of oropharyngeal cancers, with an increasing trend observed, particularly among younger age groups [2]. In Japan, a multicenter collaborative study on HPV infection and oropharyngeal cancer [3] reported an infection rate

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of approximately 50%, with HPV16 accounting for 90% of the HPV types. p16 expression, assessed through immunohistochemical staining, has served a proxy marker for HPV infection [4]. HPV-associated oropharyngeal cancers exhibit greater sensitivity to radiation therapy and chemotherapy than non-HPV-associated cancers, consequently leading to a better prognosis [5–7]. As a result, p16-positive and -negative oropharyngeal cancers are considered independent diseases based on the tumor, node, metastasis (TNM) classification (International Union Against Cancer [UICC]/American Joint Committee on Cancer [AJCC], 8th edition).

Although HPV-associated oropharyngeal cancer has a good prognosis, clinical practice reveals that cases with advanced stages or poor prognoses arising from recurrence or metastasis are frequently encountered [8]. Therefore, it is crucial to determine the prognostic risk stratification of HPV-associated oropharyngeal cancer in the histopathological examination stage, including biopsy, to aid in treatment selection and prognosis estimation. This objective can be accomplished through the utilization of appropriate histopathological biomarkers.

G protein-coupled receptors (GPCRs) are a class of cell surface receptors that receive external stimuli and regulate intracellular signaling. GPCRs are involved in physiological processes, particularly neurotransmission and immune responses [9]. A GPCR, protease-activated receptor-1 (PAR1), is highly expressed on platelets and endothelial cells and is pivotal in mediating between coagulation and inflammation and in inflammatory and fibrotic lung disease development [10]. In malignant tumors, PAR1 is crucial in carcinogenesis, angiogenesis, and metastasis [11]. In triple negative breast carcinoma, α-arrestin domain-containing protein 3 (ARRDC3) is involved in PAR1 degradation and regulation through apoptosis linked gene-2 (ALG-2)-interacting protein X (ALIX) [12]. Interestingly, genome-wide association studies reveal that uterine cervical cancer, similar to HPV-associated oropharyngeal cancer, is caused by HPV infection. Additionally, ARRDC3 is implicated in cell proliferation and susceptibility to HPV infection [13].

PAR1 is also involved in the tumor microenvironment (TME). Its expression in pancreatic adenocarcinoma contributes to the creation of a cellular microenvironment within the tumor, marked by the reduced number of cytotoxic T cells infiltrating the cancerous cells, as well as increased numbers of primitive immunosuppressor cell types [14]. The tumor-infiltrating lymphocyte (TIL) is an important TME component and reflects the antitumor immune response of the host [15, 16]. In patients with breast cancer, TIL levels can be used to predict response to preoperative adjuvant and adjuvant chemotherapy [17, 18]. Additionally, PD-L1, CD8 + TIL, and HIF-1α levels are useful biomarkers for head and neck squamous cell carcinoma (HNSCC) [19]. Therefore, evaluation of TILs is becoming progressively vital in routine pathology, primarily due to the strong correlation between TIL levels and quantitative results of immune gene expression [20]. Thus, TIL assessments may serve as a valid, inexpensive, and easily obtainable substitute for analyzing immune gene expression [21]. Recently, the International Immuno-Oncology Biomarker Working Group on Breast Cancer was established and international guidelines for the evaluation of TILs were developed [22]. International guidelines for the evaluation of TILs have also been established for solid tumors, including metastatic disease, and they can be applied to head and neck cancer [23].

In this retrospective cohort study, we evaluated the expression of p16, PAR1, and ARRDC3 in patients with oropharyngeal and hypopharyngeal cancers and assessed TIL levels using the scoring method recommended by the International Immuno-Oncology Biomarker Working Group on Breast Cancer. In addition, patients with uterine cervical cancer were compared to those with oropharyngeal cancer caused by the same HPV infection. In addition, breast cancer cell lines were evaluated to validate the reliability of this study and establish the consistency of reports in breast cancer. This step was necessary as ongoing studies on PAR1 and ARRDC3 continue to evolve.

Materials and Methods

Patients

This study involved patients with oropharyngeal, hypopharyngeal, and uterine cervical cancers at Kawasaki Medical University Hospital and Kawasaki Medical University General Medical Center who consented to have their tumor tissues (stored specimens) used in the study. Between 2006 and 2021, 79 patients with oropharyngeal, 44 with hypopharyngeal, and 42 with cervical cancers who underwent surgical treatment or chemoradiation after tissue diagnosis were enrolled. Surgical cases were selected only if a 1-cm safety zone was maintained at the time of resection and if the resection margins were negative. The exclusion criteria included second primary cancer, distant metastasis, and prior irradiation. Patient data were retrospectively collected from electronic medical records. p16 status caused differences in staging; therefore, the histologic type and grade were determined based on the TNM classification (7th edition) defined by the AJCC and UICC [24].

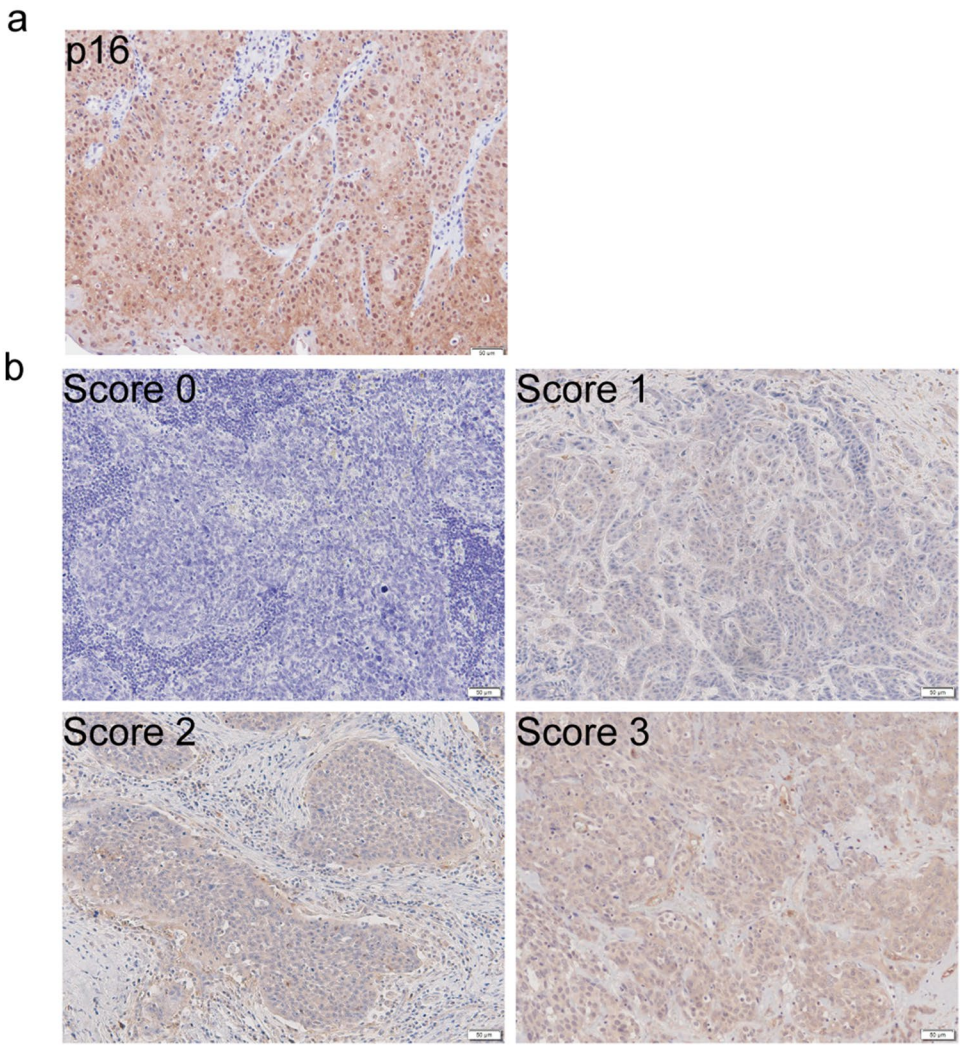


Fig. 1 Immunohistochemical staining of p16. Specimens with a nuclear expression intensity of ≥ +2/+3 and a positive distribution of ≥ 75% were p16-positive based on the American Joint Committee on Cancer (AJCC) (**a**). H-scores for alpha-arrestin domain-containing protein 3 (ARRDC3) and protease-activated receptor-1 (PAR1) were evaluated using four scores based on staining intensity (**b**)

Tissue Preparation and Staining

For pathologic diagnosis, paraffin-embedded sections were prepared and hematoxylin and eosin (H&E) staining and immunohistochemistry were performed.

Immunohistochemistry

After deparaffinization, the tissue sections were incubated with either Target Retrieval Solution (pH 9.0) (Dako, Glostrup, Denmark) or citrated (pH 6.0) buffer at 95 °C for 40 min for antigen activation in immunohistochemical staining. Antigen activation was performed via heat-induced epitope recovery (HIER). To inactivate endogenous peroxidase activity, the sections were exposed to 3% hydrogen peroxide for 5 min at 15–25 °C. The sections were washed with Tris-buffered saline (TBS). They were subsequently exposed to the primary antibodies, which included the following: p16 (EPR1473, ab108349; Abcam, Cambridge, UK; dilution 1:500, for 1 h at 15–25 °C); ARRDC3 (polyclonal, ab64817; Abcam; dilution 1:100, for 30 min at 15–25 °C); and PAR1 (polyclonal, ab32611; Abcam, dilution 1:80; at 4 °C overnight). The sections were rinsed with TBS and incubated with EnVision and the secondary antibody (Dako, Carpinteria, CA, USA; Cat. No. K4061) for 30 min at 15–25 °C. Finally, immunoreactivity was visualized by immersion in 3,3′ diaminobenzidine (DAB) for 12 min and counterstained with hematoxylin. As positive controls, p16 represented cervical cancer, and ARRDC3 and PAR1 represented the proximal tubules of normal kidney samples.

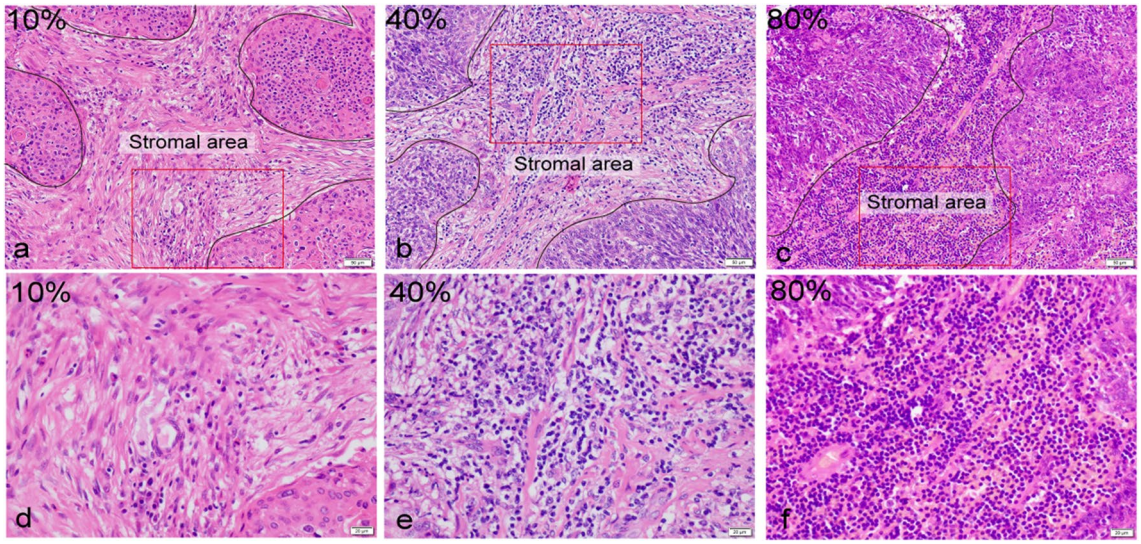


Fig. 2 Evaluating tumor-infiltrating lymphocytes. The density of tumor-infiltrating lymphocytes (TILs) was determined using hematoxylin and eosin (H&E)-stained sections. Tumor stromal areas were selected at a low magnification. The percentages of stromal areas and mononuclear cells were evaluated at magnifications ranging from ×200 to ×400. 10% (**a**), 40% (**b**), and 80% (**c**) at ×200 magnification. 10% (**d**), 40% (**e**), and 80% (**f**) at ×400 magnification. **d–f** show the images in the area enclosed by each square

Negative controls were treated similarly, without the primary antibodies.

Patients with a nuclear expression intensity of ≥ + 2/+ 3 and a positive distribution of ≥ 75% were p16-positive, based on the AJCC classification. The staining intensities of ARRDC3 and PAR1 were graded into four levels (0, negative; 1, weakly positive [staining intensity lower than the positive control]; 2, moderately positive [staining intensity equal to the positive control]; 3, strongly positive [staining intensity higher than the positive control]). The staining intensity of the entire observed field of view was semi-quantified according to the following expression: H-score (0 to 300 = 0 × of the negative cells + 1 × of the weak positive cells + 2 × of the intermediate positive cells + 3 × of the strong positive cells). One section per patient was evaluated. The entire tissue section was examined in 200 × field of view, and the average H-score of three fields of view was used as the H-score for that case (Fig. 1).

Tumor-Infiltrating Lymphocytes

TIL scoring was performed according to the Immuno-Oncology International TILs Working Group's definitions [23]. Tumor stromal areas were selected at a low magnification. The percentages of stromal areas and mononuclear cells were evaluated at magnifications ranging from ×200 to ×400. Granulocytes and polynuclear cells were excluded and evaluated in accordance with the guidelines of the TILs Working Group using light microscopy (BX53; Olympus, Tokyo, Japan). Tumors were classified into three groups based on TIL infiltration level: low: 0–10%, medium: 20–40%, and high: > 50%. Regarding prognostic analysis, the three groups were evaluated by dividing them into low, medium, and high categories, or alternatively into two groups using the median value as the cutoff (Fig. 2).

Breast Cancer Cell Lines

MCF-7 and MDA-MB-231 human breast cancer cell lines were provided by Dr. Robert Dickson (Lombardi Cancer Research Center, West Virginia, Washington DC, USA). The breast cancer cell line KPL-4 was established at the Department of Breast and Thyroid Surgery, Kawasaki Medical University, Kurashiki, Okayama, Japan [25]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 0.02% kanamycin (Meiji, Tokyo, Japan) for 3 or 4 days under normal oxygen (5% CO_2) conditions at 37 °C.

Table 1 Baseline characteristics of patients with oropharyngeal and hypopharyngeal cancers

	Oropharyngeal cancer				Hypopharyngeal cancer			
	Total ($n=79$)	p16 negative ($n=35$)	p16 positive ($n=44$)	p-value	Total ($n=44$)	p16 negative ($n=36$)	p16 positive ($n=8$)	p-value
Sex								
Male	67 (85%)	30 (86%)	37 (84%)	N/E	41 (93%)	33 (92%)	8 (100%)	N/E
Female	12 (15%)	5 (14%)	7 (16%)		3 (7%)	3 (8%)	0 (0%)	
Age (years)	65 (57–74)	69 (56–75)	63 (56–72)	0.208	68 (64–71)	70 (66–71)	65 (60–68)	0.022*
Diagnosis								
Biopsy	42 (53%)	15 (43%)	27 (61%)	0.117	31 (70%)	24 (67%)	7 (88%)	0.402
Resection	37 (47%)	20 (57%)	17 (39%)		13 (30%)	12 (33%)	1 (13%)	
Treatment								
OPE	36 (46%)	18 (51%)	18 (41%)	0.634	13 (30%)	12 (33%)	1 (13%)	0.267
CRT	31 (39%)	11 (31%)	20 (46%)		24 (55%)	17 (47%)	7 (88%)	
RT	10 (13%)	5 (14%)	5 (11%)		5 (11%)	5 (14%)	0 (0%)	
CT	2 (2%)	1 (3%)	1 (2%)		2 (5%)	2 (6%)	0 (0%)	
7th edition TNM stage								
I	11 (14%)	9 (26%)	2 (5%)	0.0348*	6 (14%)	6 (17%)	0 (0%)	0.136
II	8 (10%)	4 (11%)	4 (9%)		10 (23%)	9 (25%)	1 (13%)	
III	14 (18%)	5 (14%)	9 (21%)		4 (9%)	3 (8%)	1 (13%)	
Any IV	46 (58%)	17 (49%)	29 (66%)		24 (55%)	18 (50%)	6 (75%)	
Alcohol consumption								
Non-drinker	35 (44%)	14 (40%)	21 (48%)	0.748	14 (32%)	11 (31%)	3 (37%)	0.779
Active drinker	44 (56%)	21 (60%)	23 (52%)		30 (68%)	25 (69%)	5 (63%)	
Smoking								
Non/ex-smoker	11 (14%)	3 (9%)	8 (8%)	0.423	18 (41%)	15 (42%)	3 (37%)	N/E
Smoker	68 (86%)	32 (91%)	36 (82%)		26 (59%)	21 (58%)	5 (63%)	
Site								
Upper wall	8 (10%)	5 (14%)	3 (7%)	0.398	32 (72%)	25 (69%)	7 (88%)	0.466
Side wall	48 (61%)	19 (54%)	29 (66%)		5 (12%)	4 (11%)	1 (12%)	
Anterior wall	16 (20%)	9 (26%)	7 (16%)		7 (16%)	7 (20%)	0 (0%)	
Posterior wall	7 (9%)	2 (6%)	5 (11%)					
Histological classification								
WD	24 (30%)	15 (43%)	9 (21%)	0.081	8 (18%)	6 (17%)	2 (25%)	0.606
MD	32 (41%)	13 (37%)	19 (43%)		29 (66%)	25 (69%)	4 (50%)	
PD	23 (29%)	7 (20%)	16 (36%)		7 (16%)	5 (14%)	2 (25%)	
Double cancer								
Positive	17 (22%)	13 (37%)	4 (9%)	0.005*	18 (41%)	17 (47%)	1 (13%)	0.115
Negative	62 (78%)	22 (63%)	40 (91%)		26 (59%)	19 (53%)	7 (88%)	
Lymphatic invasion								
Positive	8 (11%)	4 (11%)	4 (9%)	N/E	3 (7%)	3 (8%)	0 (0%)	N/E
Negative	71 (89%)	31 (89%)	40 (91%)		41 (93%)	33 (92%)	8 (100%)	
Venous invasion								
Positive	11 (14%)	6 (17%)	5 (11%)	0.524	6 (14%)	6 (17%)	0 (0%)	0.573
Negative	68 (86%)	29 (83%)	39 (89%)		38 (86%)	30 (83%)	8 (100%)	
TIL score								
Low	37 (47%)	21 (60%)	16 (36%)	0.0135*	29 (66%)	25 (69%)	4 (50%)	0.325
Medium	14 (18%)	7 (20%)	7 (16%)		11 (25%)	8 (22%)	3 (38%)	
High	28 (35%)	7 (20%)	21 (48%)		4 (9%)	3 (8%)	1 (13%)	
< Median	40 (51%)	24 (69%)	16 (36%)	0.006*	25 (57%)	21 (58%)	4 (50%)	0.71
≥ Median	39 (49%)	11 (31%)	28 (64%)		19 (43%)	15 (42%)	4 (50%)	

Table 1 (continued)

	Oropharyngeal cancer				Hypopharyngeal cancer			
	Total ($n=79$)	p16 negative ($n=35$)	p16 positive ($n=44$)	p-value	Total ($n=44$)	p16 negative ($n=36$)	p16 positive ($n=8$)	p-value
Marker expression								
ARRDC3 positive	39 (49%)	14 (40%)	25 (57%)	0.176	25 (57%)	22 (61%)	3 (38%)	0.262
ARRDC3 negative	40 (51%)	21 (60%)	19 (43%)		19 (43%)	14 (39%)	5 (63%)	
PAR1 positive	45 (57%)	20 (57%)	25 (57%)	N/E	22 (50%)	18 (50%)	4 (50%)	N/E
PAR1 negative	34 (43%)	15 (43%)	19 (43%)		22 (50%)	18 (50%)	4 (50%)	

Each marker was grouped into positive and negative expression groups based on median values

Prognostic analysis was performed for three and two groups based on median values

The patients were classified into the following groups based on the TIL score: low: 0–10%; medium: 20–40%; high: > 50%

N/E not evaluable, *OPE* operation, *CRT* chemoradiotherapy, *RT* radiotherapy, *CT* chemotherapy, *PS* pyriform sinus, *PW* posterior wall, *PC* postcricoid, *WD* well differentiated, *MD* moderately differentiated, *PD* poorly differentiated

*$p < 0.05$

Cell Blocks

MCF-7, MDA-MB-231, and KPL-4 cells were cultured under normal oxygen conditions. The cells were then washed with phosphate-buffered saline (PBS), and 0.25% trypsin and 0.2% EDTA were added per 100 mm^2 of the culture dish to detach the breast cancer cells. After detachment, the cells were incubated in a CO_2 incubator at 37 °C for 7 min, and 7.5 mL of DMEM containing 10% FBS was added to the culture dish to inactivate trypsin. The detached breast cancer cells were transferred to 15-mL tubes and centrifuged at 1200 rpm for 5 min to form a pellet. The supernatant was removed, washed with PBS, and centrifuged at 1200 rpm for 5 min. After removing the supernatant, 500 µL of 10% neutral buffered formalin was added to the cell pellet, mixed well, and allowed to stand for 24 h for fixation. Formalin-fixed cell masses were embedded in paraffin, and sections of MCF-7, MDA-MB-231, and KPL-4 breast cancer cell lines were prepared. Subsequently, hematoxylin and eosin staining and PAR1 and immunostaining of ARRDC3 were performed.

Western Blotting

For protein extraction, the cells were lysed in Pierce RIPA buffer (Thermo Fisher Scientific). Direct Detect[tm] Spectrometer (Merck, Darmstadt, Germany) was used to determine total protein concentration. All proteins were separated using 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Thermo Fisher Scientific). The membranes were blocked for 1 h at 15–25 °C and incubated with primary antibodies for 8–16 h at 4 °C in blocking buffer consisting of TBS plus 5% BSA and 0.2% Tween 20. Different dilutions of the same antibodies (ARRDC3 1:2000 and PAR1 1:1000) were used for immunohistochemistry. Subsequently, the cells were incubated with a secondary antibody, goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA; lot no. L2308; dilution 1:10,000), at 15–25 °C for 1 h. The membranes were then incubated for 1 h at 15–25 °C. An Amersham Imager 680 (Thermo Fisher Scientific) was used to visualize the protein bands transferred on to the membranes. As a loading control, β-actin (Sigma-Aldrich, dilution 1:1000) was used. The band signal intensities of PAR1 and ARRDC3 and the loading control β-actin were quantified, the amounts of target protein relative to the loading control protein were calculated, and histograms were generated.

Statistical Analysis

After dividing the patients into two groups based on p16 expression, the ARRDC3, PAR1, and TIL scores were combined with clinical data and pathological parameters for statistical analysis. ARRDC3 and PAR1 expression levels were evaluated in two groups: high (> median) and low (≤ median), using the median value as the cutoff. TILs were divided into three groups (low: 0–10%, medium: 20–40%,

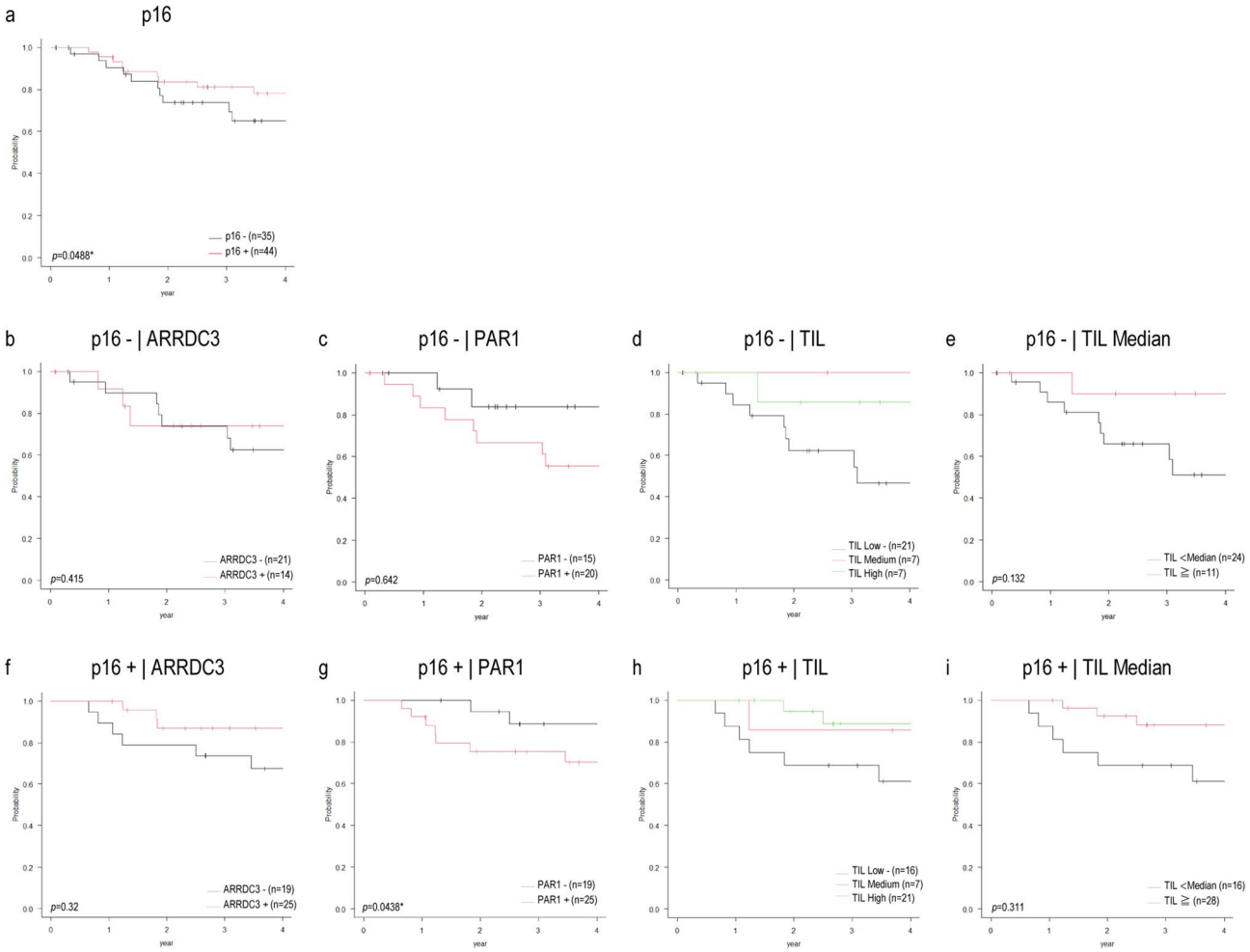


Fig. 3 Disease-specific survival (DSS) in oropharyngeal cancer. The 3-year DSS was 78%. p16-positive cases had a significantly better prognosis than the negative cases ($p=0.0488$) (**a**). The p16-negative oropharyngeal cancer subgroup ($n=35$, 44%) did not significantly differ in alpha-arrestin domain-containing protein 3 (ARRDC3), protease-activated receptor-1 (PAR1), or tumor-infiltrating lymphocyte (TIL) status (**b**–**e**). The p16-positive oropharyngeal cancer subgroup ($n=44$, 56%) had no significant differences in ARRDC3 expression and TIL status (**f**, **h**, and **i**). ($p=0.32$) (**f**). ($p=0.311$) (**i**). However, PAR1-negative cases had significantly better prognosis than the positive cases ($p=0.0438$) (**g**)

high: > 50%) and two groups: high (> median) and low (≤ median), using the median value as the cutoff.

The categorical variables are reported as number and percentage; they were evaluated using the chi-square or exact Fisher test. The median and 25th and 75th percentiles of continuous variables were calculated using the *t*-test if they showed a normal pattern of distribution. Otherwise, Mann–Whitney *U* test was used. The primary endpoint was disease-specific survival (DSS) and the secondary endpoint was disease-free survival (DFS). DSS and DFS were evaluated using Kaplan–Meier curves and log-rank tests, respectively. Cox regression analysis was used for univariate and multivariate analyses. Oropharyngeal and hypopharyngeal cancers were adjusted for TNM stage (stages I–II vs. III–IV), smoking status, alcohol consumption, venous invasion, lymphatic invasion, duplicate cancer diagnoses, site of origin, and histological differentiation. Cervical cancer was adjusted for TNM stage (stage I–II vs. III–IV), venous invasion, and lymphovascular invasion. The p16-positive and -negative cases were separately analyzed for differences in

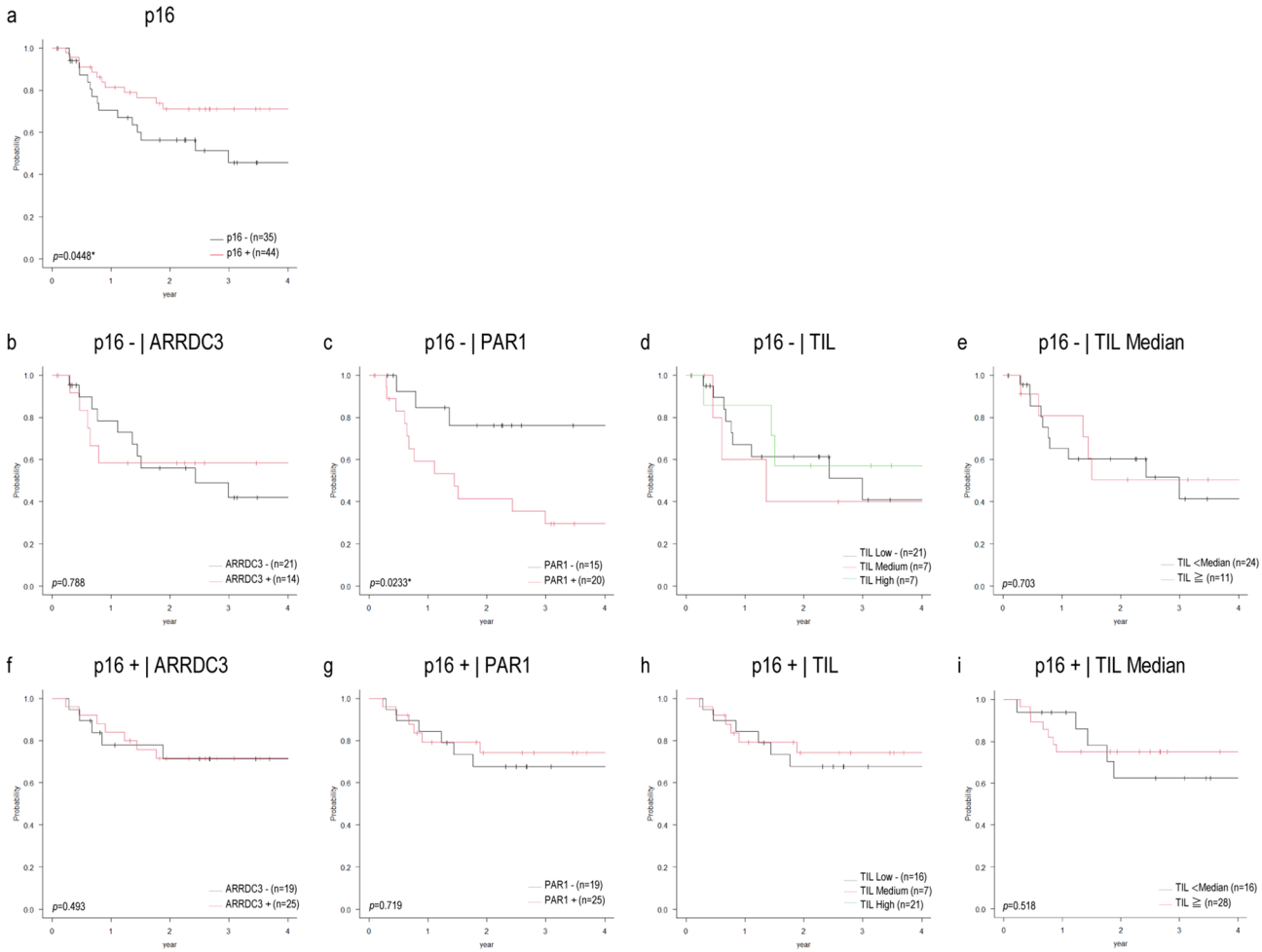


Fig. 4 Disease-free survival (DFS) in oropharyngeal cancer. The 3-year DFS was 61%. p16-positive cases had a significantly better prognosis than the negative cases ($p=0.0448$) (**a**). In the p16-negative oropharyngeal cancer subgroup ($n=35$, 44%), no significant difference existed in alpha-arrestin domain-containing protein 3 (ARRDC3) expression and tumor-infiltrating lymphocyte (TIL) levels (**b**, **d**, **e**). However, protease-activated receptor-1 (PAR1)-negative cases had significantly better prognosis than the positive cases ($p=0.0233$) (**c**). In the p16+ oropharyngeal cancer subgroup ($n=44$, 56%), ARRDC3, PAR1, and TIL levels did not significantly differ (**f**–**i**)

association with the outcome. All tests were two-tailed, and statistical significance was set at $p<0.05$.

All data were analyzed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interfaces for R (R Foundation for Statistical Computing, Vienna, Austria) [26]. To be more precise, it is a customized version of the R program commander that is designed to add statistical functions that are often used in biostatistics.

Results

Patient and Clinicopathologic Relevance

The characteristics of the patients at the time of registration for oropharyngeal, hypopharyngeal, and uterine cervical cancers are presented in Tables 1 and S1. In total, 165 patients (79 with oropharyngeal, 44 with hypopharyngeal, and 42 with uterine cervical cancer) were included. The median age of the patients at diagnosis was 65 (57–74) years

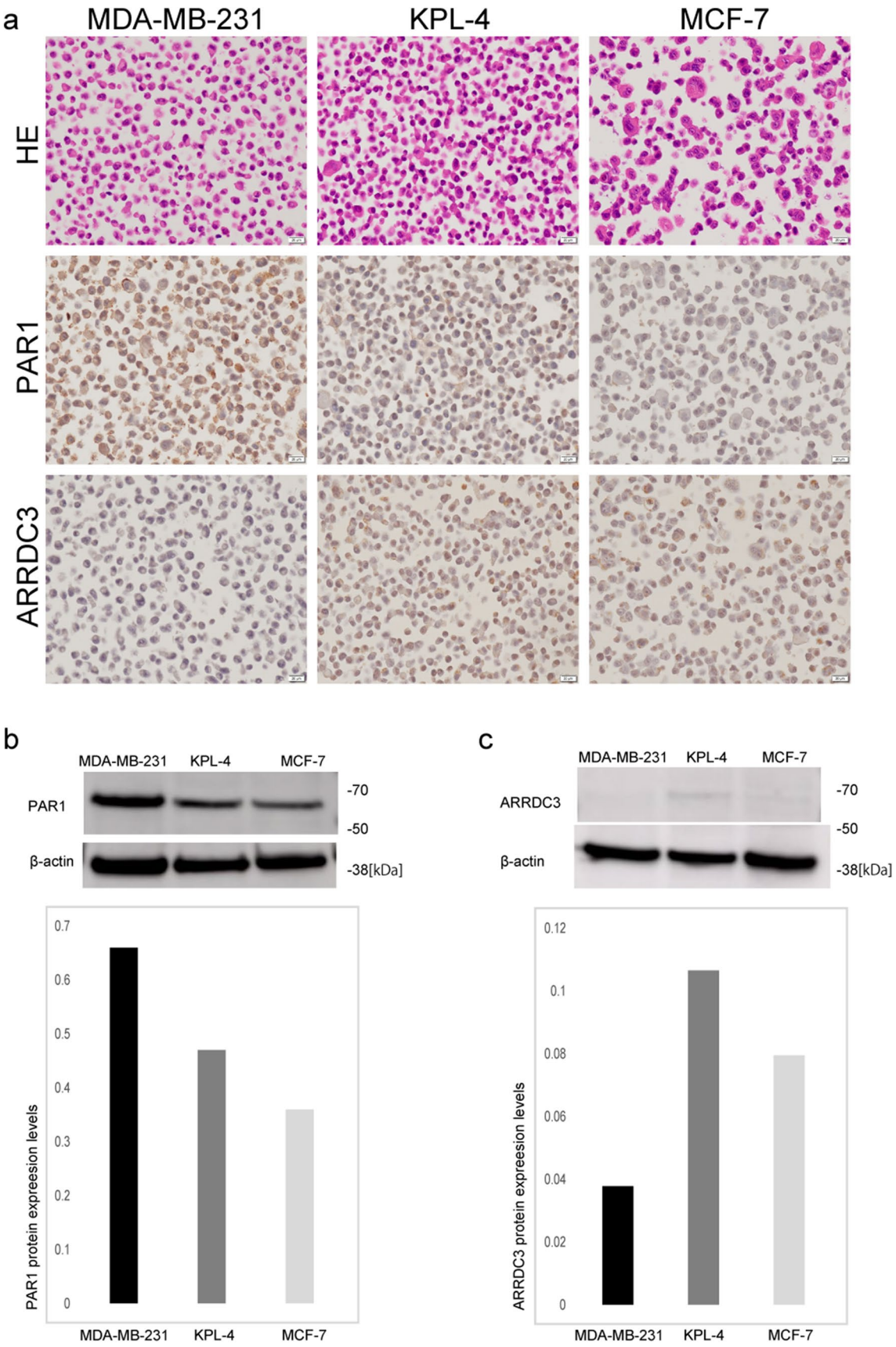
Table 2 Cox regression analysis was used for univariate and multivariate analyses

	Univariate			Multivariate		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Oropharyngeal cancer						
Disease-specific survival						
p16-negative oropharyngeal cancer						
ARRDC3 (positive vs. negative)	0.5889	0.1627–2.132	0.4198	0.2309	0.05478–0.9732	0.04583*
PAR1 (positive vs. negative)	1.326	0.4021–4.372	0.6431	4.926	0.65150–37.2500	0.1224
TIL (high vs. low)	0.3851	0.1064–1.393	0.1458	0.7193	0.16080–3.218	0.6665
p16-positive oropharyngeal cancer						
ARRDC3 (positive vs. negative)	0.578	0.1936–1.726	0.3259	0.5153	0.133900–1.9840	0.335
PAR1 (positive vs. negative)	4.228	0.9248–19.33	0.06301	11.67	1.251000–109.0000	0.03107*
TIL (high vs. low)	0.5722	0.1917–1.708	0.317	0.4985	0.111700–2.2250	0.3617
Disease-free survival						
p16-negative oropharyngeal cancer						
ARRDC3 (positive vs. negative)	1.149	0.4158–3.177	0.7884	1.234	0.188500–8.0780	0.8264
PAR1 (positive vs. negative)	3.882	1.1–13.7	0.03503*	35.68	3.62000–351.7000	0.0022*
TIL (high vs. low)	0.8143	0.2822–2.35	0.704	0.8491	0.1838000–3.9220	0.8341
p16-positive oropharyngeal cancer						
ARRDC3 (positive vs. negative)	0.7003	0.2518–1.948	0.4949	1.137	0.29700–4.351	0.8514
PAR1 (positive vs. negative)	0.8287	0.2973–2.31	0.7195	0.8602	0.29470–2.511	0.783
TIL (high vs. low)	0.7092	0.2488–2.022	0.5203	0.8236	0.22570–3.005	0.7688
Hypopharyngeal cancer						
Disease-specific survival						
p16-negative hypopharyngeal cancer						
ARRDC3 (positive vs. negative)	0.581	0.1627–2.132	0.3913	0.2123	0.04402–1.023	0.05346
PAR1 (positive vs. negative)	1.025	0.2965–3.544	0.9689	1.093	0.201800–5.9170	0.9181
TIL (high vs. low)	1.646	0.4736–5.722	0.4329	0.6117	0.088610–4.2230	0.6181
Disease-free survival						
p16-negative hypopharyngeal cancer						
ARRDC3 (positive vs. negative)	1.309	0.4019–4.26	0.6552	0.844	0.18910–3.767	0.8241
PAR1 (positive vs. negative)	1.421	0.4751–4.25	0.5296	0.9681	0.21480–4.363	0.9664
TIL (high vs. low)	1.29	0.431–3.863	0.6487	0.738	0.16600–3.281	0.6898

Univariate and multivariate time-to-event analyses for Disease-specific survival (DSS) and Disease-free survival (DFS)

Multivariate models were adjusted for TNM stage, smoking status, alcohol consumption, venous invasion, lymphatic invasion, double cancers, site of origin, and histologic differentiation

*$p < 0.05$


a
MDA-MB-231
KPL-4
MCF-7
HE
PAR1
ARRDC3
b
MDA-MB-231
KPL-4
MCF-7
PAR1
β-actin
-70
-50
-38[kDa]
PAR1 protein expreesion levels
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MDA-MB-231
KPL-4
MCF-7
c
MDA-MB-231
KPL-4
MCF-7
ARRDC3
β-actin
-70
-50
-38[kDa]
ARRDC3 protein expreesion levels
0.12
0.1
0.08
0.06
0.04
0.02
0
MDA-MB-231
KPL-4
MCF-7

◂**Fig. 5** Comparison of protease-activated receptor-1 (PAR1) and alpha-arrestin domain-containing protein 3 (ARRDC3) expression in human breast cancer tissues. From left to right, hematoxylin and eosin (H&E) staining, PAR1 expression, and ARRDC3 expression in breast cancer cell lines (MDA-MB-231, KPL-4, and MCF-7) were evaluated using cell block preparations (**a**). Western blotting (**b** and **c**). MDA-MB-231, an estrogen receptor (ER)-negative, human epidermal growth factor receptor 2 (HER2)-negative cell line with a poor prognosis, had the highest PAR1 staining intensity among the three lines and the lowest ARRDC3 staining intensity among the three cell lines. In ER-negative and HER2-positive KPL-4 cells, PAR1 staining intensity was weaker than that in MDA-MB-231 cells, and ARRDC3 had the strongest intensity among the three. MCF-7, an ER-positive, HER2-negative breast cancer cell line with a relatively good prognosis, had the lowest PAR1 staining intensity among the three strains and the higher ARRDC3 expression intensity compared to MDA-MB-231 (**a**). Western blotting showed that the PAR1 was most strongly expressed in MDA-MB-231 (**b**). ARRDC3 was weakly expressed and KPL-4 was most strongly expressed (**c**)

for oropharyngeal, 68 (64–71) years for hypopharyngeal, and 42 (35–57) years for uterine cervical cancer. Twelve patients (15%) with oropharyngeal cancer and three (7%) with hypopharyngeal cancer were women.

Pathologic parameters were evaluated, and oropharyngeal cancer was diagnosed in 42 (53%) biopsies and 37 (47%) resections, and hypopharyngeal cancer was diagnosed in 31 (70%) and 13 (30%) biopsies. Uterine cervical cancer was diagnosed in all patients using excisional specimens. Oropharyngeal cancer was treated with operation (OPE) in 36 cases (46%), chemoradiotherapy (CRT) in 31 cases (39%), radiation therapy (RT) in 10 cases (13%), and chemotherapy (CT) in 2 cases (2%). Hypopharyngeal cancer patients were treated with OPE in 13 cases (30%), CRT in 24 cases (55%), RT in 5 cases (11%), and CT in 2 cases (5%). According to the seventh edition of the AJCC staging system, 11 (14%) patients had stage I, 8 (10%) had stage II, 14 (18%) had stage III, and 46 (58%) had stage IV oropharyngeal cancer. In addition, 6 (14%) had stage I, 10 (23%) had stage II, 4 (9%) had stage III, and 24 (55%) had stage IV hypopharyngeal cancer. Of the 79 patients with oropharyngeal cancer, 68 (86%) were smokers and 44 (56%) had an alcohol consumption history. Of the 44 patients with hypopharyngeal cancer, 26 (59%) were smokers and 30 (68%) had an alcohol consumption history. Duplicate cancers occurred in 17 patients (22%) with oropharyngeal cancer and 18 (41%) with hypopharyngeal cancer.p16 was expressed in 44 (56%) patients with oropharyngeal cancer, 8 (18%) with hypopharyngeal cancer, and in all patients with cervical cancer. ARRDC3 was detected in 39 (49%), 25 (57%), and 23 (55%) patients with oropharyngeal, hypopharyngeal, and cervical cancers, respectively. PAR1 was detected in 45 (57%), 22 (50%), and 21 (50%) patients with oropharyngeal, hypopharyngeal, and cervical cancers, respectively.

A comparison of the clinicopathological parameters based on p16 expression revealed significant differences at the TNM stage ($p=0.0348$), overlapping cancers ($p=0.005$), and TIL scores (TIL3 group, $p=0.0135$; TIL2 group, $p=0.006$) for oropharyngeal cancer and age ($p=0.022$) for hypopharyngeal cancer.

Outcome Analysis

The median follow-up for patient outcomes was 3.5 (1.8–5.2) years for oropharyngeal cancer, 3.4 (1.5–4.9) years for hypopharyngeal cancer, and 6.1 (3.0–7.3) years for uterine cervical cancer. The 3-year DSS rates for patients with oropharyngeal, hypopharyngeal, and uterine cervical cancers were 78%, 75%, and 95%, respectively. The 3-year DFS rates for oropharyngeal, hypopharyngeal, and uterine cervical cancers were 61%, 65%, and 92%, respectively.

The 3-year DSS for oropharyngeal cancer was as follows: OPE: 82%, CRT: 82%, RT: 56%, CT: 50%. The 3-year DFS was as follows: OPE: 62%, CRT: 70%, RT: 43%, CT: missing values. The 3-year DSS for each hypopharyngeal cancer was as follows: OPE: 80%, CRT: 70%, RT: 80%, CT: missing values. The 3-year DFS was as follows: OPE: 66%, CRT: 64%, RT: 50%, CT: missing values.

The p16-positive oropharyngeal cancer cases had significantly better prognosis than the negative cases: 3-year DSS (p16-negative, 74% and p16-positive, 81%; $p=0.0488$) (Fig. 3a) and 3-year DFS (p16-negative, 46% and p16-positive 71%, $p=0.0448$) (Fig. 4a). However, no significant differences were observed in the prognosis of hypopharyngeal cancer: 3-year DSS (p16-negative, 70% and p16-positive, 100%, $p=0.095$) (Fig. S1a) and 3-year DFS (p16-negative, 59% and p16-positive 88%, $p=0.147$) (Fig. S2a).

A subgroup analysis was performed after dividing the patients into two groups based on p16 expression to evaluate whether ARRDC3, PAR1, and TIL score influenced disease outcome. The p16-positive oropharyngeal cancer subgroup ($n=44$, 56%) displayed no significant differences in ARRDC3 and TIL status. However, PAR1-negative cases had significantly better prognosis than the positive cases (PAR1-negative, 89% (95% confidence interval [CI]: 61–97) vs. PAR1-positive, 75% (95% CI 53–88), $p=0.0438$, Fig. 3g). At 3-year DFS, p16-negative and -positive oropharyngeal cancers did not differ noticeably in ARRDC3 and TIL expression scores. Nonetheless, PAR1-positive and p16-negative oropharyngeal cancer cases had significantly better prognoses than PAR1-negative and p16-negative oropharyngeal cancer cases (PAR1-negative, 76% (95% CI 43–92) vs. PAR1-positive 30% (95% CI 11–51), $p=0.0233$, Fig. 4c). Hypopharyngeal cancer specimens did not significantly differ in ARRDC3, PAR1, or TIL status, with or without p16 expression (Figs. S1 and S2), similar to the uterine cervical cancer specimens (Fig. S3) for the 3-year DSS and 3-year DFS.

To compare the prognostic significance of the combined immunostaining results and TIL status, univariate and multivariate analyses were performed. ARRDC3 expression correlated with DSS in p16-negative oropharyngeal cancer cases in the multivariate analysis and PAR1 expression correlated with DSS in p16-positive oropharyngeal cancer cases in the multivariate analysis. Furthermore, PAR1 correlated with DFS in p16-negative oropharyngeal cancer cases in the univariate analysis and multivariate analysis (Table 2). However, none of these factors were significant in hypopharyngeal cancer specimens (Table 2) and uterine cervical cancer specimens (Table S2).

To validate the immunohistochemical staining results, we performed immunostaining against PAR1 and ARRDC3 using blocks of the three breast cancer cell lines and compared the results with those of western blotting (Fig. 5). Both methods had consistent results, with PAR1 expressed decreased in the order of MDA-MB-231 > KPL-4 > MCF-7, and ARRDC3 expressed in the order of KPL-4 > MCF-7 > MDA-MB-231 (Fig. 5b). These results are consistent with those reported previously [12, 27, 28].

Discussion

To the best of our knowledge, this study is the first to demonstrate that p16-positive oropharyngeal cancer with high PAR1 expression is short-lived. HPV-associated oropharyngeal cancer has a better prognosis than squamous cell carcinomas of other head and neck sites. Clinical trials are underway to de-escalate treatment and spare these patients from the consequences of overtreatment [29–32], as they may present at a younger age than other head and neck squamous cell carcinomas [33]. However, an open-label, randomized, controlled phase III trial revealed that cetuximab in patients with HPV-associated oropharyngeal cancer was not more beneficial than standard cisplatin regimens in terms of reduced toxicity; rather, it displayed a prominent disadvantage in tumor control [34]. Additionally, there was no evidence of a marked difference between radiotherapy, oral surgery (TOS), and neck dissection (ND). Enrollment was stopped due to excessive toxic effects in TOS and ND [35]. Therefore, until alternative treatments based on the eighth edition staging system are validated in clinical trials, treatment decisions will be made based on the seventh edition staging system. Thus, we adopted the seventh edition system for our study. Furthermore, it is essential to clarify the prognostic risk stratification of HPV-associated positive oropharyngeal cancer and use it for treatment selection and prognostic estimation. Interestingly, even in DFS of p16-negative oropharyngeal cancer, cases with high PAR1 expression experienced earlier recurrence (univariate: HR 3.882 [1.1–13.7]; $p = 0.03503$; multivariate: HR 35.68 [3.62–351.7]; $p = 0.0022$).

PAR1 is involved in the motility and metastasis of tumor cells in malignant tumors [10]. Therefore, it may also be an important factor in HPV-unrelated oropharyngeal cancer. However, in uterine cervical cancer—also HPV-associated—no significance was found between the prognoses of both groups regarding PAR1 expression. This result could be attributed to the relatively early stage of uterine cervical cancer in the enrolled patients [TNM staging I: $n = 25$ (60%)].

ARRDC3 downregulates integrin β4 expression and inhibits breast cancer progression [27]. It is specifically involved in the degradation and regulation of PAR1 expression via ALIX in triple-negative breast cancer [12]. ARRDC3 also suppresses tumor metastasis in other cancers [36–40]. In the present study, no significant results were obtained for p16-positive oropharyngeal cancer specimens. A genome-wide study of cervical cancer (HPV-associated) suggested that ARRDC3 is involved in the invasion of HPV into cells [13]. We hope further studies will be conducted on ARRD3 in HPV-associated oropharyngeal cancer. In recent years, the evaluation of TILs has attracted attention in the head and neck cancer field.

HPV-positive oropharyngeal cancers reportedly have considerably higher numbers of CD4+ and CD8 + T cells [41, 42]. In addition, in both HPV-positive and HPV-negative tumors, high CD8 + T-cell infiltration has been correlated with a favorable clinical outcome [43, 44]. TIL is a prognostic factor in HPV-positive oropharyngeal cancers; one report revealed that patients with higher TIL levels had a 4.5-fold higher survival rate [45]. Some other studies did not reveal favorable clinical outcomes [46, 47]. Unfortunately, in this study, we did not find notable prognostic differences based on the TIL score. Therefore, TIL invasion might have different characteristics depending on the tumor site, histology, or molecular subtype [48], and the differences in the prognostic value of TIL may reflect these different biological factors. TILs should be evaluated in resected specimens [23]. In the present study, we evaluated 42 (53%) oropharyngeal and 31 (70%) hypopharyngeal cancer cases using biopsy tissue samples. Therefore, the TILs in the biopsy tissue may not be representative of the overall tumor immune infiltration and may have been inadequately evaluated. Radical cure using surgical resection is often challenging

in head and neck squamous cell carcinoma, and the availability of biopsy specimens is often limited. Restricting case selection to surgical intervention may introduce selection bias, as it tends to favor cases with relatively good general health. Thus, the prognostic relevance of TILs remains controversial. Furthermore, randomized studies with larger samples are necessary to establish the correlation between TILs and HPV. In conclusion, PAR1 is a potential biomarker for HPV-associated oropharyngeal cancer.

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Author Contributions YF: writing—review & editing. YF: methodology. FS: software. II: formal analysis. YM: data curation. MU: investigation. TA: visualization. KS: conceptualization. HH: roles/writing—original draft. TM: project administration, review & editing.

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Data Availability The datasets generated and/or analyzed during the current study are not publicly available due to ethical restrictions, but are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent for Publication Consent for publication was obtained for every individual person's data included in the study.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This trial was licensed by Kawasaki Medical University Ethics Committee (Study No. 5014-02).

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

1. Mahal BA, Catalano PJ, Haddad RI et al (2019) Incidence and demographic burden of HPV-associated oropharyngeal head and neck cancers in the United States. Cancer Epidemiol Biomark Prev 28:1660–1667. https://doi.org/10.1158/1055-9965.EPI-19-0038
2. D'Souza G, Kreimer AR, Viscidi R et al (2007) Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med 356:1944–1956. https://doi.org/10.1056/NEJMoa065497
3. Hama T, Tokumaru Y, Fujii M et al (2014) Prevalence of human papillomavirus in oropharyngeal cancer: a multicenter study in Japan. Oncology 87:173–182. https://doi.org/10.1159/000360991
4. El-Naggar AK, Westra WH (2012) p16 expression as a surrogate marker for HPV-related oropharyngeal carcinoma: a guide for interpretative relevance and consistency. Head Neck 34:459–461. https://doi.org/10.1002/hed.21974
5. Ang KK, Harris J, Wheeler R et al (2010) Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 363:24–35. https://doi.org/10.1056/NEJMoa0912217
6. O'Sullivan B, Huang SH, Su J et al (2016) Development and validation of a staging system for HPV-related oropharyngeal cancer by the International Collaboration on Oropharyngeal cancer Network for Staging (ICON-S): a multicentre cohort study. Lancet Oncol 17:440–451. https://doi.org/10.1016/S1470-2045(15)00560-4
7. Horne ZD, Glaser SM, Vargo JA et al (2016) Confirmation of proposed human papillomavirus risk-adapted staging according to AJCC/UICC TNM criteria for positive oropharyngeal carcinomas. Cancer 122:2021–2030. https://doi.org/10.1002/cncr.30021
8. Alabi O, O'Neill JP (2020) 'Good cancer gone bad:' a narrative review of HPV oropharyngeal cancer and potential poor outcomes. Eur Arch Otorhinolaryngol 277:2185–2191. https://doi.org/10.1007/s00405-020-05991-z
9. Hilger D, Masureel M, Kobilka BK (2018) Structure and dynamics of GPCR signaling complexes. Nat Struct Mol Biol 25:4–12. https://doi.org/10.1038/s41594-017-0011-7
10. Shi X, Gangadharan B, Brass LF, Ruf W, Mueller BM (2004) Protease-activated receptors (PAR1 and PAR2) contribute to tumor cell motility and metastasis. Mol Cancer Res 2:395–402
11. Arakaki AKS, Pan W-A, Trejo J (2018) GPCRs in cancer: protease-activated receptors, endocytic adaptors and signaling. Int J Mol Sci 19:1886. https://doi.org/10.3390/ijms19071886
12. Arakaki AKS, Pan W-A, Lin H, Trejo J (2018) The α-arrestin ARRDC3 suppresses breast carcinoma invasion by regulating G protein-coupled receptor lysosomal sorting and signaling. J Biol Chem 293:3350–3362. https://doi.org/10.1074/jbc.RA117.001516
13. Takeuchi F, Kukimoto I, Li Z et al (2019) Genome-wide association study of cervical cancer suggests a role for ARRDC3 gene in human papillomavirus infection. Hum Mol Genet 28:341–348. https://doi.org/10.1093/hmg/ddy390
14. Schweickert PG, Yang Y, White EE et al (2021) Thrombin-PAR1 signaling in pancreatic cancer promotes an immunosuppressive microenvironment. J Thromb Haemost 19:161–172. https://doi.org/10.1111/jth.15115
15. Pagès F, Galon J, Dieu-Nosjean M-C, Tartour E, Sautès-Fridman C, Fridman W-H (2010) Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene 29:1093–1102. https://doi.org/10.1038/onc.2009.416
16. Piersma SJ, Jordanova ES, van Poelgeest MIE et al (2007) High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer Res 67:354–361. https://doi.org/10.1158/0008-5472.CAN-06-3388
17. Denkert C, Loibl S, Noske A et al (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28:105–113. https://doi.org/10.1200/JCO.2009.23.7370

18. Bianchini G, Gianni L (2014) The immune system and response to HER2-targeted treatment in breast cancer. Lancet Oncol 15:e58-68. https://doi.org/10.1016/S1470-2045(13)70477-7
19. Zhou Z, Mu D, Zhang D et al (2020) PD-L1 in combination with CD8$^+$TIL and HIF-1α are promising prognosis predictors of head and neck squamous cell carcinoma. Cancer Manage Res 12:13233–13239. https://doi.org/10.2147/CMAR.S285691
20. Denkert C, von Minckwitz G, Brase JC et al (2015) Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. J Clin Oncol 33:983–991. https://doi.org/10.1200/JCO.2014.58.1967
21. Denkert C, Wienert S, Poterie A et al (2016) Standardized evaluation of tumor-infiltrating lymphocytes in breast cancer: results of the ring studies of the international immuno-oncology biomarker working group. Mod Pathol 29:1155–1164. https://doi.org/10.1038/modpathol.2016.109
22. Salgado R, Denkert C, Demaria S et al (2015) The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 26:259–271. https://doi.org/10.1093/annonc/mdu450
23. Hendry S, Salgado R, Gevaert T et al (2017) Assessing tumor-infiltrating lymphocytes in solid tumors: a practical review for pathologists and proposal for a standardized method from the International Immuno-Oncology Biomarkers Working Group: part 2: TILs in melanoma, gastrointestinal tract carcinomas, non-small cell lung carcinoma and mesothelioma, endometrial and ovarian carcinomas, squamous cell carcinoma of the head and neck, genitourinary carcinomas, and primary brain tumors. Adv Anat Pathol 26:311–335. https://doi.org/10.1097/PAP.0000000000000161
24. Brierly JD, Gospodarowicz MK, Wittekind C (2016) TNM classification of malignant tumours, 8th edn. Wiley-Blackwell, Hoboken
25. Kurebayashi J, Otsuki T, Tang CK et al (1999) Isolation and characterization of a new human breast cancer cell line, KPL-4, expressing the Erb B family receptors and interleukin-6. Br J Cancer 79:707–717. https://doi.org/10.1038/sj.bjc.6690114
26. Kanda Y (2013) Investigation of the freely available easy-to-use software "EZR" for medical statistics. Bone Marrow Transplant 48:452–458
27. Draheim KM, Chen H-B, Tao Q, Moore N, Roche M, Lyle S (2010) ARRDC3 suppresses breast cancer progression by negatively regulating integrin beta4. Oncogene 29:5032–5047. https://doi.org/10.1038/onc.2010.250
28. Soung YH, Pruitt K, Chung J (2014) Epigenetic silencing of ARRDC3 expression in basal-like breast cancer cells. Sci Rep 4:3846
29. Adelstein DJ, Li Y, Adams GL et al (2003) An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. J Clin Oncol 21:92–98. https://doi.org/10.1200/JCO.2003.01.008
30. Denis F, Garaud P, Bardet E et al (2004) Final results of the 94–01 French Head and Neck Oncology and Radiotherapy Group randomized trial comparing radiotherapy alone with concomitant radiochemotherapy in advanced-stage oropharynx carcinoma. J Clin Oncol 22:69–76. https://doi.org/10.1200/JCO.2004.08.021
31. Machtay M, Moughan J, Trotti A et al (2008) Factors associated with severe late toxicity after concurrent chemoradiation for locally advanced head and neck cancer: an RTOG analysis. J Clin Oncol 26:3582–3589. https://doi.org/10.1200/JCO.2007.14.8841
32. Forastiere AA, Zhang Q, Weber RS et al (2013) Long-term results of RTOG 91–11: a comparison of three nonsurgical treatment strategies to preserve the larynx in patients with locally advanced larynx cancer. J Clin Oncol 31:845–852. https://doi.org/10.1200/JCO.2012.43.6097
33. Economopoulou P, Kotsantis I, Psyrri A (2021) De-escalating strategies in HPV-associated head and neck squamous cell carcinoma. Viruses 13:1787. https://doi.org/10.3390/v13091787
34. Mehanna H, Robinson M, Hartley A et al (2019) Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial. Lancet 393:51–60. https://doi.org/10.1016/S0140-6736(18)32752-1
35. Palma DA, Prisman E, Berthelet E et al (2022) Assessment of toxic effects and survival in treatment deescalation with radiotherapy vs transoral surgery for HPV-associated oropharyngeal squamous cell carcinoma: the ORATOR2 phase 2 randomized clinical trial. JAMA Oncol 8:1–7. https://doi.org/10.1001/jamaoncol.2022.0615
36. Shen X, Sun X, Sun B et al (2018) ARRDC3 suppresses colorectal cancer progression through destabilizing the oncoprotein YAP. FEBS Lett 592:599–609. https://doi.org/10.1002/1873-3468.12986
37. Zheng Y, Lin Z-Y, Xie J-J et al (2017) ARRDC3 inhibits the progression of human prostate cancer through ARRDC3-ITGβ4 pathway. Curr Mol Med 17:221–229. https://doi.org/10.2174/1566524017666170807144711
38. Chen Y, Tian D, Chen X et al (2021) ARRDC3 as a diagnostic and prognostic biomarker for epithelial ovarian cancer based on data mining. Int J Gen Med 14:967–981. https://doi.org/10.2147/IJGM.S302012
39. Xiao J, Shi Q, Li W et al (2018) ARRDC1 and ARRDC3 act as tumor suppressors in renal cell carcinoma by facilitating YAP1 degradation. Am J Cancer Res 8:132–143
40. Yan J, Shi L, Lin S, Li Y (2021) MicroRNA-624-mediated ARRDC3/YAP/HIF1α axis enhances esophageal squamous cell carcinoma cell resistance to cisplatin and paclitaxel. Bioengineered 12:5334–5347. https://doi.org/10.1080/21655979.2021.1938497
41. Ward MJ, Thirdborough SM, Mellows T et al (2014) Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. Br J Cancer 110:489–500. https://doi.org/10.1038/bjc.2013.639
42. Partlová S, Bouček J, Kloudová K et al (2015) Distinct patterns of intratumoral immune cell infiltrates in patients with HPV-associated compared to non-virally induced head and neck squamous cell carcinoma. Oncoimmunology 4:e965570. https://doi.org/10.4161/21624011.2014.965570
43. Balermpas P, Rödel F, Rödel C et al (2016) CD8+ tumour-infiltrating lymphocytes in relation to HPV status and clinical outcome in patients with head and neck cancer after postoperative chemoradiotherapy: a multicentre study of the German cancer consortium radiation oncology group (DKTK-ROG). Int J Cancer 138:171–181. https://doi.org/10.1002/ijc.29683
44. Näsman A, Romanitan M, Nordfors C et al (2012) Tumor infiltrating CD8$^+$ and Foxp3$^+$ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer. PLoS ONE 7:e38711. https://doi.org/10.1371/journal.pone.0038711
45. Ruangritchankul K, Sandison A, Warburton F et al (2019) Clinical evaluation of tumour-infiltrating lymphocytes as a prognostic factor in patients with human papillomavirus-associated oropharyngeal squamous cell carcinoma. Histopathology 75:146–150. https://doi.org/10.1111/his.13873
46. Kong CS, Narasimhan B, Cao H et al (2009) The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. Int

J Radiat Oncol Biol Phys 74:553–561. https://doi.org/10.1016/j.ijrobp.2009.02.015
47. Rittà M, Landolfo V, Mazibrada J et al (2013) Human papillomavirus tumor-infiltrating T-regulatory lymphocytes and P53 codon 72 polymorphisms correlate with clinical staging and prognosis of oropharyngeal cancer. New Microbiol 36:133–144
48. De Meulenaere A, Vermassen T, Aspeslagh S, Vandecasteele K, Rottey S, Ferdinande L (2016) TILs in head and neck cancer: ready for clinical implementation and why (not)? Head Neck Pathol 11:354–363. https://doi.org/10.1007/s12105-016-0776-8