# 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 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

81 can be accomplished through the utilization of appropriate histopathological biomarkers.

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

94 PAR1 is also involved in the tumor microenvironment (TME). Its expression in
95 pancreatic adenocarcinoma contributes to the creation of a cellular microenvironment within
96 the tumor, marked by the reduced number of cytotoxic T cells infiltrating the cancerous cells,
97 as well as increased numbers of primitive immunosuppressor cell types [14]. The tumor-
98 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, TIL levels were evaluated using the scoring method recommended by the International Immuno-Oncology Biomarker Working Group on Breast Cancer. 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].

### *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. 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 + l × 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 d under normal oxygen (5 % $CO_2$) conditions at 37 °C.

***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). The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) 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 %, high: >50 %) and two groups: high (>median) and low

225 (≤median), using the median value as the cutoff.

226 The categorical variables are reported as number and percentage; they were evaluated
227 using the chi-square or exact Fisher test. The median and 25th and 75th percentiles of
228 continuous variables were calculated using the *t*-test if they showed a normal pattern of
229 distribution. Otherwise, Mann–Whitney U test was used. The primary endpoint was disease-
230 specific survival (DSS) and the secondary endpoint was disease-free survival (DFS). DSS and
231 DFS were evaluated using Kaplan–Meier curves and log-rank tests, respectively. Cox
232 regression analysis was used for univariate and multivariate analyses. Oropharyngeal and
233 hypopharyngeal cancers were adjusted for TNM stage (stages I–II vs. III–IV), smoking status,
234 alcohol consumption, venous invasion, lymphatic invasion, duplicate cancer diagnoses, site of
235 origin, and histological differentiation. Cervical cancer was adjusted for TNM stage (stage I–
236 II vs. III–IV), venous invasion, and lymphovascular invasion. The p16-positive and -negative
237 cases were separately analyzed for differences in association with the outcome. All tests were
238 two-tailed, and statistical significance was set at $p < 0.05$.

239 All data were analyzed using EZR (Saitama Medical Center, Jichi Medical University,
240 Saitama, Japan), a graphical user interfaces for R (R Foundation for Statistical Computing,
241 Vienna, Austria) [26]. To be more precise, it is a customized version of the R program
242 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 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 (47 %), 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.

**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 | PS 32 (72%) | 25 (69%) | 7 (88%) | 0.466 |
| **Side wall** | 48 (61%) | 19 (54%) | 29 (66%) | | PW 5 (12%) | 4 (11%) | 1 (12%) | |
| **Anterior wall** | 16 (20%) | 9 (26%) | 7 (16%) | | PC 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%) | 0.52 4 | 6 (14%) | 6 (17%) | 0 (0%) | 0.5 73 |
| **Negative** | 68 (86%) | 29 (83%) | 39 (89%) | | 38 (86%) | 30 (83%) | 8 (100%) | |
| **TIL score** | | | | | | | | |
| **Low** | 37 (47%) | 21 (60%) | 16 (36%) | 0.01 35* | 29 (66%) | 25 (69%) | 4 (50%) | 0.3 25 |
| **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%) | |
| **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.

N/E: not evaluable.

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%.

OPE: operation; CRT: chemoradiotherapy; RT: radiotherapy; CT: chemotherapy; PS: pyriform sinus; PW: posterior wall; PC: post-cricoid; WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated.

* $p<0.05$

**Table S1.** Baseline characteristics of patients with uterine cervical cancer

| | Total (n = 42) |
|---|---|
| Age | 42 (35–57) |
| Diagnose | |
| resection | 42 (100%) |
| Treatment | |
| OPE+AC | 24 (57%) |
| OPE | 12 (27%) |
| NAC + OPE+AC | 3 (7%) |
| OPE+RT | 2 (6%) |
| CRT+OPE+AC | 1 (3%) |
| TNM staging | |
| I | 25 (60%) |

| | |
|---|---|
| II | 9 (21%) |
| III | 8 (19%) |
| Differentiation | |
| keratinizing type | 24 (57%) |
| non-keratinizing type | 18 (43%) |
| Lymphatic invasion | |
| Positive | 19 (45%) |
| Negative | 23 (55%) |
| Venous invasion | |
| Positive | 10 (24%) |
| Negative | 32 (76%) |
| TIL score | |
| Low | 20 (48%) |
| Medium | 11 (26%) |
| High | 11 (26%) |
| median negative | 23 (55%) |
| median positive | 19 (45%) |

| | |
|---|---|
| Marker expression | |
| ARRDC3-positive | 23 (55%) |
| ARRDC3-negative | 19 (45%) |
| PAR1-positive | 21 (50%) |
| PAR1-negative | 21 (50%) |

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

The patients were classified into the following groups based on the TIL score: Low: 0%–10%; Medium: 20%–40%; High: >50%.

Prognostic analysis was evaluated in three and two groups based on median values

OPE: operation; CRT: chemoradiotherapy; RT: radiotherapy; AC: adjuvant chemotherapy; NAC; neoadjuvant chemotherapy

***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 %,

300 respectively.

301 The 3-year DSS for oropharyngeal cancer was as follows: OPE: 82%, CRT: 82%, RT:
302 56%, CT: 50%. The 3-year DFS was as follows: OPE: 62%, CRT: 70%, RT: 43%, CT: missing
303 values. The 3-year DSS for each hypopharyngeal cancer was as follows: OPE: 80%, CRT: 70%,
304 RT: 80%, CT: missing values. The 3-year DFS was as follows: OPE: 66%, CRT: 64%, RT: 50%,
305 CT: missing values.

306 The p16-positive oropharyngeal cancer cases had significantly better prognosis than the
307 negative cases: 3-year DSS (p16-negative, 74 % and p16-positive, 81 %; p = 0.0488) (Fig. 3a)
308 and 3-year DFS (p16-negative, 46 % and p16-positive 71 %, p = 0.0448) (Fig. 4a**)**. However,
309 no significant differences were observed in the prognosis of hypopharyngeal cancer: 3-year
310 DSS (p16-negative, 70 % and p16-positive, 100 %, p = 0.094) (Fig. S1a) and 3-year DFS (p16-
311 negative, 59 % and p16-positive 88 %, p = 0.147) (Fig. S2a).

312 A subgroup analysis was performed after dividing the patients into two groups based on
313 p16 expression to evaluate whether ARRDC3, PAR1, and TIL score influenced disease
314 outcome. The p16-positive oropharyngeal cancer subgroup (n = 44, 56 %) displayed no
315 significant differences in ARRDC3 and TIL status. However, PAR1-negative cases had
316 significantly better prognosis than the positive cases (PAR1-negative, 89 % (95 % confidence
317 interval [CI]: 61–97) vs. PAR1-positive, 75 % (95 % CI: 53–88), p = 0.0438, Fig. 3g). At 3-
318 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 (Fig. 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. In oropharyngeal cancer, PAR1 expression correlated with DSS in p16-negative cases in the univariate analysis and ARRDC3 expression correlated with DSS in p16-negative cases in the multivariate analysis. Furthermore, PAR1 correlated with DSS in p16-negative cases and PAR1 correlated with DFS in p16-negative and -positive cases in the multivariate analysis (Table 2). However, none of these factors were significant in hypopharyngeal 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

337 expressed in the order of KPL-4 > MCF-7 > MDA-MB-231 (Fig. 5b). These results are

338 consistent with those reported previously [12,27,28].

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

| **Oropharyngeal cancer** | | | | | | |
|---|---|---|---|---|---|---|
| | Univariate | | | Multivariate | | |
| | HR | 95% CI | p-value | HR | 95% CI | p-value |
| 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 | 0.0458* |
| 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 | 0.0630 1 | 11.67 | 1.251000–109.0000 | 0.0310 7* |
| 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 | 0.0350 3* | 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 | | | | | | |
| | Univariate | | | Multivariate | | |
| | HR | 95% CI | p-value | HR | 95% CI | p-value |
| 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 | 0.0534 6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 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 |

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

341 (DFS)

342 Multivariate models were adjusted for TNM stage, smoking status, alcohol consumption, venous invasion,

343 lymphatic invasion, double cancers, site of origin, and histologic differentiation

344 * $p<0.05$

345 **Table S2.** Cox regression analysis for univariate and multivariate analyses

| **Uterine cervical cancer** | | | | | | |
|---|---|---|---|---|---|---|
| | Univariate | | | Multivariate | | |
| | HR | 95% CI | p-value | HR | 95% CI | p-value |
| **Disease-specific survival** | | | | | | |
| **ARRDC3 (positive vs. negative)** | 0.8452 | 0.05286–13.51 | 0.9053 | 0.7204 | 0.03833–13.540 | 0.8266 |
| **PAR1 (positive vs. negative)** | 0.8452 | 0.05286–13.51 | 0.9053 | 0.7204 | 0.03833–13.540 | 0.8266 |
| **TIL (high vs. low)** | 0.7087 | 0.04421–11.36 | 0.8078 | 0.7375 | 0.04519–12.040 | 0.8308 |
| **Disease-free survival** | | | | | | |
| **ARRDC3 (positive vs. negative)** | 0.8209 | 0.1156–5.831 | 0.8436 | 0.4455 | 0.05758–3.447 | 0.4387 |
| **PAR1 (positive vs. negative)** | 0.8622 | 0.1213–6.128 | 0.8822 | 0.3645 | 0.02849–4.665 | 0.4378 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **TIL (high vs. low)** | 0.715 | 0.1004–5.101 | 0.738 | 0.742 | 0.09467–5.828 | 0.777 |

Univariable and multivariable time-to-event analyses for OS and DFS

Multivariable models were adjusted for TNM stage, venous invasion, lymphatic invasion

**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

362 trials, treatment decisions will be made based on the seventh edition staging system. Thus, we
363 adopted the seventh edition system for our study. Furthermore, it is essential to clarify the
364 prognostic risk stratification of HPV-associated positive oropharyngeal cancer and use it for
365 treatment selection and prognostic estimation. Interestingly, even in DFS of p16-negative
366 oropharyngeal cancer, cases with high PAR1 expression experienced earlier recurrence
367 (univariate: HR, 3.882 [1.1–13.7]; p = 0.03503; multivariate: HR, 35.68 [3.62–351.7]; p = 0.
368 0022).

369 PAR1 is involved in the motility and metastasis of tumor cells in malignant tumors [10].
370 Therefore, it may also be an important factor in HPV-unrelated oropharyngeal cancer. However,
371 in uterine cervical cancer—also HPV-associated—no significance was found between the
372 prognoses of both groups regarding PAR1 expression. This result could be attributed to the
373 relatively early stage of uterine cervical cancer in the enrolled patients (TNM staging I: n = 25
374 (60 %)).

375 ARRDC3 downregulates integrin β4 expression and inhibits breast cancer progression
376 [27]. It is specifically involved in the degradation and regulation of PAR1 expression via ALIX
377 in triple-negative breast cancer [12]. ARRDC3 also suppresses tumor metastasis in other
378 cancers [36–40]. In the present study, no significant results were obtained for p16-positive
379 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.

## Compliance with Ethical Standards


### Funding

This study was funded by Research Project Grants from Kawasaki Medical University (grant number R04G-001). The funding agency had no involvement in the design of the study, the collection, analysis, or interpretation of the data, or the writing of the manuscript.


### Conflict of Interest

The authors declare that they have no conflict of interest.

### 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.

**Consent for Publication**

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

**Author Contributions**

Yoshinori Fujita: Writing - review & editing. Yujiro Fukuda: Methodology. Fumiaki Sanuki: Software. Isao Irei: Formal analysis. Yasumasa Monobe: Data curation. Masako Uno: Investigation. Takeshi Akisada: Visualization. Koishiro Shimoya: Conceptualization. Hirotaka Hara: Roles/Writing - original draft. Takuya Moriya: Project administration, review & editing.

**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.

434

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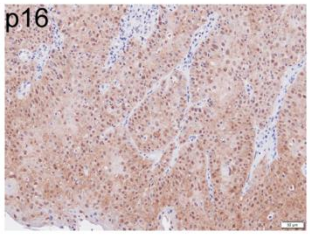
593

**Figure Legends**

**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)

a




b

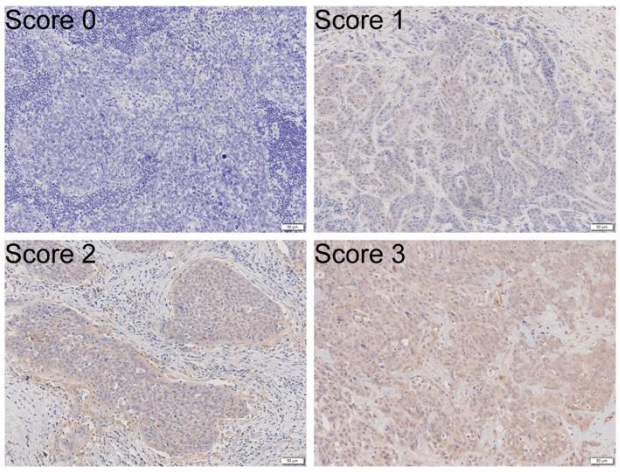


**Fig. 2** Evaluating tumor-infiltrating lymphocytes

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

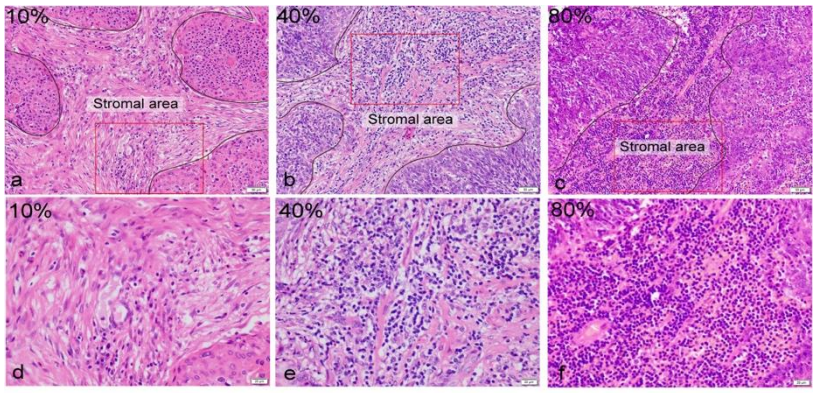


**Fig. 3** Disease-specific survival (DSS) in oropharyngeal cancer

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. (p = 0.415) (b). (p = 0.642) (c). (p = 0.132) (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)

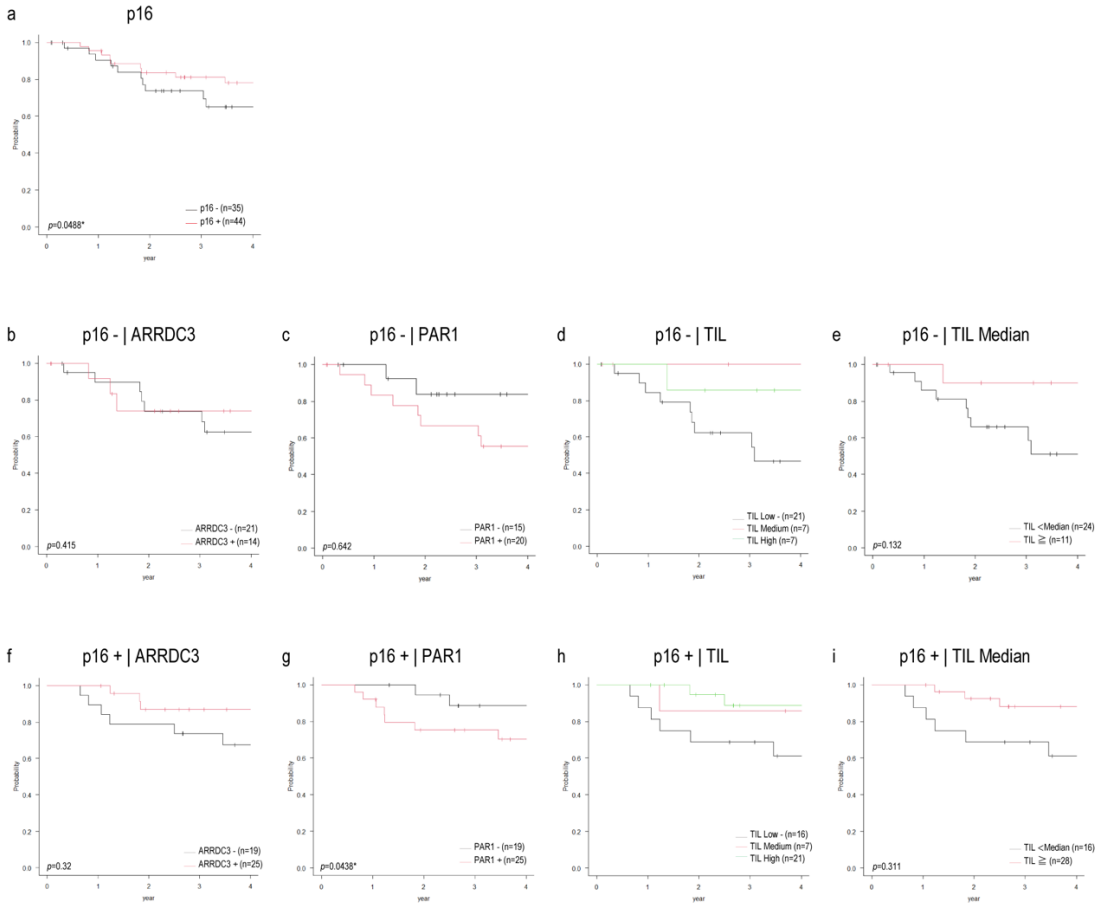


**Fig. 4** Disease-free survival (DFS) in oropharyngeal cancer

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)

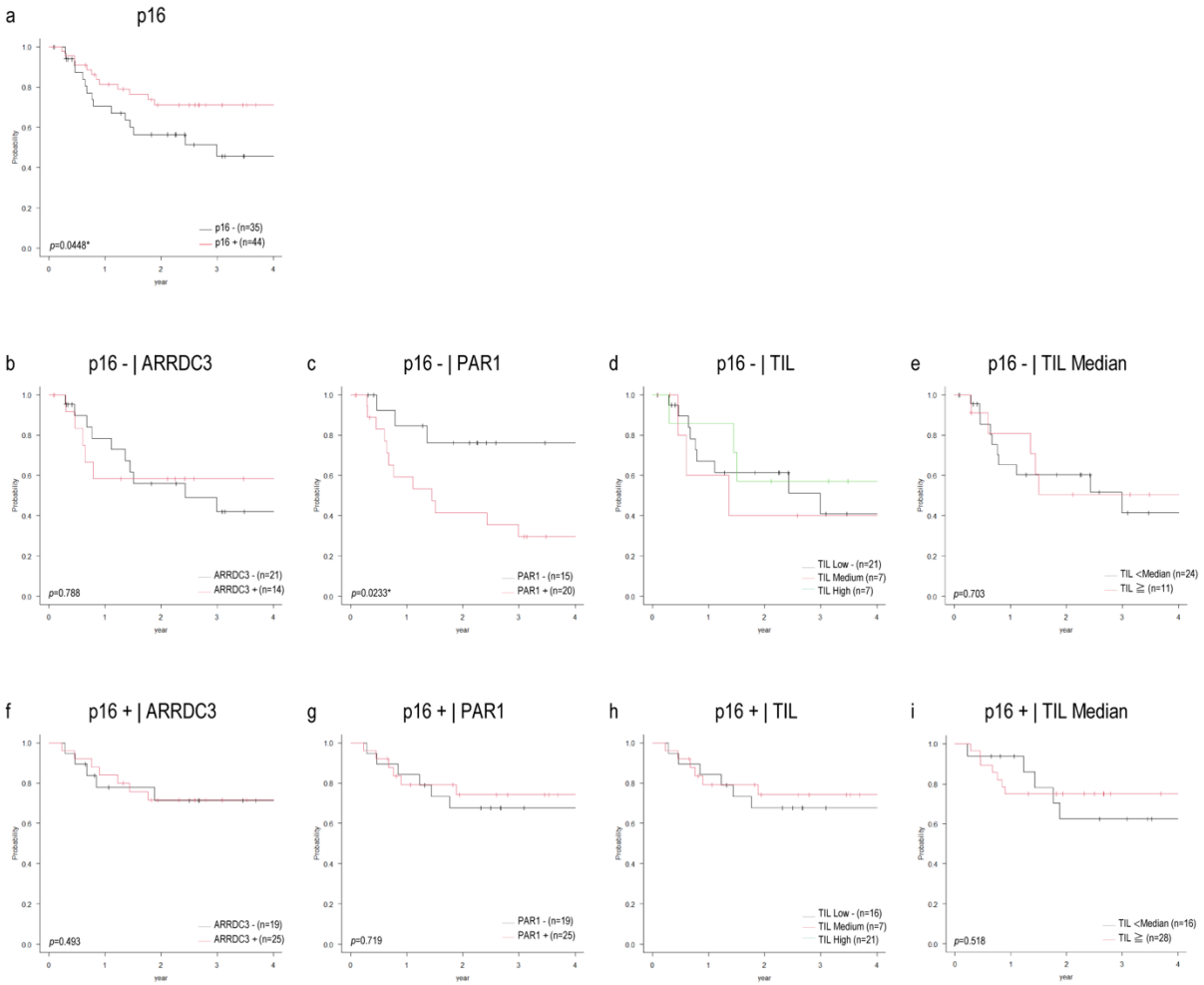
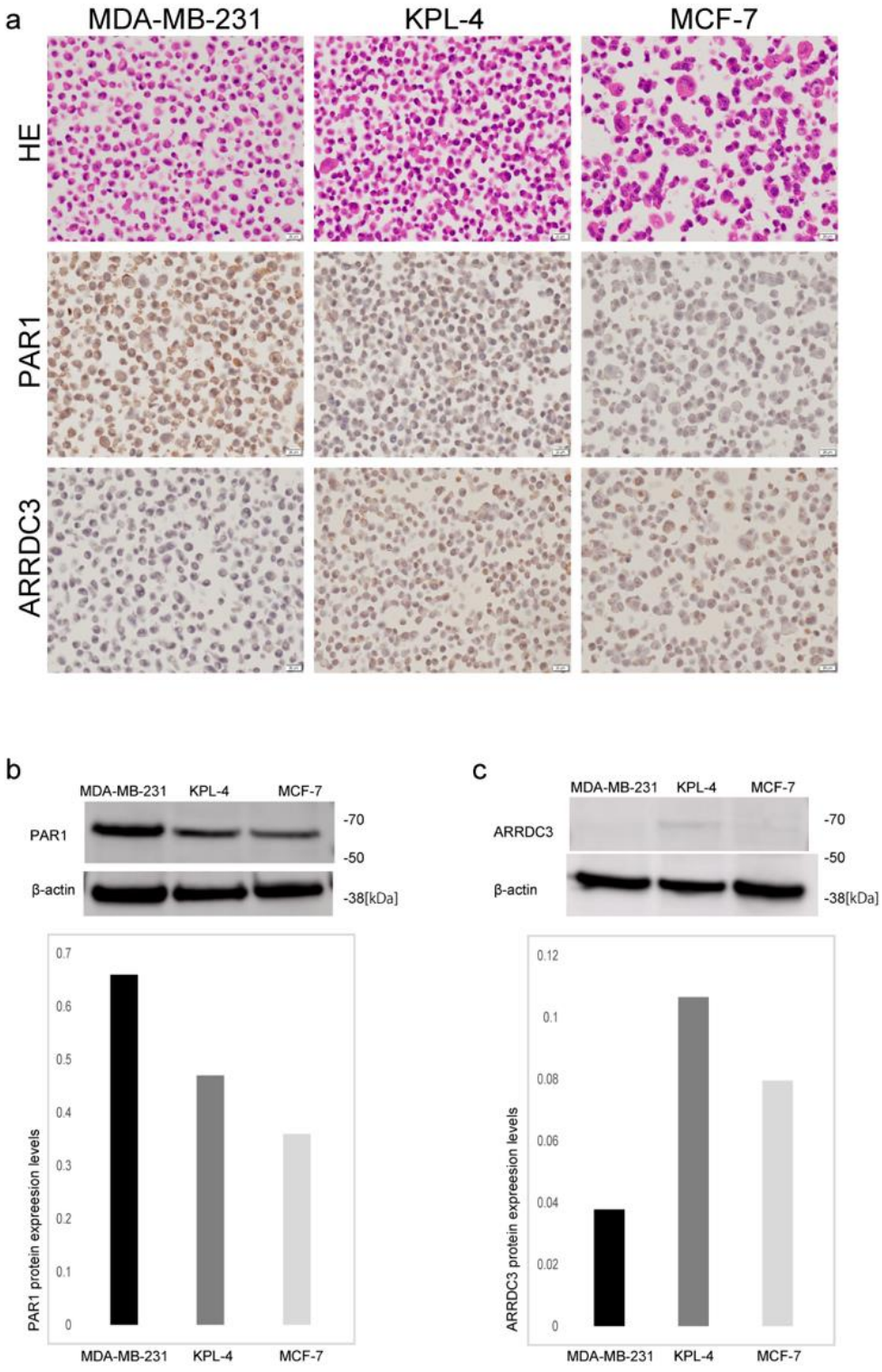


**Fig. 5** Comparison of protease-activated receptor-1 (PAR1) and alpha-arrestin domain-containing protein 3 (ARRDC3) expression in human breast cancer tissues

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 (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 highest 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).


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

652

653

**Supplementary Figures**

**Fig. S1** Disease-specific survival (DSS) in hypopharyngeal cancer

The 3-year DSS was 75 %.

No significant difference existed in survival between patients with and without p16 expression (p = 0.095) (a). However, all patients with p16-positive results (n = 8; 18 %) survived during observation.

In the p16-positive hypopharyngeal cancer subgroup (n = 36, 82 %), no predominant differences were observed in alpha-arrestin domain-containing protein 3 (ARRDC3) and protease-activated receptor-1 (PAR1) expression or tumor-infiltrating lymphocyte (TIL) status (b–e).

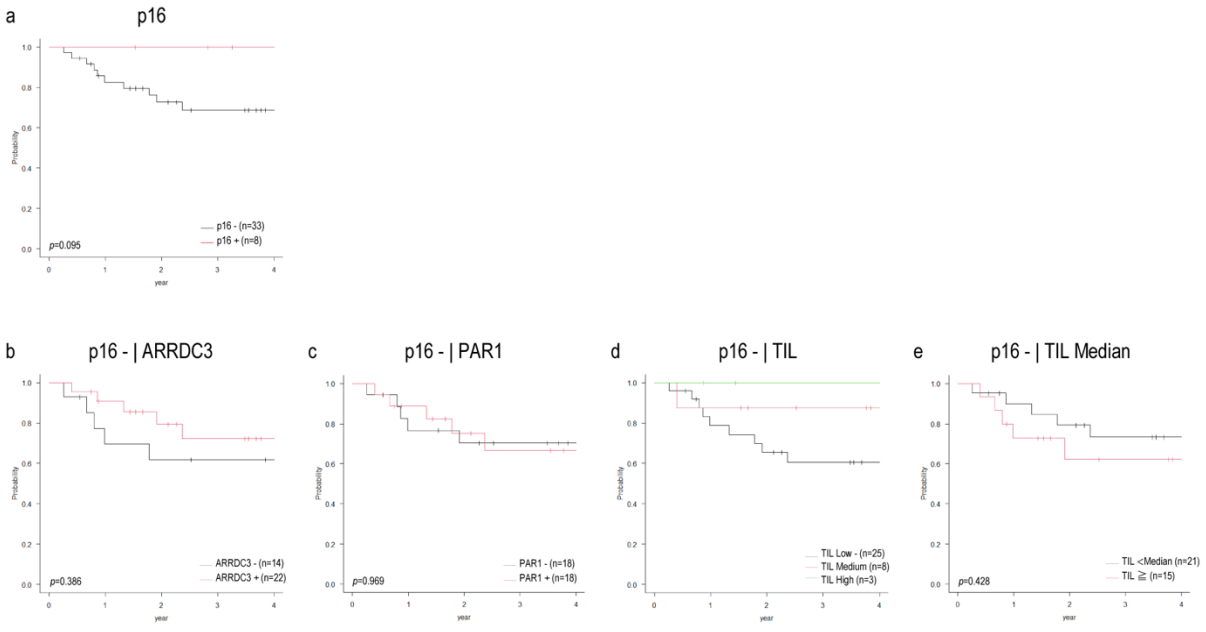


**Fig. S2** Disease-free survival (DFS) in hypopharyngeal cancer

The 3-year DFS was 65 %.

No significant difference was observed in survival between patients with and without p16 expression (p = 0.147) (a).

In the p16 hypopharyngeal cancer subgroup (n = 36, 82 %), no predominant differences existed in alpha-arrestin domain-containing protein 3 (ARRDC3) and protease-activated receptor-1 (PAR1) expression or tumor-infiltrating lymphocyte (TIL) status (b–e).

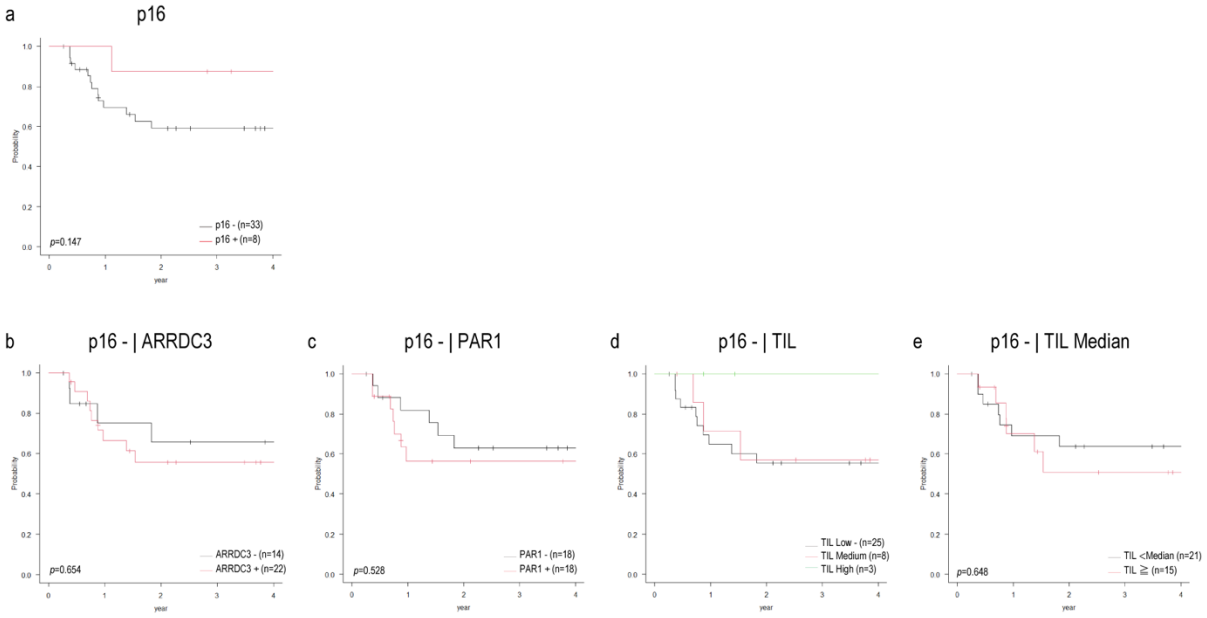


**Fig. S3** Disease-specific survival (DSS) and disease-free survival (DFS) in cervical cancer

The 3-year DSS and DFS were 95 % and 92 %, respectively.

No significant differences were observed in alpha-arrestin domain-containing protein 3 (ARRDC3) and protease-activated receptor-1 (PAR1) expression or tumor-infiltrating lymphocyte (TIL) status between the DSS and DFS groups.