Expression patterns of uPAR, TF and EGFR and their potential as targets for molecular imaging in oropharyngeal squamous cell carcinoma

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Abstract. The clinical introduction of molecular imaging for the management of oropharyngeal squamous cell carcinoma (OPSCC) relies on the identification of relevant cancer-specific biomarkers. The application of three membrane-bound receptors, namely urokinase-type plasminogen activator receptor (uPAR), tissue factor (TF) and EGFR have been previously explored for targeted imaging and therapeutic strategies in a broad range of solid cancers. The present study aimed to investigate the expression patterns of uPAR, EGFR and TF by immunohistochemistry (IHC) to evaluate their potential for targeted imaging and prognostic value in OPSCC. In a retrospective cohort of 93 patients with primary OPSCC, who were balanced into the 45 human papillomavirus (HPV)-positive and 48 HPV-negative groups, the IHC-determined expression profiles of uPAR, TF and EGFR in large biopsy or tumor resection specimens were analyzed. Using the follow-up data, overall survival (OS) and recurrence-free survival were measured. Specifically, associations between survival outcome, biomarker expression and clinicopathological factors were examined using Cox proportional hazards model and log-rank test following Kaplan-Meier statistics. After comparing the expression pattern of biomarkers within the tumor compartment with that in the adjacent normal tissues, uPAR and TF exhibited a highly tumor-specific expression pattern, whereas EGFR showed a homogeneous expression within the tumor compartment as well as a consistent expression in the normal mucosal epithelium and salivary gland tissues. The positive expression rate of uPAR, TF and EGFR in the tumors was 98.9, 76.3 and 98.9%, respectively. No statistically significant association between biomarker expression and survival outcome could be detected. Higher uPAR expression levels had a trend towards reduced OS according to results from univariate analysis (P=0.07; hazard ratio=2.01; 95% CI=0.92-4.37). Taken together, these results suggest that uPAR, TF and EGFR may be suitable targets for molecular imaging and therapy in OPSCC. In particular, uPAR may be an attractive target owing to their high positive expression rates in tumors and a highly tumor-specific expression pattern.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer globally, with an incidence of 890,000 new cases in 2018 (1). For oropharyngeal squamous cell carcinoma (OPSCC), a rising incidence rate has been reported over recent decades (2). This rising incidence has been observed in particular in the sub-group of patients with disease related to human papilloma virus (HPV) infection (2). Classically, treatment of OPSCC mainly involves chemo- and/or radiotherapy (3). However, a primary surgical treatment strategy has been recently established for early-stage primary disease and recurrent cases (3). Molecular imaging as a form of applied health technology is a field that is developing rapidly. Optimization of existing modalities or translation of novel modalities is currently in progress, with the aim of

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improving imaging for the staging and treatment of cancer (4). In particular, targeted optical imaging has been explored intensively over the past decade, since this modality allows for the real-time intraoperative visualization of tumor extension and margins (5). This in turn may aid in addressing the major challenge of inadequate tumor margins across a range of solid cancers that are treated surgically (5). For surgically treated OPSCC, the reported pooled rate of tumor-positive margins is 8% (6). Identification of molecular targets that are highly expressed in cancer and absent or minimally expressed in adjacent healthy tissues is key to the efficacy of molecular imaging (7). To evaluate potential targets for imaging purposes, studies investigating the exact expression pattern within the tumor compartment and the demarcation boundary with the non-cancerous tissue at the margins in appropriately-sized cohorts of patients are required (8).

Key parameter for the applicability of a target are tumor specificity and the expression rate in most if not all types of cancer. The present study investigated the expression pattern of urokinase-type plasminogen activator receptor (uPAR; also known as CD87), EGFR and tissue factor (TF; also known as CD142) in OPSCC. These are membrane-bound receptors that are considered to be candidate targets for imaging purposes in several solid cancers, including HNSCC (9-11) For uPAR and EGFR, numerous clinical studies with targeted probes for positron emission tomography and optical imaging are currently in progress (ClinicalTrials.gov Identifiers: NCT02945826, NCT02965001, NCT02960724, NCT02755675 and NCT03134846).

The plasminogen activator (PA) system serves a central role in cancer invasion and metastasis, which is mediated through pericellular proteolytic activity to degrade the extracellular matrix at the invasive front of a tumor. uPAR is expressed by both cancer cells and tumor-associated stromal cells. High expression levels in tumors and limited expression of uPAR in non-cancerous tissues have been previously reported in various cancers, including oral cavity squamous cell carcinoma (OSCC) (12-15) In addition, higher expression levels of uPAR have been associated with poorer survival and more aggressive disease in numerous types of cancer including breast, bladder and colorectal cancer (16-18).

The role of EGFR in HNSCC has been extensively explored, primarily for antibody-based targeted therapy. However, it also emerged as a candidate for targeted imaging over the past decade (19,20) EGFR is closely associated with tumor growth, since it is expressed by tumor cells in addition to various normal tissues in the human body (21).

An established relationship between cancer and thrombosis and venous thromboembolic events has been extensively reported. TF form part of the extrinsic coagulation cascade and it has been reported to be highly expressed in several solid tumors (22). In the tumor microenvironment, TF participates in multiple oncogenic signaling pathways that stimulate tissue remodeling and tumor angiogenesis in the invasive process; TF also modulates the immune response within the tumor compartment and assists tumor cells to escape the host immune system (23). Preclinical data has been reported to support the rationale for targeting TF using imaging and therapy (11,24).

Therefore, the present study aimed to investigate the expression patterns of uPAR, EGFR and TF by immunohisto-chemistry (IHC) to evaluate their potential for targeted imaging and possible prognostic value in OPSCC.

Materials and methods

Patients and tumor characteristics. From an established retrospective database of patients with primary OPSCC diagnosed between January 2000 and January 2012 in the department of Otolaryngology, Head & Neck surgery & Audiology at Rigshospitalet (Copenhagen, Denmark), a balanced cohort of 48 (52%) HPV-positive and 46 (48%) HPV-negative patients were randomly assembled. The inclusion criteria were primary OPSCC with preserved large biopsies or diagnostic resection specimens being available for IHC expression analysis. Exclusion criteria were insufficient tumor tissue for IHC analysis or incomplete dataset in the database. All specimens were collected prior to non-surgical treatment if this was indicated. Because HPV is a well-known powerful prognostic marker of OPSCC outcome, a balanced selection based on HPV status was performed. Both p16 and HPV-DNA status was available. The dataset in this current study has been part of previous publications and assays for the analysis of p16 and HPV-DNA have previously been described (25). Briefly, for p16 detection by IHC, the anti-P16 clone E6HA OptiView DAB IHC Detection Kit (Roche Diagnostics); for HPV-DNA analysis, DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Inc.) and PCR was performed using General Primers GP5+/GP6+. The 8th version of the TNM Union For International Cancer Control (UICC8) staging manual was applied (26). The recorded clinicopathological characteristics and treatment modalities are provided in Table I. The present study was approved by the Ethical Committee of the Capital Region of Denmark (protocol H-15016322; Copenhagen, Denmark) and was conducted in accordance with The Declaration of Helsinki.

IHC. At the time of collection, fresh tissue was fixed in 10% buffered formalin at room temperature for 24 h and then embedded in paraffin. The paraffin-embedded tissue blocks were sliced into 4-μm thick serial sections. Deparaffinization was performed with xylene for 15 min followed by dehydration using an ethanol gradient (from 99 to 70%, then demineralized water). IHC staining was performed on a Ventana Benchmark Ultra semi-automated autostainer (Roche Diagnostics). All antibody protocols have been previously optimized on positive and negative control tissues. The following antibodies were used: anti-cytokeratin (CK) AE1/AE3+8/18 (Pan Cytokeratin Plus; cat. no. 162; 1:200; Biocare Medical, LLC), mouse anti-human uPAR R2 (1:20,000; in-house antibody; Finsen Laboratory), anti-EGFR (Confirm anti-EGFR 5B7; cat. no. 790-4347; ready-to-use; Ventana Medical Systems, Inc.; Roche Diagnostics) and anti-TF (anti-TF; cat. no. 4509; 1:150; American Diagnostica, Inc.). Antigen retrieval for uPAR was performed with proteinase K (1:50 in Tris-HCl; cat. no. S3004; DAKO, Agilent Technologies, Inc.) for 8 min at room temperature, followed by 100˚C heating with Cell Conditioning 1 buffer (CC1; Ventana Medical Systems, Inc.; Roche Diagnostics) for 16 min. For TF, EGFR and CK,

Table I. Clinicopathological characteristics of the patients with oropharyngeal squamous cell carcinoma.

Clinicopathological characteristic	N (%) or median (range)
Sex	
Female	24 (26)
Male	69 (74)
Age, years	60 (46-84)
Tumor location	
Pharynx wall	8 (9)
Palatine tonsils	70 (75)
Lingual tonsils	12 (13)
Soft palate	3 (3%)
HPV-DNA	
Positive	45 (48)
Negative	48 (52)
Smoking status	
Never smoker	16 (17)
Previous	38 (41)
Current smoker	39 (42)
Tumor differentiation	
Non-keratinizing	23 (25)
High	3 (3)
Modrate	37 (40)
Low	30 (32)
T stage	
T1	16 (17)
T2	45 (48)
T3	14 (15)
T4	18 (19)
N stage	
N0	24 (26)
N1	43 (46)
N2a/2b/2c	22 (24)
N3	4 (4)
UICC8 TMN stage	
S1	36 (39)
S2	20 (22)
S3	16 (17)
S4	21 (23)
Treatment	
RT	33 (35)
RCT	46 (49)
Surgery	8 (9)
Surgery + RT	1 (2)
Surgery + RCT	1 (2)
Palliation	4 (4)
Recurrence	
No	74 (80)
Yes	19 (20)
Local	6 (6)
Regional	6 (6)
Locoregional	1 (2)
Distant	6 (6)

Table I. Continued.

Clinicopathological characteristic	N (%) or median (range)
Second primary	
No	79 (85)
Yes	14 (15)

HPV, human papillomavirus; RCT, radiochemotherapy; RT, radiotherapy.

heat-induced epitope retrieval was performed at 100˚C in CC1 buffer for 32 min. The blocking step was performed using Peroxidase Blocking Solution (cat. no. S2023; DAKO, Agilent Technologies, Inc.) for 8 min at room temperature, followed by 2% BSA (cat. no. A7906; Sigma-Aldrich; Merck KGaA) blocking for 10 min at room temperature. For uPAR and TF, EnVision mouse HRP-conjugated EnVision FLEX+ secondary antibodies were used at room temperature for 40 min (cat. no. K8002; ready-to-use; DAKO; Agilent Technologies, Inc.), followed by detection with DAB and DAB+ Chromogen Solution (cat. no. K4065; diluted according to manufacturer's instructions; DAKO, Agilent Technologies, Inc.). For EGFR and CK, ultraView Universal DAB Detection Kit combined with ultraView Kit+ amplification (cat. nos. 760-500 and 760-080, respectively; Roche Diagnostics) and OptiView DAB IHC Detection Kit (cat. no. 760-700; Roche Diagnostics), respectively, were applied for detection.

In addition, separate sections were also stained with H&E for a 60 min protocol including deparaffinization, dehydration as described above, then stained in hematoxylin (cat. no. 854183; Capital Region, Hospital Farmacy) for five minutes, rinsed and stained with eosin (cat. no. 854653; Capital Region, Hospital Farmacy) for three minutes at room temperature. Digital images of all IHC slides were acquired using a Axio Scan.Z1 (Carl Zeiss AG) brightfield microscope slide scanner.

Expression analysis and scoring system. Five slides from each individual tumor were reviewed and scored by two experienced head and neck pathologists (KK and GL) blinded to the clinical dataset. In case of any discrepant assessment of IHC target expression, individual slides would be reviewed together to produce a consensus score. For each tumor, the extent of tumor compartmentalization and differentiation was evaluated using H&E and CK staining images. For uPAR, TF and EGFR respectively, IHC staining was semi-quantitatively assessed for intensity (I-score) and proportional expression (P-score; that is, the proportion of cells with positive biomarker expression) within the tumor compartment by assessment of the whole tumor section at lowest magnification. The I-score was stratified as 0-3 (none, weak, moderate and strong intensity, respectively) whereas the P-score was stratified as 0-3 (0-10, 11-50, 51-75 and 76-100%, respectively). The P-score was estimated based on the observed target expression on tumor cells, stromal cells and inflammatory cells within the tumor compartment. By adding the I-score and P-score, a composite

semi-quantitative score (PI-score) was calculated, as proposed by Allred *et al* (27). The PI-score is a 7-point system stratified from 0 to 6. If present, IHC expression in adjacent non-cancerous tissues would also be qualitatively characterized. To estimate the expression rate of uPAR, TF and EGFR within the cohort, expression would be considered positive if the PI-score was >1. To assess the possible prognostic value of the three targets, the PI-score was dichotomized into high and low expression based on the mean value for each target for association analyses of associations. PI-scores <3 was used for the dichotomization of uPAR and TF, whereas PI-scores <4 was used for EGFR.

Statistical analysis. For associations between biomarker expression and each of the clinicopathological variables, Pearson's χ^2 test or Fisher's exact test (for low number of events) was applied; for age comparison, an unpaired t-test was performed. For survival statistics, overall survival (OS) was defined as time from the date of diagnosis until death of any cause. Recurrence-free survival (RFS) was defined as time from the date of diagnosis until the date of recurrence confirmed by biopsy, death by any cause. Kaplan-Meier curves were applied for survival plots and 5-year survival estimates, where log-rank test was used for comparison between the groups. Using the Cox proportional hazards model, univariate and multivariate estimates of hazard ratios (HRs) were calculated for OS and RFS following adjustment for sex, age, smoking status, HPV status, tumor location, T-site, T-stage, N-stage, UICC8 TNM-stage, tumor differentiation and biomarker expression. P<0.05 was considered to indicate a statistically significant difference. Data analysis was performed in the SAS software package, version 6.1 (SAS Institute, Inc.) and R statistics (version 3.6.1).

Results

Basal patient characteristics. In this cohort of 93 patients, the median age at diagnosis was 60 years (range, 40-84 years), where 69 of the patients (74%) were men. The median follow-up calculated from the day of diagnosis was 4.8 years (range, 0.03-14.2 years). The tumor subsites were palatine tonsils (75%), base of the tongue (13%), pharyngeal wall (9%) and soft palate (3%). At follow up, January 2020, 51 patients were alive, and 42 patients were deceased, 25 (27%) of whom succumbed to OPSCC. During follow-up, 19 (20%) patients had recurrence confirmed by biopsy after primary treatment. The median time to recurrence was 9 months.

Target expression analysis. The positive expression rates in tumors based on the PI-score (PI>1) for uPAR, TF and EGFR were calculated to be 98.9, 76.3 and 98.9%, respectively. For the I-score, positive staining (I>0) for uPAR, TF and EFGR was present in 100, 89.3 and 100% of the specimens. The exact extension of the tumor compartment, and the margin demarcation from adjacent non-cancerous tissues were evaluated on the H&E and CK stainings and then compared to the topographic distribution of the IHC stainings for each of the three biomarkers. For uPAR a tumor-specific staining pattern was observed, with enhanced expression within the tumor compartment but limited or absent expression in the
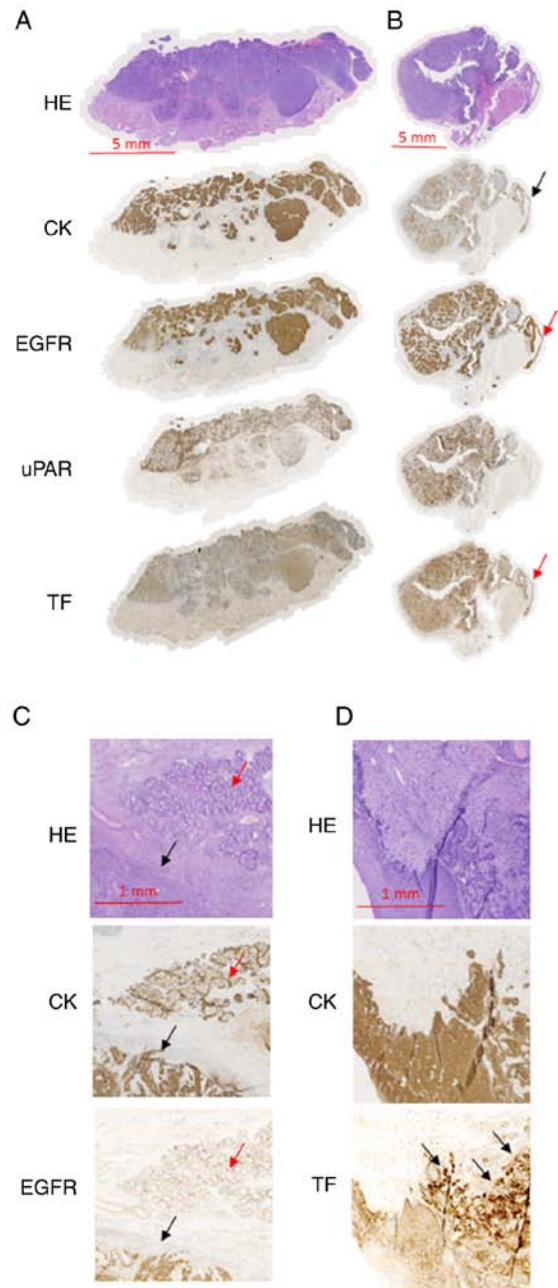

Figure 1. Biomarker expression patterns. Representative images of adjacent sections from four different tumor resection specimens from the palatine tonsil. Staining for H&E and CK shows the outlining of the tumor compartment. (A) In tumor A, tumor-specific expression pattern is seen for all three targets, EGFR, uPAR and TF with an accurate demarcation of the cancerous tissue and absence of staining in the adjacent normal tissues when compared with the H&E and CK stainings. (B) In tumor B, normal epithelium could be seen on the right side of the section, which stained strongly positive for CK (black arrow) and positive staining was also observed for EGFR and TF (red arrows). (C) An example of salivary duct tissues (red arrow) present adjacent to the tumor margin (black arrow). Positive staining in salivary tissue is seen for both CK and EGFR. (D) An example of intense staining of TF at the invasive front of the tumor (black arrow). C and D demonstrate the regular presence of EGFR and TF expression outside the tumor compartment, which may render them inappropriate for utilization for cancer imaging. CK, cytokeratin; TF, tissue factor; uPAR, urokinase-type plasminogen activator receptor.

adjacent non-cancerous tissues (Fig. 1). In the tumor compartment, membranous uPAR staining was seen on tumor cells as well as on stromal cells. Tumor-associated stromal uPAR

Table II. Cox proportional hazards model on survival outcome from univariate and multivariate statistics.

Clinicopathological characteristic	Overall survival				Recurrence-free survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (CI)	P-value	HR (CI)	P-value	HR (CI)	P-value	HR (CI)	P-value
Sex								
Female	Ref.		Ref.		Ref.		Ref.	
Male	1.03 (0.51-2.05)	0.94	1.16 (0.46-2.91)	0.75	0.91 (0.33-2.54)	0.86	0.75 (0.18-3.05)	0.69
Age								
Risk per 1-year increase in age	1.06 (1.03-1.10)	<0.01	1.08 (1.03-1.13)	<0.01	1.05 (1.00-1.10)	0.05	1.05 (0.98-1.13)	0.14
Tumor location								
Palatine and lingual tonsils	Ref.		Ref.		Ref.		Ref.	
Other	1.57 (0.70-3.54)	0.28	1.51 (0.48-4.79)	0.48	0.41 (0.06-3.09)	0.39	0.51 (0.05-5.20)	0.57
Human papillomavirus status								
Positive	Ref.		Ref.		Ref.		Ref.	
Negative	4.23 (2.08-8.63)	<0.01	5.48 (1.11-27.05)	0.04	1.73 (0.70-4.32)	0.24	1.31 (0.17-10.21)	0.8
Smoking status								
Never smoker	Ref.		Ref.		Ref.		Ref.	
Previous or current smoker	2.29 (0.82-6.43)	0.11	0.95 (0.24-3.81)	0.94	1.96 (0.45-8.48)	0.37	5.21 (0.44-62.31)	0.19
T-stage								
T1	Ref.		Ref.		Ref.		Ref.	
T2	0.80 (0.33-1.91)	0.61	2.11 (0.60-7.38)	0.24	0.87 (0.23-3.29)	0.84	0.99 (0.21-4.76)	0.99
T3	0.26 (0.05-1.23)	0.09	0.73 (0.12-4.23)	0.72	0.67 (0.11-4.01)	0.66	0.68 (0.06-7.09)	0.75
T4	3.17 (1.28-7.85)	0.01	2.64 (0.66-10.56)	0.17	3.54 (0.87-14.38)	0.08	10.27 (0.50-210.62)	0.13
N-stage								
N0	Ref.		Ref.		Ref.		Ref.	
N1	0.72 (0.33-1.57)	0.41	0.93 (0.31-2.78)	0.9	1.19 (0.37-3.86)	0.78	1.23 (0.22-6.81)	0.81
N2	1.42 (0.63-3.19)	0.39	0.73 (0.20-2.66)	0.64	1.58 (0.42-5.87)	0.5	1.56 (0.24-10.07)	0.64
N3	3.72 (1.02-13.56)	0.05	2.57 (0.42-15.74)	0.31	3.22 (0.36-28.83)	0.3	1.79 (0.09-33.93)	0.7
UICC8 TNM stage								
1	Ref.		Ref.		Ref.		Ref.	
2	0.60 (0.19-1.91)	0.39	0.19 (0.03-1.15)	0.07	1.00 (0.24-4.17)	1	0.73 (0.10-5.25)	0.75
3	3.21 (1.35-7.62)	<0.01	1.68 (0.36-7.84)	0.51	3.71 (1.17-11.70)	0.03	2.64 (0.28-24.56)	0.4
4	4.45 (2.03-9.76)	<0.01	0.95 (0.12-7.44)	0.96	2.17 (0.58-8.12)	0.25	0.15 (0.00-7.73)	0.35
Urokinase-type plasminogen activator receptor								
Low	Ref.		Ref.		Ref.		Ref.	
High	2.01 (0.92-4.37)	0.07	0.87 (0.32-2.31)	0.77	1.20 (0.43-3.34)	0.72	0.88 (0.27-2.91)	0.83

Table II. Continued

| Clinicopathological characteristic | Overall survival | | | | Recurrence-free survival | | | |
| | Univariate | | Multivariate | | Univariate | | Multivariate | |
	HR (CI)	P-value	HR (CI)	P-value	HR (CI)	P-value	HR (CI)	P-value
Tissue factor								
Low	Ref.		Ref.		Ref.		Ref.	
High	0.75 (0.38-1.50)	0.42	1.17 (0.50-2.76)	0.72	1.60 (0.64-3.97)	0.31	2.39 (0.67-8.51)	0.18
EGFR								
Low	Ref.		Ref.		Ref.		Ref.	
High	1.37 (0.72-2.61)	0.34	1.55 (0.73-3.28)	0.25	1.33 (0.52-3.37)	0.55	2.11 (0.68-6.53)	0.19

HR(CI), hazard ratio (95% confidence interval); Ref, reference (equal to 1).

expression was also observed on macrophages, neutrophils, fibroblasts. At the mucosal margin, there was a sharp demarcation between carcinoma and adjacent epithelium, with the absence of uPAR expression in the unaffected epithelial lining. At the deep margins, uPAR staining delineated the tumor compartment. In addition, in the majority of cases, a narrow rim of expression was present in the stroma at the invasive border. Intense uPAR staining was frequently observed at the invasive front of the tumor and in the microscopic tumor buds separated from the primary tumor. In 10 (11%) of the specimens, uPAR positivity was also found on neutrophilic granulocytes, where pronounced inflammatory infiltration or abscess formation was discovered outside the tumor compartment.

TF demonstrated a tumor-specific expression pattern when positive, though in some cases adjacent normal epithelium also demonstrated weak positivity. As a general pattern, more intense TF staining was typically seen at the invasive front at the deep margin (Fig. 1).

For EGFR, strong and homogeneous expression was observed in the tumor compartment, but high expression could also be observed in the normal mucosal epithelium (Fig. 1). EGFR expression in the salivary tissue was consistently positive in the ductal epithelium (Fig. 1).

Survival and recurrence analysis. A Cox proportional hazards univariate and multivariate analysis was performed for RFS and OS (Table II). Age, HPV status and UICC8 TNM stage were identified to be independent prognostic factors for OS according to the univariate analysis, but only age and HPV status maintained significance in the multivariate analysis (Table II). Only increase in TNM staging for more advanced stage 3 and 4 disease was associated with RFS according to univariate analysis. For the three biomarkers, high uPAR PI-scores had a non-significant trend towards reduced OS according to univariate analysis only (Table II). In addition, Kaplan-Meier survival curves were generated with univariate log-rank tests performed to assess the association of the PI-scores of the three biomarkers with the OS and RFS (Fig. 2). For the entire cohort, the 5-year OS and RFS was 59 and 76%, respectively. For HPV-positive and HPV-negative patients, the respective 5-year OS was 77 and 41%, whereas the respective 5-year RFS was 81 and 67%. For patients with a high and low PI-scores for uPAR, the 5-year OS was 57 and 68%, respectively, whereas the RFS was 74 and 79% for patients with high and low PI-scores, respectively (Fig. 2). In addition, separate tests were performed for the P-score and I-score but no statistically significant association with OS or RFS could be detected (data not shown).

Biomarker association analysis. Possible associations between clinicopathological parameters and high or low PI-scores of uPAR, EGFR and TF were next investigated by univariate analysis (Table III). No statistically significant relationships were be detected.

Discussion

The present study investigated the expression patterns and applicability of three candidate receptor targets in
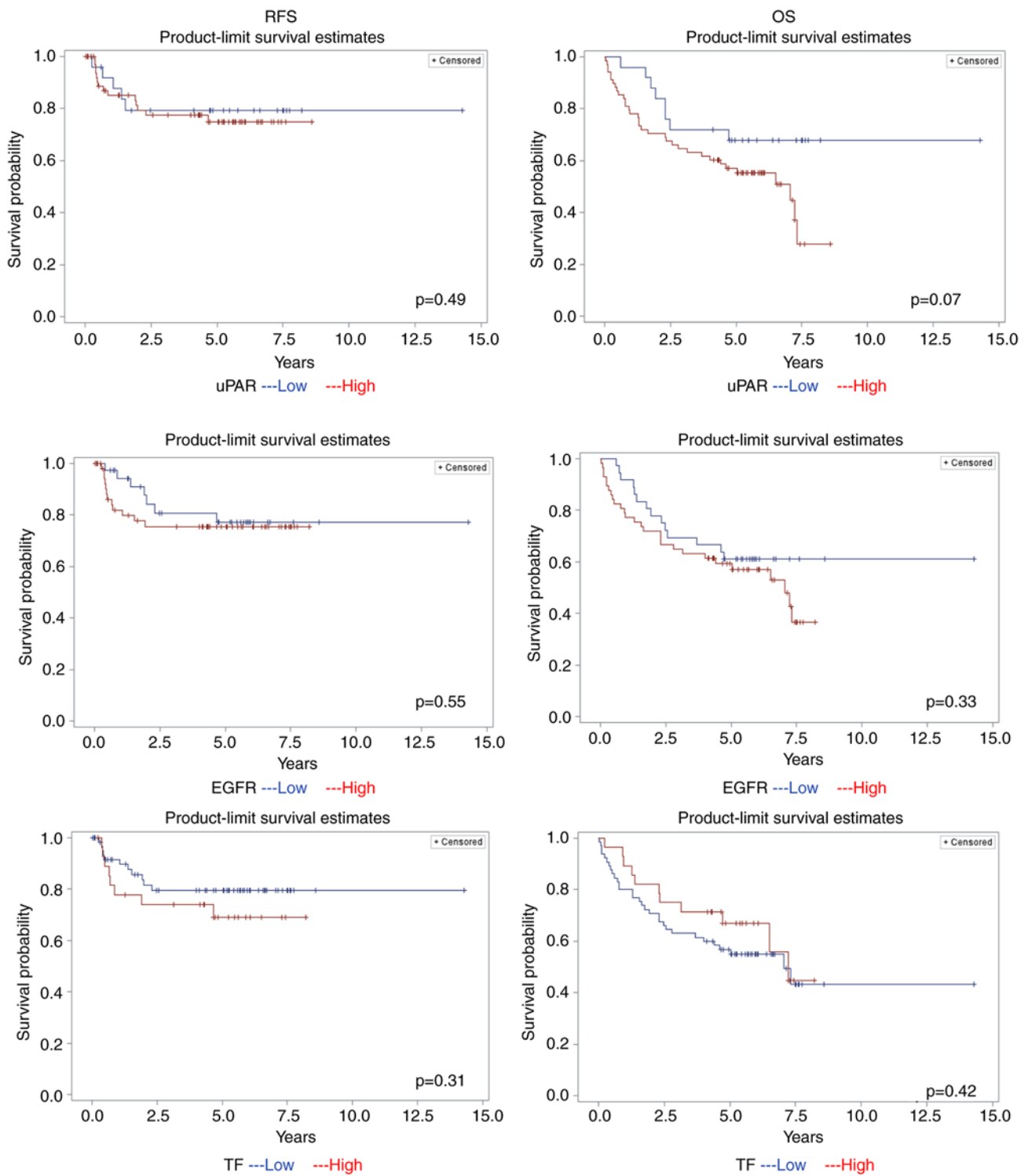
Figure 2. Survival analysis following stratification by biomarker expression. Kaplan-Meier survival curves showing OS and RFS after stratification by high and low expression levels of EGFR, uPAR and TF. OS, overall survival; RFS, recurrence-free survival; TF, tissue factor; uPAR, urokinase-type plasminogen activator receptor.

OPSCC for targeted molecular imaging based on IHC staining in large biopsy or tumor resection specimens. To the best of our knowledge, such topographic data on uPAR and TF expression for this large sub-site type of HNSCC has not been previously reported. Expression of EGFR in OPSCC has been previously described in a number of other studies (28-30) An important finding from the present study was that uPAR in particular appeared to be a promising

target in OPSCC due to its highly tumor-specific expression profile with limited expression in the non-cancerous regions adjacent to the tumor compartment. Although a tumor-specific expression pattern could also be observed for TF, the positive expression rate in the whole cohort was lower (76%). EGFR exhibited a high positive expression rate and a homogeneous expression pattern within the tumor compartment, equivalent to high receptor densities, rendering it a

Table III. Univariate analysis of the level of biomarker expression and clinicopathological characteristics. Biomarker expression based on PI-score.

Clinicopathological characteristic	Urokinase-type plasminogen activator receptor			Tissue factor			EGFR		
	High, n (%)	Low, n (%)	P-value	High, n (%)	Low, n (%)	P-value	High, n (%)	Low, n (%)	P-value
Sex									
Male	51 (55)	18 (19)		22 (24)	47 (51)		42 (45)	27 (29)	
Female	17 (18)	7 (8)	0.77	6 (6)	18 (19)	0.53	15 (16)	9 (10)	0.89
Age			0.29			0.44			0.37
Tumor location									
Pharynx wall	1 (1)	7 (7)		8 (9)	0 (0)		3 (3)	5 (5)	
Palatine tonsils	18 (19)	52 (56)		48 (52)	22 (24)		26 (28)	44 (47)	
Lingual tonsils	4 (4)	8 (9)		7 (8)	5 (5)		7 (7)	5 (5)	
Soft palate	2 (2)	1 (1)	0.31	2 (2)	1 (1)	0.23	0 (0)	3 (3)	0.27
Human papilloma virus-DNA									
Positive	35 (49)	15 (16)		16 (17)	32 (34)		28 (30)	20 (22)	
Negative	35 (38)	10 (11)	0.33	12 (13)	33 (35)	0.48	29 (31)	16 (17)	0.55
Smoking status									
Never	27 (29)	8 (9)		14 (15)	21 (23)		21 (23)	14 (15)	
Ever	41 (44)	17 (18)	0.5	14 (15)	44 (47)	0.11	39 (63)	22 (24)	0.84
Tumor differentiation									
G1-G2	17 (18)	9 (10)		6 (6)	20 (22)		12 (13)	14 (15)	
G3-G4	51 (55)	16 (17)	0.29	22 (24)	45 (48)	0.36	45 (48)	22 (24)	0.06
T-stage									
T1-T2	42 (45)	19 (20)		17 (18)	44 (47)		61 (66)	26 (28)	
T3-T3	26 (28)	6 (6)	0.2	11 (12)	21 (23)	0.52	22 (24)	11 (28)	0.28
N-stage									
N0	16 (17)	8 (9)		6 (6)	18 (19)		15 (16)	9 (10)	
N+	52 (56)	17 (18)	0.41	22 (24)	47 (51)	0.53	42 (45)	27 (29)	0.89
TNM stage									
S1-S2	39 (42)	17 (18)		21 (23)	35 (38)		34 (37)	22 (24)	
S3-S4	29 (31)	9 (32)	0.35	7 (8)	30 (32)	0.06	23 (25)	14 (15)	0.89
Recurrence									
No	54 (58)	20 (22)		20 (22)	54 (58)		45 (48)	29 (31)	
Yes	14 (15)	5 (5)	0.95	8 (9)	11 (12)	0.2	12 (13)	7 (8)	0.85

Pearson's χ^2 or Fisher's exact test (for low numbers) was used, except for age (as a continuous variable), in which an unpaired t-test was performed.

potential marker for targeted strategies. However, EGFR did not show a tumor-specific expression pattern, instead having regular expression levels in the adjacent normal epithelium and salivary gland tissues. Therefore, EGFR may be a less attractive target for molecular imaging. Our group previously performed a similar study of the expression of uPAR, TF and EFGR in OSCC (31); in comparison, the microscopic expression patterns and tumor-specificity of these three biomarkers appears very similar to the findings in OPSCC. This may indicate that individual targeted molecular strategies against either uPAR, TF or EGFR may be applied in OSCC and OPSCC, and possibly in HNSCC in general, which expands the utility of individual-targeted imaging agents or therapeutics.

It has been reported previously that targeted intraoperative optical imaging allows for a lower detection threshold for tumor deposits in the μm-range (10). Therefore, it is highly likely that this technology can be used to guide resection with adequate margins using biomarkers with tumor-specific expression patterns. In a clinical study using optical imaging in HNSCC by coupling with optically labeled antibodies against EGFR, Gao *et al* (32) previously demonstrated the potential use of targeted optical imaging for margin evaluation in the post-operative histopathological work-up. In addition, this

previous study also reported tracer uptake in non-cancerous tissues outside the tumor compartment, such as the salivary gland tissues (32). The challenge of EGFR expression in normal tissues compared with targeted tumor imaging has been discussed by several previous studies, which underlines the need for exploring novel biomarkers in different types of cancer that exhibit highly tumor-specific expression (33-35)

The identification of biomarkers for targeting purposes with a high positive expression rate is of importance for ensuring applicability and reproducibility in highly heterogenous populations. Van Oosten *et al* (8) previously suggested a lower expression rate of 80% for qualifying a given biomarker as clinically relevant for a targeted imaging strategy. Collecting preoperative biopsies for assessing biomarker expression in individual patients, which is already performed during immunotherapeutic procedures, is another alternative to imaging (36). However, it is a less feasible strategy if the targeted imaging of biomarkers with lower positive expression rates is anticipated. Baart *et al* (33) proposed the simultaneous use of several targeting agents against different biomarkers to achieve maximal agent-to-target binding in the same patient.

The role of the PA system in HNSCC has been previous reported, especially for OSCC and to a lesser extent for laryngeal SCC (37,38) However, it has not been previously described for OPSCC (37,38). In addition, little is known about TF in HNSCC, though a tumor-specific expression pattern in OSCC, very similar to the expression pattern found in this study, has been reported previously by our group (31). Since the mucosal lining in both the oral cavity and the pharynx is non-keratinizing squamous epithelium, it is likely that the same biomarker expression profiles are present in both locations (39). Depending on the IHC scoring system used, the positive expression rate of uPAR in OSCC has been reported to be between 39 and 100% (38,40) which is in line with a positivity rate of 98.9% in the present study. The positive expression rate for TF in OSCC was reported to be 58% (31), which is lower compared with the positive expression rate of 76.9% in OPSCC seen in the current study.

The present study did not detect an association between the expression levels of uPAR, TF and EFGR with HPV status, where the microscopic distribution of these three biomarkers did not reveal any differences between sections with and without HPV. It is well documented that HPV-positive and -negative tumors are two distinctly different diseases in terms of pathogenesis and prognosis, such that the carcinogenesis process in the presence of HPV infection can interact with the immune (41,42) Both uPAR and TF have been reported to serve a role in immunological regulation and responses in the tumor microenvironment (17,23) However, the expression of only three receptors within a highly complex biological system was studied in the present study, which may explain why no relationship with HPV status could be observed.

In the present cohort, HPV-positive patients had significantly more favorable survival outcome. In the survival analysis, the level of uPAR, TF and EGFR expression did not significantly associate with any of the survival outcomes tested. Only high uPAR expression had a non-significant trend towards reduced OS. Other previous studies of OSCC and other types of cancer, like breast or bladder cancer, have found that high uPAR or uPA expression significantly associated with reduced survival and more aggressive histopathological tumor physiology (43,44) A possible reason for the lack of prognostic significance in terms of uPAR in OPSCC expression in the present study may be the small sample size and the inherent lack of granularity and accuracy in the IHC-based scoring. Nevertheless, these methodological shortcomings should equally influence all three molecular targets. Therefore, they indicate that of the three markers tested, uPAR is the most prognostic. However, the present study was limited by a relatively small sample size to perform robust association analyses on survival statistics.

In conclusion, results from the present study suggest that uPAR, TF and EGFR are suitable targets for molecular imaging and therapy in OPSCC. uPAR in particular may be an attractive target owing to its highly tumor-specific expression pattern and association with prognosis. For TF, preselection of patients may be necessary due to a lower positivity rate.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AC and CG designed the study in collaboration with KK, GL, KJ, BWC, JM, AK and CvB. CG and JSJ collected tissue specimens and established the dataset. AC and CG have assessed the authenticity of the raw data. KK, GL and KJ conducted the immunohistochemistry. KK and GL performed the histopathological scoring. AC, CG and JSJ performed the statistical data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Approval for this study was obtained from the Ethical Committee of the Capital Region of Denmark (protocol H-15016322; Copenhagen, Denmark). The Dataset was anonymized prior to analysis. The Ethical Committee waived the need to obtain consent to participate from the patients due to the circumstance that a great proportion of the cohort had succumbed to disease or were severely ill, where revealing that information about this project would lead to more distress in the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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