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Original contribution

Tumor-stroma ratio is a promising prognostic classifier in oropharyngeal cancer☆,☆☆

Alhadi Almangush DDS, PhD [a,b,c,d,*], Lauri Jouhi MD, PhD [e], Caj Haglund MD, PhD [f,g], Jaana Hagström DDS, PhD [h,a,i], Antti A. Mäkitie MD, PhD [e,b,j], Ilmo Leivo MD, PhD [k,l]

[a] *Department of Pathology, University of Helsinki, FI-00014 Helsinki, Finland*
[b] *Research Program in Systems Oncology, Faculty of Medicine, University of Helsinki, Helsinki, Finland*
[c] *Department of Pathology, University of Turku, Turku, Finland*
[d] *Faculty of Dentistry, Misurata University, Misurata, Libya*
[e] *Department of Otorhinolaryngology - Head and Neck Surgery, University of Helsinki and Helsinki University Hospital, FI-00029 HUS, Helsinki, Finland*
[f] *Research Programs Unit, Translational Cancer Medicine, University of Helsinki, 00014, Helsinki, Finland*
[g] *Department of Surgery, University of Helsinki and Helsinki University Hospital, Helsinki, Finland*
[h] *Department of Oral Pathology and Radiology, University of Turku, Turku University Hospital, Turku, Finland*
[i] *Research Programs Unit, Translational Cancer Medicine, University of Helsinki, Helsinki, Finland*
[j] *Division of Ear, Nose and Throat Diseases, Department of Clinical Sciences, Intervention and Technology, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden*
[k] *Institute of Biomedicine, Pathology, University of Turku, 20520 Turku, Finland*
[l] *Turku University Central Hospital, 20521 Turku, Finland*

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Summary Tumor-stroma ratio (TSR) has been analyzed in many tumor types. To date, the clinical significance of TSR has not been investigated in oropharyngeal squamous cell carcinoma (OPSCC). We used a recently introduced recommendation for the assessment of TSR in a large cohort of 182 patients with OPSCC treated at the Helsinki University Hospital. The percentage of tumor-associated stroma was estimated in hematoxylin and eosin (HE)–stained sections and categorized into 2 groups: "stroma-high" (>50%) and "stroma-low" (≤50%). In multivariable analysis, TSR had a significant association with patient survival as stroma-high tumors showed worse disease-free survival (hazard ratio [HR] = 3.22, 95% confidence interval [CI] = 1.43-7.26, P = .005),

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* Corresponding author. Department of Pathology, University of Helsinki, Haartmaninkatu 3 (P.O. Box 21), FIN-00014, Helsinki, Finland.
E-mail address: alhadi.almangush@helsinki.fi (A. Almangush).

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disease-specific survival (HR = 2.48, 95% CI = 1.29-4.74, $P = .006$), and overall survival (HR = 2.23, 95% CI = 1.29-3.85, $P = .004$). The prognostic value of TSR was superior to the Tumor-Node-Metastasis classification. In addition, the significant prognostic value of TSR was demonstrated when analyzing human papillomavirus (HPV)−positive and HPV-negative cases separately ($P < .05$). In conclusion, TSR is a powerful prognostic indicator in OPSCC. It can be assessed quickly without additional costs using standard HE slides. Owing to its simplicity and reproducibility, TSR can be implemented in routine pathology diagnostics and reporting. Patients with stroma-rich tumors have an increased risk of recurrence and cancer-related mortality and may benefit from appropriate intensive treatment strategies with close follow-up.

1. Introduction

The rising incidence of oropharyngeal squamous cell carcinoma (OPSCC), particularly in Western societies, is largely driven by human papillomavirus (HPV) infection. This increase is expected to continue in the near future, although vaccinations may eventually interfere with such development [1]. Fortunately, the survival rate of OPSCC is clearly better among patients with HPV-positive tumors. However, it must be remembered that the survival of HPV-positive patients may also be negatively influenced by other factors, such as smoking. Thus, there are a notable number of OPSCC patients with poor survival, and this indicates a need for reliable classifiers to accurately predict tumor behavior independent of the HPV status. This would allow for the selection of the best options during personalized treatment planning.

The Tumor-Node-Metastasis (TNM) classification is the mainstay in decision making of many cancers including OPSCC. However, shortcomings have been reported in the TNM staging of OPSCC [2]. To empower the TNM staging, powerful prognostic parameters/biomarkers have been incorporated into the TNM classification of some tumor types, including tumors of the head and neck [3]. Of note, none of these parameters were related to stromal characteristics. Similarly, the current World Health Organization histologic grading of OPSCC categorizes tumors without assessing their stromal compartment [4], and thus stroma is not considered in clinical decision making.

Tumor-stroma ratio (TSR) is defined as the proportion of the area of remodeled stroma associated with tumor islands related to the total area of the tumor [5−9]. Stromal cells of the tumor microenvironment consist of non-neoplastic cells, including fibroblasts, endothelial cells, and immune cells embedded in the extracellular matrix [10]. Activities of these cells may reportedly promote cancer recurrence and metastasis [11]. In addition, stromal cells, together with cancer cells, have an essential role in determining the composition of immune cell infiltrates in tumors [12]. A growing body of evidence emphasizes the significance of tumor stroma in the behavior and prognostication of many malignancies [13,14], including head and neck cancers [5].

Up to date, no criteria for the evaluation of stromal characteristics have been included in the pathology reports of OPSCC. It remains of high clinical relevance to assess the prognostic significance of the information provided by stromal parameters in OPSCC. Here, for the first time, we analyze the significance of TSR in a large cohort of OPSCC using hematoxylin and eosin (HE)−stained sections.

2. Materials and methods

The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of the Helsinki University Hospital (HUS). A total of 331 patients treated for primary oropharyngeal cancer at the HUS (Helsinki, Finland) during the years 2000-2009 were considered in this study. We excluded 149 patients who had concurrent head and neck cancers, had received palliative treatment, had had earlier treatments for head and neck cancer, had histologies other than SCC, or whose tumor tissues were not available. This left a total of 182 cases of OPSCC that were eligible for analysis of TSR. In included cases, samples were retrieved before radiotherapy or chemoradiotherapy in 180 cases, while samples of 2 cases were available as post-treatment specimens only. In all cases, we evaluated the resection specimen except in 15 cases where we used preoperative representative biopsies due to the unavailability of post-operative samples. A combination of HPV in situ hybridization (ISH) and p16 immunohistochemistry was used to classify the tumors as either HPV positive or HPV negative, as described in our previous study [15]. The Ventana Inform HPV ISH assay was carried out with a high-risk HPV probe detecting high-risk HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66, and iVIEW Blue detection kit in Benchmark XT series Stainer (Tucson, Arizona). For this assay, we used 5-μm-thick tissue sections and an extended Ventana cell conditioning solution (CC2) and pretreatment with ISH protease 3 (incubation time 32 min). The tumor was considered HPV positive if tumor

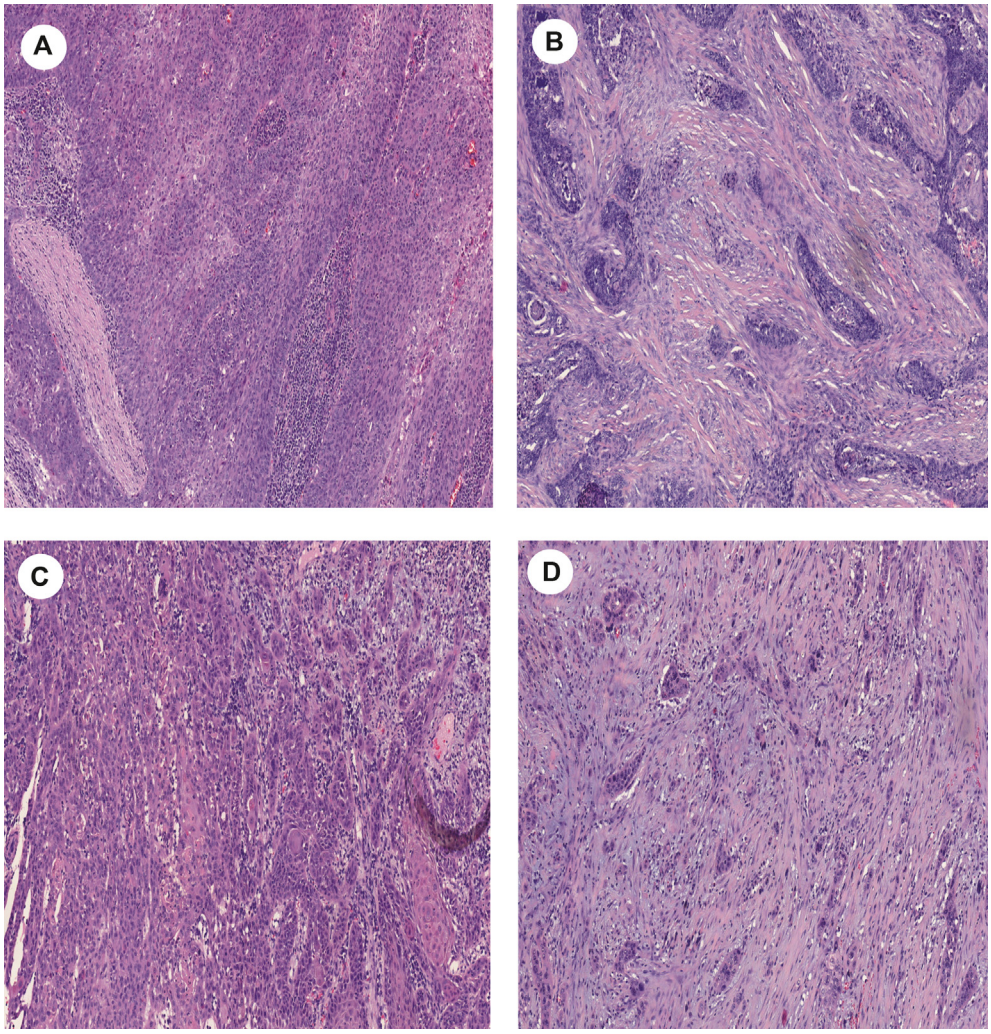

Fig. 1 Tumor-stroma ratio scores in oropharyngeal cancer (10× magnification). A, Human papillomavirus (HPV)-positive tumor with stroma-low score. B, HPV-positive tumor with stroma-high score. C, HPV-negative tumor with stroma-low score. D, HPV-negative tumor with stroma-high score.

cell nuclei were positive using ISH. For p16 immunohistochemistry, the primary antibody was monoclonal mouse antihuman p16INK4a (9517 CINtec Histology Kit, MTM laboratories, Germany). The tumor was considered p16 positive when more than 70% of the tumor cells showed strong nuclear and cytoplasmic immunopositivity.

We used a conventional light microscope to evaluate TSR. Initially, areas with the highest amounts of tumor-associated stroma were selected using low magnification (×5 objective). Then higher magnification (×10 objective) was used to evaluate the amount of tumor-associated stroma in a selected field that had cancer cells present on the four sides of the microscopic field [16,17]. The percentage of stroma in the selected field compared with the cancer cell component was estimated at 10% intervals (ie, 10, 20, 30% … etc.). Tumors showing >50% tumor-associated stroma were classified as "stroma-high," while tumors with ≤50% stroma were considered as "stroma-low", as previously described [16,17] and presented in Fig. 1. In case of heterogeneity of the TSR (ie, a tumor having both stroma-high and stroma-low areas), the stroma-high areas were deemed as decisive, as previously advised [16]. This means that if only one microscopic field presented as stroma-high, this field was considered decisive. Areas of necrosis, preexisting lymphoid tissue, or any other normal structures were left out of the microscopic field. If this was not possible, these irrelevant tissues (ie, necrotic tumor, lymphoid stroma, etc.) were excluded when estimating tumor-associated stroma.

A training session was arranged for the assessment of TSR by an experienced head and neck pathologist (I.L.) familiarized with the scoring criteria and the method. Two

observers (A.A. and I.L.) blinded to clinicopathologic information then scored the cases. Furthermore, a consensus agreement review session between the observers was conducted to agree on tumors with TSR % at the borderline cut-off point (ie, around 50%).

2.1. Statistical analysis

We used cross-tabulation and χ^2 test to analyze the relationship between TSR and clinicopathologic features. A Kaplan–Meier estimate and log-rank test were used for survival analyses. Univariable and multivariable analyses were conducted using Cox regression. Hazard ratio (HR), 95% confidence interval (CI), and *P* were reported in both analyses. Disease-free survival was measured from the completion of primary treatment until any recurrence or last follow-up. Overall survival was measured from the completion of primary treatment to death of any cause or last follow-up. Disease-specific survival was measured from the completion of primary treatment to death of disease or last follow-up. All statistical analyses were performed using IBM SPSS Statistics (version 28).

3. Results

The median follow-up time was 4.48 years (range, 3.51–5.00 years). There were 140 (76.9%) men and 42 (23.1%) women. Interobserver variability between the observers was measured with the kappa coefficient test, and there was a good agreement between the observers ($\kappa = 0.752$, $P < .001$). There was an association between TSR and the T classification ($P = .017$). However, no significant association ($P > .05$) was found between TSR and other clinicopathologic factors, including gender, tumor grade, HPV status, N classification, and the applied treatment (Table 1).

Kaplan–Meier survival curves showed a significant prognostic value for TSR in disease-free survival, disease-specific survival, and overall survival ($P < .001$, Figs. 2A, 3A, and 4A). Patients with stroma-high tumors showed a significantly worse survival. For disease-free survival, TSR remained statistically significant in both the HPV+ cases ($P = .005$, Fig. 2B) and the HPV− cases ($P = .024$, Fig. 2C). Similarly, for disease-specific survival, TSR was a significant prognosticator in both the HPV+ cases ($P = .006$, Fig. 3B) and HPV− cases ($P = .022$, Fig. 3C). In overall survival, TSR was again a significant prognosticator in both the HPV+ ($P = .03$, Fig. 4B) and HPV− cases ($P = .005$, Fig. 4C).

Cox regression analysis of the univariable analysis (Table 2) showed that TSR was associated with disease-free survival (HR 3.49, 95% CI 1.66-7.35, $P < .001$), disease-specific survival (HR 2.80, 95% CI 1.54-5.09, $P < .001$), and overall survival (HR 2.46, 95% CI 1.50-4.04, $P < .001$). In multivariable modeling (Table 2), in the stroma-high versus stroma-low cases, TSR was significantly associated with disease-free survival with an HR of 3.22 (95% CI 1.43-7.26, $P = .005$) and with disease-specific survival with an HR 2.48 (95% CI 1.29-4.74, $P = .006$). The other prognostic parameters, including TNM stage, did not reach statistical significance in multivariable analyses (Table 2). Similarly, in overall survival (Table 2), TSR was a dominant prognostic parameter with an HR of 2.23 (95% CI 1.29-3.85, $P = .004$). These results from multivariable analyses indicate that TSR is a prognostic parameter independent of the routinely considered parameters, including tumor stage and HPV status.

4. Discussion

The prominent presence of tumor-associated stroma is one of the hallmarks of cancer progression, and it can be assessed simply by evaluating TSR in HE-stained sections. Tumor-associated stroma has been associated with aggressive behavior in many solid tumors [18–23], including some types of head and neck cancer [5]. Notably, to date, no study has analyzed TSR in OPSCC. Here, we report for the first time on the prognostic significance of TSR in OPSCC.

It is well known that cancer cells are in close contact with stromal tissues of the tumor microenvironment which has modulatory roles in cancer behavior [24]. Mesenchymal stem cells are recruited into tumor stroma [25] and seem to be among key players in carcinogenesis. Interestingly, a co-injection of tumor cells and cancer-associated fibroblasts (which are among the main cellular components of the stroma) caused increased invasion and metastasis in vivo when compared to a co-injection of tumor cells and normal fibroblasts [25]. Furthermore, it has been speculated that tumors with a rich tumor-associated stroma may be influenced by stromal growth-promoting properties of various growth factors leading to more aggressive tumor behavior [7].

It is important to note that the prognostic impact of TSR in the present study was independent of the routinely considered parameters, as indicated by the multivariable analyses (Table 2), where TSR was included in the same Cox regression model as tumor stage and HPV status. Analysis of the results on disease-free, disease-specific, and overall survival (Table 2) indicated that TSR has a powerful prognostic value as demonstrated by hazard ratio, 95% CI, and *P* (Table 2). Specifically, patients with a stroma-rich tumor had a 3.22 times higher risk of recurrence and 2.48 times higher risk of OPSCC-related death compared with those who had a stroma-low tumor. Furthermore, the prognostic value of TSR was statistically significant when the cases were divided into HPV-positive and HPV-negative groups (Figs. 2B and C, 3B and C, and 4B and C). Indeed, the significance of HPV status as a prognosticator for

Table 1 Association between tumor-stroma ratio and clinicopathologic parameters of oropharyngeal squamous cell carcinoma.

Variable	Total	Tumor-stroma ratio		*P*
		Stroma-low	Stroma-high	
	N = 182	Number (%) 111 (61.0%)	Number (%) 71 (39.0%)	
Age				1.00
Young (<60 y)	101	62 (61.4%)	39 (38.6%)	
Old (≥60 y)	81	49 (60.5%)	32 (39.5%)	
Stage				.532
Early (I-II)	27	18 (66.7%)	9 (33.3%)	
Advanced (III-IV)	155	93 (60.0%)	62 (40.0%)	
Gender				.719
Male	140	84 (60.0%)	56 (40.0%)	
Female	42	27 (64.3%)	15 (35.7%)	
Alcohol intake				.547
No	53	35 (66.0%)	18 (34.0%)	
Previously	21	12 (57.1%)	9 (42.9%)	
Yes	33	18 (54.5%)	15 (45.5%)	
Smoking				.224
Never	20	12 (60.0%)	8 (40.0%)	
Former	46	32 (69.6%)	14 (30.4%)	
Currently	85	46 (54.1%)	39 (45.9%)	
T classification				**.017**
T1	35	29 (82.9%)	6 (17.1%)	
T2	68	41 (60.3%)	27 (39.7%)	
T3	40	22 (55.0%)	18 (45.0%)	
T4	39	19 (48.7%)	20 (51.3%)	
N classification				1.00
N0	35	21 (60.0%)	14 (40.0%)	
N+	147	90 (61.2%)	57 (38.8%)	
Grade				.100
I	15	11 (73.3%)	4 (26.7%)	
II	70	36 (51.4%)	34 (48.6%)	
III	97	64 (66.0%)	33 (34.0%)	
HPV status				.543
Positive	91	58 (63.7%)	33 (36.3%)	
Negative	91	53 (58.2%)	38 (41.8%)	
Tumor site[a]				.812
Anterior wall	56	31 (55.4%)	25 (44.6%)	
Lateral wall	104	66 (63.5%)	38 (36.5%)	
Posterior wall	3	2 (66.7%)	1 (33.3%)	
Superior wall	19	12 (63.2%)	7 (36.8%)	
Treatment				.524
Sx ± (C)RT	120	71 (59.2%)	49 (40.8%)	
(C)RT ± Sx	62	40 (64.5%)	22 (35.5%)	

Abbreviations: Sx, surgery; CRT, chemoradiotherapy; RT, radiotherapy; HPV, human papillomavirus.
Value in bold indicates that $P < 0.05$.
[a] The tumor sites were analyzed separately for the anterior wall (base of tongue and vallecula), lateral wall (palatine tonsils, tonsillar fossa, and tonsillar pillars), posterior wall, and superior wall (soft palate and uvula).

OPSCC is well documented in earlier studies and in the present one.

The usability of routine HE staining in the evaluation of TSR makes it easily applicable in pathology practice. Of note, we found a good level of agreement between the observers regarding the assessment of TSR. This is in line with the previously published studies on TSR, where a good to perfect interobserver agreement was repeatedly reported. For example, Vangangelt et al. [21] in their recent study on a large cohort of breast tumors and Lv et al. [18] in a study of hepatocellular cancer reported a Kappa value of 0.87. Similarly, Smit et al. [26] in a study of lung cancer

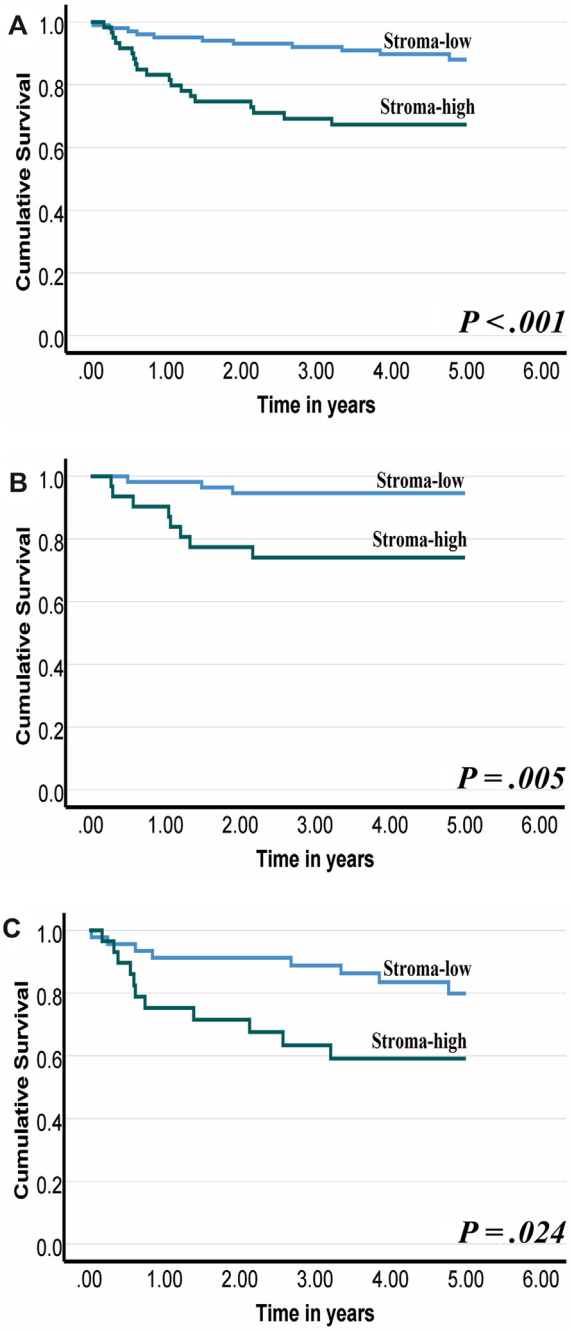

Fig. 2 Kaplan-Meier analysis of disease-free survival in oropharyngeal cancer patients with stroma-low tumors versus stroma-high tumors. A, All cases combined. B, Analysis of HPV-positive cases separately. C, Analysis of HPV-negative cases separately.

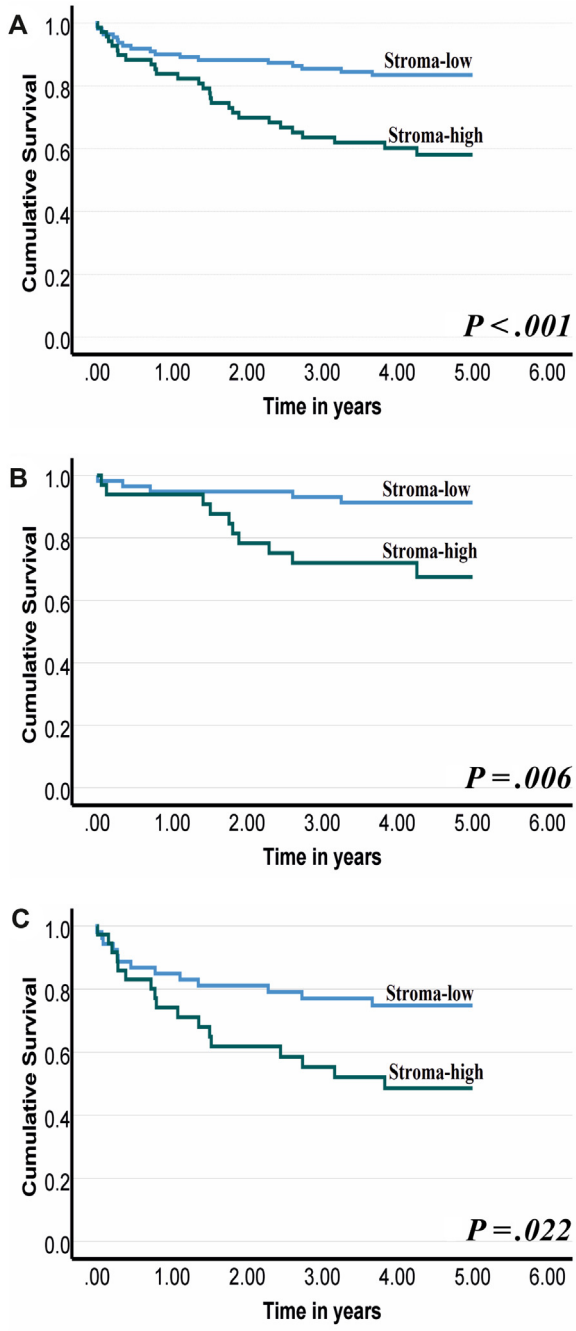

Fig. 3 Kaplan-Meier analysis of disease-specific survival in oropharyngeal cancer patients with stroma-low tumors versus stroma-high tumors. A, All cases combined. B, Analysis of HPV-positive cases separately. C, Analysis of HPV-negative cases separately.

reported a Kappa value of 0.75. Furthermore, Souza da Silva et al. [23] in a study of colorectal cancer and Zong et al. [22] in a study of cervical cancer reported a Kappa value of 0.81.

Interestingly, methods for standardized assessment of TSR have been recently proposed by van Pelt et al. for colon cancer [16] and by Hagenaars et al. [17] for breast cancer. Both articles describe guidelines that can be useful

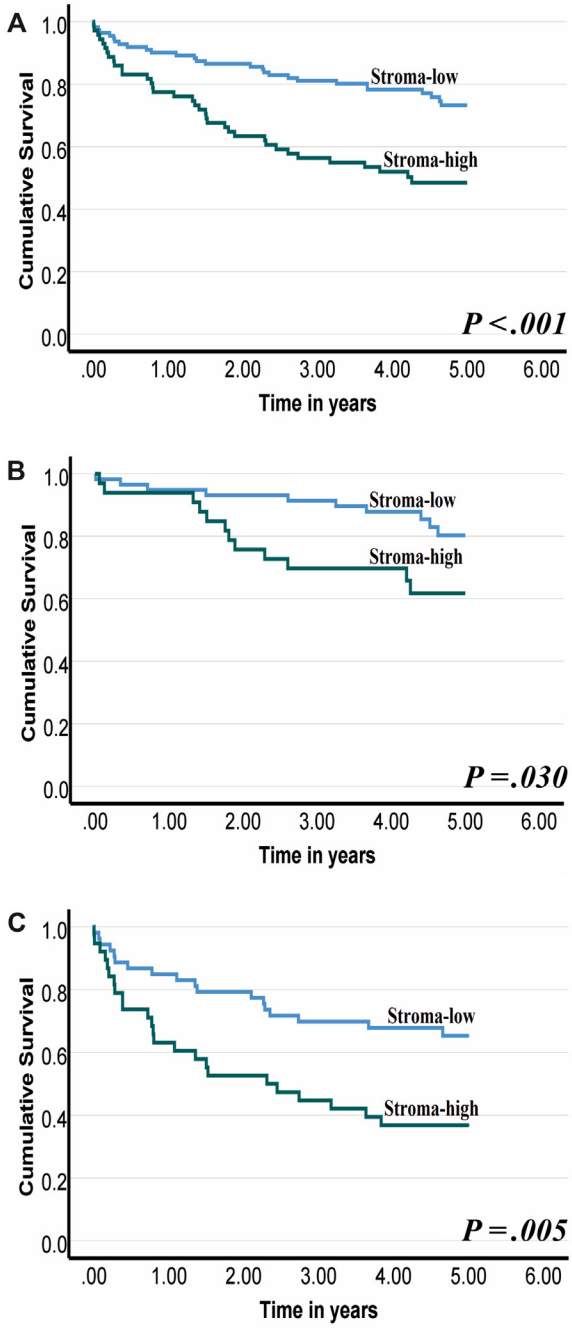

Fig. 4 Kaplan-Meier analysis of overall survival in oropharyngeal cancer patients with stroma-low tumors versus stroma-high tumors. A, All cases combined. B, Analysis of HPV-positive cases separately. C, Analysis of HPV-negative cases separately.

also for a standardized assessment of TSR in OPSCC. This includes, firstly, and at low magnification (×5) to select the area with the highest amount of stroma with cancer cells present in the four sides. Then a higher magnification (×10) is applied to estimate the stroma in this field in percentages (ie, 10%, 20%, or 30% … etc.). That is followed by classification of the tumor as "stroma-high" if having >50% tumor-associated stroma or as "stroma-low" if having ≤50% tumor-associated stroma. TSR needs to be assessed in a microscopic field with no preexisting lymphoid tissue, necrotic tissue, muscle tissue, and no large vessels presented. However, if this is not the case in a particular tumor, such areas must be excluded. Indeed, when assessing TSR in oropharyngeal cancer, there are challenging situations. For example, if there is only preexisting lymphoid tissue around the tumor parenchyma or the amount of tumor-associated stroma is scarce or not clearly identified. Such cases may require the use of a higher magnification to carefully recognize areas of tumor-associated stroma and the exclusion of preexisting lymphoid tissue from assessments. Another situation that is sometimes challenging is to assess TSR in small preoperative biopsies. In such cases, it is necessary to have representative samples and to carefully examine all of them. Assessment of TSR in preoperative biopsies has been reported recently with promising results in laryngeal cancer [27], breast cancer [28], and rectal cancer [29]. However, further studies are required on preoperative biopsies in oropharyngeal cancer.

The application of information technology (IT) platforms in daily practice of pathology and biomarker evaluation is cost-effective, provides precise quantification, and can reduce interobserver variability [30]. Of note, the use of digital pathology and machine learning—based assessment of stromal features has been reported for the evaluation of TSR in some cancers including colorectal [30] and gastric cancers [31]. Results on those cancers strongly support the use of digital assessment of TSR. Therefore, in future studies, such a method should be developed and validated for TSR in OPSCC.

In a recent study, Lewis et al. [32] did not find a significant relationship between HPV status and the morphologic features of OPSCC. Similarly, the present study did not find a significant correlation between HPV status and TSR (Table 1). The morphologic diversity within OPSCC subtypes (especially HPV+ tumors) is well known and might be the reason behind the lack of relationship between HPV status and morphologic features [32]. Shortcomings of this study include a relatively short median follow-up time in our cohort (median follow-up time, 4.48 years). In addition, IT-based systems for the evaluation of TSR were not used in our study. Therefore, there is a need for further studies to validate our findings on TSR in OPSCC, to allow for longer follow-up times, and to develop IT-based systems for the assessment of TSR in OPSCC.

Accumulated evidence on the prognostic significance of TSR originating from several research groups has allowed for meta-analyses in colorectal [6], gastric [7], lung [8], and oral cancers [5]. A definitive conclusion of all these meta-

Table 2 Disease-free survival, disease-specific survival, and overall survival analyses of the prognostic significance of tumor-stroma ratio (TSR) and clinicopathologic parameters of 182 patients treated for oropharyngeal squamous cell carcinoma.

Parameter	Univariable analysis		
	Disease-free survival	Disease-specific survival	Overall survival
	HR (95% CI), *P*	HR (95% CI), *P*	HR (95% CI), *P*
Gender			
Male	Reference	Reference	Reference
Female	2.08 (0.80-5.39), $P = .13$	2.19 (0.99-4.88), $P = .054$	1.50 (0.85-2.64), $P = .16$
Smoking			
Never	Reference	Reference	Reference
Former	1.69 (0.47-6.15), $P = .42$	1.66 (0.46-6.05), $P = .44$	1.24 (0.48-3.21), $P = .65$
Currently	1.89 (0.56-6.42), $P = .31$	3.29 (1.01-10.7), $P = .048$	2.36 (1.01-5.53), $P = .048$
T classification			
T1	Reference	Reference	Reference
T2	1.58 (0.51-4.89), $P = .43$	1.97 (0.74-5.27), $P = .18$	1.92 (0.84-4.43), $P = .12$
T3	2.34 (0.74-7.47), $P = .15$	1.79 (0.62-5.26), $P = .28$	2.44 (1.03-5.81), $P = .044$
T4	2.35 (0.69-8.04), $P = .17$	3.62 (1.31-9.96), $P = .013$	4.18 (1.79-9.76), $P = .001$
N classification			
N0-N1	Reference	Reference	Reference
N2-N3	1.55 (0.59-4.01) $P = .37$	2.09 (1.05-4.19), $P = .037$	1.49 (0.89-2.48), $P = .129$
HPV status			
Positive	Reference	Reference	Reference
Negative	2.59 (1.26-5.35), $P = .010$	2.51 (1.38-4.56), $P = .003$	2.46 (1.52-3.98), $P < .001$
Treatment			
Sx ± (C)RT	Reference	Reference	Reference
(C)RT ± Sx	1.24 (0.62-2.49), $P = .551$	1.01 (0.56-1.82), $P = .98$	1.13 (0.71-1.81), $P = .604$
Tumor-stroma ratio			
Stroma-low	Reference	Reference	Reference
Stroma-high	3.49 (1.66-7.35), $P < .001$)	2.80 (1.54-5.09), $P < .001$	2.46 (1.50-4.04), $P < .001$
Multivariable analysis			
Parameter	**Disease-free survival**	**Disease-specific survival**	**Overall survival**
	HR (95% CI), *P*	**HR (95% CI), *P***	**HR (95% CI), *P***
Smoking			
Never	Reference	Reference	Reference
Former	1.11 (0.28-4.32), $P = .89$	1.34 (0.36-5.05), $P = .66$	1.09 (0.38-3.14), $P = .88$
Currently	0.96 (0.26-3.59), $P = .96$	1.77 (0.50-6.20), $P = .374$	1.53 (0.57-4.10), $P = .40$
T classification			
T1	Reference	Reference	Reference
T2	0.98 (0.29-3.24), $P = .97$	1.27 (0.46-3.56), $P = .64$	1.28 (0.50-3.27), $P = .60$
T3	1.45 (0.41-5.07), $P = .57$	1.48 (0.49-4.48), $P = .49$	1.97 (0.75-5.20), $P = .17$
TNM stage			
Early stage	Reference	Reference	Reference
Advanced stage	1.07 (0.39-2.89), $P = .89$	1.62 (0.62-4.19), $P = .32$	1.84 (0.78-4.35), $P = .17$
HPV status			
Positive	Reference	Reference	Reference
Negative	2.05 (0.85-4.94), $P = .11$	1.97 (0.93-4.15), $P = .07$	1.81 (0.97-3.39), $P = .06$
Tumor-stroma ratio			
Stroma-low	Reference	Reference	Reference
Stroma-high	3.22 (1.43-7.26), $P = .005$	2.48 (1.29-4.74), $P = .006$	2.23 (1.29-3.85), $P = .004$

Abbreviations: HR, hazard ratio; CI, confidence interval; HPV, human papillomavirus; Sx, surgery; CRT, chemoradiotherapy; RT, radiotherapy.

analyses indicates that TSR is a powerful prognosticator and an important parameter in outcome prediction. Similarly, the present study on OPSCC demonstrated a powerful prognostic value for TSR. Importantly, the assessment of TSR is cost-effective, fast, and requires only minor effort from the pathologist. The present findings should be validated in independent large multicenter cohorts to allow for consideration of TSR in clinical decision making.

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