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Reproducibility and validation of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies

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ABSTRACT

Background: Tumour stroma ratio (TSR) in histological sections of resected oesophageal adenocarcinomas proved to be a prognostic factor for patients' survival. The objectives of this study were to assess inter- and intraobserver agreement for TSR scoring on biopsy material and to validate these biopsy results with the results derived from surgical specimens.

Methods: Biopsies and surgical specimens of 91 patients with oesophageal adenocarcinoma were available. TSR was determined on the original haematoxylin–eosin (H&E) tissue sections from primary tumour biopsies. To assess interobserver variation, TSR was scored by three pathologists as 0–25%, 25–50%, 50–75% or 75–100%. A second scoring was done to examine intraobserver variation. The definitive TSR biopsy score was compared with the corresponding resection specimen score. Kappa statistics were applied to evaluate agreement.

Results: Biopsies of 10 (11%) patients were rejected because of poor quality. For 81 TSR biopsy scores, interobserver correlations ranged between 0.239 and 0.486 ($P < 0.001$ for all). By classifying scores into two groups (<50% and $\geqslant$50%), interobserver correlations ranged between 0.372 and 0.886 ($P < 0.001$ for all). Intraobserver agreement was substantial to near-perfect ($\kappa = 0.780$–0.848; $P < 0.001$ for all). Definitive TSR biopsy score showed moderate correlation with TSR scores on surgical specimens ($\kappa = 0.506$), but it was an independent prognostic factor for survival.

Conclusion: Reproducibility of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies was good. The ease of TSR scoring on H&E sections together with its correlation with patients' survival may have clinical relevance in this era of neoadjuvant therapy.

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1. Introduction

Partial oesophagectomy is the mainstay in potentially curative treatment for oesophageal cancer. Postoperative mortality has decreased substantially in recent years, mainly as a result of more precise preoperative staging and better patient selection.[1–3] Still, many patients present with recurrences within two years after surgery and even in specialised high volume centres, 5-year survival rates rarely exceed 40%.[4–6]

Histopathological factors, such as extracapsular lymph node involvement and possibly genetic expression profiling of the tumour, are in relation with metastatic potential and survival.[7,8] The tumour micro-environment, including the supportive stromal component, plays a crucial role in the progression, growth and spread of cancers.[9–11] Earlier, we found that the amount of stroma in direct relation to the tumour was an independent prognostic factor for survival in colon cancer[12,13] and breast cancer.[14]

Subsequently, we have investigated the tumour stroma ratio (TSR) on histological sections of oesophagectomy specimens.[15] In a set of 93 patients who underwent resection for oesophageal adenocarcinoma, a significant difference in survival time was observed between patients with a high TSR (⩾50%) and patients with a low TSR (<50%). Patients with a low TSR showed a significantly worse survival. Since neoadjuvant therapies are increasingly used, and patient selection for multimodality treatment becomes even more important, assessment of prognostic factors is preferably done before the start of treatment. Therefore, TSR scoring should be validated on biopsy specimens.

The objectives of the present study were: (1) to assess inter- and intraobserver agreement for TSR scoring on oesophageal adenocarcinoma biopsies, and (2) to correlate these biopsy results with the results derived from the surgical specimens and with survival data.

2. Material and methods

2.1. Tissue selection

From the database of the Comprehensive Cancer Centre Leiden (CCCL), we had previously selected a consecutive series of 93 patients with oesophageal adenocarcinoma who underwent resection with curative intent between 1990 and 2004 at the Leiden University Medical Centre (LUMC) or the Reinier de Graaf Gasthuis in Delft (RdGG).[15] Patients who were treated with neoadjuvant therapy were excluded, as were patients who died within 30 d after surgery. Patient, tumour and treatment characteristics were retrieved from the original patient files of the CCCL database. Original pathology reports were retrospectively reviewed and any discrepancies with the CCCL database were checked on the original patient material by a pathologist (VS). For all patients, the haematoxylin–eosin (H&E) stained sections of the primary tumour biopsies were retrieved from the Department of Pathology in the hospital where the primary diagnosis had been established. All specimens were handled in a coded fashion, according to national ethical guidelines ('Code for Proper Secondary Use of Human Tissue', Dutch Federation of Medical Scientific Societies).

2.2. Histopathological protocol

On microscopic examination, the 5 μm H&E sections of the biopsies specimens were routinely analysed, and the section showing the most invasive part was selected. From this section, the TSR was visually estimated on the basis of morphological characteristics. The assessment was done using a 5–10× microscope objective (50–100× total magnification). In case of tumour heterogeneity, those areas with the lowest TSR were considered of worse prognostic value and therefore deemed decisive. Stromal tissue fragments without any tumour cells were considered not to have an apparent relation to the tumour and excluded for estimation. Other tissue components (e.g., necrosis, inflammation and blood vessels) were discounted. The estimate was then recorded as the TSR. TSR was defined as very low (<25%), low (⩾25% and <50%), high (⩾50% and <75%) or very high (⩾75%) (Fig. 1). Tumour node metastasis (TNM) staging was done according to the International Union against Cancer (UICC) guidelines version 6.[16]

Using this protocol, the sections were independently assessed by three experienced pathologists (VS, MLvV and JSN), on two occasions separated by a 2–4-week time period.

Hence, there were six TSR scores for each biopsy. A definitive diagnosis in discrepantly scored biopsies was established on the basis of four or more similar scores. If less than four scores were similar, the score was regarded as ambiguous and a consensus diagnosis should be reached with the use of a multihead microscope.

2.3. Follow-up

All patients had a regular follow-up schedule consisting of 3-monthly visits during the first 2 years after surgery and 6-monthly visits thereafter. Routine radiologic examinations were not performed. Follow-up data were collected until death or July 2009. When necessary, the patient's general practitioner was contacted for additional information.

2.4. Statistics

Statistical calculations were performed using SPSS version 15.0 (Statistical Package for the Social Sciences, Chicago, IL). Differences between groups were assessed using the Mann–Whitney test for continuous variables and the χ^2 test or Fisher's exact test for categorical variables. The Kappa statistic (κ) was used to assess inter- and intraobserver agreement of TSR scoring. A κ of 0.0 or less was considered to represent poor agreement, 0.01–0.20 slight agreement, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–1.00 near-perfect agreement.[17] To compare TSR biopsy scores with the scores from resection specimens of the same patients, previously described results were used.[15]

Survival was calculated from the date of surgery using the Kaplan–Meier method. For overall survival analysis, events were defined as death from any cause. Differences in survival distributions were tested with log rank statistics. The Cox proportional hazards model was used to determine the Hazard Ratio (HR) of explanatory variables on overall survival. Variables achieving a probability value of less than 0.05 in the univariate analysis were subsequently introduced in a

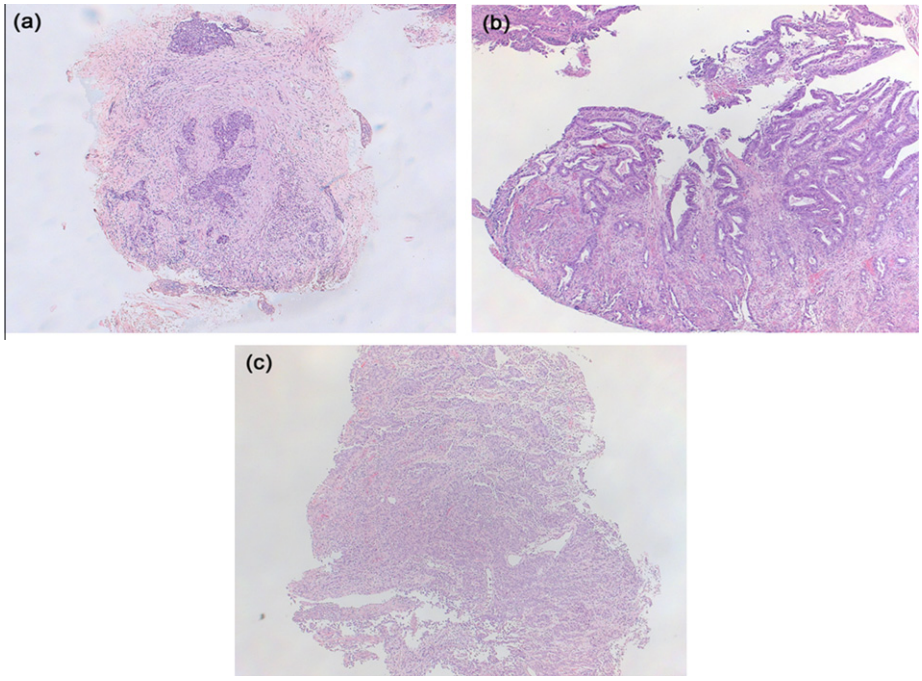


Fig. 1 – Histological views illustrating different tumour stroma ratio (TSR) scores in oesophageal adenocarcinoma biopsies (H&E, 50× total magnification). (a) TSR estimated as ⩾25% to <50%: TSR low. (b) TSR estimated as ⩾50% to <75%: TSR high. (c) TSR estimated as ⩾75%: TSR very high. Abbreviations: H&E, haematoxylin and eosin; TSR, tumour stroma ratio.

multivariate stepwise proportional-hazard analysis (Cox model) to identify those variables significantly associated with survival. Results are given as hazard ratios with their 95% confidence interval. *P*-values <0.05 (2-sided) were considered statistically significant.

3. Results

3.1. *Patient demographics*

Archival biopsy material was available for 91 of the 93 patients who underwent oesophagectomy. Biopsies of 10 (11%) patients were unanimously rejected because of the poor quality of the tissue specimen. The median age of the 81 study patients was 64 (range 37–82) years. There were 66 men (81.5%) and 15 women (18.5%). Median follow-up was 23 (range 3–220) months.

3.2. *Histopathological features*

Overall, there were 486 TSR biopsy scores (81 slides, three observers and two scores per observer for each slide). A definitive TSR score could be reached in all 81 slides without the use of a multihead microscope. There were 29 (36%) slides

Table 1 – Interobserver agreement between three pathologists for TSR biopsy scores of 81 patients with oesophageal adenocarcinoma.

	Pathologists	Agreement	κ	*P*
TSR biopsy score 1				
(<25%, ⩾25% to <50%, ⩾50% to <75%, or ⩾75%)	1 and 2	64%	0.392	<0.001
	1 and 3	72%	0.410	<0.001
	2 and 3	69%	0.486	<0.001
(<50% or ⩾50%)	1 and 2	95%	0.818	<0.001
	1 and 3	98%	0.886	<0.001
	2 and 3	93%	0.710	<0.001
TSR biopsy score 2				
(<25%, ⩾25% to <50%, ⩾50% to <75%, or ⩾75%)	1 and 2	57%	0.346	<0.001
	1 and 3	72%	0.411	<0.001
	2 and 3	51%	0.239	<0.001
(<50% or ⩾50%)	1 and 2	86%	0.589	<0.001
	1 and 3	90%	0.507	<0.001
	2 and 3	81%	0.372	<0.001

Abbreviations: TSR, tumour stroma ratio.

Table 2 – Intraobserver agreement amongst three pathologists for TSR biopsy scores of 81 patients with oesophageal adenocarcinoma.

Pathologist	TSR biopsy score 1	TSR biopsy score 2				Agreement (<25%, ⩾25% to <50%, ⩾50% to <75%, or ⩾75%)	κ	P	Agreement (<50% or ⩾50%)	κ	P
		<25%	⩾25% to <50%	⩾50% to <75%	⩾75%						
1	<25%	–	–	–	–						
	⩾25% to <50%	–	11	0	0						
	⩾50% to <75%	–	0	2	5	79%	0.509	<0.001	98%	0.848	<0.001
	⩾75%	–	2	10	51						
2	<25%	–	–	–	–						
	⩾25% to <50%	–	15	0	0						
	⩾50% to <75%	–	4	21	4	74%	0.603	<0.001	93%	0.787	<0.001
	⩾75%	–	2	11	24						
3	<25%	–	–	–	–						
	⩾25% to <50%	–	6	2	1						
	⩾50% to <75%	–	0	15	7	84%	0.683	<0.001	96%	0.780	<0.001
	⩾75%	–	0	3	47						

Abbreviations: TSR, tumour stroma ratio.

with an unanimous score (six corresponding scores). In 20 cases (25%), five scores were similar and in 32 patients (40%), four scores were the same.

The definitive TSR score ranged from low (in 12 patients) to very high (in 53 patients). There were no scores between 0 and 25% (very low).

3.3. *Interobserver agreement (Table 1)*

Kappa values for interobserver agreement ranged from 0.392 to 0.486 for the first score and from 0.239 to 0.411 for the second score ($P < 0.001$ for all correlations). By classifying scores into two groups (<50% and ⩾50%), kappa values improved to 0.818, 0.886 and 0.710 for the first score, and to 0.589, 0.507 and 0.372 for the second score ($P < 0.001$ for all correlations).

3.4. *Intraobserver agreement (Table 2)*

Intraobserver agreement was moderate to substantial (range 0.509–0.683 [$P < 0.001$ for each]). Using a cut-off value of 50%, intraobserver agreement was substantial to near-perfect with an agreement between 93% and 98% (range 0.780–0.848).

3.5. *Correlation of TSR biopsy score with TSR surgical specimen score (Table 3)*

In 66 (81%) of 81 subjects, the definitive TSR score of the biopsy material was the same as the TSR score on histological sections of the oesophagectomy specimen ($\kappa = 0.506$, $P < 0.001$). The scores were discrepant in 15 cases: all of them with a biopsy score ⩾50%, whereas the surgical specimen showed TSR lower than 50%.

3.6. *Correlation of TSR biopsy score with other clinicopathologic parameters (Table 4)*

There were no significant differences between the TSR <50% group and the TSR ⩾50% group regarding patient, tumour and treatment characteristics except for M status and radicality of resection.

Median survival for patients with a low TSR was 14 months (95% confidence interval [C.I.] 9–20 months) compared to 26 months (95% C.I. 13–39 months) for patients with a high TSR. Overall survival at 3 years was 17% in the TSR <50% group and 45% in the TSR ⩾50% group ($P = 0.024$; Fig. 2). In the univariate model, pT-status, pN-status, lymph node ratio, extracapsular lymph node involvement, and radicality of the resection were also significantly related to overall survival (Table 5). Tumour location, surgical approach, pM status and differentiation grade were not significantly correlated to survival. In multivariate analysis, TSR biopsy score remained an independent prognostic variable for overall survival together with extracapsular lymph node involvement and radicality of the resection.

For disease-free survival, similar results were found. Median disease-free survival was 11 months (95% C.I. 6–16 months) in the TSR <50% group, and 19 months (95% C.I. 14–24 months) in the TSR ⩾50% group ($P = 0.040$).

In the subgroup of 56 patients with M0R0 disease, overall survival was significantly better in the TSR high group than in the TSR low group (43 months versus 21 months; $P = 0.043$), whereas there were no significant differences be-

Table 3 – Correlation between biopsy results and results derived from surgical specimens regarding TSR scoring in 81 patients with oesophageal adenocarcinoma.

Definitive TSR biopsy score	Final TSR score on resection specimen		
	<50%	⩾50%	Total
<50%	12 (15%)	0 (0%)	12
⩾50%	15 (19%)	54 (67%)	69
Total	27	54	81

Abbreviations: TSR, tumour stroma ratio.

Table 4 – Patient, tumour and treatment characteristics for 81 patients with oesophageal adenocarcinoma in relation to TSR biopsy score.

Characteristics	Total (n = 81)		TSR < 50% (n = 12)		TSR ⩾ 50% (n = 69)		P value
	No. of patients	%	No. of patients	%	No. of patients	%	
Gender							0.687
Male	66	82	9	75	57	83	
Female	15	19	3	25	12	17	
Median age at surgery in years (range)							0.433
Tumour location	64	37–82	66	43–80	64	37–82	0.228
Oesophagus	41	51	8	67	33	48	
GOJ	40	49	4	33	36	52	
Surgical approach							0.726
Transhiatal	59	73	8	67	51	74	
Transthoracic	22	27	4	33	18	26	
pT status							0.324
pT1	13	16	1	8	12	17	
pT2	16	20	1	8	15	22	
pT3	52	64	10	83	42	61	
pN status							0.454
pN0	35	43	4	33	31	45	
pN1	46	57	8	67	38	55	
Lymph node ratio							1.000
<0.2	49	61	7	58	42	61	
⩾0.2	32	40	5	42	27	39	
Extracapsular LNI							0.190
Node negative	36	44	7	58	22	32	
No extracapsular LNI	29	36	1	8	15	21	
Extracapsular LNI	16	20	4	33	32	46	
pM status							**0.020**
pM0	79	98	10	83	69	100	
pM1a	2	3	2	17	0	0	
pTNM stage							0.114
I–II	44	54	4	33	40	58	
III–IV	37	46	8	67	29	42	
Differentiation grade							0.999
Well	7	9	1	8	6	9	
Moderate	40	49	6	50	34	49	
Poor	34	42	5	42	29	42	
Radicality							**0.006**
R0	56	69	4	33	52	75	
R1 or R2	25	31	8	67	17	24	

Abbreviations: TSR, tumour stroma ratio; GOJ, gastro-oesophageal junction; pTNM, pathologic tumour node metastasis; LNI, lymph node involvement; R0, microscopically radical resection; R1, microscopically irradical resection; R2, macroscopically irradical resection.

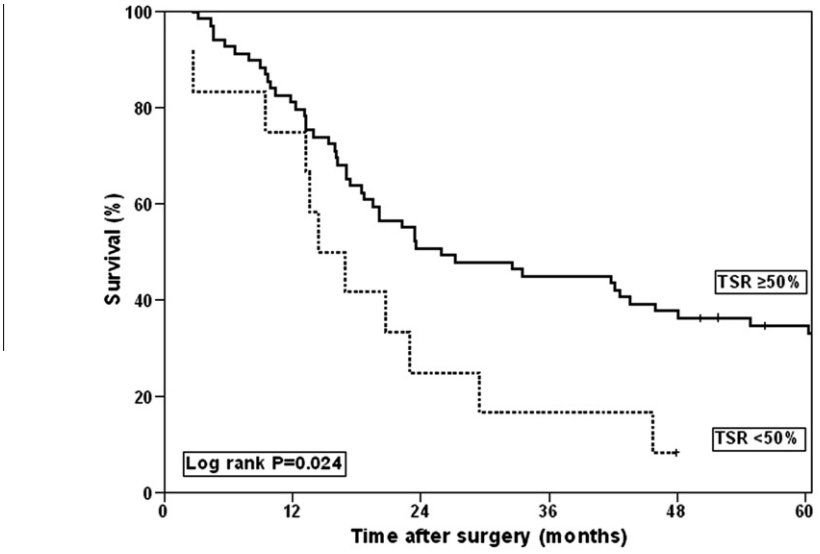
tween the two groups regarding patient, tumour and treatment characteristics.

For the 15 patients with a discrepant TSR score between the biopsy and the resection specimen, median survival was 12 months (95% C.I. 8–17 months), whereas the 66 patients with similar scores had a median survival of 33 months (95% C.I. 13–53 months).

4. Discussion

Our study results showed that TSR biopsy scoring in patients with oesophageal adenocarcinoma was reproducible. Biopsy results demonstrated moderate correlation with results derived from surgical specimens. The definitive TSR biopsy score was an independent prognostic factor for survival. A high score (TSR ⩾50%) was associated with better survival.

Concordant with our previous study results on surgical resection specimens, scoring of TSR on biopsy material was easy on H&E stained sections. Interobserver agreement ranged from fair to moderate when scores were categorised into four categories. Dividing the scores into two groups (<50% and ⩾50%), correlation coefficients improved. The rationale of using 50% as a cut-off value has been discussed previously.[12,13,15] In earlier research, the 50% level of TSR was



TSR ≥50%	69	56	35	31	26	21
TSR <50%	12	9	3	2	0	0
Total	81	65	38	33	26	21

Fig. 2 – Overall survival for 81 patients who underwent oesophageal resection for adenocarcinoma in relation to TSR biopsy score. The numbers in the box refer to the number of patients at risk at 12-month intervals. Abbreviations: TSR, tumour stroma ratio.

determined on the basis of a maximum discriminating power for survival. Intraobserver agreement was substantial to near-perfect using the cut-off value of 50%. This rate of agreement was comparable to that found in our previous study on oesophageal resection specimens ($\kappa = 0.835$, 95% C.I. 0.717–0.952).[15]

Definitive TSR biopsy scores and TSR surgical specimen scores were the same in 81% of the patients. All disagreement cases had a TSR biopsy score of $\geqslant$50% and a TSR surgical specimen score of <50%. The low survival rate in this group of patients with discrepant TSR scores suggests that more (if not all) of these cases have been misclassified on their biopsy material. In heterogeneous tumours, those areas with lowest TSR were chosen for analysis. The fact that biopsies reveal only parts of the tumour may explain why fewer patients had a low TSR score in the biopsy group than in the resection group. This sampling issue is a drawback to the method of TSR scoring on biopsies.

As in the study on oesophagectomy specimens, the definitive TSR score in the present study on biopsy specimens was significantly associated with survival. This phenomenon might be explained by biological processes involved in oesophageal cancer invasion and metastasis. The composition of tumours differs amongst patients and has been related to different survival in breast cancer, lung cancer, skin cancer, prostate cancer and colorectal cancer.[14,18–21] In most of these studies, the higher the stroma percentage or desmoplastic response, the poorer patient's outcome. There are several theories as to why a low TSR may lead to a worse prognosis. First, it has been hypothesised that tumours with a greater proportion of stroma are able to produce more growth factors leading to a greater tumour burden. Second, it has been suggested that the relative amount of desmoplastic fibrosis reduces the accessibility of tumours to the immune response by encapsulating the malignant cells and preventing their destruction.[22] Our findings support the hypothesis that tumour-associated stroma influences tumour biological behaviour in a negative manner (poor prognosis).

Tumour cells can change their adjacent stroma by producing stroma-modulating growth factors to create a supportive environment for tumour progression. Therefore, in patients with a low TSR, the presence of more stroma in the tumour may have resulted in the production of factors that promote this tumour progression and consequently, in a higher rate of R1 resections.[23] An additional survival analysis on the patients with M0 R0 disease showed that TSR still matters in this subgroup of potentially curative patients. Although the majority of patients with a high risk TSR had undergone an incomplete resection and some had distant metastases, survival remained significantly better in the TSR $\geqslant$50% group.

Some limitations to our study should be noted. First, we used a semi-quantitative visual estimation of the stromal component rather than measuring the components objectively. Second, a small proportion of the biopsies (11%) had to be rejected because of poor tissue quality. Finally, it should be noted that the three pathologists did not meet prior to the start of the study to discuss the criteria that they used for the scoring of tumour stroma ratio. Nevertheless, the method used appeared to be robust and reproducible. A prospective biopsy study is ongoing to validate the present results.

Table 5 – Cox univariate and multivariate analysis for overall survival in 81 patients who underwent resection for oesophageal adenocarcinoma.

	Univariate analysis			Multivariate analysis		
	HR	95% C.I.	P value	HR	95% C.I.	P value
TSR biopsy score			**0.028**			**0.042**
⩾50%	1.000	Ref.	–	1.000	Ref.	
<50%	2.118	1.086–4.127	**0.028**	2.042	1.164–4.328	
Surgical approach			0.065			
Transhiatal	1.000	Ref.	–			
Transthoracic	1.621	1.035–2.752	0.065			
pT status			**0.014**			0.761
pT1	1.000	Ref.	–	1.000	Ref.	
pT2	1.341	0.537–3.350	0.530	1.168	0.454–2.384	
pT3	2.678	1.233–5.817	**0.013**			
pN status			**0.005**			0.780
pN0	1.000	Ref.	–	1.000	Ref.	
pN1	2.141	1.253–3.660	**0.005**	1.108	0.540–2.271	
Lymph node ratio			<**0.001**			0.084
<0.2	1.000	Ref.	–	1.000	Ref.	
⩾0.2	3.144	1.852–5.339	<**0.001**	1.880	0.919–3.845	
Extracapsular LNI			**0.001**			**0.016**
Node negative	1.000	Ref.	–	1.000	Ref.	
No extracapsular LNI	1.994	1.104–3.602	**0.022**	2.421	1.179–4.970	
Extracapsular LNI	3.504	1.781–6.895	<**0.001**			
pM status			0.183			
pM0	1.000	Ref.	–			
pM1a	2.656	0.630–11.185	0.183			
Differentiation grade			0.064			
Well	1.000	Ref.	–			
Moderate	1.091	0.422–2.823	0.858			
Poor	1.977	0.758–5.156	0.163			
Radicality			<**0.001**			<**0.001**
R0	1.000	Ref.	–	1.000	Ref.	
R1	3.279	1.808–5.949	<**0.001**			
R2	6.454	2.703–15.413	<**0.001**	3.095	1.664–5.755	
Adjuvant therapy			0.154			
No	1.000	Ref.	–			
Yes	2.349	0.726–7.601	0.154			

Abbreviations: HR, hazard ratio; C.I., confidence interval; TSR, tumour stroma ratio; pTNM, pathologic tumour node metastasis; LNI, lymph node involvement; R0, microscopically radical resection; R1, microscopically irradical resection; R2, macroscopically irradical resection.

Recent research has focussed on identifying prognostic markers that select patients who may benefit from neoadjuvant treatment. The identification of patients with a poor prognosis based on TSR biopsy scoring could easily be used in routine diagnostic practice. Following the results of the present study, we conclude that patients with a low tumour stroma ratio could benefit from the development of new effective agents that inhibit the interaction between stromal and epithelial cells. The development of these agents will depend on our understanding of the underlying mechanisms of stroma induction and formation in tumours.[22]

Conflict of interest statement

None declared.

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