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Vangangelt, K.M.H.; Tollenaar, L.S.A.; Pelt, G.W. van; Kruijf, E.M. de; Dekker, T.J.A.; Kuppen, P.J.K.; ... ; Mesker, W.E.

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Kiki M.H. Vangangelt, Lisanne S.A. Tollenaar, Gabi W. van Pelt, Esther M. de Kruijf, Tim J.A. Dekker, Peter J.K. Kuppen, Rob A.E.M. Tollenaar and Wilma E. Mesker

Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

The tumor–stroma ratio (TSR) has previously been found to be a strong prognostic parameter in primary breast cancer tumors. Since the presence of tumor cells in lymph nodes is important for clinical decision making, the influence of TSR in the primary breast tumor combined with the TSR in tumor-positive lymph nodes on prognosis was evaluated. Women with invasive breast cancer without distant metastasis who underwent an axillary lymph node dissection between 1985 and 1994 at the Leiden University Medical Center were retrospectively analyzed. TSR assessment was performed on hematoxylin and eosin stained tissue slides. In total, 87 (45.5%) primary tumors were scored as stroma-low and 104 (54.5%) as stroma-high. Patients with a high stromal percentage in the primary tumors had a statistically significant worse relapse free period (RFP) compared to stroma-low tumors (HR 1.97, 95% CI 1.37–2.82, $p < 0.001$). A total number of 915 lymph nodes were assessed for TSR. In 101 (52.9%) patients, heterogeneity was observed between stroma percentage category in primary tumor and lymph nodes. The combination of TSR of the primary tumor combined with TSR of tumor-positive lymph nodes strengthened each other as independent prognostic parameter for RFP ($p = 0.019$). Patients with primary tumor stroma-low/lymph nodes stroma-low tumors showed strongly improved RFP rates compared to patients with primary tumor stroma-high/lymph node stroma-high tumors with 10-year percentages of 58 *versus* 8%, respectively. Assessing the TSR on tumor-positive lymph nodes can provide additional prognostic information. Stromal activation strongly differs between primary tumors and lymph node metastasis.

Introduction

In patients with invasive breast cancer, the presence of regional lymph node (LN) metastasis is one of the most important prognostic parameter for long-term prognosis.[1] Careful evaluation of LN status is crucial to decide whether patients should undergo an axillary lymph node dissection (ALND) or axillary radiotherapy and also plays a large role in deciding on adjuvant chemotherapy. As breast cancer is a heterogeneous disease,[2] distinguishing patients that need more aggressive therapy from patients that would benefit from a more conservative approach remains a difficult challenge. Prognostic parameters derived from the stromal compartment might therefore provide an important tool. The interaction between tumor cells and cells in the tumor microenvironment has gained significant interest in the last two decades. The tumor stroma consists of inflammatory cells, capillaries, fibroblasts and extracellular matrix.[3] Fibroblasts that surround and infiltrate the primary tumor (PT), the so-called cancer associated fibroblasts (CAFs) are believed to play a key role in tumor progression by secreting chemokines and growth factors. This may lead to increased cancer cell proliferation, increased motility and invasiveness, enhanced angiogenesis and tumor promoting inflammation.[4,5]

Based on the analysis of hematoxylin and eosin (H&E) stained histologic slides, our research group developed an internationally validated prognostic tool, the tumor–stroma ratio (TSR), that assesses the amount of stromal proliferation within the borders of the PT. This parameter has shown to be of high prognostic value in several types of epithelial neoplasms, including breast cancer,[6–10] colon cancer,[11–14] gastric cancer[15] and esophageal cancer.[16] These studies have invariably shown a worse prognosis in patients with so-called stroma-high tumors compared to patients with stroma-low tumors.

The additional prognostic value of TSR assessment in metastatic LNs for disease free survival (DFS) in patients with stage III colorectal cancer was published by Van Pelt *et al.*[17] By our knowledge, the influence of stromal growth in LNs affected by breast cancer has not yet been investigated. The objective of this current study was to evaluate the prognostic value of TSR in the primary tumor combined with TSR in tumor-positive LNs in primary breast tumors compared to TSR in primary breast tumors alone.

Key words: breast cancer, tumor–stroma ratio, lymph nodes, prognosis

Additional Supporting Information may be found in the online version of this article.

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Correspondence to: Dr. Wilma E. Mesker, Department of Surgery, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands, Tel.: +31715262987, E-mail: w.e.mesker@lumc.nl

What's new?

Measuring the amount of stroma in tumor- positive lymph nodes, not just only in the primary tumor, can better predict breast cancer relapse. In recent years, researchers have increasingly looked to the tumor microenvironment for prognostic clues. Here, the authors compared the prognostic value of the TSR in primary breast tumors alone with TSR from both primary tumor and tumor-positive lymph nodes. Risk of relapse for patients with high amount of stroma at the primary tumor was 75%, while a combination of stroma-high primary tumor and stroma-high lymph nodes correlated with 92% relapse rate after 10 years of follow-up.

Material and Methods

Study population

The patients included in this study were selected from a database consisting of patients with invasive breast cancer without distant metastasis, who were primary treated with surgery between 1985 and 1994 at the Leiden University Medical Center. Patient data were assessed retrospectively (N = 677). Only patients who underwent an axillary lymph node dissection were included in this study. Patients with a history of cancer (other than basal cell carcinoma or cervical carcinoma in situ), bilateral breast cancer or absence of resected tissue slides were excluded, leaving 193 patients for analysis. The resected tumors were graded by an

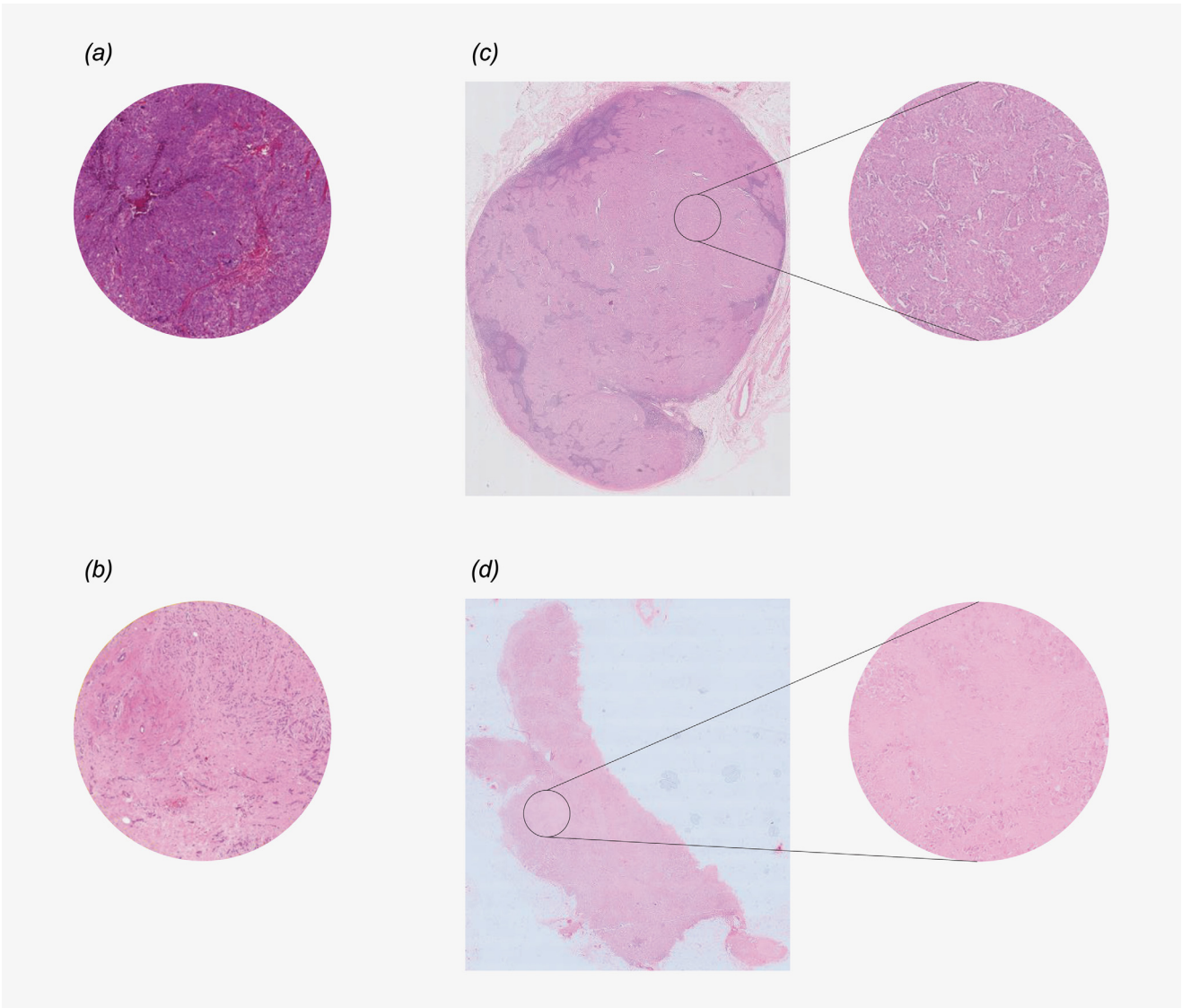

Figure 1. Examples of TSR in breast cancer. Lymph nodes were scanned with an automated scanning system (Philips Ultra Fast Scanner 1.6 RA) at 20× magnification. (*a*) PT stroma-low, (*b*) PT stroma-high, (*c*) tumor-positive LN stroma-low, (*d*) tumor-positive LN stroma-high. Abbreviations: TSR, tumor–stroma ratio; PT, primary tumor; LN, lymph node. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1. Patient characteristics and statistically significant difference between stroma-low and stroma-high primary tumors calculated by χ^2 test

Characteristics	*N*	Stroma-low (%) (*N* = 87)	Stroma-high (%) (*N* = 104)	*p*-value
Age (in years)				
‹40	15	9 (10.3)	6 (5.8)	0.364
›40–60	94	39 (44.8)	55 (52.9)	
›60	82	39 (44.8)	43 (41.3)	
Grade				
I	18	5 (5.7)	13 (12.5)	0.170
II	85	37 (42.5)	48 (46.2)	
III	88	45 (51.7)	43 (41.3)	
Histological type				
Ductal	171	83 (96.5)	88 (85.4)	0.010
Lobular	18	3 (3.5)	15 (14.6)	
Tumor stage				
pT1	42	16 (18.6)	26 (26.3)	0.449
pT2	109	54 (62.8)	55 (55.6)	
pT3/4	34	16 (18.6)	18 (18.2)	
Nodal stage				
pN1	148	75 (86.2)	73 (70.2)	0.011
pN2	11	1 (1.1)	10 (9.6)	
pN3	32	11 (12.6)	21 (20.2)	
ER status				
Negative	83	40 (47.1)	43 (44.8)	0.760
Positive	98	45 (52.9)	53 (55.2)	
PR status				
Negative	86	36 (42.4)	50 (51.0)	0.241
Positive	97	49 (57.6)	48 (49.0)	
HER2 status				
Negative	118	57 (82.6)	61 (82.4)	0.978
Positive	25	12 (17.4)	13 (17.6)	
Surgery with or without RT				
MST without RT	62	30 (34.5)	32 (30.8)	0.860
MST with RT	63	28 (32.2)	35 (33.7)	
BCS without RT	0	0 (0)	0 (0)	
BCS with RT	76	29 (33.3)	37 (35.6)	
Chemotherapy				
No	127	52 (59.8)	75 (72.1)	0.072
Yes	64	35 (40.2)	29 (27.9)	
Hormonal therapy				
No	136	61 (70.1)	75 (72.1)	0.761
Yes	55	26 (29.9)	29 (27.9)	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; MST, mastectomy; RT, radiotherapy; BCS, breast conserving therapy.

experienced breast cancer pathologist using the current pathological standards. TSR assessment of the primary breast tumors is described earlier.[9] All samples 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).

TSR assessment

TSR was visually assessed by conventional light microscopy on 5 μm routine H&E stained slides. First, the PT and LNs were evaluated with a 5× objective in order to identify the most stroma-rich tissue area(s). The most stroma abundant area was selected and assessed with a 10× objective. Only

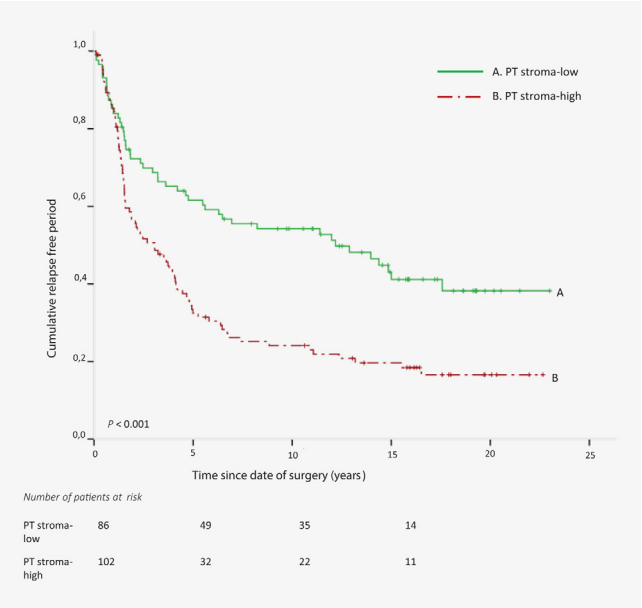

Figure 2. Kaplan–Meier analysis for relapse free period of patients with stroma-low PTs and stroma-high PTs. Abbreviations: PT, primary tumor. [Color figure can be viewed at wileyonlinelibrary.com]

tumor fields with tumor cells present at all borders of the image field were eligible. Stroma percentage was scored by increments of 10%. A stroma percentage ≤50% was categorized as stroma-low and a stroma percentage >50% was considered stroma-high (Fig. 1). Positive LNs were identified as stroma-high if at least one of the LNs had a stroma percentage of >50% (Fig. 1). Lymph node metastases of >0.2 mm but ≤2 mm were defined as micrometastases. In case of micrometastasis the TSR was evaluated in a smaller image field as long as tumor cells were present at all borders.

Statistical analysis

IBM Statistics v23.0 (SPSS, Inc., an IBM Company, Chicago, IL) was used to perform statistical analyses.

Cohen's Kappa value was used to assess the inter-observer agreement. A value above 0.6 was considered as valid. A χ^2 test was used for the evaluation of statistically significant differences for categorical variables between patients with stroma-high or stroma-low tumors. For numerical variables (lymph node yield) distribution was tested for normality using the Shapiro–Wilk test. Statistically significant differences of non-parametric variables were analyzed using the Mann–Whitney *U* test. The primary endpoint was relapse free period (RFP), which was defined as time from date of surgery until local, regional or distant recurrence of breast cancer. Patients who died or were lost to follow-up were censored at the last date of which they were known to be recurrence free and/or alive. The definition of secondary endpoint overall survival (OS) was time from date of surgery until death from any cause. Kaplan–Meier curves were compared to log-rank tests in order to assess differences in RFP. Univariate and multivariate analysis for RFP and OS were calculated by Cox proportional hazard analyses. Parameters with a *p*-value of less than 0.10 in univariate analysis were entered in multivariate analysis. For all analyses a *p*-value of less than 0.05 was considered statistically significant. Effect modification was evaluated by adding interaction in Cox regression analysis.

Results

Patients

In total, H&E slides derived from 193 breast cancer patients could be evaluated for TSR. Two patients were excluded due to poor quality of LN tissue slides, leaving 191 patients for analysis. The study group consisted of women with a median age at time of diagnosis of 57.4 years (range 27.5–87.6 years). The median follow-up period was 7.3 years (range 0.2–23.0 years). Table 1 provides a detailed overview of the patient characteristics.

Prognostic value of the TSR in the primary tumor

In total, 87 (45.5%) PTs were determined to be stroma-low and 104 (54.5%) as stroma-high. Patients with stroma-high PTs had a statistically significant worse RFP compared to stroma-low tumors (hazard ratio (HR) 1.97, 95% confidence interval (CI) 1.37–2.82, $p < 0.001$) (Fig. 2). After 10 years of follow-up, 75% of patients with stroma-high tumors developed a recurrence compared to 46% of patients with stroma-low tumors. The multivariate analysis showed that TSR in the PT is a statistically significant independent prognostic factor for RFP (HR 1.70, 95% CI 1.16–2.49, $p = 0.006$) (Table 2) and OS (HR 1.49, 95% CI 10.4–2.14, $p = 0.029$) (Supporting Information Table S1). In the stroma-high group statistically significant more patients had a tumor of lobular type and a higher nodal stage (Table 1). The TSR assessment of the PTs in the total group of patients was previously published by our group.[9] The tissue slides were scored in a blinded fashion by a second observer with a Cohen's kappa of 0.85 (almost perfect agreement).

TSR in tumor-positive lymph nodes

In total, 915 LNs were analyzed (range 1–18 per patient). A patients' LNs were categorized as stroma-high if at least one of the LNs had a stroma percentage of >50%. The LNs of 160 (83.8%) patients were scored as stroma-low and 31 as stroma-high (16.2%). Stroma-low PTs and stroma-low LNs were seen in 73 patients (38.2%). Stroma-high PTs and stroma-high LNs were seen in 17 patients (8.9%). In 101 (52.9%) patients, heterogeneity was observed between stroma percentage category in primary tumor and lymph nodes. No interaction between the TSR in the PTs and LNs was found as also for TSR in LNs and nodal status. The Mann–Whitney *U* test did not show a statistically significant difference between lymph node yield (not normally distributed) and the TSR category of LNs. In 10 patients only

Table 2. Univariate and multivariate analysis for relapse free period calculated by Cox proportional hazard analysis

		Univariate analysis			Multivariate analysis TSR PT			Multivariate analysis TSR PT and LNs		
Characteristics	***N***	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Age (in years)										
‹40	15	1		0.868						
›40–60	94	1.17	0.62–2.22							
›60	82	1.10	0.57–2.12							
Grade										
I	18	1		0.745						
II	85	0.99	0.53–1.85							
III	88	1.14	0.61–2.11							
Histological type										
Ductal	171	1		0.131						
Lobular	18	1.52	0.88–2.60							
Tumor stage										
pT1	42	1		0.472						
pT2	109	0.88	0.58–1.34							
pT3/4	34	1.17	0.69–1.98							
Nodal stage										
pN1	148	1		0.001	1		0.610	1		0.674
pN2	11	2.46	1.27–4.77		1.42	0.71–2.84		1.35	0.67–2.71	
pN3	32	1.90	1.23–2.93		1.11	0.68–1.82		1.13	0.69–1.84	
ER status										
Negative	83	1		0.311						
Positive	98	1.21	0.84–1.73							
PR status										
Negative	86	1		0.311						
Positive	97	0.83	0.59–1.19							
HER2 status										
Negative	118	1		0.331						
Positive	25	0.76	0.43–1.33							
Surgery with or without RT										
MST without RT	62	1		0.017	1		0.039	1		0.050
MST with RT	63	1.62	1.05–2.48		1.65	1.04–2.63		1.64	1.03–2.62	
BCS without RT	0									
BCS with RT	66	0.94	0.61–1.47		0.99	0.63–1.55		1.02	0.64–1.61	
Chemotherapy										
No	127	1		‹0.001	1		0.004	1		0.004
Yes	64	0.47	0.32–0.70		0.53	0.35–0.82		0.53	0.35–0.82	
Hormonal therapy										
No	136	1		0.488						
Yes	55	0.87	0.59–1.29							
TSR										
Stroma-low	87	1		‹0.001	1		0.006			
Stroma-high	104	1.97	1.37–2.82		1.70	1.16–2.49				
TSR PT combined with LNs										
PT low/LN low	73	1		0.001				1		0.019

(Continues)

Table 2. Continued

		Univariate analysis			Multivariate analysis TSR PT			Multivariate analysis TSR PT and LNs		
Characteristics	***N***	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
PT low/LN high	14	1.78	0.86–3.68	0.12				1.58	0.76–3.30	0.223
PT high/LN low	87	2.04	1.37–3.04	‹0.001				1.75	1.15–2.65	0.009
PT high/LN high	17	2.86	1.56–5.24	0.001				2.41	1.29–4.49	0.006

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; MST, mastectomy; RT, radiotherapy; BCS, breast conserving therapy; TSR, tumor–stroma ratio; PT, primary tumor; LN, lymph nodes.

micrometastases were observed. These small tumor fields consisted of tumor cells for more than 90%. Thirty percent of the LNs were scored in a blinded fashion by a second observer with a Cohen's kappa of 0.79.

Prognostic value of TSR in primary tumor combined with tumor-positive lymph nodes

The TSRs of the PT and positive LNs were combined to evaluate the possibility of an additional prognostic effect. The four different combinations of TSR (PT stroma-low/LNs stroma-low, PT stroma-low/LNs stroma-high, PT stroma-high/LNs stroma-low and PT stroma-high/LNs stroma-high) were plotted for the RFP with an overall *p*-value of 0.001 (Fig. 3). The patient characteristics of these four groups were described in Supporting Information Table S2. Patients with PT stroma-low/LNs stroma-low showed better 10-year RFP rates compared to patients with PT stroma-high/LNs stroma-high with percentages of 58 *versus* 8%, respectively. These analyses show the strong prognostic impact of high amounts of stroma in the PT as well as LNs with regard to RFP. Multivariate analysis showed that the combination of TSR in PT and LNs is an independent prognostic factor for RFP ($p = 0.019$) (Table 2). A non-statistically significant trend was seen in favor for stroma-low PT/stroma-low LNs for OS ($p = 0.084$) (Supporting Information Table S1).

Discussion

This is the first study investigating the TSR in tumor-positive LNs in patients with invasive breast cancer. Patients with LN metastases were previously considered to be immediately eligible for adjuvant chemotherapy, irrespective of other clinicpathological parameters. As studies have shown that patients with 1–3 positive LNs do not necessarily have a worse prognosis compared to node-negative tumors, subsequent guidelines have since stated that LN involvement in itself is not a reason for adjuvant chemotherapy.[18] Further research is, however, needed to further refine the prognosis of lymph node-positive patients, both to omit chemotherapy in some cases or possibly to escalate chemotherapy for others.

Analogous to our work regarding the prognostic implication of stromal proliferation in PTs, we investigated the added significance of assessing stroma in breast cancer positive LNs. We found that incorporating the TSR of LNs combined with the TSR of the corresponding PT provided a superior prediction of RFP compared to the TSR of the PT alone. When TSR is solely evaluated in the PT, the disease recurrence rate after 10 years is 75% in primary stroma-high tumors whereas the number is 46% in primary stroma-low tumors. When the TSR of the LNs is added to these two groups, a group of patients with a high risk can be identified, namely PT stroma-high/LNs stroma-high. Considering the fact that this patient group has a recurrence rate of 92% after 10 years, this method seems capable of identifying a group of patients with a worse prognosis.

An interesting result is the strong discrepancy between TSRs in the PT with those of the LNs of the same patients. In 101 (52.9%) patients heterogeneity was observed between the stroma percentage category in the PT and LNs. Only a small proportion of patients was scored as stroma-high when evaluating the LNs ($N = 31$), which is in stark contrast to the fairly large amount of stroma-high PTs ($N = 104$). Consequently, a high number of patients with stroma-high tumors presented with stroma-low LN metastases. This finding might be reflective of differential activity of signaling processes across primary and metastatic tumors. The formation of genetically and

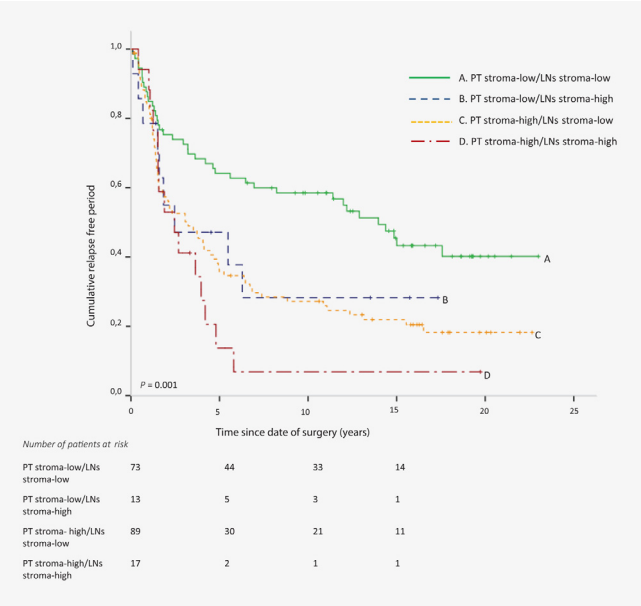

Figure 3. Kaplan–Meier analysis for relapse free period of patients with PT stroma-low/LNs stroma-low, PT stroma-low/LNs stroma-high, PT stroma-high/LNs stroma-low, PT stroma-high/LNs stroma-high. Abbreviations: PT, primary tumor; LN, lymph node. [Color figure can be viewed at wileyonlinelibrary.com]

transcriptionally distinct subclones of tumor cells that arise during tumor evolution might have an influence on both the activation of tumor-associated stroma as well as tumor cell dissemination. In the current study, we found that at least one LN with a high amount of stroma was predictive for a statistically significant decreased RFP.

A previously published study by Van Pelt *et al.* also showed the additional value of TSR in lymph nodes. The authors concluded that the assessment of TSR in the PT combined with the TSR in metastatic LNs has an additional value with regards to the prediction of DFS in patients treated with adjuvant therapy for stage III colon cancer.[17] Incorporating the TSR in clinical practice has certain advantages compared to other potential biomarkers. TSR scoring can be carried out on standard H&E slides and is performed by visually eyeballing the tissue area during standard pathological assessment. TSR scoring takes less than a minute and requires no additional costs. Implementation of this method in daily practice is therefore an easy and non-expensive option. The concordance of the inter-observer variability has been high between researchers from our group, which is confirmed in the current study.[6,10,14]

The patients for this study were primary treated with surgery between 1985 and 1994 and are part of a well characterized treatment cohort with long-term follow-up. However, this obviously means that modern-day adjuvant chemotherapy and hormonal regimens and selection of these treatment modalities according to current guidelines were not applied to this dataset. This is reflected by the relatively poor prognosis of the included patients compared to currently treated patient groups. Therefore, before definitive conclusions can be made regarding the prognostic and therapeutic implication of tumoral LN fibrosis, validation of the current results in modern-day cohorts should be undertaken.

Lastly, according to treatment guidelines, breast cancer patients first undergo a sentinel lymph node biopsy (SLNB) in case of no suspicion of positive lymph nodes by ultrasound or clinical examination.[1] Depending on the presence of LN metastasis an ALND will be performed. Evaluation of TSR in a tumor-positive LN dissected during sentinel node procedure is interesting. A recent publication from Giuliano *et al.* showed that less invasive SLNB alone was non-inferior to predicting overall survival compared to ALND in women with T1 or T2 tumors, no palpable axillary adenopathy and 1 or 2 positive sentinel LNs.[19] Evaluation of TSR in sentinel nodes could be an important next step to evaluate if this clinical prognostic marker can select patients who will benefit from ALND or axillary radiotherapy.

Conclusion

The TSR is a simple, fast and cheap method. Assessing the TSR on tumor-positive LNs can provide further prognostic stratification in breast cancer patients. Stromal activation strongly differs between PTs and LN metastases, likely reflecting heterogeneity of tumor stroma metastasis process.

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