Applications of the tumor-stroma ratio: Towards clinical implementation

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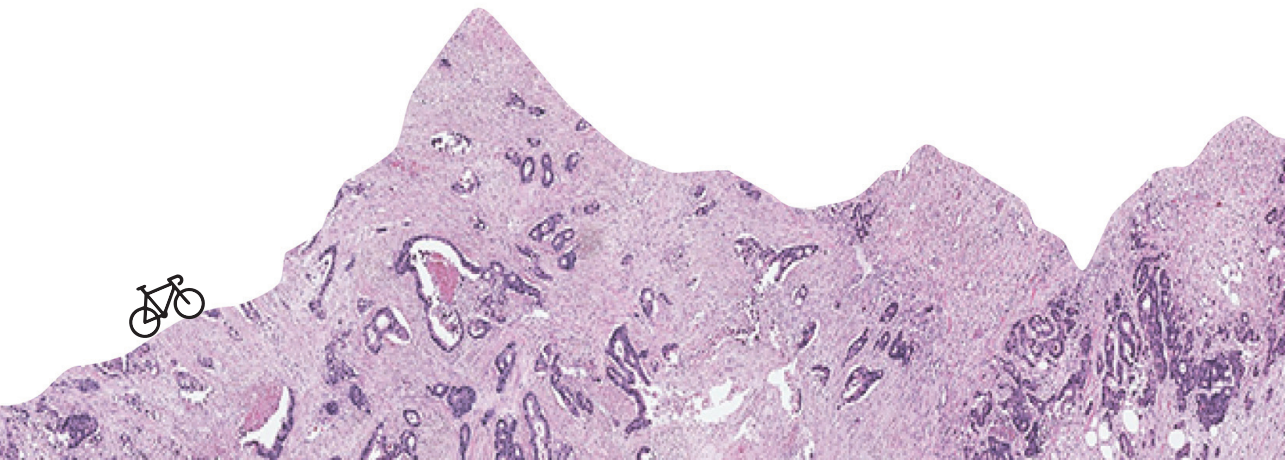
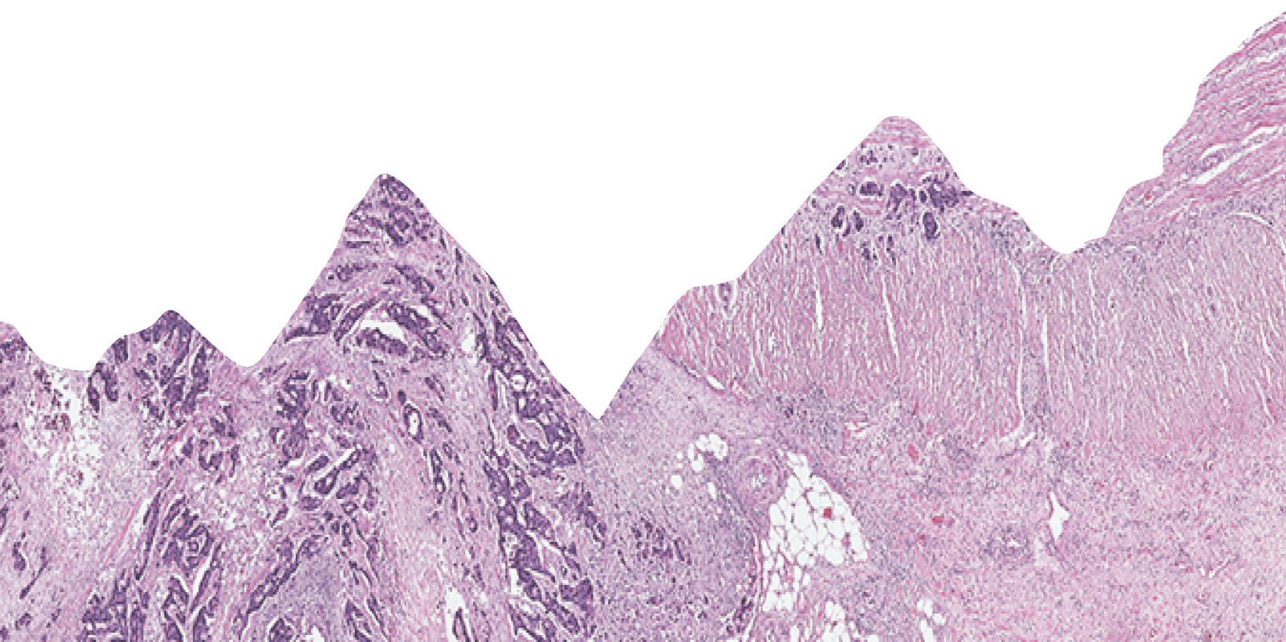


CHAPTER 1

General introduction and thesis outline



The tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC) provides prognostic information and aids in clinical decision making [1-3]. It is developed to define the anatomical extent of tumor spread, making epidemiological studies comparable, and is commonly used for clinical decision making since it has prognostic impact. However, it's becoming more evident that additional prognostic factors are needed [4]. For example, 5-25% of stage II colorectal cancer patients will develop recurrence of disease within 5 years. In addition, patients with stage IIB have a worse prognosis compared to stage IIIA colorectal cancer patients, which could lead to undertreatment (stage II) or overtreatment (stage III) [5]. Previously proposed additional biomarkers have been based on tumor cell characteristics, like tumor cell morphology, molecular pathways, gene expression and the tumor immune response [6-8]. A disadvantage of some of these is the high costs of generating genetic and transcriptomic data.

After years of focusing on the malignant cells to understand the role of tumor suppressors and oncogenic factors in the transformation to malignancy, nowadays the so-called tumor microenvironment (TME) is subject of investigation as well. It is increasingly known that the malignant cell relies on this stromal part of the tumor and therefore does not act alone. Intratumoral stroma within the TME is variable and different cell-types like infiltrating immune cells, cancer-associated fibroblasts, endothelial cells and pericytes all play a role in supporting malignant transformation, invasion of the tumor and metastasis [9, 10]. Moreover, intratumoral stroma has been associated with reduced chemotherapy delivery [11] and increased chemotherapy resistance [12], and consequently could play a role in patient treatment outcome.

A prognostic biomarker based on the TME is the tumor-stroma ratio (TSR), which is the main subject of this thesis. This biomarker is based on microscopic pathological analysis on conventional hematoxylin and eosin (H&E)-stained paraffin sections and assessment is fast, cheap and reliable. It has been shown to link patients with a high stromal content to worse prognosis. Since 2007, when Mesker et al. first published the TSR to be of prognostic value for survival of stage I-III colon cancer patients, many studies have validated this finding in different types of epithelial cancer [13-32].

An extensive literature overview on the background of tumor stroma and the tumor-stroma ratio is given in **Chapter 2,** followed by a detailed description of the assessment of the tumor-stroma ratio in **Chapter 3**, including possible pitfalls and recommendations.

Metastatic lymph nodes have important prognostic implications. In colorectal cancer, lymph node-negative patients have a more than 20% higher survival rate after 5 years compared to lymph node-positive patients [5]. Furthermore, research has shown that not only the number of positive lymph nodes is important for patient outcome, but also the composition of the microenvironment within the lymph node metastasis [33]. In **Chapter 4** we evaluated the TSR in metastatic lymph nodes of stage III colon cancer patients to investigate the possible additional prognostic value. Moreover, it was of interest whether the TSR in the lymph nodes was associated with the TSR in the primary tumor. These same research questions have also been investigated within a cohort of primary breast cancer patients. In **Chapter 5** the results of these studies on the prognostic importance of the TSR in metastatic lymph nodes have been summarized.

The search for prognostic and predictive biomarkers for implementation in routine clinical diagnostics has already resulted in several biomarkers which nowadays are used in the clinic to characterize colorectal tumors and determine specific treatment. Amongst them are the microsatellite instability (MSI) status and mutations in *BRAF* and *KRAS*. MSI status is the most consistently used biomarker for colorectal cancer prognosis in clinical practice, whereas *BRAF* and *KRAS* mutations are primarily used in the metastatic setting. However, in general, colorectal cancer patients are being treated in the adjuvant setting regardless of clinical or molecular characteristics, with heterogenous response to treatment. Patients with a *BRAF* mutation are known to have a poor prognosis and to not respond well to standard chemotherapy or *BRAF* targeted therapy [34, 35]. Studies showed the existence of different colorectal cancer subtypes [6], and even more specifically, two *BRAF* mutant subtypes [36]. Hence, the need for understanding the biology of *BRAF* mutated colorectal cancer and recognizing the different subtypes to select the most effective treatment for these patients. Unfortunately, most of the methods to detect these subtypes are based on gene expression arrays, which are, as stated before, difficult and expensive to implement clinically, whereas the TSR is easier to implement with little additional costs. Therefore, as described in **Chapter 6,** we investigated whether the TSR in combination with the *BRAF* mutation status could select for a subgroup of patients who might need a different treatment approach.

Due to changes in the tissue composition induced by neoadjuvant chemo- and/or radiotherapy, patients who are treated prior to surgery need to be excluded for TSR scoring [37-40]. As a consequence, rectal cancer and esophageal cancer patients, who often receive neoadjuvant therapy, are usually excluded from TSR studies. Analyzing the TSR in biopsies to predict the

prognosis of the patient might be an alternative, although the TSR cannot be determined at the most invasive front. However, in esophageal cancer, for instance, it has been shown that the TSR score assessed on biopsies could still be used as an independent prognostic factor for survival [41]. Especially for stroma-high cases it might be of interest to use the TSR as prognosticator, as the correlation between biopsies and primary tumors was found to be 100%. Another highly interesting approach to use the TSR in biopsies, is to see if it could aid in the prediction of the response to neoadjuvant treatment. This might prevent unnecessary neoadjuvant therapy for patients who are likely not to respond well to the treatment and therefore could continue directly with resection. This potential use of the TSR was investigated in both rectal cancer (**Chapter 7**) as well as in esophageal cancer (**Chapter 8**) patients.

Finally, the research published in this thesis is summarized and discussed in **Chapter 9**. The future perspectives on how to continue this line of research towards implementation for patient care are described here as well. **Chapter 10** describes the summary, discussion and future perspectives in Dutch.

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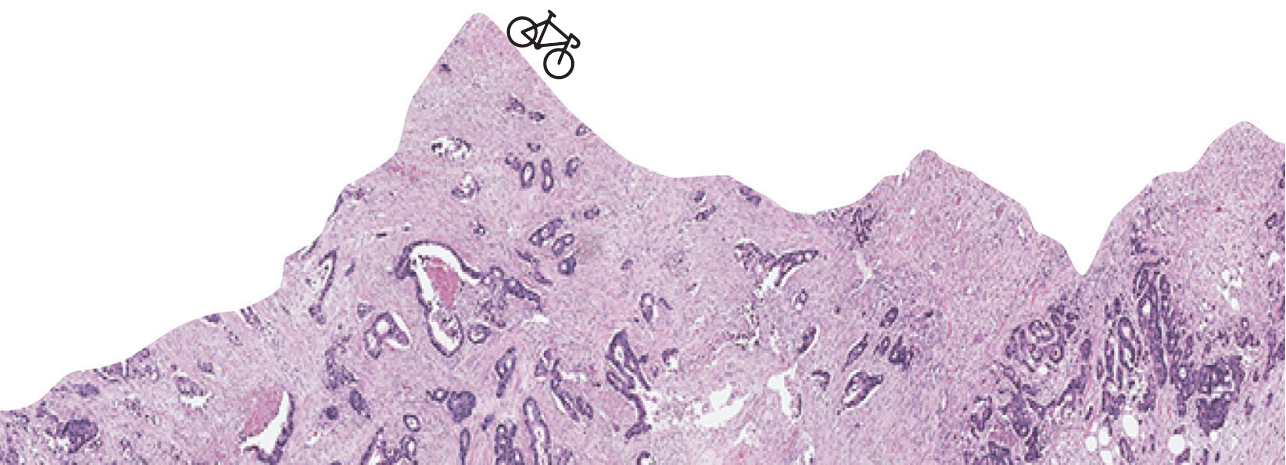

CHAPTER 2

The tumor-stroma ratio in colon cancer; The biological role and its prognostic impact

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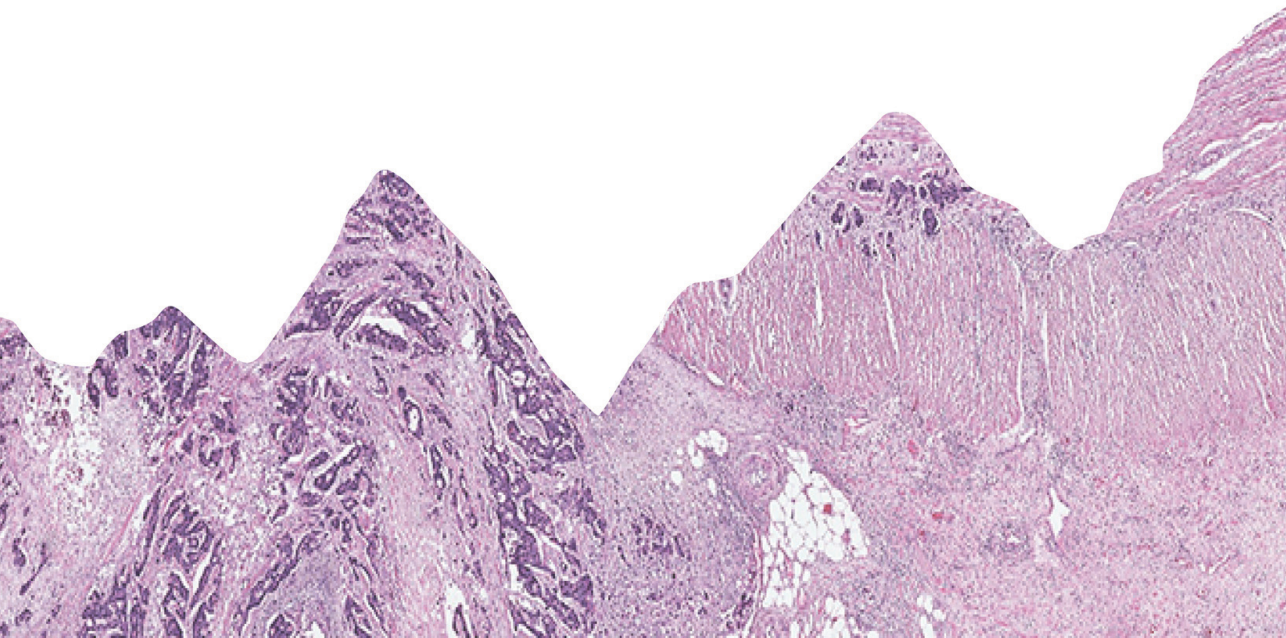
*both authors contributed equally

Histopathology 2018 Aug;73(2):197-206



Abstract

The tumor microenvironment consists of a complex mixture of non-neoplastic cells including fibroblasts, immune cells and endothelial cells embedded in the proteins of the extracellular matrix. The tumor microenvironment plays an active role in tumor behavior. By interacting with cancer cells, it influences disease progression and the metastatic capacity of the tumor. Tumors with a high amount of stroma correspond to poor patient prognosis. The tumor-stroma ratio (TSR) is a strong independent prognostic tool in colon cancer and provides additional value to the current clinically used tumor-node-metastasis classification. The TSR is assessed on conventional hematoxylin and eosin-stained paraffin sections at the invasive front of the tumor. Here we review studies demonstrating the prognostic significance of the TSR in solid epithelial tumors with a focus on colon cancer. Moreover, the biological role of the tumor microenvironment during tumor progression and invasion will be discussed as well as the attempts to target the tumor stroma for therapeutic purposes. We suggest that the TSR can be implemented with little effort and without additional costs in current routine pathology diagnostics owing to its simplicity and reliability.

Keywords

Colon cancer, TNM classification, tumor microenvironment, tumor-stroma ratio

Introduction

The tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC) is most commonly used in clinical decision making to define the extent of tumor progression. The TNM provides prognostic information and aids in treatment decision [1-3]. However, clinical outcome varies between patients with colon cancer within the same TNM stage. For instance, 5-25% of stage II patients still develop recurrence of disease within 5 years. In addition, patients with stage IIB have a worse prognosis compared to stage IIIA colon cancer patients, leading in some cases to undertreatment of stage II patients and overtreatment of stage III patients [4].

The current TNM classification is based on anatomical extent, but there is need for additional prognostic and/or predictive markers [5]. Additional biomarkers have been proposed based on tumor cell characteristics, including tumor cell morphology, molecular pathways, genetic mutations, cell of origin and gene expression (see below), as well as the tumor immune response (Figure 1) [6]. A drawback of some of these is the high cost of genetic and transcriptomic data, whereas standard pathological assessment using microscopical analysis is fast, cheap and reliable. A biomarker that is based on microscopical analysis is therefore desirable. The tumor-stroma ratio (TSR), also referred to as the tumor-stroma percentage, is assessed on conventional hematoxylin and eosin (H&E)-stained paraffin sections at the invasive front of the tumor and links patients with high stromal reaction to worse prognosis. The TSR has been reported as a strong independent prognostic tool in colon cancer as well as in other epithelial cancers [7-24]. The importance of the tumor stroma is emphasized in the recent consensus molecular subtypes (CMS) classification of colorectal cancer (CRC). The CMS1 – 4 was assessed based on transcriptome analysis of CRC. Tumors classified as CMS4 were characterized by a worse prognosis, activated transforming growth factor (TGF)-β and increased stromal content [6]. Two studies showed that stromal cells contribute extensively to the mesenchymal phenotype of aggressive CRC categorized as CMS4 [25, 26].

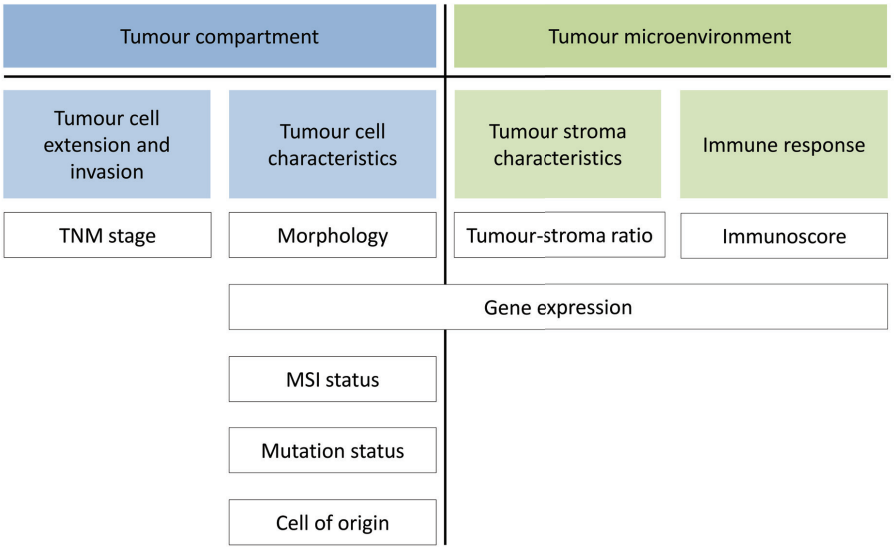


Figure 1. Distinct colorectal cancer classifications based on tumor compartment and tumor microenvironment.

The tumor stroma consists of a complex mixture of non-neoplastic cells including fibroblasts, immune cells and endothelial cells embedded in the proteins of the extracellular matrix (ECM). The activated form of fibroblasts, the so-called cancer-associated fibroblasts (CAFs), are the predominant cell type in the tumor stroma and are involved in tumor progression and invasion. Stromal cells supply the tumor with growth factors, cytokines and metabolites and stimulate blood vessel formation (Figure 2). In this way the tumor stroma contributes to tumorigenesis and induction of epithelial-mesenchymal transition (EMT) in cancer cells [27]. This explains why a tumor with a high stromal content reflects a prometastatic phenotype of cancer cells and that the interaction between cancer and stromal cells affects disease outcome and response to therapy [28, 29]. However, the biological mechanism of cancer cells recruiting and activating fibroblasts is not completely understood.

Here we will give an overview of the prognostic value of the TSR in colon cancer as well as in other epithelial cancers types. Moreover, the biological role of the tumor microenvironment during tumor progression and invasion will be discussed, as well as the attempts to target the tumor stroma for therapeutic purposes.

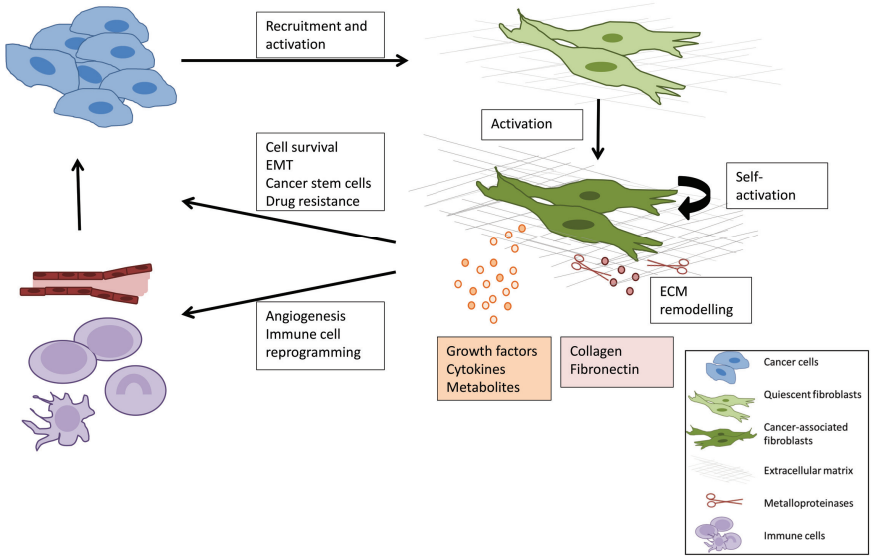


Figure 2. A simplistic scheme of the direct and indirect effect of cancer-associated fibroblasts (CAFs) on cancer cells. The activation of CAFs through transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF) induces angiogenesis, reprograms immune cells in the tumor microenvironment and leads to cancer cell survival. Also, the secretion of cytokines and different soluble molecules by CAFs induces cancer cell survival, epithelial-mesenchymal transition (EMT), stem cell properties and drug resistance in cancer cells.

Methodology of the tumor-stroma ratio

The TSR is evaluated based on routine 5-µm thick H&E sections using conventional microscopy. The intratumoral stroma formation is assessed at the invasive part of the tumor, which is most determinative for tumor progression. This was decided in a study of colon cancers in which multiple H&E slides from different areas of the tumor were available for scoring. Heterogeneity in the percentage of stroma was observed throughout the tumor and the highest stroma percentages were observed in the tumor areas with the deepest invasion in the bowel wall (higher T-stage) [8]. For retrospective studies, the slide with the most invasive part of the tumor generally corresponds to the slide used in routine pathology to determine the T-status and is indicated in the pathology report.

Areas covered with the largest amount of stroma are selected using a x2.5 or x5 objective. Using the x10 objective, image fields are scored in increments of 10%. Tumor cells are to be present at the four borders of the selected image field (Figure 3). Identifying one single image-field with high stroma content is decisive for a final stroma classification. A statistically determined cut-off value of 50% distinguishes between stroma-high (>50%) and stroma-low (≤50%) patients [8]. Using these criteria, scoring of the TSR is relatively easy resulting in a low inter-observer variation in different published validation studies (Table 1)[7-9, 12, 30].

The TSR is estimated adequately in resection specimens of patients operated for a primary epithelial tumor, including mucinous tumors. However, patients pretreated with chemo- and/or radiotherapy are generally excluded from TSR scoring. Therapy induces changes in tissue arrangements as cell morphology and composition, resulting in stromal formation surrounding the tumor [31-34]. Analyzing the TSR in biopsies to assess the prognostic value of the patient is an alternative for patients pre-treated with chemo- and/or radiotherapy (see below).

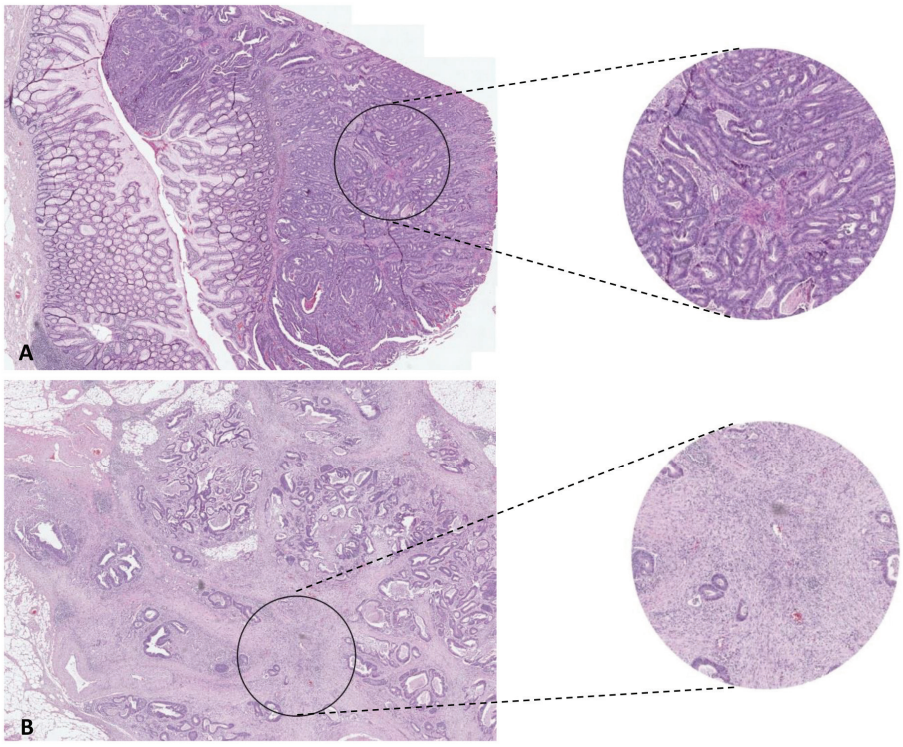


Figure 3. Examples of stroma-low (A; 20% stroma) and stroma-high (B; 90% stroma) hematoxylin and eosin (H&E) stained paraffin sections at the most invasive part of primary colon cancers.

Tumor-stroma ratio, a prognostic factor in colon cancer

TSR in primary colon cancer

Multiple studies, performed and validated by different research groups, demonstrate that the TSR is a robust prognostic factor in colon cancer. In 2007, Mesker *et al*. developed the TSR for patients with stage I – III disease, and found that patients with tumors with a high stromal content had a significantly worse overall survival ($P < 0.001$) and disease free survival ($P < 0.001$), independently of T-stage and N-stage [8]. The studies of Huijbers *et al*., Park *et al*. and van Pelt *et al*. found comparable results for overall and disease free survival ($n = 710$, $P = 0.002$ and $P < 0.001$), cancer specific survival ($n = 250$, $P = 0.009$), and disease free survival ($n = 102$, $P = 0.038$), respectively [7, 10, 11]. West *et al*.'s research group used a semi-automated method to investigate the prognostic value of the relative proportion of tumor at the luminal surface. Although a different method compared to the TSR, they found a comparable cut-off value of 47%, leading to similar results [12] (Table 1). Both Park *et al*. and West *et al*. included rectal cancer patients who did not receive neoadjuvant therapy. However, their results were comparable with studies only investigating colon cancer patients (from caecum to sigmoid colon).

The adverse prognostic impact of high tumor stroma is observed in both early disease and advanced colon cancer. As patients with stage II colon cancer have highly variable outcomes, the TSR is a useful tool to select patients who are at risk of developing recurrence of disease or metastases. Consequently, this subpopulation might also be considered for adjuvant therapy, a decision based currently on the American Society of Clinical Oncology (ASCO) criteria including T4 tumor stage, the number of lymph nodes examined (<10), poor tumor differentiation, presence of lymphatic, vascular and/or perineural invasion and perforation of the bowel wall. The study of Huijbers *et al*. investigated the TSR next to the ASCO criteria to select high risk stage II colon cancer patients. They found that the TSR improved the ASCO criteria and reclassified 14% of the patients as high-risk, thereby dropping the rate of undertreated patients from 6% to 4% [7]. This suggests that adjuvant therapy might be considered in stage II patients with high tumor stroma content. Further research should assess the effectiveness of adjuvant therapy in stroma-high patients.

Table 1. Characteristics of tumor stroma studies in colorectal cancer.

Study	Number of patients	Stage	Outcome (HR (95%CI))	Interobserver variation
Mesker *et al.*, 2007	122	I-III	OS: 3.74 (2.32-6.01), *P* < 0.001 DFS: 4.18 (2.63-6.65), P < 0.001	NS
Mesker *et al.*, 2009	135	I-II	OS: 2.73 (1.73-4.30), *P* < 0.001 DFS: 2.43 (1.55-3.82), *P* < 0.002	κ = 0.6-0.7 (3 observers)
Huijbers *et al.*, 2013	710	II-III	OS: 1.71 (1.22-2.41), *P* = 0.002 DFS: 1.95 (1.45-2.61), *P* < 0.001	κ = 0.89
West *et al.*, 2010a	145	I-IV	CSS: 2.09 (1.09-4.00), *P* = 0.017	κ = 0.97
Park *et al.*, 2014	250	I-III	CCS: 1.84 (1.17-2.92), *P* = 0.009	κ = 0.81
van Pelt *et al.*, 2016	102	III	DFS PT: 1.98 (1.04-3.77), *P* = 0.038 DFS PT+LNs: 2.85 (1.33-6.10), *P* = 0.007	κ = 0.73
Hynes *et al.*, 2017	445	II-III	CSS: 1.45 (0.92-2.29) OS: 1.49 (1.02-2.20)	κ = 0.5-1.0 (4 observers)

[a]West et al. used a cut-off point of 47% with a semi-automated method.

Abbreviations: NS, not stated; HR, hazard ratio; CI, confidence interval; OS, overall survival; DFS, disease free survival; CSS, cancer specific survival; PT, primary tumor; LNs, lymph nodes

TSR in metastatic lymph nodes of colon cancer

The prognostic implications of metastatic lymph nodes have been widely established. Lymph node-negative patients have a 5-year survival rate of more than 58% (stage IIC), decreasing to 35% when lymph nodes are involved (stage IIIC) [4].

Although lymph node involvement has proven its importance, all studies investigating the TSR in colon cancer patients have found the TSR to be a prognostic factor independent of the N-status [7, 8, 10-12]. Moreover, evaluation of the TSR in metastatic lymph nodes of stage III colon cancer patients has recently been shown to be of additional prognostic value. A strong heterogeneity of TSR between lymph nodes of a single patient was observed, and it was found that the presence of abundant stroma in at least one lymph node already contributed significantly to the prognostic information initially learned solely from the primary tumor (*P* = 0.007) [11]. These findings emphasize that not only the number of positive lymph nodes but also the composition of the microenvironment within the lymph node metastasis is important for patient outcome [35].

TSR in pre-operative biopsies

As mentioned previously, patients pretreated with chemo- and/or radiotherapy are not eligible for tumor stroma scoring due to therapy related stromal formation. As a consequence, rectal cancer patients, who often receive neoadjuvant therapy, are usually excluded from TSR studies.

Scoring the TSR on biopsies of neoadjuvantly treated patients might be a good alternative, although the TSR cannot be determined at the most invasive front. In esophageal cancer, for instance, TSR score assessed on biopsies was an independent prognostic factor for survival, in line with the TSR in primary tumors [36]. The TSR scores of the primary tumor and the matching presurgical biopsy correlated in 81% of the cases. The remaining discrepant scores were stroma-high primary tumors while the matching biopsy was assessed as stroma-low, thereby underestimating the TSR and leading to false-negative selection. However, as the biopsies showed a high correlation with matching resection material, especially for stroma-high cases (100% correlation), biopsies could be used for prediction of patient outcome. Eventually, it would be of interest if the TSR scores of biopsies could be used to predict the response to neoadjuvant treatment.

The biological mechanism of the tumor stroma in colon cancer

The tumor microenvironment formation

A high stromal content is a reflection of the highly activated interaction between tumor and stromal cells. During tumor progression, specific molecular changes in colon cancer cells cause the recruitment and activation of surrounding stromal cells by releasing soluble growth factors, metabolites and cytokines [37]. Two main cancer cell-secreted growth factors are TGF-β and platelet-derived growth factor (PDGF), which have been largely acknowledged to mediate the conversion of normal fibroblasts into CAFs (Figure 2) [37-39]. Mitogenic factors secreted by fibroblasts include hepatocyte growth factor [27], fibroblast growth factors, epidermal growth factor family members and chemokine ligand 12 [40]. In addition, a number of studies analyzing transcriptomic data have reported that the activation level of CAFs present in the tumor showed prognostic value in colorectal cancer [26, 41, 42].

The TGF-β signaling pathway is considered a central player during tumor progression. The pathway exerts a dual role: its activation can function as a tumor suppressor by inducing apoptosis in normal cells and early stage cancers and can later promote tumorigenesis. The paradox that high levels of TGF-β1 correlate with poor prognosis can partially be explained by the fact that the tumor stroma remains highly responsive to the growth factor. TGF-β-activated CAFs secrete a range of growth factors that support tumor growth and induce a mesenchymal phenotype in cancer cells [37].

The role of the tumor microenvironment in tumor progression

Various mechanisms have been proposed to explain how the tumor microenvironment contributes to tumor progression, tumor invasion and metastasis, for instance by (i) impacting the proliferation and survival of cancer cells, (ii) increasing their stem-like properties and favoring EMT [27, 38, 43], (iii) rewiring the tumor metabolism [40] and/or (iv) stimulating metastatic dissemination (Figure 2). *In-vivo* studies demonstrated that co-injection of cancer cells and CAFs or mesenchymal stem cells lead to an increased tumor growth, invasion and metastasis compared to co-injection of cancer cells with normal fibroblasts [44, 45].

The tumor stroma provides a nourishing environment that maintains cancer stem cells (CSCs) in a tumor. CSCs are characterized by an activated Wnt pathway and the nuclear translocation of the oncoprotein β-catenin. Vermeulen *et al.* showed that colon cancer cells located at the tumor invasive front acquire an increased stem-like state due to stromal fibroblasts activating the Wnt pathway, compared to cancer cells located in the central part of the tumor. These results suggest that CAFs foster stemness of cancer cells [27]. Tumors with an increased number of CSCs are predictive of a negative patient outcome due to intratumoral heterogeneity [28, 29]. Furthermore, stem-like properties acquired by premetastatic cancer cells are linked to EMT induction, a process where cancer cells lose epithelial characteristics and acquire mesenchymal properties. It was found in several studies that the tumor stroma, in particular myofibroblasts, can induce EMT in cancer cells via cell-to-cell contact [45, 46].

In addition, soluble factors secreted by cancer cells participate in the metabolic reprograming of CAFs. CAFs rely on aerobic glycolysis, a metabolism comparable to that of highly proliferating cells. The metabolic alteration in CAFs, in its turn, probably promotes the cancer cell metabolic adaptation [47]. The tumor stroma can impact the aggressive behavior of cancer cells not only through cell-cell contact and auto- and paracrine signaling but also through mechanical pressure. Due to the abundant ECM and the high number of CAFs, the tumor stroma forms a physical barrier around the tumor that increases the interstitial pressure and hypoxia in the tumor. Cancer cells respond to hypoxic conditions through the up-regulation of hypoxia-inducible factor 1α, a master transcription factor that activates a whole range of genes involved in angiogenesis, migration, metabolism, tumor invasion and metastasis [48].

Targeting the stromal compartment

While tumor cells have been the main therapeutic target in the past, different components of the tumor microenvironment, such as immune cells and angiogenesis, have been targeted

recently. Based on the understanding of the tumor stroma, oncogenic pathways activated in the tumor microenvironment, CAF markers and their soluble molecules can be targeted therapeutically [42]. For instance, the TGF-β pathway is highly increased in fibroblasts of stroma-high tumors. Based on preclinical studies, different TGF-β targeting agents were used in clinical trials, such as the TGF-β receptor kinase inhibitor galunisertib (rectal adenocarcinoma NCT02688712, Phase II), showing both negative as well as positive results. The dual function of the signaling pathway makes it a challenging target [49]. For an extensive summary of TGF-β targeting drugs, see the review by Colak *et al* [50]. Another activated signaling pathway is the PDGFR pathway which can be targeted by imatinib anticancer drug. The ongoing ImPACCT clinical trial investigates the efficacy of the drug in patients with colon cancer characterized as CMS4, described in Ubink *et al* [51].

Therapeutically targeting CAFs can also promote anti-tumor response and it could be used in combination with standard therapy in order to target both CAFs and cancer cells. For instance, sibrotuzumab is an antibody that inactivates the CAF marker FAP. Clinical trials have failed however to show clinical efficacy in metastatic colorectal cancer [52].

Furthermore, the tumor microenvironment exerts an important influence on therapy response. Previous preclinical and clinical studies showed that tumors with high stromal content become resistant to therapy. Lotti *et al*. demonstrated that chemotherapy-treated CAFs promoted tumor-initiating cells and tumor growth *in vivo* [53]. Similar results were found in endothelial cells able to induce chemoresistance in CRC cells [54]. Consistent with the preclinical studies, a correlation was found between poor prognosis and increased amount of stroma in tumors pretreated with radio- and/or chemotherapy [55, 56]. Song *et al*. showed in a randomized clinical trial that CRC patients at stages II-III of the CMS4 subtype did not benefit from adjuvant oxaliplatin [57]. Furthermore, a retrospective study showed that patients with rectal cancer of the CMS4 subtype had poor response to radiotherapy [26].

Acquiring further insights in the complexity between the cancer cells and its microenvironment may provide novel tumor stroma-targeted therapy as well as a better understanding of drug resistance.

TSR ratio in solid epithelial tumors

The prognostic value of the TSR reaches further than colon cancer; it is also observed in a range of different other solid epithelial tumors [13-24]. Recently, an elaborated meta-analysis was

conducted on 14 studies with 4238 patients to study the TSR on prognosis in solid tumors. The authors identified that stroma-high tumors were associated with worse overall survival and disease-free survival in colon cancer, breast cancer, ovarian cancer, non-small cell lung cancer, nasopharyngeal cancer, esophageal cancer and hepatocellular cancer [58]. However, two articles studying early stage cervical cancer found contradictory results [17, 22]. The study by Pongsuvareeyakul *et al*. did not reveal an independent prognostic value of the TSR [22]. This might be explained by the fact that this study had a small number of recurrences and death, which might reduce the ability of statistical analysis. Furthermore, in contrast to the study by Liu *et al*., Pongsuvareeyakul *et al*. only included cervical adenocarcinoma patients and no squamous carcinoma patients, suggesting that histological types of cervical cancer might have a different impact on prognosis. This should be further investigated. Similar to colon cancer, the TSR method also has a high interobserver agreement in a variety of studies of other epithelial cancer types (Table 2) [13-22, 59]. The use of the TSR across tumor types emphasizes the robustness of the method.

Table 2. Characteristics of tumor stroma studies in other types of epithelial cancers, which adapted the method described in this paper and reported an interobserver variation.

Study	Number of patients	Stage	Type of cancer	Interobserver variation
Courrech Staal *et al*., 2010	93	I-IV	Esophageal	κ = 0.84
De Kruijf *et al*., 2011	574	I-III	Breast	κ = 0.85
Moorman *et al*., 2012	124	I-III	Breast (triple-negative)	κ = 0.74
Dekker *et al*., 2013	403	I-III	Breast	κ = 0.80
Wang *et al*., 2013	95	I-III	Esophageal	κ = 0.84
Gujam *et al*., 2014	361	I-III	Breast	κ = 0.83
Liu *et al*., 2014	184	I-II	Cervical	κ = 0.81
Zhang *et al*., 2014	93	I-IV	Nasopharyngeal	κ = 0.85
Lv *et al*., 2015	300	I-IV	Liver	κ = 0.87
Pongsuvareeyakul *et al*., 2015	131	I-II	Cervical	κ = 0.78
Li *et al*., 2017	51	II-IV	Gallbladder	κ = 0.85

Daily diagnostic practice

Many prognostic biomarkers have been, or are currently, under investigation for implementation in routine clinical diagnostics. For instance, mutations in *BRAF* and *KRAS* and the microsatellite instability (MSI)-status are well-known prognostic and predictive markers used in the clinic to

characterize colorectal tumors and determining specific treatment. Besides its prognostic value, the TSR might be used as an additional high-risk factor to select patients for adjuvant therapy. We believe that stroma-high tumors should be treated accordingly. However, there is as yet no information how stroma-high tumors will respond to adjuvant therapy.

Potential prognostic markers as the Immunoscore [60], tumor budding [61] and the CMS classification [6] have been proposed for implementation in daily practice. In order for a biomarker to be implemented into the clinic it has to show clinical relevance. Also, feasibility and ease to use are important factors.

In our opinion, it is time to combine biomarkers which integrate different aspects of the tumor biology, including the interaction with the tumor microenvironment. In addition to the clear evidence of the prognostic value of the TSR, a critical advantage of the TSR lays within its simplicity, reproducibility and low costs. Therefore, the TSR method is applicable for all pathology centers.

Further research

Automation

An automated scoring method of the TSR is under development, which will lead to a standardized protocol with optimal reproducibility. In 2014, Bianconi *et al.* showed the possibility to discriminate between tumor epithelial and stroma in colorectal cancer patients, with an accuracy of almost 97% using an automated image analysis system. However, this study was based on an image database that consisted of small parts of tissue samples instead of whole tumor slides. The challenge for automated scoring will be to detect the areas containing the highest amount of stroma using whole slide imaging [62]. A disadvantage of an automated scoring method is the increase of cost and time due to the acquirement of a slide scanner and software. However, the digitalization of the pathology workflow asks for automated scoring of the TSR. Therefore, the automation of the method is almost inevitable.

Prospective multicenter study

The TSR has been discussed by the TNM Evaluation Committee (UICC) and the College of American Pathologists (CAP), who stated that it has the potential to be included in the TNM staging algorithm. In order to reach this, the reproducibility of the TSR method is currently being validated in a large European multicenter study. In parallel, a prospective cohort will be used to validate the potential value of the TSR as a selection tool for high-risk patients.

Conclusion

It is well established that the interaction between cancer cells and its microenvironment is involved in tumor progression and metastasis. The TSR probably reflects this interaction. CAFs constitute the most abundant cell type in the tumor stroma, and this cell population releases a cascade of growth factors promoting tumorigenesis. The tumor stroma is able to induce stem cell-like properties and EMT in colon cancer cells, making the cancer cell acquire prometastatic capacities. Acquiring further insights in the complexity between the cancer cells and its microenvironment may provide novel tumor stroma-targeted therapy and understanding of drug resistance.

Given the current understanding of the tumor stroma, colon cancer should not solely be categorized based on tumor cell characteristics, but also according to the tumor microenvironment. The TSR has been proven to have prognostic relevance in colon cancer patients. Combining this knowledge, it would suggest that the TSR should be added to the current TNM classification. Owing to its simplicity, reliability and low costs, the TSR score can be implemented with little efforts in current routine diagnostics of the pathologist.

Conflicts of interest

The authors declare no conflicts of interest.

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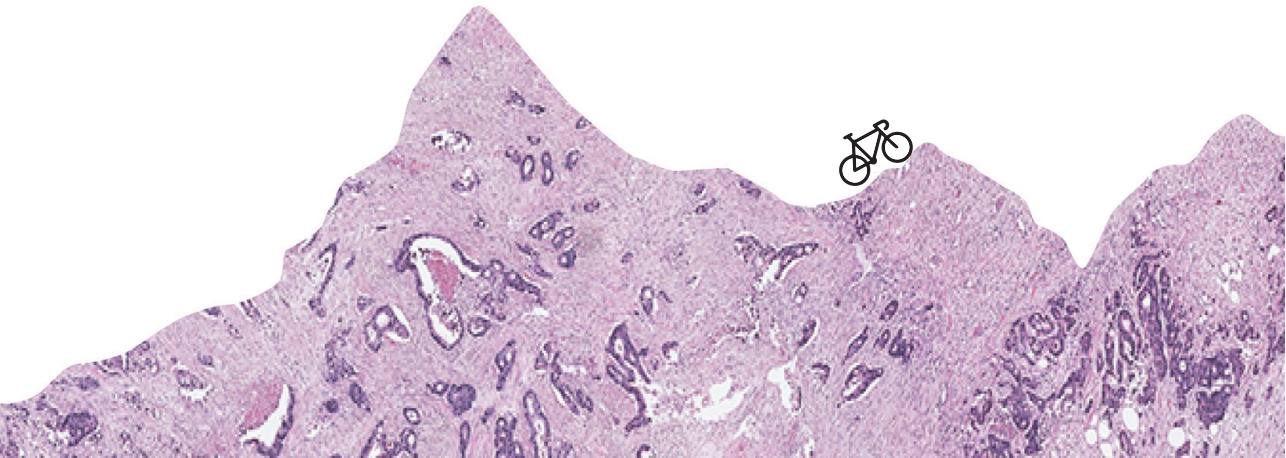

CHAPTER 3

Scoring the tumor-stroma ratio in colon cancer: Procedure and recommendations

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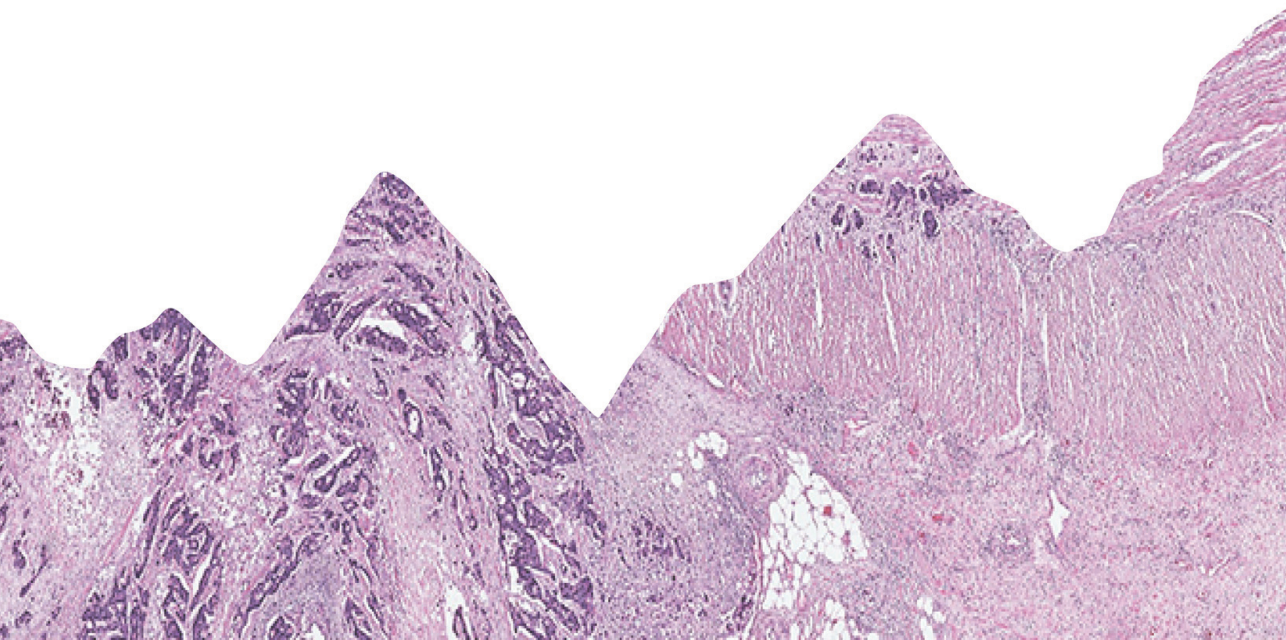

Abstract

The tumor-stroma ratio (TSR) has been reported as a strong, independent prognostic parameter in colon cancer as well as in other epithelial cancer types, and may be implemented to routine pathology diagnostics. The TSR is an easy technique, based on routine hematoxylin and eosin stained histological sections, estimating the amount of stroma present in the primary tumor. It links tumors with high stromal content to poor prognosis. The analysis time is less than two minutes with a low inter-observer variation. Scoring of the TSR has been validated in a number of independent international studies. In this manuscript, we provide a detailed technical description of estimating the TSR in colon cancer, including examples, pitfalls and recommendations.

Introduction

For many years the choice of optimal treatment of cancer has mostly been based on clinico-pathological characteristics, such as patient age and performance status, tumor type, malignancy grade, tumor size, and the presence of regional or distant metastases [1]. Current research in biomarker development is focusing more and more on the tumor microenvironment. Molecular biomarkers based on tumor characteristics have been developed, but one should not ignore valuable information provided by the tumor microenvironment, i.e. the stromal compartment of the tumor. Tumor-stroma plays an important role in cancer initiation and progression, in that the stroma interacts with nonmalignant cells as well as with malignant cells at different stages of tumorigenesis, ranging from tumor onset to invasion and metastasis [2].

As shown by our research group, the morphological evaluation of the tumor microenvironment in conventional, routine hematoxylin and eosin (H&E) stained tissue sections provides valuable information with high prognostic impact. Epithelial malignant tumors from patients with unfavorable prognosis have been documented to show a high proportion of stroma (> 50% stroma = stroma-high), whereas tumors with abundant carcinoma tissue (≤ 50% stroma = stroma-low) are associated with a better prognosis. This phenomenon has led to the development of the tumor-stroma ratio (TSR) as a prognostic parameter. Evaluation of this parameter in large patient series has confirmed its prognostic value for several types of cancers including colon [3-6], breast [7-9] and esophageal carcinomas [10]. International groups have validated our results for colon and breast cancer, and additionally, found the same prognostic value in other types of epithelial cancer, e.g. cervical and lung cancer [11-21]. The TSR scoring technique has been shown to be highly reproducible, with inter-observer kappa-values ranging from 0.68 to 0.97 (Table 1). Owing its simplicity and reliability, the TSR may add significant prognostic information to the currently used TNM classification and is well-suited and cost-effective for implementation in routine diagnostics by the pathologist.

In this paper, we describe in detail the technical protocol of determining the TSR in colon cancer, including examples, pitfalls and recommendations.

Table 1. An overview of tumor-stroma ratio studies reporting an inter-observer score.

Study	Number of patients	Stage	Type of cancer	Inter-observer variation[a]
Mesker et al., 2009 [5]	135	I-II	Colon cancer	κ = 0.6-0.7[b] (3 observers)
Courrech-Staal et al., 2010 [10]	93	I-IV	Esophageal cancer	κ = 0.84[b]
West et al., 2010 [17]	145	I-IV	Colorectal cancer	κ = 0.97
De Kruijf et al., 2011 [7]	574	I-III	Breast cancer	κ = 0.85[b]
Moorman et al., 2012 [13]	124	I-III	Breast cancer	κ = 0.74[b]
Wang et al., 2012 [15]	95	I-III	Esophageal squamous cell cancer	κ = 0.84[b]
Huijbers et al., 2013 [3]	710	II-III	Colon cancer	κ = 0.89[b]
Dekker et al., 2013 [8]	403	I-II	Breast cancer	κ = 0.80[b]
Downey et al., 2014 [24]	180	I-III	Breast cancer (ER+)	κ = 0.70
Park et al., 2014 [14]	250	I-III	Colorectal cancer	κ = 0.81[b]
Liu et al., 2014 [11]	184	I-II	Cervical cancer	κ = 0.81[b]
Zhang et al., 2014 [19]	93	I-IV	Nasopharyngeal cancer	κ = 0.85[b]
Gujam et al., 2014 [21]	361	I-III	Breast cancer	κ = 0.83[b]
Lv et al., 2015 [12]	300	I-IV	Hepatocellular cancer	κ = 0.87[b]
Pongsuvareeyakul et al., 2015 [32]	131	I-II	Cervical	κ = 0.78[b]
van Pelt et al., 2016 [6]	102	III	Colon cancer	κ = 0.73[b]
Li et al., 2017 [33]	51	II-IV	Gallbladder	κ = 0.85[b]
Roeke et al., 2017 [9]	737	I-III	Breast cancer	κ = 0.68[b]

[a]Kappa value

[b]Study in which the method described in this paper was used for scoring the TSR.

Method

Slide selection

Slides of the primary tumor are selected from the most invasive part of the colon adenocarcinoma (i.e. the slides used in routine pathology to determine the T-status). For retrospective studies, these slides are mostly indicated in the pathology report, and if not, all available tumor slides are collected and analyzed. In case of more slides to be analyzed from the most invasive part of the tumor, the section with the highest percentage of stroma is scored and decisive for the final estimation of the TSR.

Histopathological scoring

H&E stained tissue sections from the primary tumor of 4 µm thickness are analyzed by conventional microscopy. Areas appearing to have the highest amount of stroma are selected using the x2.5 or the x5 lens. Hereafter, an area where both tumor and stromal tissue are present within this vision-site is selected using a x10 objective. Tumor cells are to be present at all borders of the selected image field (Fig. 1). The amount of stroma tissue is estimated per 10% increment (10, 20, 30% etc.) per image field. For statistical analysis, stromal ratio groups are divided in stroma-high and stroma-low groups. Stroma-high is defined as > 50% stromal area, and stroma-low as ≤ 50% stromal area in the histological section, as determined *a priori* to have maximum discriminative power [4]. Even if there is only one image-field with a stroma-high score, this image-field is decisive.

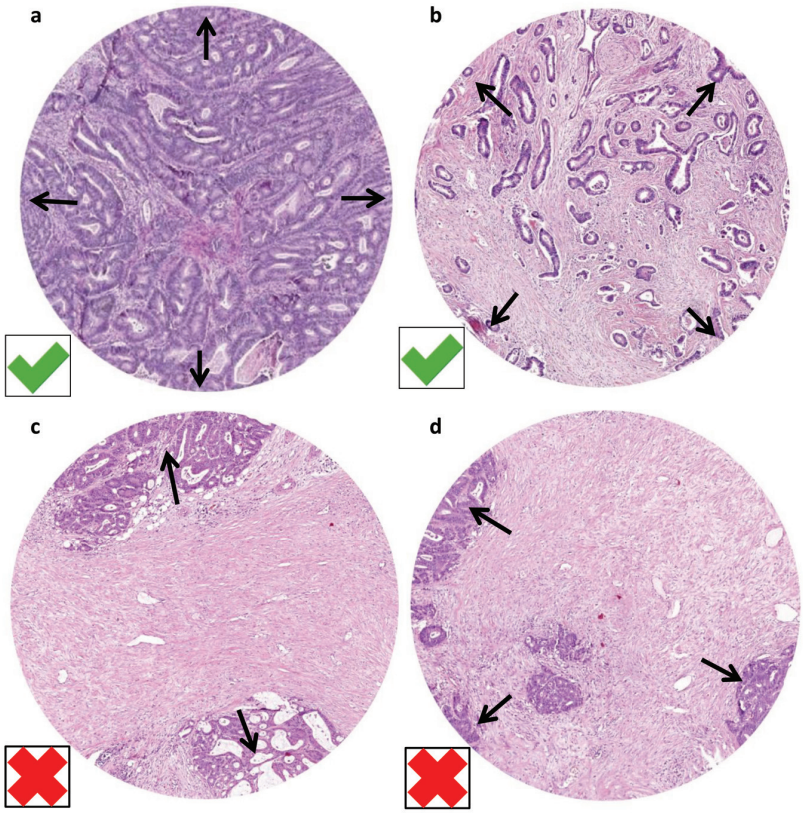

Figure 1. Examples of a stroma-low (**a**) and stroma-high (**b**) colon carcinoma, which meet the criteria for the presence of vital tumor cells on all 4 sides of the field of vision (arrows) and are thus correct for scoring. When tumor cells are only present at two (**c**) or three (**d**) sides of the field of vision (mucus is not included in estimating TSR), these areas are not suitable for scoring (Images displaying the microscopic view, all images 100x magnification).

When scoring the TSR, misinterpretations while estimating the percentage of stroma can occur due to general issues, as well as based on specific histological issues. Both are discussed below.

General issues

Different oculars

In daily practice, different microscopes are available with different lens specifications, leading to different area sizes of the field of vision. With most used oculars having a diameter ranging from 18 mm to 22 mm, the area of the field of vision will range from 2.54 mm^2 to 3.80 mm^2. However, only in exceptional cases a larger field of vision will make it able to meet the criterion of tumor cells needing to be present at all borders, whereas with a smaller field of vision this might not be possible, or *vice versa*. For scoring the TSR this has not lead to any major differences in scoring percentages.

Quality of H&E staining

An important factor for determining the TSR is the quality of the H&E stain. When the stain is too pale or too intense it is difficult to distinguish the stromal tissue from the smooth muscle tissue of the bowel wall. This may happen, when using too thin or too thick histologic sections, respectively.

If the TSR scoring cannot be carried out optimally due to the quality of the stain, it is recommended to re-stain the section before scoring the TSR.

Only one possibly stroma-high area (stromal component > 50%)

In case there is only one area/field of vision that might be categorized as stroma-high, but doubt remains (even after consulting a second observer), we recommend to consider the total composition of the whole tissue section with the x2.5 or x5 objective to classify that particular case. However, if there is no doubt that the one and only field is stroma-high (or consensus can be reached), the case is classified as stroma-high.

Histological issues

It is always preferred to score a field of vision in which no muscle tissue, necrotic tissue, and/or large blood vessels are present, but as this might not always be the case, we discuss the options below and provide our recommendations, also regarding other tissue qualities (see Table 2 for a summary).

Mucinous adenocarcinomas

In mucinous cancers, it can be very difficult to estimate the TSR correctly. The mucus is allowed to be present in the field of vision, but has to be visually ignored from scoring (Table 2, Fig. 2a, Supplementary fig. 1). It may also be possible to determine the TSR in the non-mucinous area of a mucinous tumor's deepest penetration of the bowel wall.

Infiltration with inflammatory cells

Heavy inflammation is often encountered within the stromal component in the tumor microenvironment of colon adenocarcinomas, and can be included in the TSR scoring as part of the stroma. However, lymphoid follicles may represent an integrated part of the "native" histology of the large bowel, and thus may not constitute a response to the expanding epithelial tumor within the tumor microenvironment. Thus, we recommend areas with lymphocytic follicles/aggregates to be avoided or else visually ignored from scoring (Fig. 2b).

Necrotic tissue

Necrotic tissue or areas with pure neutrophilic inflammation, which may indicate necrosis, should be left out of the microscopic scoring field. If this is not possible, the necrotic parts will have to be visually ignored for scoring, as for the mucus in mucinous tumors (Table 2, Fig. 2c).

Lumen

Almost all tissue sections from colon adenocarcinomas will contain areas of glandular lumen. These areas should be ignored for scoring (Supplementary fig. 1).

Smooth muscle tissue of the bowel wall

Smooth muscle tissue should be left out of the microscopic field (Fig. 2d). If this is not possible, the smooth muscle cells will have to be visually ignored for scoring (Table 2).

In T2-, T3- and T4-staged adenocarcinomas of the colon, the tumor cells invade into or through the muscular layer of the colon. This can cause a mix-up of stromal cells and smooth muscle cells, which in some cases can be very hard to distinguish from one another. To enable an accurate scoring, we recommend performing an immunohistochemical desmin stain for these particular cases (Supplementary fig. 2).

Blood vessels

Blood vessels are part of the stroma, and small vessels should therefore be included in the scoring, being a part of the neo-angiogenesis in the tumor micro-environment. However, fields

of vision with native, large blood vessel(s) (i.e. thick smooth muscle wall of more than 3 layers of smooth muscle cells) should be replaced by another area for scoring, or, if this is not possible, the large vessel(s) should be visually ignored in the scoring (Supplementary fig. 3a).

Hyalinization

Hyalinization is a change in consistency of the collagenous matrix in the stromal tumor tissue, which gives the tissue a 'glassy' appearance. Being a part of the stroma, it should be included in the scoring (Supplementary fig. 3b).

Tumor budding

Tumor budding occurs very often at the invasive front of adenocarcinomas of the colon [22]. Therefore, it is likely that cell clusters are located in a field of vision chosen for scoring the TSR. These very small cell clusters can sometimes be hard to distinguish in H&E stained sections, and they may, falsely, be ignored as adenocarcinoma cells in the TSR scoring. In those particular cases, when the (suspected) presence of budding cells makes it difficult to categorize the TSR estimate as low or high, it is recommended to perform an immunohistochemical cytokeratin stain (e.g. AE1/AE3) to identify these malignant epithelial tumor cells (Supplementary fig. 4).

Table 2. Summary of the difficulties occurring during scoring the tumor-stroma ratio in colon adenocarcinomas with recommendations on how to act on them.

Difficulty	Recommendation
Mucinous tumor	Mucus should be ignored for scoring[a]
(Abundant) inflammatory cell infiltration	Infiltration with inflammatory cells is not an exclusion criteria and can be included in the scoring
Necrotic tissue	Necrotic tissue should be left out of the microscopic field. If this is not possible, the necrotic parts will have to be ignored for scoring[a]
Smooth muscle tissue	Smooth muscle tissue should not be considered for scoring. In case it is not possible to select a suitable field without smooth muscle tissue (*e.g.* in stage II tumors), this tissue compartment should be ignored for scoring.[a] A desmin stain may be of assistance
Glandular lumen	Areas of glandular lumens are ignored for scoring[a]
Blood vessels	Small vessels are included as part of the stroma. Large vessels with a muscular wall (> 3 layers of smooth muscle cells) should be avoided or else ignored for scoring[a]
Tumor budding cells	Budding adenocarcinoma cells should be separated from the surrounding stroma, and may be highlighted by a cytokeratin stain (AE1/AE3 is recommended) in problematic cases
Hyalinization	Part of the stroma and therefore included for scoring

[a]To ignore areas for scoring: the microscopic field minus the tissue that has to be visually ignored is set at 100%. The stroma percentage has to be determined from only the solid (= neoplastic + vital stromal compartment) tissue parts.

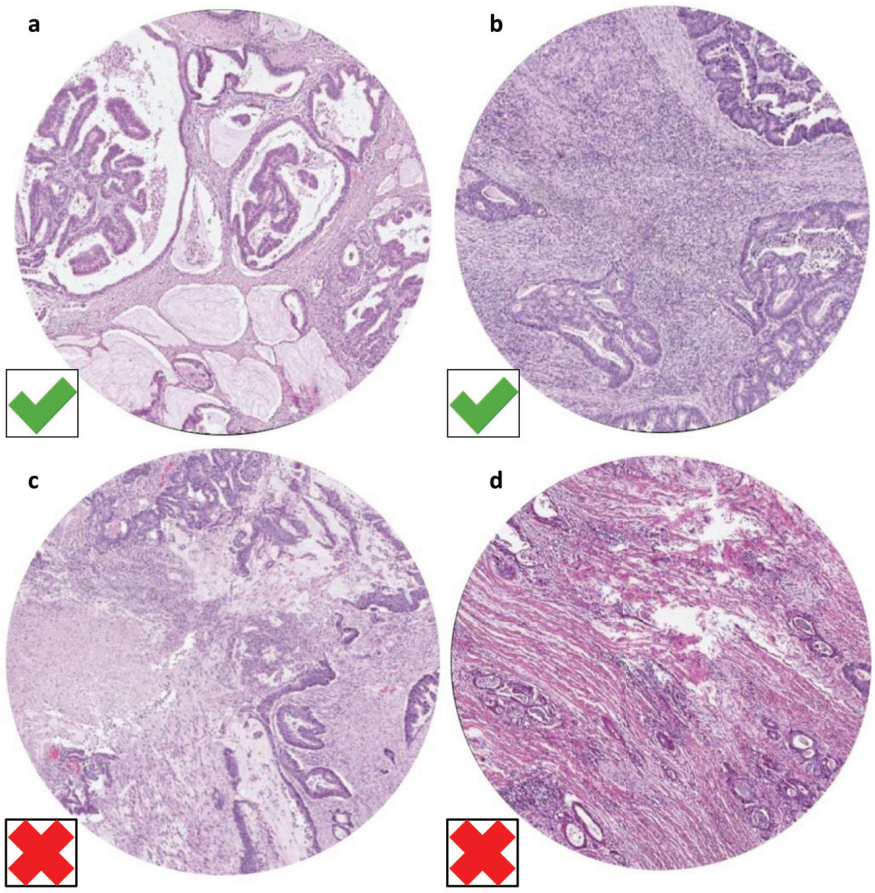

Figure 2. Examples of infiltration of a mucinous colon carcinoma (**a**) and inflammatory cells (**b**), which both meet the criteria for scoring. For the mucinous colon carcinoma, the mucus has to be ignored for scoring. Fields of vision with necrotic tissue (**c**) and smooth muscle tissue (**d**) do not meet the scoring criteria and should not be considered for scoring (Images displaying the microscopic view, all images 100x magnification).

Discussion

The high interest for the TSR, with sometimes differently used approaches of the protocol, calls for a standardized and easily implemented protocol. Although the technique described in this paper is focused on colon cancer, multiple studies have proven its robustness and usefulness for other types of solid epithelial cancers (Table 1). Our method and suggested protocol can therefore also be applied to these tumors. This also includes non-neoadjuvantly treated rectum carcinomas, as Park et al. showed in their study [14].

Scoring the TSR is a robust method, which only takes little extra time and costs, and has potential to be implemented in daily practice. The method is highly reproducible with low inter-observer variation (Table 1). Nevertheless, some difficulties may appear during scoring, as discussed in this paper. In our experience, the biggest challenge is to distinguish between stromal tissue and smooth muscle fibers, particularly in stage II colon adenocarcinomas. In challenging cases, we recommend performing a desmin stain. Being an intermediate filament, desmin is expressed in both smooth and skeletal muscle myocytes. Although scoring the TSR is in general an easy to apply method, in any case of difficulties in scoring, or doubt by the observer, one may consult a second observer to his/her own need, according to the usual practice encountering challenging morphologies.

Also in case of a stroma percentage at or around the cut-off point of 50%, consulting a second observer could be of help when in doubt. In addition, the total composition of the whole tissue section viewed with a x5 objective could be considered to make a final decision.

Scoring of the TSR in colon adenocarcinomas is performed on the tissue slide from the most invasive part of the tumor, which is the slide used in routine pathology to determine the T-status. This was decided after a study of colon cancers in which multiple H&E slides from different areas of the tumor were available for scoring. Although heterogeneity was seen in the percentage of stroma throughout the tumor, the highest stroma percentages were seen in the tumor areas with the deepest penetration in the bowel wall (higher T-stage) [4].

Most studies have validated our findings of the prognostic impact of the TSR in various kinds of malignant epithelial tumors. However, three studies have not been able to demonstrate validation of the TSR [23-25]. Discrepancies were caused by a different interpretation of the TSR scoring method. Instead of using the highest stroma percentage, these studies used either the mean percentage in case of heterogeneity [23], only one area of 9 mm^2 at the tumor leading or non-leading edge [24], or the mean percentage of 5 image fields from not only the deepest invasive margin but also adjacent tumor areas [25]. The latter two studies both used semi-automated image analysis.

Experimental Design

Automated digitized estimation of the TSR allows for a broader and highly standardized application, and two international groups have actually validated our results using automated image analysis systems [17, 26]. Although this approach might increase reproducibility, such equipment is rather costly, and not accessible at all pathological departments yet. In addition, scanning and analyzing using an automated image analysis system takes approximately 20 min per slide. In contrast, visual microscopic scoring of the intra-tumor stroma ratio can easily

be performed as a routine for conventional morphological diagnosis, and therefore only takes a little extra time (< 2 min). Moreover, validation studies have independently reported an inter-observer reproducibility of substantial to almost perfect between two independent observers (Table 1). However, in the scope of digitizing the pathology workflow, automated scoring of the TSR would suit the diagnostic approach.

Limitations

Assessment of the TSR can be adequately estimated in patients operated for a primary epithelial malignant neoplasm. Neoadjuvant treatments with chemo- and/or radiotherapy induce changes to the cellular morphology and composition of the tumor microenvironment, and result in stromal formation surrounding the tumor [27-30]. Therefore, patients pre-treated with chemo- and/or radiotherapy should be excluded for TSR analysis. For these patients, analyzing pre-treatment biopsies might be a good alternative, although the TSR cannot be determined at the most invasive front. As biopsies for colon cancer are rare, this might not apply for these cases. However, the method described in this manuscript can be used for several other epithelial cancer types, for which taking biopsies is more common practice. This has been nicely demonstrated for example for esophageal cancer, with the TSR scores of the tumor resection correlating with the matching pre-surgical biopsy TSR scores in 81% of the cases studied. In discrepant cases, the biopsy scores were stroma-low, whereas the surgical removed tumors were scored stroma-high, thereby underestimating the TSR. For stroma-high cases, however, a 100% correlation was found. Moreover, TSR biopsy scores showed to be an independent prognostic factor for survival [31], which motivates more investigation into the prognostic and predictive impact of TSR in pre-treatment biopsies from malignant epithelial tumors.

Compliance with Ethical Standards

This work did not involve human participants, therefore no informed consent was obtained. The tissue examples shown in this work are strictly used for illustration, and 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).

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Conflicts of interest

R. Al Dieri is the director general of the European Society of Pathology. All other authors declare no conflicts of interest.

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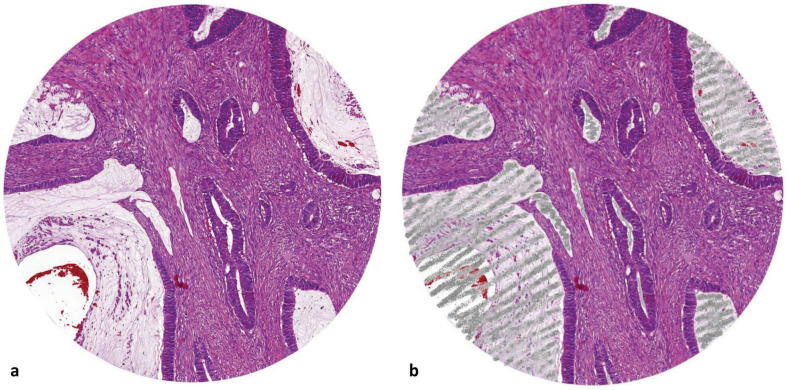
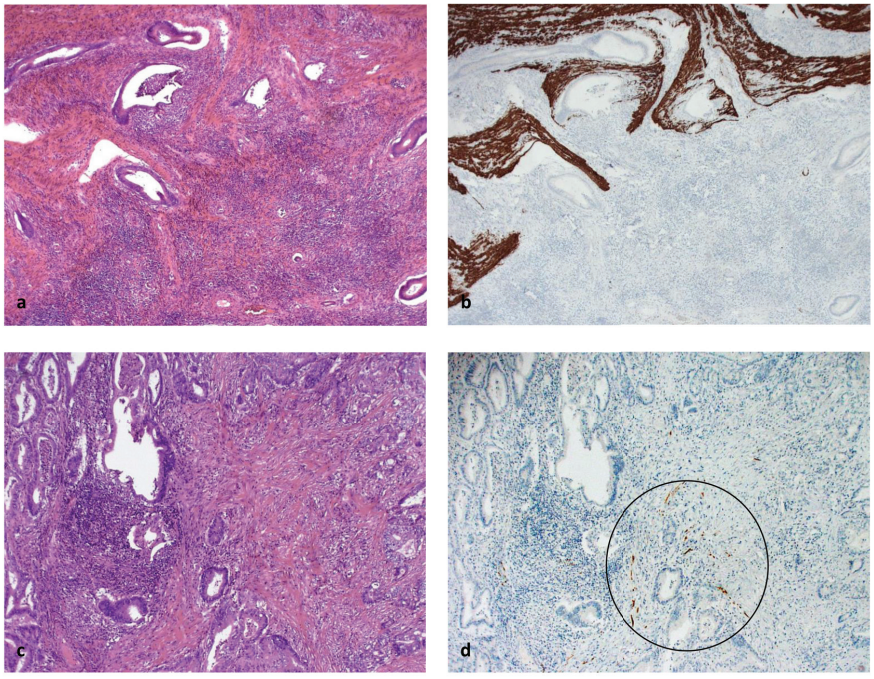
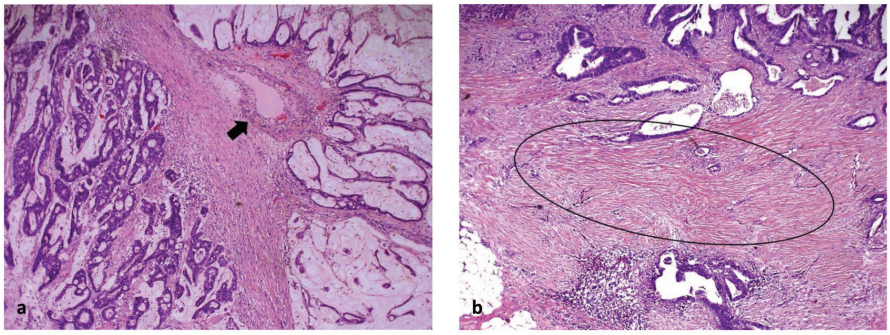
Supplementary Figures



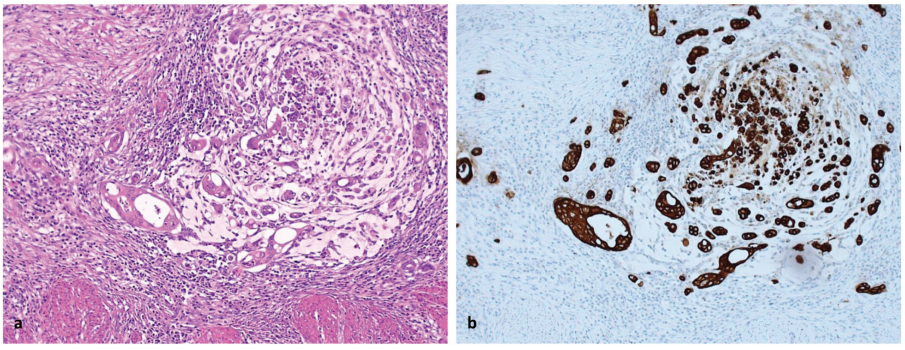
Supplementary Figure 1. Example of a field of vision which contains mucus areas and lumen that need be visually excluded for scoring (a). In figure (b) the areas to be ignored are marked in grey (Both images 100x magnification).



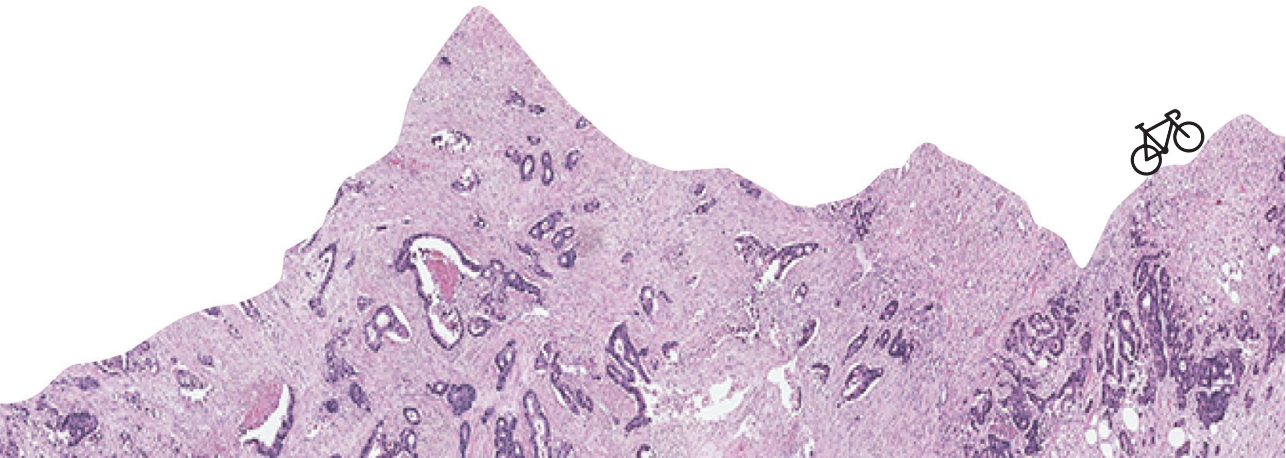
Supplementary Figure 2. Example of an adenocarcinoma of the colon, showing desmoplasia and inflammation in the HE-stained tissue section, encountering difficulties in discerning smooth muscle when estimating the TSR (**a**). This problem can be solved by using a desmin stain (**b**). In some cases the stroma may seem rather cellular, imitating smooth muscle cells (**c**). However, in this case a desmin stain discloses only a few positive, brown spindling smooth muscle cells (**d**, circle)(All images 100x magnification).



Supplementary Figure 3. Example of a partly mucinous adenocarcinoma (**a**), where difficulties in excluding mucin may influence the TSR-estimation, which may be further exaggerated by excluding possible smooth muscle and a large native vessel (arrow) in the H&E-stained section. Figure (**b**) shows a colon adenocarcinoma with stromal hyalinization (especially within the oval circle), which may be hard to differentiate from smooth muscle (Both images 40x magnification).



Supplementary Figure 4. In this close view from a colon adenocarcinoma, the H&E-stained section (**a**) discloses heavy inflammation, and the impression of a rather dense budding population of tumor cells. However, the latter are hard to visualize, whereas the smooth muscle coat (lower and right part of the figure) is easy to grasp. The use of a cytokeratin AE1/AE3 stain (**b**) solves this obstacle for estimating TSR (Both images 100x magnification).



CHAPTER 4

Stroma-high lymph node involvement predicts poor survival more accurately for patients with stage III colon cancer

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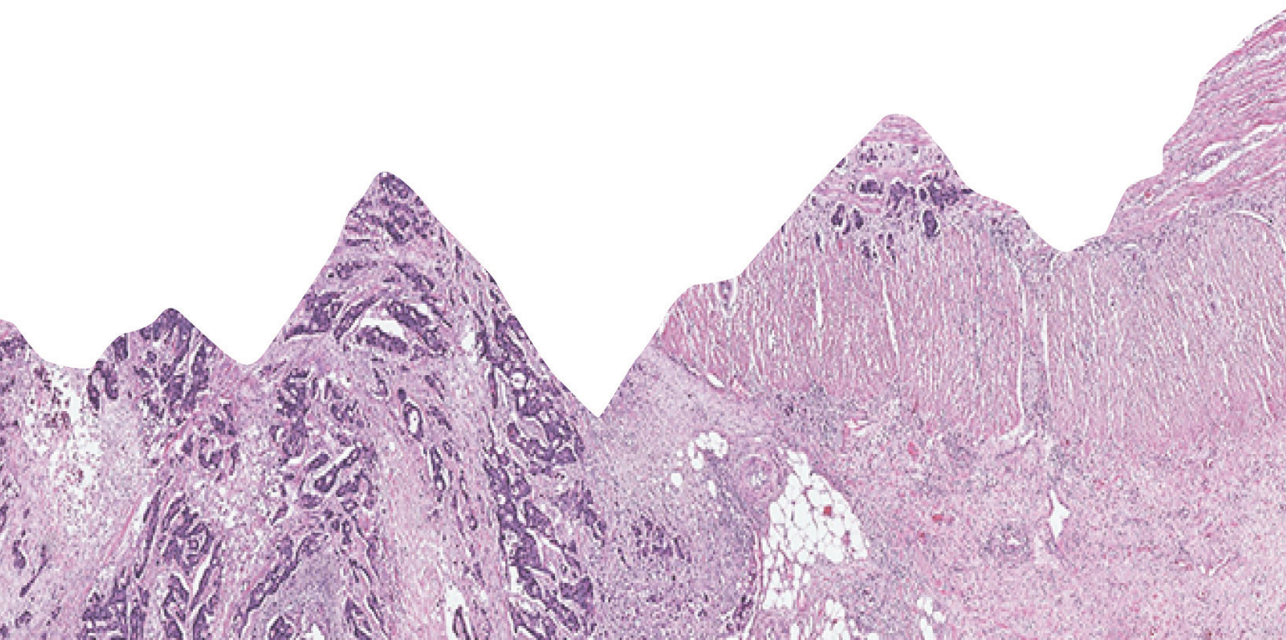

Abstract

Objective The tumor microenvironment has ample impact on the behavior of the malignant process in colon cancer (CC). Patients with a high percentage of stroma within the primary tumor, determined by the tumor-stroma ratio (TSR), have a poor prognosis. In metastatic lymph nodes from patients with stage III CC, the TSR is heterogeneous, but the impact on patients' prognosis is unknown.

Methods Hematoxylin and eosin stained tissue slides of primary tumor (PT) and associated lymph nodes (LNs) metastases from 102 patients with stage III CC were analyzed for the TSR. Stroma-high (>50% stroma) and stroma-low (≤50% stroma) groups were evaluated with respect to disease free survival (DFS).

Results Of 102 analyzed primary tumors, 47 (46.1%) scored as stroma-high and 55 (53.9%) as stroma-low. In total 33 patients had at least one stroma-high LN and 69 patients had one or more stroma-low LNs. Interestingly, 28 patients (27.5%) had both stroma-high and stroma-low LNs, and in another 44 cases the TSR between PT and LNs differed: 29 patients had a stroma-high PT but stroma-low LNs, and 15 patients *vice versa*. As a result of the combination of the TSR analysis of the PT and the involved metastatic LNs, 62 patients (60.8%) were classified as stroma-high and 40 (39.2%) as stroma-low, restaging 14.7% of the patients to stroma-high with a significantly worse 5-year DFS compared to stroma-low patients (59% *versus* 82%, HR = 2.83 (95%CI 1.34–5.97), $P = 0.006$). In multivariate analysis, the TSR retained its independent prognostic impact (HR = 2.85 (95%CI 1.33-6.10), $P = 0.007$).

Conclusion The presence of abundant stroma in metastatic LNs from patients with stage III CC adds to the prognostic information learned from the primary tumor alone and supports selective patient treatment.

Keywords

Colon cancer, disease free survival, histology, lymph node, tumor-stroma ratio

Introduction

One of the important determinants of prognosis for patients with colon cancer (CC) is lymph node involvement. For patients with a stage I or II tumor, the 5-year survival rate is more than 58% (stage IIC), but decreases to 35% (stage IIIC) when lymph nodes are involved [1]. Adjuvant chemotherapy has been shown to improve survival in patients with locoregional nodal metastases after resection, as it reduces the risk of death by an absolute 10% after 8 years [2]. However, not all stage III CC patients have aggressive disease and need to be treated, although identifying this group remains problematic.

Main factors contributing to intra-tumor heterogeneity have been well described at morphological, molecular and genomic levels. Heterogeneity between primary CC tumors and corresponding metastases has been reported on the level of biomarkers as well as genetic aberrations [3-6]. The intra-tumoral heterogeneity is believed to be the origin of the selection process during metastatic progression. Tumor progression is not only driven by the malignant cells, but also by altered communication between neoplastic cells and non-malignant cell populations, including fibroblasts, endothelial and inflammatory cells in the tumor stroma. The so-called infiltrating and surrounding fibroblasts, also known as cancer-associated fibroblasts (CAFs), play an important role. CAFs remodel the extracellular matrix (ECM) and secrete chemical factors, which all together promote the transformation process by encouraging tumor growth, angiogenesis, inflammation and metastasis and contribute to drug resistance [7]. Therefore, by ignoring the stromal compartment, valuable prognostic information is lost. The analysis of hematoxylin and eosin (H&E) stained histologic slides reveals that the stromal compartment provides more information than previously thought. The tumor-stroma ratio (TSR) has been shown by our group to be prognostic in several types of malignant epithelial neoplasms including colon cancer [8-10], breast cancer [11, 12] and esophageal cancer [13, 14] and has, moreover, also been validated by various independent, international groups [15-18].

In the current study we analyzed patients with stage III CC to: 1. Evaluate the difference regarding the stroma between the primary tumor (PT) and metastatic lymph nodes (LNs); and 2. Determine the additional prognostic value of the TSR in lymph node metastasis.

Materials and Methods

Patients

The patient cohort consisted of patients with colon cancer from Leiden University Medical Center (LUMC), the Netherlands, and Vejle Hospital, Denmark. All patients were diagnosed between 1996 and 2011 and underwent complete surgical resection (R0) of stage III CC followed by adjuvant chemotherapy. Patients with histologically proven TNM stage III (any T, N1 or N2, M0) without gross or microscopic evidence of residual disease were included. Patients with a history of cancer other than basal cell carcinoma or cervical carcinoma *in situ* or with multiple synchronous colon tumors were excluded, as well as patients who died within two months after surgery. Clinico-pathological data and outcome characteristics of these patients are shown in Table 1.

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). The Danish series of patients were included after approval from the Scientific Ethical Committee of Southern Denmark (ID#-20140117) and the Danish Data Agency according to Danish law, and the tissue used for research was confirmed not to be included in the Danish Registry of Human Tissue Utilization.

Histopathological scoring

Tissue samples, consisting of 5 µm H&E stained histologic sections from the most invasive part of the PT (i.e. the slides used in routine pathology to determine the T-status) and the corresponding metastatic LNs, were analyzed by conventional microscopy. Areas appearing to have the largest amount of stroma were selected using a 2.5x or 5x objective. Hereafter, an area where both tumor and stromal tissue were present within this vision-site was selected using a 10x objective. Tumor cells were to be present at all borders of the image field. Two investigators estimated the tumor-stroma ratio in a blinded manner. In case of an inconclusive score, and consensus could not be reached, a third observer was decisive. Scoring percentages were given *per* tenfold (10%, 20%, 30% etc.) *per* image-field.

In case one of the metastatic LNs from a patient was stroma-high, the final score for the LNs was also considered stroma-high. When examining the four different groups (PT-low/LN-low, PT-low/LN-high, PT-high/LN-low and PT-high/LN-high), we observed that the PT-low/LN-high group had the worst outcome, supporting the large impact of TSR in the metastatic LNs (data not shown).

Table 1. Characteristics of total patient population and stratified for each cohort, or TSR-group

	Total		Leiden		Vejle			Stroma-low		Stroma-high		
	***N*=102**	**%**	***N*=47**	**%**	***N*=55**	**%**	***P*-value**	***N*=55**	**%**	***N*=47**	**%**	***P*-value**
Sex												
Male	58	56.9	28	59.6	30	54.5	0.609	34	61.8	24	51.1	0.274
Female	44	43.1	19	40.4	25	45.5		21	38.2	23	48.9	
Age												
<70	70	68.6	35	74.5	35	63.6	0.240	36	65.5	34	72.3	0.455
>=70	32	31.4	12	25.5	20	36.4		19	34.5	13	27.7	
Grade												
Low	7	6.9	6	12.8	1	1.8	**0.042***	1	1.8	6	12.8	0.067*
Medium	60	58.8	22	46.8	38	69.1		35	63.6	25	53.2	
High	26	25.5	10	21.3	16	29.1		16	29.1	10	21.3	
Missing	9	8.8	9	19.1	0	0.0		3	5.5	6	12.8	
Histological type												
Adenocarcinoma	87	85.3	38	80.9	49	89.1	0.240	45	81.8	42	89.4	0.336
Mucinous	13	12.7	7	14.9	6	10.9		8	14.5	5	10.6	
Signet ring cell carcinoma	2	2.0	2	4.3	0	0.0		2	3.6	0	0.0	
Site of primary tumor‡												
Left	62	60.8	28	59.6	34	61.8	0.817	28	50.9	34	72.3	**0.027**
Right	40	39.2	19	40.4	21	38.2		27	49.1	13	27.7	
T-stage												
T2/T3	90	88.2	41	87.2	49	89.1	0.772	52	94.5	38	80.9	**0.032**
T4	12	11.8	6	12.8	6	10.9		3	5.5	9	19.1	
N-stage												
N1	68	66.7	32	68.1	36	65.5	0.779	35	63.6	33	70.2	0.482
N2	34	33.3	15	31.9	19	34.5		20	36.4	14	29.8	

Table 1. Continued

	Total		Leiden		Vejle			Stroma-low		Stroma-high		
	***N*=102**	**%**	***N*=47**	**%**	***N*=55**	**%**	***P*-value**	***N*=55**	**%**	***N*=47**	**%**	***P*-value**
MSI status												
MSS	49	48.0	28	59.6	21	38.2	0.472*	28	50.9	21	44.7	0.148*
MSI	7	6.9	5	10.6	2	3.6		6	10.9	1	2.1	
Missing	46	45.1	14	29.8	32	58.2		21	38.2	25	53.2	
TSR Primary tumor												
Stroma-low	55	53.9	22	46.8	33	60.0	0.183					
Stroma-high	47	46.1	25	53.2	22	40.0						
TSR Lymph nodes												
Stroma-low	69	67.6	28	59.6	41	74.5	0.107	40	72.7	29	61.7	0.235
Stroma-high	33	32.4	19	40.4	14	25.5		15	27.3	18	38.3	
TSR PT + LNs												
Stroma-low	40	39.2	14	29.8	26	47.3	0.071					
Stroma-high	62	60.8	33	70.2	29	52.7						

[1]Right-sided tumors were defined as those originating proximal to the splenic flexure and left-sided as those originating distal to the splenic flexure.

Bold indicates values with a significant difference P < 0.05.

Abbreviations: MSI = Micro Satellite Instability; DFS = Disease Free Survival; TSR = Tumor-stroma ratio; PT = Primary tumor; LNs = Metastatic lymph nodes.

**P*-value excluding missing data.

We therefore decided that for combining the lymph node TSR with the TSR of the primary tumor, a patient was considered stroma-high when either the PT and/or the metastatic LNs were stroma-high. In case of a low TSR in the PT as well as in the metastatic LNs, the patient was considered stroma-low.

Statistics

Statistical analysis was performed using IBM SPSS software version 20.0. Our primary endpoint was disease free survival (DFS), which was defined as the time from the date of primary surgery until the date of death or to the date of first loco-regional or distant recurrence or the date of a second primary tumor. If no recurrence occurred, DFS was calculated as the time period until the date of last follow-up.

Stroma-high was defined as > 50% stroma surface area and stroma-low as ≤ 50% stroma surface area, as determined *a priori* to have maximum discriminative power [9]. Inter-observer variability was analyzed using the Cohen's kappa coefficient. Analysis of the survival curves was performed using Kaplan-Meier Survival Analysis and differences in survival distributions were tested using log-rank statistics. Cox regression was used for univariate and multivariate analyses. Variables with a p-value <0.1 in univariate analysis were included in the multivariate analysis. P-values <0.05 were considered statistically significant.

Results

Patients

A total of 53 LUMC patients and 55 from Vejle hospital were included in the study. There were no significant clinicopathologic differences between the Dutch and Danish cohorts, except for tumor grade (Table 1). Six patients (5.9%) had to be excluded due to poor quality of histological tissue, resulting in a total study population of 102 patients. Additional patient information, including survival data, was collected after scoring all samples for the TSR. The study cohort comprised 58 men and 44 women, with a median age of 65 years (range 31-79 years). Of all patients, 70 (68.6%) were younger than 70 years of age, and 32 patients (31.4%) were older (Table 1).

Scoring tumor-stroma ratio

Out of 102 analyzed PTs, 47 (46.1%) were scored as stroma-high and 55 (53.9%) as stroma-low. There were no significant differences for clinicopathologic characteristics between the two

groups, except for location of the primary tumor and T-status (Table 1). For the PT, the observers agreed on classification in 87% of all cases. In the other 13% of cases consensus was reached or a third observer was decisive. For the metastatic LNs agreement was reached in 84% cases, consensus was reached or a third observer was decisive in the remaining 16%. Cohen's kappa coefficient revealed a substantial inter-observer agreement in classification for the PT as well as the LNs (κ = 0.73 and 0.68, respectively).

Heterogeneity

When analyzing the TSR in the LNs, we observed that the metastasizing process of the PT to the LNs is a heterogeneous process (Figure 1). Interestingly, 28 patients (27.5%) had both stroma-high as well as stroma-low LNs. In 44 cases, the TSR between the PT and the LNs was different between stroma-high and stroma-low: 29 patients had a stroma-high PT but stroma-low LNs, and 15 patients *vice versa*.

Relation with Outcome

Primary Tumor

The stroma-high population had a significantly worse DFS compared to the stroma-low patients (HR = 1.89 (95%CI 1.00-3.56), *P* = 0.046) (Figure 2A, table 2), with a 5-year DFS of 61% *versus* 74% (stroma-high *versus* stroma-low, respectively). In multivariate analysis the TSR remained a significant prognostic variable (HR = 1.98 (95%CI 1.04-3.77), *P* = 0.038)(Table 2).

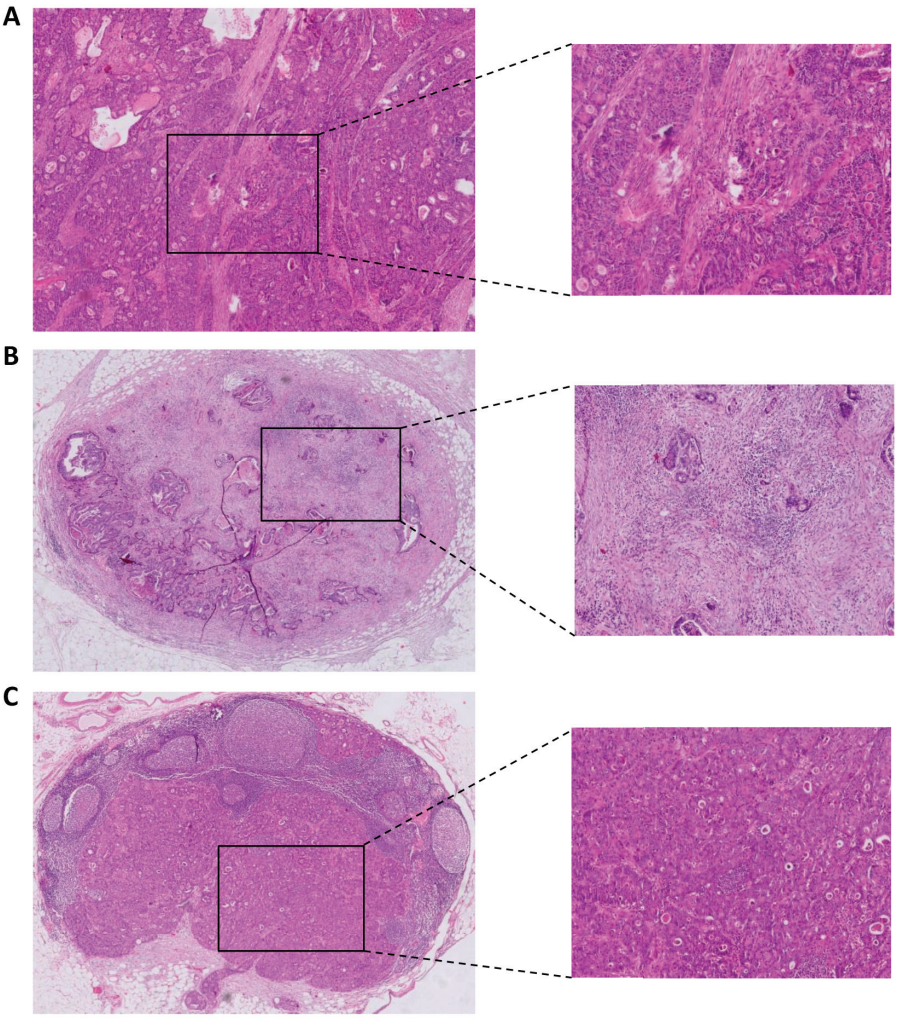

Figure 1 Heterogeneity between tumor-stroma ratio of primary tumor (A) and corresponding metastatic lymph nodes (B, C). Hematoxylin and eosin stained sections of a stroma-low primary tumor (A), and two corresponding metastatic lymph nodes, one stroma-high (B) and one stroma-low (C).

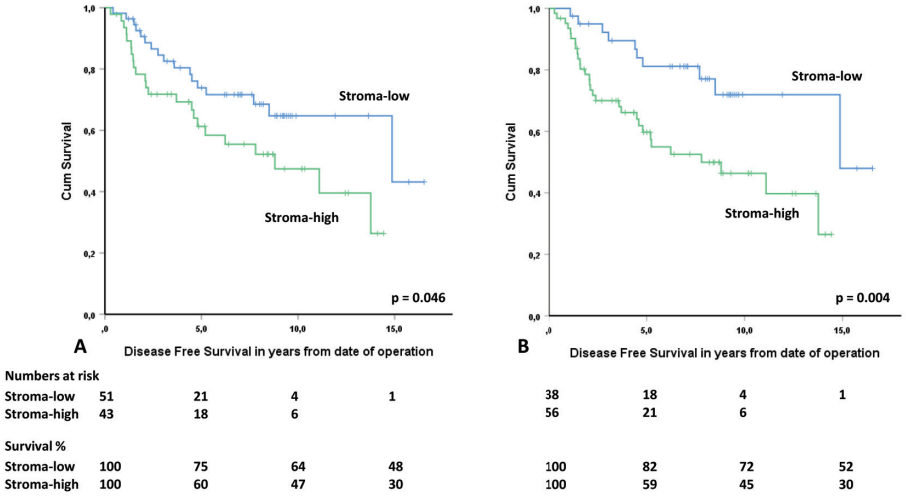

Figure 2 Kaplan-Meier disease free survival curves regarding stroma score of primary tumor (A) and combined analysis of primary tumor and associated metastatic lymph nodes (B).

Lymph node involvement and combined analysis

A total of 1398 LNs were examined (median 13 *per* patient; range 3-49), of which 348 (median 2 *per* patient; range 1-17) contained metastasis from the PT, and 68 patients had stage N1 and 34 had N2. In total 33 patients had at least one metastatic LN with a high amount of stroma, and were therefore considered stroma-high. The remaining 69 patients had one or more metastatic LNs with only a low TSR.

As a result of combining the stroma analysis of the PT and the involved LNs, 62 patients (60.8%) were classified as stroma-high and 40 (39.2%) as stroma-low. This resulted in restaging of 14.7% of stroma-low patients to the stroma-high group, increasing the DFS of the remaining stroma-low patients from 74% to 81% for 5-year DFS. In the stroma-high population, patients had a worse 5-year DFS compared to the stroma-low population (60% *versus* 81%, HR = 2.83 (95%CI 1.34–5.97), *P* = 0.004) (Figure 2B, Table 2). In multivariate analysis the combined TSR remained a significant prognostic variable (HR = 2.85 (95%CI 1.33-6.10), *P* = 0.007)(Table 2).

Table 2. Uni- and multivariate analysis regarding DFS

		Univariate analysis			Multivariate analysis		
	N	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Sex							
Male	58	1					
Female	44	0.822	0.435-1.555	0.547			
Age							
<70	70	1			1		
>=70	32	1.733	0.912-3.293	0.093	1.915	1.007-3.641	**0.047**
Grade							
Low	7	1					
Medium	60	1.106	0.261-4.696	0.891			
High	26	1.331	0.291-6.085	0.712			
Histological type							
Adenocarcinoma	87	1					
Mucinous	13	0.945	0.368-2.425	0.907			
Signet ring cell carcinoma	2	0.553	0.072-4.243	0.569			
Site of primary tumor†							
Left	62	1					
Right	40	1.370	0.734-2.557	0.323			
T-stage							
T2/T3	90	1					
T4	12	1.918	0.843-4.361	0.120			
N-stage							
N1	68	1			1		
N2	34	2.006	1.059-3.798	**0.033**	1.992	1.051-3.778	**0.035**
MSI status							
MSS	49	1					
MSI	7	0.705	0.160-3.115	0.645			
TSR PT‡							
Stroma-low	55	1			1		
Stroma-high	47	1.893	0.999-3.588	**0.046**	1.978	1.037-3.774	**0.038**
TSR PT+LNs‡							
Stroma-low	40	1			1		
Stroma-high	62	2.825	1.338-5.965	**0.006**	2.850	1.331-6.104	**0.007**

†Right-sided tumors were defined as those originating proximal to the splenic flexure and left-sided as those originating distal to the splenic flexure.

‡TSR PT and TSR PT+LNs have been analyzed in two separate models, both adjusted for age and N-stage

Bold indicates values with a significant difference $P < 0.05$.

Abbreviations: MSI = Micro Satellite Instability; DFS = Disease Free Survival; TSR = Tumor-stroma Ratio; PT = Primary tumor; LNs = Metastatic lymph nodes.

Discussion

In this study we analyzed the TSR, primarily used for analysis of the PT, in metastatic LNs from patients with stage III CC. The number of metastatic LNs evaluated in surgical specimens of CC has risen significantly over the past two decades. However, according to a study of Parsons *et al.,* this improvement has not been associated with an increase in higher-staged cancers [19], raising the question whether the absolute number of metastatic LNs should be evaluated as the primary basis for estimating prognosis or if a different approach should be considered. As we have shown in this study, the analysis of the TSR of metastatic LNs has an additional value with respect to the disease free survival of adjuvantly treated patients with stage III CC. Although the metastasizing process to the LNs is very heterogeneous, the presence of just one metastatic LN with a high amount of stroma is enough to predict a worse DFS. This might indicate that a different treatment approach is necessary for patients classified as stroma-high compared to patients in the stroma-low group.

Cancer research for the development of targeted therapies has focused largely on genetic and epigenetic abnormalities of the epithelial component of solid tumors. Recent approaches to predict recurrence or benefit from therapy focus on gene signature profiles using microarray gene analysis. New colorectal cancer (CRC) subtypes have been identified by three independent research groups [20-22]. All groups identified one subtype associated with poor prognosis, and more importantly, this subtype was recently observed to associate with a high stromal content [23]. This finding is in line with our observation that patients with a stroma-high tumor have a worse prognosis. Moreover, the recent identification of mechanisms of therapeutic resistance that were mainly conferred by changes in the tumor microenvironment, indicates the importance of the development of therapies targeting the non-cancer stromal cells, like fibroblasts and extracellular matrix components [24].

Many prognostic and predictive biomarkers have been, or are currently under investigation for possible implementation in routine clinical diagnostics. Markers such as *BRAF*, *KRAS* and *NRAS* are well-known prognostic (*BRAF*) and predictive (*RAS*, for metastatic CRC) markers used in the clinic, whereas serial measurement of carcinoembryonic antigen is the standard for disease monitoring. Also, multiple markers have been associated with resistance or sensitivity to therapy; *RAS* mutations and *BRAF* mutations are already known to cause resistance to anti-EGFR therapy, but recently also PTEN- and PI3K-mutations, miR-181a and IGF2 overexpression have been found to be predictive for response to anti-EGFR therapy [25]. Although these markers

might contribute to further characterization of the tumor, therefore facilitating the selection of treatment for the individual patient, the techniques used to determine these markers, like gene expression arrays or next generation sequencing, are time consuming and accompanied with high costs. Moreover, for gene expression array analyses, it is common practice to select those parts of the tissue in which tumor cells form the major component, as admixtures of stroma and inflammatory cells will lead to masking of amplifications and deletions. This may lead to exclusion of stroma-high tumors, which may form a selection bias for patients with a better prognosis. On the contrary, determining the TSR is easy, has high reproducibility and low inter-observer variation, and is not associated with extra costs. In addition, the TSR has also been discussed by the TNM Evaluation Committee and the College of American Pathologists, who stated that our observations are important and novel and have the potential to be included in the TNM staging algorithm. They advocated validation in a prospective, multicenter study, development of a consensus agreement and a quality assessment program. Therefore, a reliability and reproducibility study will be conducted among national and international pathologists. An e-learning module will be developed with a quality assessment program in the framework of the European Society of Pathology EQA program. At the same time further improvement of the technique by an automated method is currently being developed to obtain an even more robust measurement, which is essential for estimating the cut-off threshold, as well as an even higher reproducibility.

Although the extent of nodal involvement (i.e. N1 *versus* N2) is a known predictor for survival amongst stage III CC patients [26, 27], in this study we found no correlation between N-status and TSR (Table 1), and both variables were found to be independent prognostic parameters. Also the MSI status has been proven to be a predictive marker for the survival of colon cancer patients. In the current study this was not found, possibly due to the fact that almost half of the study cohort had an unknown MSI status. However, in previous studies we have already shown that the TSR is a prognostic parameter, independent of MSI status [8, 9].

In this study we also found a strong heterogeneity within the metastasizing process of the stroma based on visual evaluation, whereas several studies have investigated the expression levels of different prognostic markers in CRC and corresponding LN metastases on the molecular level [28-30]. In concordance with our data, the expression patterns of some of these markers also showed to be heterogeneous between the PT and LN metastases. For example, the expression of p53 has been documented to be similar between PT and LN metastases [28, 30],

whereas EGFR expression differed. This difference in EGFR expression indicates that the PT does not reflect the situation in LN metastases, which might have important clinical implications [29].

Although there have been studies published which describe the expression of biomarkers in the stroma of metastatic LNs, as discussed above, to our knowledge, this is the first study investigating the amount of stroma present in LN metastases from patients with CC. In this study we have shown that the analysis of the TSR in metastatic lymph nodes has an additional value with respect to disease free survival in patients with stage III CC. Taking tumor heterogeneity into consideration, this parameter might be used as a marker to select patients for therapy targeting the stromal compartment of the tumor.

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Conflicts of Interest and Source of Funding

The authors have declared no conflicts of interest. No funding has been received.

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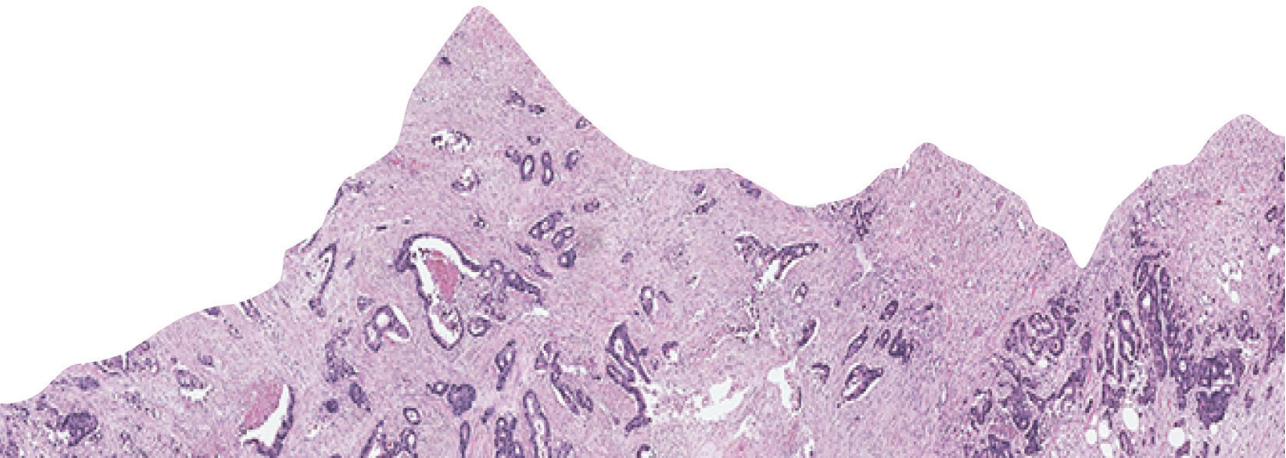

CHAPTER 5

Tumor stroma as contributing factor in the lymph node metastases process?

Wilma E Mesker, Gabi W van Pelt, Rob AEM Tollenaar

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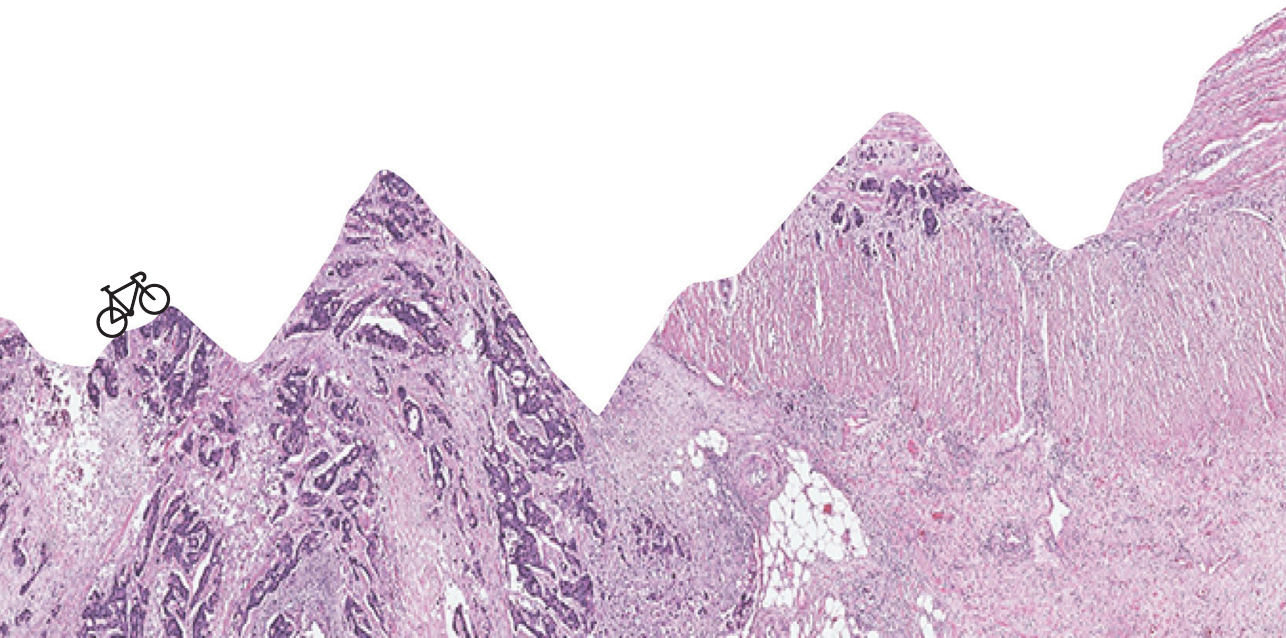

Editorial

Tumor associated stroma as part of the tumor microenvironment has increasingly gained interest and acceptance in the field of patients prognostication and treatment. Stroma is not the innocent bystander as previously thought but co-orchestrates the metastases process. The amount of stroma in the primary tumor (PT) is a strong prognostic parameter for breast, colon and other epithelial malignancies. The so called tumor-stroma ratio (TSR) distinguishes between patients with good and worse outcome of disease [1-4].

The presence of tumor cells in lymph nodes (LN) is important for clinical decision making. In recent papers we have shown that tumor associated fibroblasts are present in high amounts in the lymph nodes from patients with breast and colon cancer [3, 5]. The high stromal scores (>50% per image field) correspond with the aggressive behavior of the tumor. Patients with a high amount of stromal cells in one or more lymph nodes showed a worse overall and disease free survival. Patients with a low amount of stroma showed statistically significant good outcomes. What was surprising is that the observed metastases process was heterogenous, meaning that some lymph nodes were occupied with less tumor cells but many fibroblasts, whereas also the opposite was observed within the same patient. Based on these findings we might say that tumor associated fibroblasts have the capacity to metastasize or can accompany metastasizing tumor cells. However, the fact that in some cases only fibroblasts were seen in mostly tumor-free lymph nodes, makes the latter less likely.

This strong heterogeneity within the metastasizing process of the stroma was observed using microscopical investigation of routine stained tissue slides but other studies have investigated expression levels on the molecular level and validated our findings [6-9]. In studies to determine prognostic markers for colorectal cancer and investigating the corresponding LN metastases, the expression patterns of some of the markers showed to be heterogeneous between the PT and LN metastases. While the expression of p53 has been documented to be similar between PT and LN metastases, the EGFR and HER2 expression differed significantly [6, 8, 9]. These differences in EGFR and HER2 expression indicate that the PT does not accurately reflect the metastatic situation and we need the information of the LN metastases, which might have important clinical implications.

For colon cancer stage III it was shown that the analysis of the TSR in metastatic LNs is of additional value with respect to survival time of the patients and can be considered as guide for selective treatment to overcome over- and undertreatment [5].

Breast cancer patients with LN metastases were previously immediately eligible for adjuvant chemotherapy, irrespective of other clinico-pathological 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. 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 relapse free period (RFP) and a group of patients with a notably high risk could be identified. The fact that this patient group showed a recurrence rate of 92% after 10 years, considers this method most capable of identifying patients with a worse prognosis [3].

An interesting observation is the strong discrepancy between TSR in the PT with those of the LNs of the same patient. In more than 50% of the patients heterogeneity was observed between the stroma category in the PT and LNs. This finding might be reflective of differential activity of the signaling processes across primary and metastatic tumors. The formation of genetically and transcriptionally distinct sub clones 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.

Taking tumor heterogeneity into consideration the TSR might be used as a marker to specifically select patients for therapy. Mechanisms of therapeutic resistance were recently identified, which were mainly conferred by changes in the tumor microenvironment. For future patient treatment regimens this might indicate the development of new therapies targeting the non-cancer stromal cells [10].

Incorporating the TSR in clinical practice has clear advantages compared to other potential biomarkers. TSR scoring can be carried out on standard H&E slides and is performed by visually eyeballing of the tissue 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.

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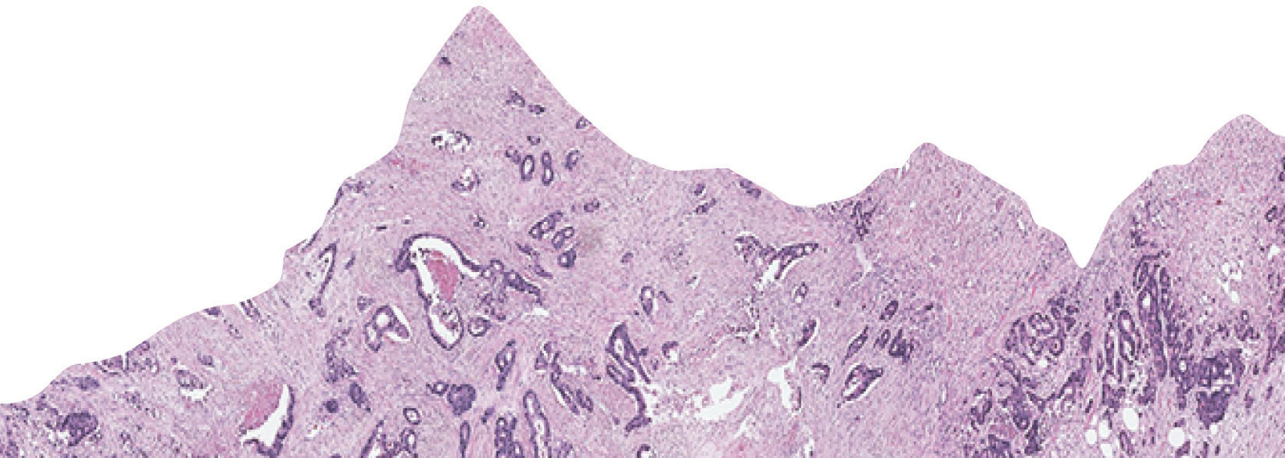

CHAPTER 6

Differential prognostic value of tumor-stroma ratio depending on *BRAF* mutation in colorectal cancer

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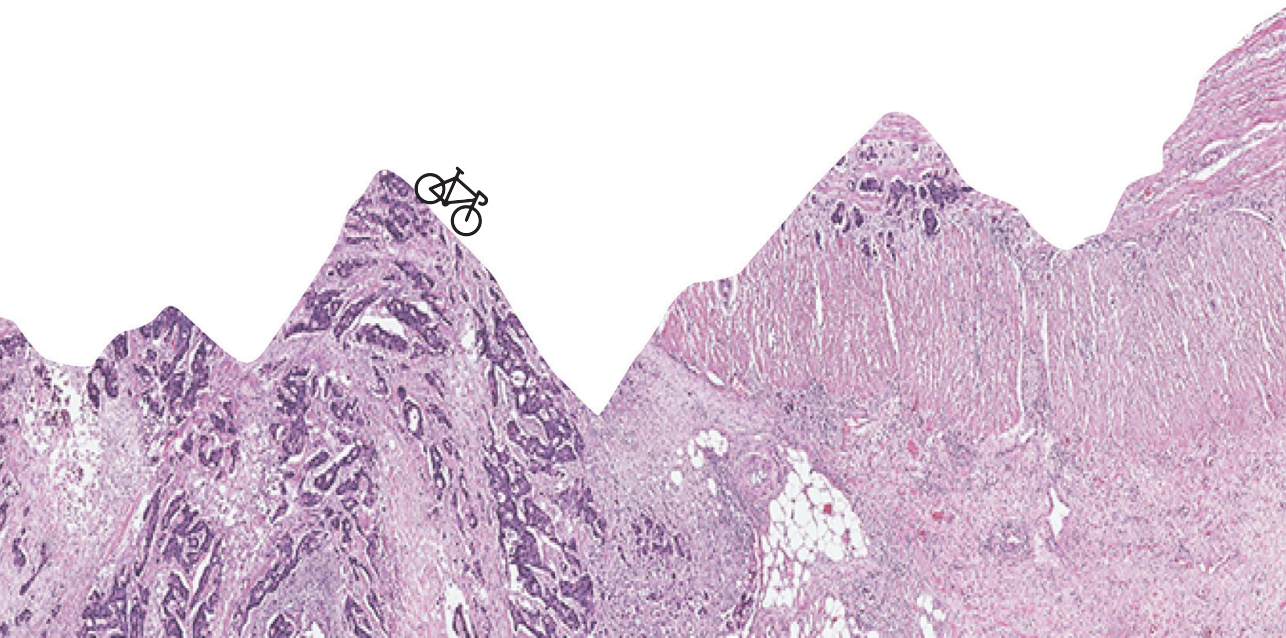
In preparation



Abstract

Background The tumor-stroma ratio (TSR) and *BRAF* mutation are prognosticators for poor survival in colorectal cancer (CRC). Here we investigated whether both features are independently prognostic and whether their combination might provide further stratification.

Methods The study cohort consisted of stage II-III CRC patients from two large randomized clinical trials: Quasar2 and Avant. TSR and *BRAF-V600E* mutation were previously determined for both trials. Stroma-high (>50%) and stroma-low (≤50%) groups combined with *BRAF-V600E* were evaluated for outcome using 5 years disease free survival (DFS).

Results In a total cohort of 2118 patients successfully profiled for both biomarkers, a single prognostic model adjusted by clinical variables showed both TSR and *BRAF-V600E* to be independent prognosticators for 5 years DFS (HR 1.59, 95%CI 1.32-1.90, $p < 0.001$; HR 1.66, 95%CI 1.24-2.22, $p = 0.001$, respectively). The combination of both biomarkers resulted in 1302 stroma-low/*BRAF* wt (62%), 150 stroma-low/*BRAF* mut (7%), 599 stroma-high/*BRAF* wt (28%) and 67 stroma-high/*BRAF* mut (3%) cases. No difference was found for survival within the stroma-low group based on *BRAF-V600E*. However, within the stroma-high group patients with *BRAF-V600E* mutation had a significant worse survival compared to patients with wild type *BRAF* (HR 2.24, 95%CI 1.49-3.36, $p < 0.001$), where the survival fraction was 71% and 53%, respectively. Accordingly, the interaction between TSR and *BRAF-V600E* was found to be significant ($p = 0.031$). No heterogeneity was found between the two clinical trials.

Conclusion TSR and *BRAF-V600E* might provide independent prognostic information that synergistically results in a subtype with very poor outcome in stage II-III CRC patients. While these results require further external validation, they suggest the combination of these two simple biomarkers may be more useful for patient stratification than assessed individually.

Keywords

BRAF, colorectal cancer, survival, tumor-stroma ratio

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer related death in Europe [1] and its heterogenous outcome needs to be improved. Metastatic CRC shows extremely poor survival, hence the current standard of care is treatment with chemotherapy after surgery where possible. However, outcome in non-metastatic CRC is variable so it requires a decision about whether to give adjuvant treatment or not, which will be key for the subsequent outcome of each patient. In general, such decision is mostly based on the size of the tumor (T-stage) and the involvement of lymph nodes (N-stage). The only molecular variable that is used for this selection is microsatellite instability (MSI), a hypermutable phenotype caused by defects in the DNA mismatch repair machinery [2], that shows better survival in stage II patients. However, other biomarkers have consistently been associated with survival in CRC. One of the most promising ones is the tumor-stroma ratio (TSR) as it has the potential to be implemented easily and with little extra costs into daily diagnostics. TSR can be determined on routine hematoxylin and eosin (H&E) slides and it has been shown that patients with a tumor containing a high amount of stroma (>50%) have a worse prognosis compared to patients with less stromal cells in the primary tumor [3-5]. Another known biomarker for poor prognosis in CRC is *BRAF-V600E* in the metastatic setting [6, 7] and within MSI-patients in the adjuvant setting [8-11]. This mutation is found in ~10% of CRC cases and accounts for most mutations in the gene [12]. *BRAF-V600E* mutation is strongly associated with other molecular and clinical factors such as CpG Island Methylator Phenotype, MSI, right sidedness and female gender [13]. These may also provide outcome information at different degrees and further research may be able to refine their usefulness as prognostic biomarkers.

To better understand CRC biology and its clinical heterogeneity, many efforts for classification into different molecular subtypes have been performed. The best example is the development of the consensus molecular subtypes (CMS) from the transcriptome. Four subtypes were identified: CMS1, mainly composed of MSI tumors and immune activated CRCs; CMS2, the canonical subtype with marked WNT and MYC signaling activation; CMS3 representing epithelial tumors with metabolic dysregulation; and CMS4, which is the mesenchymal subtype with prominent TGF-β activation, stromal infiltration and angiogenesis [14]. CMS4 was the only subtype found to be prognostic, which is extremely consistent with the association of TSR with poor outcome. Regarding *BRAF-V600E*, over 70% of tumors with such mutation were classified as CMS1 and 17% as the stromal subtype CMS4. Due to the heterogeneity within *BRAF* mutants Barras et al. specifically investigated this subset of tumors and showed two distinct

transcriptomic subtypes for *BRAF-V600E* mutants, called BM1 and BM2 [15]. Interestingly, BM1 has an increased epithelial-mesenchymal transition (EMT) compared to BM2, and indeed nearly all CMS4 *BRAF* mutants were classified as BM1 and most CMS1 as BM2. Accordingly, BM1 subtype showed poorer survival than BM2, although the difference was not found to be significant, maybe due to lack of statistical power in a relatively small cohort of *BRAF-V600E* mutants.

Due to the different biology reported among *BRAF* mutants that is strongly associated with the stromal subtype CMS4, we investigated the relationship in clinical outcome between TSR and *BRAF-V600E* mutation.

Patients and methods

Study population

Due to the relatively low numbers of CRC patients showing both high TSR and *BRAF-V600E* mutation, this study combines two similar clinical trials. All clinical data were collected retrospectively. None of the patients received neoadjuvant therapy.

The Quick and Simple and Reliable (Quasar) 2 cohort

The Quasar2 study is an international phase III randomized trial of adjuvant capecitabine ± bevacizumab after complete surgical resection of high-risk stage II and stage III CRC patients. In total 1941 patients were included in the original study between 2005 and 2010 [16]. For the current study cohort a total of 1272 H&E slides from United Kingdom patients were available. Only patients who did not receive neoadjuvant treatment were eligible for the study. Ethical approval for patient recruitment and sample collection was approved centrally and at all recruiting centers. Ethical approval for anonymized tumor molecular analysis was granted by Oxfordshire Research Ethics Committee B (Approval No 05/Q1605/66).

Avant cohort

The Avant trial is an adjuvant phase 3 randomized controlled trial that enrolled high-risk stage II and stage III colon cancer patients. All patients were treated with curative intent, including surgery (prior to randomization) followed by adjuvant chemotherapy in one of three assigned treatment arms (FOLFOX-4, FOLFOX4 with bevacizumab or XELOX with bevacizumab) [17]. Between December 2004 and June 2007, 3451 patients were included in the Avant trial of which 1213 patients had H&E slides available for our study cohort. The Avant trial was performed in accordance with the declaration of Helsinki. Protocol approval was obtained from the ethics

review committees or institutional review boards at participating sites. Patients provided written informed consent before study participation. For our study, archival material was used in an anonymized matter, therefore no additional informed consent was needed.

BRAF-V600E mutation

Detection of the *BRAF-V600E* mutation of Quasar2 patients was performed as published in the original report [16]. Briefly, DNA was extracted from tumor samples using the DNeasy Kit (Qiagen, Germany) after digestion with proteinase K. Standard direct DNA sequencing was performed for *BRAF* mutations in exon 15. Reactions were visualized with Mutation Surveyor software (Softgenetics, USA) and the *BRAF-V600E* mutation was annotated. For the Avant trial, the mutational analysis was performed locally in the 330 participating hospitals using their own methods.

From this point forward *BRAF-V600E* mutation will be referred to as *BRAF* mutation.

TSR assessment

The TSR was assessed as previously described in detail from either physical or scanned H&Es [18]. In short, 4 µm H&E stained sections from the most invasive part of the primary tumor were analyzed. A x2.5 or x5 objective was used to select the area with the highest amount of stroma. With this area, using a x10 objective, the percentage of stroma present within the field of vision was estimated per 10-fold. Tumor cells had to present at all sides of the field of vision (Figure 1). The highest percentage of stroma was decisive, even if only one image field contained a high amount of stroma.

Each cohort was scored independently by two observers, blinded for clinical data and outcome (Qusasar2 by GvP and AH; Avant by GvP and SZ). All cases were scored using conventional microscopy, except for 160 cases of the Quasar2 cohort, which were scored using digital images of the slides using Aperio ImageScope (Leica, The Netherlands). For digital scoring, the same protocol was followed as with conventional microscopy, however a circular annotation of 3.46 mm^2 was used to mimic the field of vision of a 100x magnification of most used microscopes. TSR percentages were dichotomized into stroma-low (≤50% stroma) and stroma-high (>50% stroma). The results of TSR for each separate cohort have been published previously [19, 20]. However, for the Quasar2 cohort 240 additional eligible cases were added for this study.

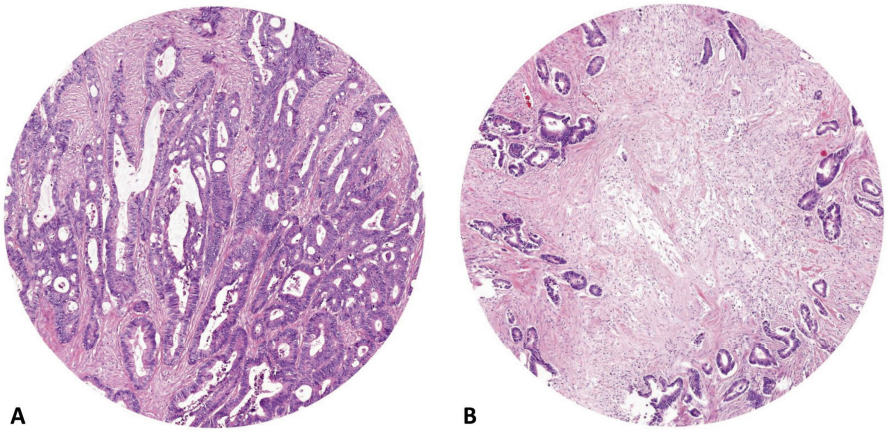


Figure 1. Examples of a stroma-low (A) and a stroma-high (B) tumor (100x magnification).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 25 (IBM, The Netherlands). Primary endpoint was disease free survival (DFS), which was defined as the time from randomization to the date of death or a recurrence event. If no event occurred DFS was calculated as time from randomization till last date of follow up. DFS was right-censored at 5 years. Differences in categorical variables between patient, tumor and treatment characteristics were analyzed using the Chi-square test. Analyses of survival curves were performed using Kaplan-Meier plots. The Cox proportional hazard model was used to determine the Hazard Ratio (HR) of explanatory variables for DFS in univariate and multivariate analyses. Covariates were selected for adjustment in the multivariate model if they showed a p-value of <0.1 in univariate analysis (age, gender, stage, MSI) or a clear clinical relevance (treatment, cohort). Analyses are reported according to the REMARK guidelines [21].

Results

Patients

H&E slides were available for TSR assessment in 2485 patients (Quasar2 n=1272, Avant n=1213). After exclusion of 117 patients (5%) due to technical or biological reasons, a total of 2368 patients were included in the final analyses (Figure 2). Both trials showed similar frequencies for all variables where the majority of patients were male (57%), between 50 and 70 years of age (65%) and had stage III disease (74%), while 13% showed MSI (Table 1).

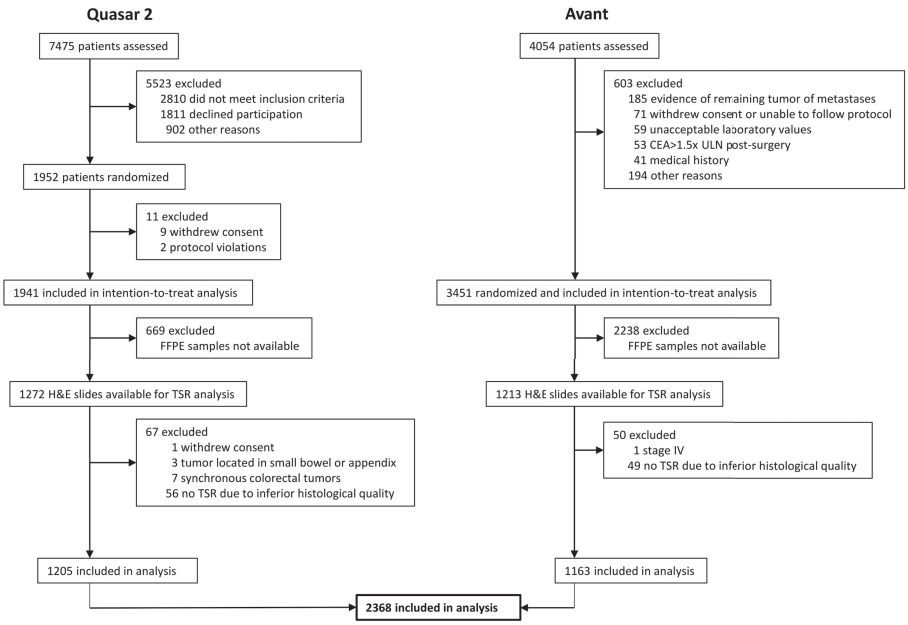


Figure 2. Consort flow diagram of the study.

The Quasar2 and Avant cohorts had 407 (33%) and 339 (29%) patients classified as stroma-high, respectively, resulting in a total of 746 stroma-high patients (32%). Furthermore, Quasar2 and Avant contained 139 (13%) and 78 (7%) *BRAF* mutant cases, respectively, for a total of 217 (10%)(Table 1). The distribution of patient and tumor characteristics stratified for TSR and *BRAF* mutation showed significant differences consistent with the prior literature (Suppl. Tables 1 and 2, respectively). No association was found between both biomarkers.

Table 1. Patient, tumor and treatment characteristics, stratified by cohort.

	Total N = 2368 (%)	Quasar 2 N = 1205 (%)	Avant N = 1163 (%)
Gender			
Female	1022 (43)	507 (42)	515 (44)
Male	1346 (57)	698 (58)	648 (56)
Age			
0-50	373 (16)	112 (9)	261 (22)
51-64	1013 (43)	482 (40)	531 (46)
65-70	525 (22)	284 (24)	241 (21)
>70	457 (19)	327 (27)	130 (11)
Stage			
II	617 (26)	420 (35)	197 (17)
III	1751 (74)	785 (65)	966 (83)
Adjuvant treatment			
Control arm	973 (41)	583 (48)	390 (34)
Experimental arm	1395 (59)	622 (52)	773 (67)
MSI status			
MSS	1867 (79)	964 (80)	912 (78)
MSI	270 (11)	149 (12)	121 (10)
Missing	222 (9)	92 (8)	130 (11)
TSR			
Stroma-low	1622 (69)	798 (66)	824 (71)
Stroma-high	746 (32)	407 (34)	339 (29)
***BRAF* status**			
Wildtype	1901 (80)	928 (77)	973 (84)
Mutant	217 (9)	139 (12)	78 (7)
Missing	250 (11)	138 (12)	112 (10)
5 years DFS status			
No event	1840 (78)	870 (72)	970 (83)
Event	528 (22)	335 (28)	193 (17)

Abbreviations: MSS microsatellite stable, MSI microsatellite instability, TSR tumor-stroma ratio, DFS disease free survival.

Survival by TSR and BRAF

In each cohort, a prognostic model adjusted for age, gender, stage, adjuvant treatment and MSI showed that patients with a stroma-high tumor had significantly worse 5 yr DFS compared to patients with a stroma-low tumor (Quasar2 HR 1.57, 95%CI 1.25-1.97, $p < 0.001$; Avant HR 1.59, 95%CI 1.17-2.16, $p = 0.003$)(Suppl. Figure 1A-B; Suppl. Table 3). In a similar multivariate survival analysis, *BRAF* mutation was an independent prognostic factor within the Quasar2 cohort (HR 1.74, 95%CI 1.24-2.44, $p = 0.001$), but not within Avant (HR 1.36, 95%CI 0.77-2.40, $p = 0.296$)(Suppl. Figure 1C-D, Suppl. Table 3), although in the subset of MSS samples it showed an expected trend for poor prognosis (Suppl. Figure 1F). Accordingly, both cohorts were combined for further survival analyses.

Table 2. Uni- and multivariate analysis for 5 year DFS for TSR and *BRAF* in the total cohort.

	Univariate analysis					Multivariate analysis				
	N	N events	HR	95% CI	p-value	N	N events	HR	95% CI	p-value
Age										
<=50	373	60	1			313	50	1		
51-64	1013	216	1.36	1.02-1.81	**0.036**	901	197	1.20	0.88-1.65	0.246
65-70	525	129	1.60	1.18-2.17	**0.003**	452	112	1.31	0.93-1.83	0.121
>70	457	123	1.77	1.30-2.42	**<0.001**	404	104	1.25	0.88-1.77	0.211
Gender										
Female	1022	203	1			891	179	1		
Male	1346	325	1.22	1.02-1.46	**0.026**	1179	284	1.20	0.99-1.45	0.060
Stage										
II	617	102	1			551	94	1		
III	1751	426	1.60	1.29-1.99	**<0.001**	1519	369	1.71	1.36-2.16	**<0.001**
Cohort										
Quasar2	1205	335	1			1057	293	1		
Avant	1163	193	0.58	0.49-0.69	**<0.001**	1013	170	0.57	0.46-0.69	**<0.001**
Adjuvant treatment										
Control	973	216	1			838	184	1		
Experimental	1395	312	1.02	0.86-1.21	0.854	1232	279	1.14	0.94-1.38	0.177
MSI status										
MSS	1876	444	1			1816	426	1		
MSI	270	43	0.66	0.48-0.90	**0.009**	254	37	0.54	0.38-0.78	**0.001**
TSR										
Stroma-low	1622	309	1			1416	275	1		
Stroma-high	746	219	1.65	1.39-1.97	**<0.001**	654	188	1.54	1.28-1.86	**<0.001**
BRAF										
wt	1901	407	1			1859	402	1		
mut	217	61	1.39	1.06-1.82	**0.017**	211	61	1.65	1.23-2.20	**0.001**

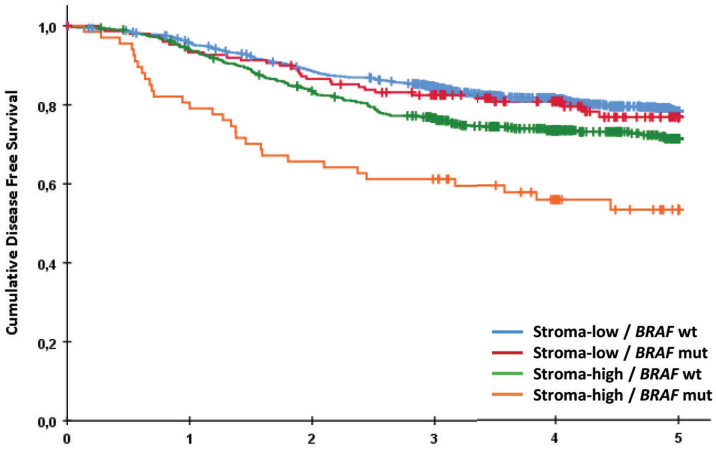
Abbreviations: DFS disease free survival, MSS microsatellite stable, MSI microsatellite instable, wt wildtype, mut mutant, TSR tumor-stroma ratio, HR hazard ratio, CI confidence interval.

In the total cohort, patients with a stroma-high tumor had significantly worse 5 yr DFS compared to patients with a stroma-low tumor (HR 1.65, 95%CI 1.39-1.97, p < 0.001). For *BRAF* mutant patients, a significant difference was also seen for a worse 5 yr DFS compared to *BRAF* wild type tumors (HR 1.39, 95%CI 1.06-1.82, p = 0.017). In a single multivariate model including both TSR and *BRAF* with other relevant factors, both biomarkers were independently prognostic (TSR HR 1.54, 95%CI 1.28-1.86, p < 0.001, *BRAF* HR 1.65, 95%CI 1.23-2.20, p = 0.001)(Table 2). A significant interaction on DFS was found between TSR and *BRAF* mutation (p = 0.031).

Combination of TSR and BRAF

Of all patients, 2118 (89%) were profiled for both biomarkers. Their combination resulted in 1302 stroma-low/*BRAF* wt (62%), 150 stroma-low/*BRAF* mut (7%), 599 stroma-high/*BRAF* wt (28%) and 67 stroma-high/*BRAF* mut (3%) cases. The distribution of patient characteristics stratified for the 4 combinations did not show any significant difference in gender, age, stage or treatment arm (Suppl. Table 4). However, MSI was more frequent in stroma-high within *BRAF* mutants but not within *BRAF* wild types (p<0.001 and p=0.128, respectively).

No significant difference for DFS was found between patients with a stroma-low *BRAF* mutant tumor compared to stroma-low *BRAF* wildtype (HR 1.34, 95%CI 0.90-1.99, p = 0.146, multivariate analysis; 5 year survival rates 77% versus 78%, respectively). However, patients with a stroma-high tumor regardless of their *BRAF* mutation status showed worse survival than stroma-low/*BRAF* wild type (stroma-high *BRAF* wildtype HR 1.45, 95%CI 1.19-1.77, p <0.001; stroma-high *BRAF* mutant HR 3.05, 95%CI 2.07-4.49, p < 0.001, multivariate analysis)(Table 3, Figure 3). In contrast with stroma-low patients, where no difference in DFS was found between patients with a *BRAF* wildtype tumor and patients with a *BRAF* mutated tumor, patients with a stroma-high tumor and a *BRAF* mutation had significantly worse DFS compared to stroma-high *BRAF* wildtype patients (HR 2.24, 95%CI 1.49-3.36, p < 0.001, multivariate analysis), with survival rates of 53% vs 71%, respectively.



Numbers at risk						
Stroma-low/ *BRAF* wt	1296	1230	1092	841	457	124
Stroma-low / *BRAF* mut	150	139	123	97	59	18
Stroma-high / *BRAF* wt	597	557	477	354	198	60
Stroma-high / *BRAF* mut	67	54	44	35	21	7

Figure 3. Kaplan-Meier curves of 5 year disease free survival of the total cohort for the combination of TSR and *BRAF* mutation. Numbers at risk are mentioned below the graph.

MSI subgroup analysis

Based on the different frequency of MSI in the 4 combinations together with the known different survival of *BRAF* depending on MSI, we also performed a prognostic analysis of TSR/*BRAF* in relation to MSI for each cohort separately as well as the total cohort. Multivariate analyses revealed MSS tumors to show similar differences as in the original analyses for each cohort as well as the total cohort, although *BRAF* mutant MSS tumors lost formal significance in the Avant cohort (Suppl. Figure 1, Suppl. Figure 2, Suppl. Table 5). The number of samples and events in MSI tumors was low and the models were not significant although stroma-high/BRAF mutant tumors showed a similar trend as in MSS.

Table 3. Uni- and multivariate analysis for 5 year DFS for the combination of TSR and *BRAF* in the total cohort.

	Univariate analysis					Multivariate analysis				
	N	**N events**	**HR**	**95% CI**	**p-value**	**N**	**N events**	**HR**	**95% CI**	**p-value**
Age										
<=50	373	60	1			313	50	1		
51-64	1013	216	1.36	1.02-1.81	**0.036**	901	197	1.20	0.88-1.64	0.256
65-70	525	129	1.60	1.18-2.17	**0.003**	452	112	1.31	0.93-1.83	0.123
>70	457	123	1.77	1.30-2.42	**<0.001**	404	104	1.23	0.87-1.74	0.241
Gender										
Female	1022	203	1			891	179	1		
Male	1346	325	1.22	1.02-1.46	**0.026**	1179	284	1.20	1.00-1.45	0.054
Stage										
II	617	102	1			551	94	1		
III	1751	426	1.60	1.29-1.99	**<0.001**	1519	369	1.71	1.36-2.15	**<0.001**
Cohort										
Quasar2	1205	335	1			1057	293	1		
Avant	1163	193	0.58	0.49-0.69	**<0.001**	1013	170	0.56	0.46-0.69	**<0.001**
Adjuvant treatment										
Control	973	216	1			838	184	1		
Experimental	1395	312	1.02	0.86-1.21	0.854	1232	279	1.14	0.94-1.38	0.176
MSI status										
MSS	1876	444	1			1816	426	1		
MSI	270	43	0.66	0.48-0.90	**0.009**	254	37	0.56	0.39-0.81	**0.002**
TSR / *BRAF*										
Stroma-low / *BRAF* wt	1302	248	1			1271	244	1		
Stroma-low / *BRAF* mut	150	31	1.10	0.76-1.60	0.619	145	31	1.34	0.90-1.99	0.146
Stroma-high / *BRAF* wt	599	159	1.46	1.20-1.78	**<0.001**	588	158	1.45	1.19-1.77	**<0.001**
Stroma-high / *BRAF* mut	67	30	2.91	1.99-4.24	**<0.001**	66	30	3.05	2.07-4.49	**<0.001**

Abbreviations: DFS disease free survival, MSS microsatellite stable, MSI microsatellite instable, wt wildtype, mut mutant, TSR tumor-stroma ratio, HR hazard ratio, CI confidence interval.

Discussion

This study shows that patients with a stroma-high *BRAF* mutated tumor have significantly worse 5 yr DFS compared to all other patients and supports a synergistic effect of both biomarkers for poor outcome. Remarkedly, within stroma-low tumors the *BRAF* mutation does not affect DFS, whereas in stroma-high tumors it does. Our results suggest that the poor prognosis reported for *BRAF* mutants may be confined specifically to stroma-high tumors, while TSR may be a broader biomarker. However, as all our patients were treated with adjuvant therapy it is currently unclear whether chemotherapy may be responsible for such poor outcome. Preclinical and clinical studies have shown that tumors with high stromal content become resistant to therapy. Cancer associated fibroblasts, which play an important role in the tumor microenvironment, treated with chemotherapy can promote tumor-initiating cells and tumor growth *in vivo*, whereas endothelial cells were found to be able to induce chemoresistance in CRC cells [22, 23]. Furthermore, a subgroup analysis within a randomized clinical trial showed that stage II-III CRC patients of the CMS4 subtype did not benefit from an adjuvant regimen with fluorouracil, leucovorin and oxaliplatin [24]. Our results would be consistent with a chemoresistance effect in high-stroma tumors although the lack of untreated patients in our two clinical trials precludes any conclusions.

Further research is needed to understand the biological reasons behind this synergistic association. A possible hypothesis would be that carcinomas concomitant for *BRAF* mutation and stroma-high may arise from serrated adenomas as they are mostly *BRAF* mutants that are directed to a mesenchymal subtype [25]. Interestingly, the serrated pathway gives rise to both MSI and MSS carcinomas. Although our outcome analyses within MSI tumors are unconclusive due to low number of patients and events, they are consistent with this combined biomarker being independent of MSI status and hence arising from serrated adenomas.

BRAF confers poor prognosis specifically in tumors displaying a MSS phenotype. Interestingly, our results suggest that the subtype composed of high-stroma and *BRAF* mutation may be independent of MSI. Accordingly, such subtype in MSI tumors may have worse prognosis relative to the other MSI CRCs, although that might result in survival rates similar to other subtypes in MSS patients. To date no single biomarker has been shown to provide strong stratification. However, the analysis of combinations of biomarkers well described in literature showing mild stratification may provide deeper insights. Some compound biomarkers have already been suggested for CRC, such as the combination of MSI with tumor infiltrating lymphocytes [26]

or stroma with ploidy [27]. Intriguingly, these compound biomarkers and ours are composed of epithelial and microenvironment features suggesting there may be crosstalk between these compartments with biological and clinical implications. Meta-analysis from several large cohorts may be able to decipher the real biological associations and the most relevant biomarkers for clinical implementation. In this regard, some attempts have recently been made [10, 28, 29] although larger efforts are required. While such analyses are unlikely to provide biomarkers that accurately predict relapse events, they can easily improve current information based on staging to decide which patients may or may not benefit from chemotherapy and focus research on targeted therapies for specific subtypes refractory to current cytotoxic treatments.

BRAF testing is not routinely performed in non-metastatic CRC. However, this might be reconsidered as there is increasing evidence that *BRAF* mutations are associated with poor prognosis in the adjuvant setting[8, 11, 30-32] and our results further refine such associations. In addition, our results call for further investigation into treatment alternatives for patients with stroma-high and *BRAF* mutation. The outcome of such patients treated with adjuvant cytotoxic therapies as their current standard of care is very poor, showing survival rates that are relatively close to unselected patients in first-line treatment of metastatic CRC [33]. Although single *BRAF* inhibitors have not succeed in CRC due to late resistance, combination therapy with two or three inhibitors has recently obtained promising results in metastatic CRC [34]. Patients with *BRAF* mutation and stroma-high may have more room for benefit from such a combined therapy than stroma-low, which shows outcome patterns more similar to other subtypes.

To our knowledge only one report has previously looked at outcome stratifying by the combination of *BRAF* mutation and TSR which showed negative results in a cohort of 1183 cases from the Quasar clinical trial [35]. However, TSR was measured with a technically different method analyzing larger areas than ours which were not selected by stromal content. We consider this selection extremely important to capture the most relevant biological information regarding stromal infiltration as opposed to only surrounding stromal presence. Importantly, the clinical setting of this cohort is totally different as Quasar is mostly composed of stage II patients randomized between adjuvant fluorouracil/folinic acid chemotherapy and observation. As previously discussed, it is unclear whether chemotherapy or even stage may be confounding factors so this study and ours are not inconsistent.

The strength of our study is the use of two large high-quality randomized clinical trials. Both are similar in experimental set-up where all patients received adjuvant therapy with similar regimens. No outcome differences were found between treatment arms in either trial and survival patterns were similarly consistent [16, 17]. However, our limitation is the lack of a validation cohort which would need to be equally large due to the relatively low number of patients showing concomitant high-stroma and *BRAF* mutation. Accordingly, even if our results are consistent in two different clinical trials, additional cohorts are required to better assess our current findings. It would also be of particular interest to analyze large cohorts of patients without any adjuvant treatment to understand whether such poor prognosis may be exacerbated by current cytotoxic regimens.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

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Supplementary tables

Supplementary table 1. Patient, tumor and treatment characteristics of total cohort, stratified for TSR.

	Total ***N*** **= 2368 (%)**	**Stroma-low** ***N*** **= 1622 (%)**	**Stroma-high** ***N*** **= 746 (%)**	**p-value**
Gender				
Female	1022 (43)	703 (43)	319 (43)	0.791
Male	1346 (57)	919 (57)	427 (57)	
Age				
0-50	373 (16)	263 (16)	110 (15)	0.398
51-64	1013 (43)	705 (44)	308 (41)	
65-70	525 (22)	348 (22)	177 (24)	
>70	457 (19)	306 (19)	151 (20)	
Stage				
II	617 (26)	413 (26)	204 (27)	0.332
III	1751 (74)	1209 (75)	542 (73)	
Adjuvant treatment				
Control arm	973 (41)	651 (40)	322 (43)	0.164
Experimental arm	1395 (59)	971 (60)	424 (57)	
MSI status				
MSS	1876 (79)	1260 (78)	616 (83)	**<0.002***
MSI	270 (11)	207 (13)	63 (8)	
Missing	222 (9)	155 (10)	67 (9)	
BRAF **status**				
Wildtype	1901 (80)	1302 (80)	599 (80)	0.849*
Mutant	217 (9)	150 (9)	67 (9)	
Missing	250 (11)	170 (11)	80 (11)	
5 years DFS status				
No event	1840 (78)	1313 (81)	527 (71)	**0.001**
Event	528 (22)	309 (19)	219 (29)	

Abbreviations: TSR tumor-stroma ratio, MSS microsatellite stable, MSI microsatellite instability, DFS disease free survival.

*without missing values.

Supplementary table 2. Patient, tumor and treatment characteristics of total cohort, stratified for *BRAF*.

	Total ***N* = 2118 (%)**	***BRAF* wildtype** ***N* = 1901 (%)**	***BRAF* mutant** ***N* = 217 (%)**	**p-value**
Gender				
Female	911 (43)	801 (42)	110 (51)	**0.017**
Male	1207 (57)	1100 (58)	107 (49)	
Age				
0-50	326 (15)	308 (16)	18 (8)	**<0.001**
51-64	918 (43)	841 (44)	77 (36)	
65-70	465 (22)	402 (21)	63 (29)	
>70	409 (19)	350 (18)	59 (27)	
Stage				
II	561 (27)	503 (27)	58 (27)	0.935
III	1557 (74)	1398 (74)	159 (73)	
Adjuvant treatment				
Control arm	860 (41)	781 (41)	79 (36)	0.190
Experimental arm	1258 (59)	1120 (59)	138 (64)	
MSI status				
MSS	1816 (86)	1701 (90)	115 (53)	**<0.001***
MSI	254 (12)	158 (8)	96 (44)	
Missing	48 (2)	42 (2)	6 (3)	
TSR				
Stroma-low	1452 (69)	1302 (69)	150 (69)	0.878
Stroma-high	666 (31)	599 (32)	67 (31)	
5 years DFS status				
No event	1650 (78)	1494 (79)	156 (72)	**0.030**
Event	468 (22)	407 (21)	61 (28)	

Abbreviations: MSS microsatellite stable, MSI microsatellite instability, TSR tumor-stroma ratio, DFS disease free survival.
*without missing values.

Supplementary table 3. Uni- and multivariate analysis for 5 year DFS of TSR and *BRAF* for separate cohorts.

		Univariate analysis					Multivariate analysis*				
		N	**N events**	**HR**	**95% CI**	**p-value**	**N**	**N events**	**HR**	**95% CI**	**p-value**
Quasar 2	**TSR**										
	Stroma-low	798	195	1			739	181	1		
	Stroma-high	407	140	1.53	1.23-1.90	**<0.001**	374	131	1.57	1.25-1.97	**<0.001**
	BRAF										
	wt	928	248	1			920	246	1		
	mut	139	47	1.35	0.99-1.84	0.061	137	47	1.74	1.24-2.44	**0.001**
Avant	**TSR**										
	Stroma-low	824	114	1			728	106	1		
	Stroma-high	339	79	1.78	1.33-2.37	**<0.001**	305	69	1.59	1.17-2.16	**0.003**
	BRAF										
	wt	973	159	1			939	156	1		
	mut	78	14	1.14	0.66-1.97	0.638	74	14	1.36	0.77-2.40	0.296

Abbreviations: TSR tumor-stroma ratio, wt wildtype, mut mutation, HR hazard ratio, CI confidence interval.

*Adjusted for age, gender, stage, treatment, MSI.

Supplementary table 4. Patient, tumor and treatment characteristics of total cohort, stratified for TSR/*BRAF*.

	Total ***N* = 2118 (%)**	**Low / wt** ***N* = 1302 (%)**	**High / wt** ***N* = 599 (%)**	**p-value**	**Low / mut** ***N* = 150 (%)**	**High / mut** ***N* = 67 (%)**	**p-value**
Gender							
Female	911 (43)	545 (42)	256 (43)	0.727	77 (51)	33 (49)	0.883
Male	1207 (57)	757 (58)	343 (57)		73 (49)	34 (51)	
Age							
0-50	326 (15)	215 (17)	93 (16)	0.553	13 (9)	5 (8)	0.586
51-64	918 (43)	586 (45)	255 (43)		57 (38)	20 (30)	
65-70	465 (22)	266 (20)	136 (23)		40 (27)	23 (34)	
>70	409 (19)	235 (18)	115 (19)		40 (27)	19 (28)	
Stage							
II	561 (27)	339 (26)	164 (27)	0.539	41 (27)	17 (25)	0.868
III	1557 (74)	963 (74)	435 (73)		109 (73)	50 (75)	
Adjuvant treatment							
Control arm	860 (41)	521 (40)	260 (43)	0.175	55 (37)	24 (36)	1.000
Experimental arm	1258 (59)	781 (60)	339 (57)		95 (63)	43 (64)	
MSI status							
MSS	1816 (86)	1154 (89)	547 (91)	0.128*	67 (45)	48 (72)	**<0.001***
MSI	254 (12)	117 (9)	41 (7)		78 (52)	18 (27)	
Missing	48 (2)	31 (2)	11 (2)		5 (3)	1 (2)	
5 years DFS status							
No event	1650 (78)	1054 (81)	440 (74)	**<0.001**	119 (79)	37 (55)	**<0.001**
Event	468 (22)	248 (19)	159 (27)		31 (21)	30 (45)	

Abbreviations: TSR tumor-stroma ratio, MSS microsatellite stable, MSI microsatellite instability, wt wildtype, mut mutant, DFS disease free survival.

*without missing values.

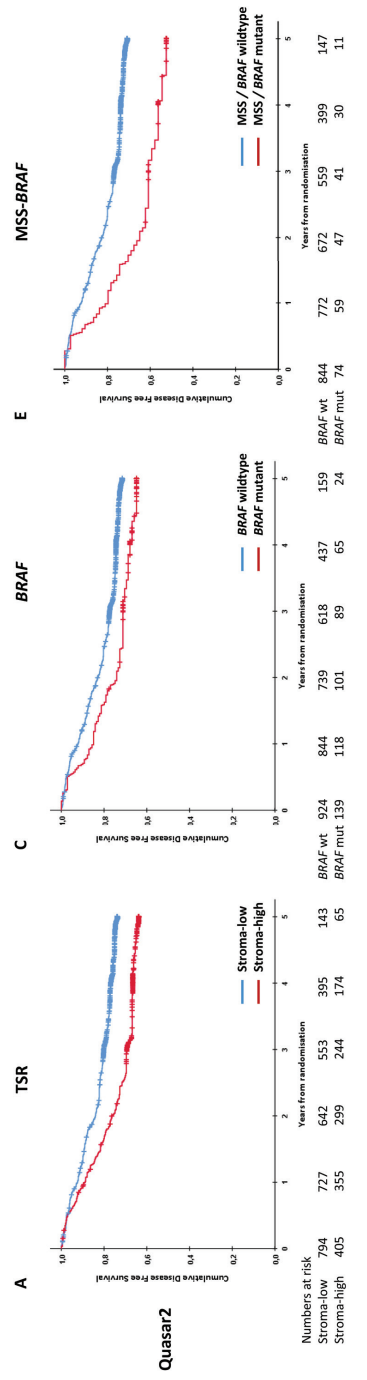
Supplementary table 5. Multivariate analysis for 5 year DFS stratified for MSI status for separate and total cohorts.

		Quasar2					Avant					Total cohort				
		N	N events	HR	95% CI	p-value	N	N events	HR	95% CI	p-value	N	N events	HR	95% CI	p-value
MSS*	**TSR**															
	Stroma-low	629	161	1			631	96	1			1260	257	1		
	Stroma-high	335	120	1.58	1.24-2.00	**<0.001**	281	67	1.66	1.21-2.26	**0.002**	616	187	1.61	1.33-1.95	**<0.001**
	BRAF															
	wt	847	233	1			854	147	1			1701	380	1		
	mut	74	34	1.94	1.35-2.78	**<0.001**	41	12	1.65	0.91-2.99	0.097	115	46	1.89	1.39-2.57	**<0.001**
	TSR / *BRAF*															
	Stroma-low / *BRAF* wt	557	138	1			597	89	1			1154	227	1		
	Stroma-low / *BRAF* mut	43	16	1.64	0.97-2.75	0.064	24	5	1.25	0.51-3.09	0.630	67	21	1.55	0.99-2.42	0.057
	Stroma-high / *BRAF* wt	290	95	1.44	1.11-1.87	**0.006**	257	58	1.58	1.14-2.20	**0.007**	547	153	1.50	1.22-1.84	**<0.001**
	Stroma-high / *BRAF* mut	31	18	3.24	1.97-5.32	**<0.001**	17	7	3.15	1.45-6.85	**0.004**	48	25	3.32	2.19-5.02	**<0.001**
MSI*	**TSR**															
	Stroma-low	110	20	1			97	10	1			207	30	1		
	Stroma-high	39	11	1.77	0.83-3.78	0.142	24	2	1.10	0.23-5.13	0.908	63	13	1.48	0.76-2.88	0.253
	BRAF															
	wt	73	13	1			85	9	1			158	22	1		
	mut	63	13	1.93	0.79-4.73	0.150	33	2	0.34	0.07-1.71	0.190	96	15	1.12	0.54-2.31	0.767
	TSR / *BRAF*															
	Stroma-low / *BRAF* wt	52	10	1			65	7	1			117	17	1		
	Stroma-low / *BRAF* mut	49	8	1.46	0.51-4.23	0.484	29	2	0.40	0.08-2.08	0.273	78	10	0.90	0.39-2.10	0.810
	Stroma-high / *BRAF* wt	21	3	0.96	0.25-3.71	0.947	20	2	1.15	0.23-5.74	0.866	41	5	0.91	0.33-2.53	0.858
	Stroma-high / *BRAF* mut	14	5	5.20	1.41-19.1	**0.013**	4	0	0	0.00-∞	0.991	18	5	2.25	0.72-7.01	0.164

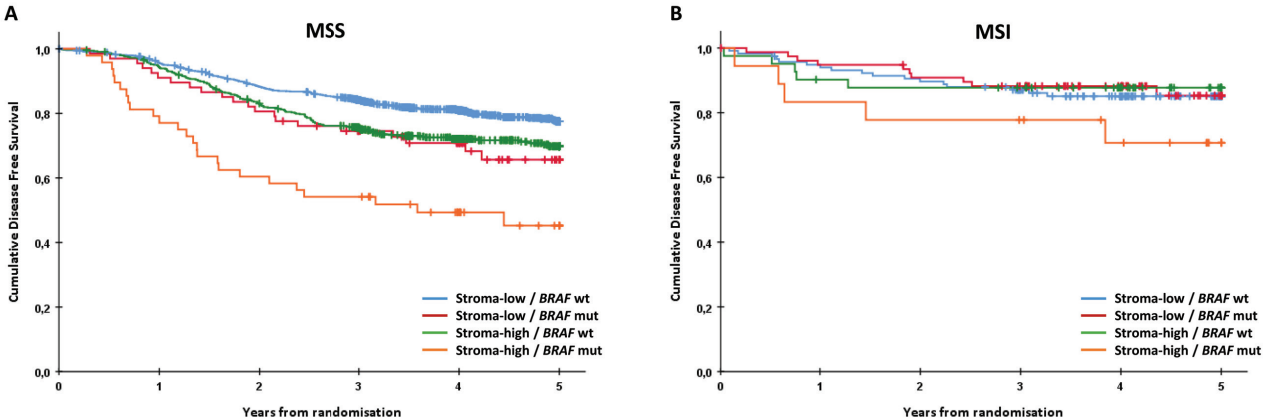
Abbreviations: DFS disease free survival, MSS microsatellite stable, MSI microsatellite instability, wt wildtype, mut mutant, TSR tumor-stroma ratio, HR hazard ratio, CI confidence interval

*TSR, *BRAF* and TSR / *BRAF* were all independently adjusted for age, gender, stage and adjuvant treatment.

Supplementary figures



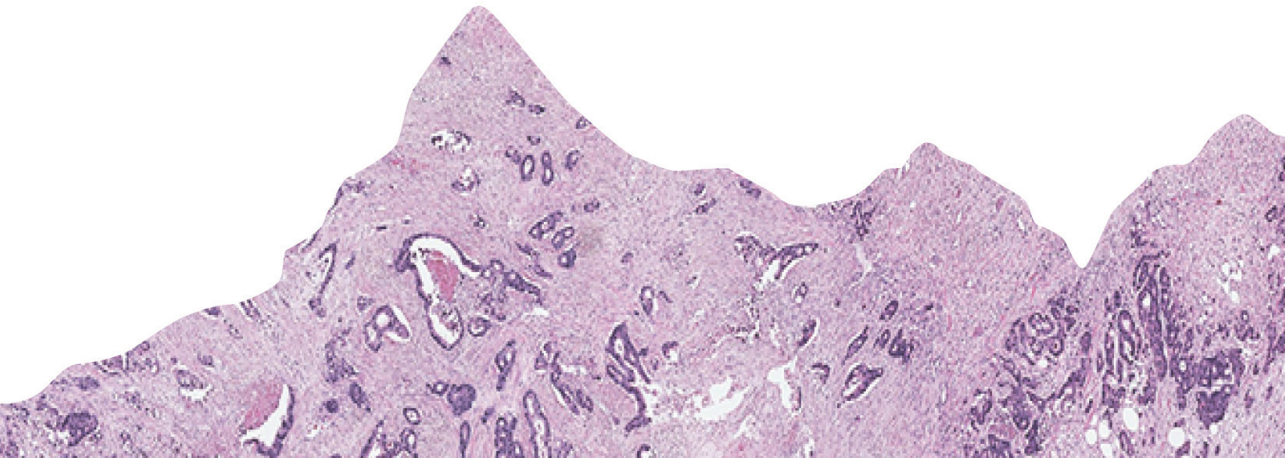
Supplementary Figure 1. Kaplan-Meier curves of 5 years disease free survival for TSR (A - B, Quasar2 and Avant, respectively), *BRAF* (C - D, Quasar2 and Avant, respectively) and MSS-*BRAF* (E – F, Quasar2 and Avant, respectively). Numbers at risk are mentioned below the graphs.



Numbers at risk						
Stroma-low/ *BRAF* wt	1149	1090	965	743	406	110
Stroma-low / *BRAF* mut	67	61	51	39	25	7
Stroma-high / *BRAF* wt	546	511	434	319	180	57
Stroma-high / *BRAF* mut	48	38	29	22	13	4

117	110	100	80	45	12
78	73	68	54	33	10
41	36	34	27	14	3
18	15	14	12	8	3

Supplementary Figure 2. Kaplan-Meier curves of 5 years disease free survival for MSS (A) and MSI (B) separately for the combination of TSR and *BRAF* mutation within the total cohort. Numbers at risk are mentioned below the graphs.



CHAPTER 7

Tumor-stroma ratio as predictor of response to neoadjuvant chemoradiotherapy in rectal cancer

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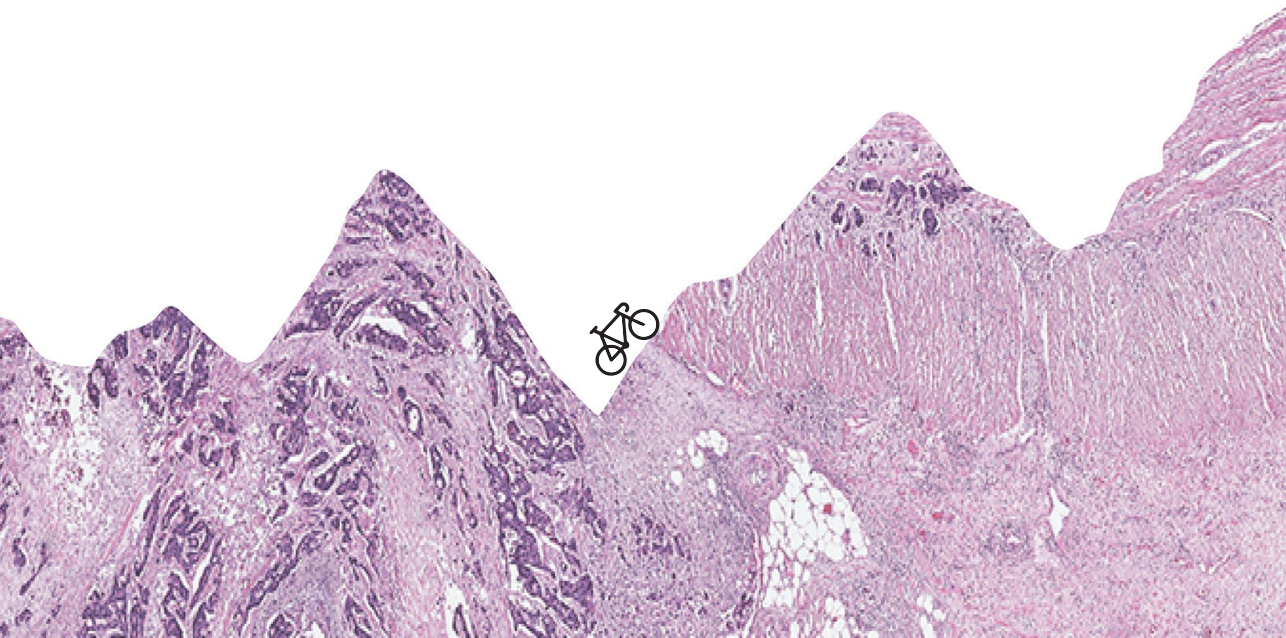
Submitted



Abstract

Purpose There are no predictive markers for response to neoadjuvant chemoradiotherapy (nCRT) in rectal cancer patients. The tumor-stroma ratio (TSR) has proven to be a prognostic marker in several types of cancer, but its value in predicting pathologic complete response (pCR) in rectal cancer patients treated with nCRT remains unknown.

Methods The study cohort consisted of patients with rectal adenocarcinoma who received nCRT followed by surgery. Hematoxylin and eosin (H&E) stained sections of diagnostic biopsies were digitally assessed for TSR by two independent investigators. Patients were categorized in stroma-low (TSR ^{3} 50%) and stroma-high (TSR < 50%) groups for further analyses. The tumor regression grade (TRG) was assessed on H&E stained sections of the resected primary tumor specimens to determine pathologic response.

Results A total of 76 patients were included in this study, of which 37 patients (49%) were categorized as stroma-low and 39 (51%) as stroma-high. Eighteen patients (24%) had a pCR (TRG 1) to capecitabine-based chemoradiotherapy. pCR was numerically higher in stroma-low patients (32%, 95%CI 19%-50%) as compared to stroma-high tumors (15%, 95%CI 6%-31%; odds ratio 2.61, P = 0.09). At 6 years follow-up, relapse-free survival rate was 83% (95%CI 71%-96%) in stroma-low patients and 53% (95%CI 29%-97%) in stroma-high (hazard ratio 0.46, P = 0.10).

Conclusion TSR may help predict pCR and long-term relapse rate in rectal cancer patients receiving standard nCRT, with stroma-high patients presenting poor outcomes. The digital pathology assessment of TSR will facilitate validation studies and implementation in daily practice.

Keywords

Biopsy, neoadjuvant chemoradiotherapy, pathologic response, prediction, rectal cancer, tumor-stroma ratio

Introduction

In Europe, approximately 30% of patients diagnosed with colorectal cancer (CRC) in 2018 suffered from invasive rectal adenocarcinoma, with a mortality rate of 40% [1]. The incidence of rectal cancer is increasing, particularly in the younger population [2]. Currently, the recommended treatment for patients with high-risk locally advanced rectal carcinoma is neoadjuvant chemoradiotherapy (nCRT) [3]. In approximately 20% of the patients, nCRT leads to a complete pathological response (pCR), which is associated with better long-term outcomes [4, 5]. It is debatable whether these patients need resection of the primary tumor or can be offered an active wait-and-see approach [6-8]. In contrast, non-responders to nCRT will have higher risk of local and systemic relapse [4, 5] while retaining all potential side effects of the treatment. Hence, the importance to define biomarkers that predict whether or not a patient with rectal cancer will achieve pCR with standard nCRT.

Clinical factors, including carcinoembryonic antigen (CEA) levels at diagnosis, tumor size, clinical T- and N-stage, distance of the tumor from the anal margin, and the time interval from nCRT to surgery, are associated with response to nCRT in rectal cancer. In addition, some pathological features have been shown to predict poor response to nCRT, like tumor differentiation, absence of circumferential tumor margin involvement, mucinous type and the presence of macroscopic ulceration [9-12].

Imaging modalities such as ^{18}F-labelled 3'-deoxy-3'-fluorothymidine (FLT) positron emission tomography (PET) and ^{18}F-fluorodeoxyglucose (FDG)-PET combined with computed tomography (CT) have limited value in predicting response in patients with rectal cancer treated with nCRT [13, 14]. Microarray studies showed promising results in different cohorts, but implementation in routine clinical practice is difficult [15, 16]. Furthermore, radiomics and transcriptomics markers have substantial costs.

In the last decade it has been recognized that tumor growth patterns and inflammatory response are strong determinants of prognosis in CRC [17]. Huang et al. showed that tumor microenvironment features may also play a role in predicting tumor response to nCRT [18]. They evaluated both local tumor microenvironment (tumor-infiltrating lymphocytes (TILs), intratumor budding (ITB)) as well as the systemic inflammatory environment (neutrophil-to-lymphocyte ratio, C-reactive protein) and found that the combination of CD8+ intraepithelial TILS and ITB was an independent predictive factor for the pathological response to nCRT in rectal cancer patients.

A simple method to assess the tumor microenvironment on routine hematoxylin and eosin (H&E) stained sections of biopsies (or primary tumors), is the tumor-stroma ratio (TSR). It has prognostic value in multiple types of epithelial cancers like colon, breast and gastric adenocarcinomas [19-23]. A high stroma component (low TSR) is related to worse patient outcomes after curative treatment. In the current study we investigated whether TSR, determined in biopsy specimens before nCRT, could aid in predicting therapy response in rectal cancer patients.

Material and Methods

Patients

In a prospective patient cohort of 82 consecutive patients with rectal cancer from the Vall d'Hebron University Hospital, Barcelona, Spain, we retrospectively analyzed the impact of TSR on clinical outcomes. All patients were diagnosed with clinical stage II-III rectal carcinoma between 2011 and 2018 and were treated according to standard-of-care protocols (neoadjuvant chemoradiotherapy followed by surgery). Radiotherapy consisted of a total dose of 50.4 Gy, given in 28 fractions of 1.8 Gy, 5 fractions per week. Concurrent chemotherapy consisted of capecitabine alone or in combination with oxaliplatin. A minimum follow-up of 12 months from surgery to last follow-up in patients alive was required.

This research has been approved by the local ethics committee of the Vall d'Hebron University Hospital. All samples were handled in a coded de-identified fashion, according to national data privacy regulations.

Tumor-stroma ratio (TSR)

The TSR was determined on digital H&E biopsy sections using NanoZoomer Digital Pathology (NDP.view 2, Hamamatsu, Hamamatsu City, Shizuoka, Japan). The area with the highest amount of stroma was selected, using a circular annotation of 3.46 mm^2. This annotation mimics the microscopical scoring with a 10x objective on most commonly used microscopes. The amount of stroma present in the selected area was visually estimated in increments of 10%. Tumor cells were to be present at the four borders of the selected area. Identifying one single area with high stroma content was decisive for a final stroma classification. Patients were categorized in two groups, i.e. stroma-low (TSR ^{3} 50%) and stroma-high (TSR < 50%) (Fig. 1). A detailed TSR scoring protocol has been published previously [24]. All sections were independently scored by two observers (GP, SZ), blinded for any clinical information.

The response to nCRT was assessed on the resection specimens by experienced gastrointestinal pathologists using the tumor regression grade (TRG) defined by Mandard [25]. This classification is defined by 5 categories. TRG 1 is defined as complete regression with no residual cancer but only fibrosis through all layers of the rectum wall and is called pathologic complete response (pCR); TRG 2 is characterized by scattered residual cancer cells or groups of cells within the fibrosis; TRG 3 shows an increase of residual cancer cells but fibrosis predominates; TRG 4 is characterized by residual cancer outgrowing the fibrosis, and TRG 5 is defined by absence of any regressive changes.

Statistical analysis

Statistical analysis was performed using IBM SPSS software version 25 (Armonk, New York, USA) and R statistical software version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria). Differences in categorical variables between patient, tumor and treatment characteristics for the TSR groups were analyzed using the Fisher's exact test. For comparison of continuous variables, the Mann-Whitney U test was used. Inter-observer variability was analyzed using the Cohen's kappa coefficient. For the predictive correlative endpoint of TRG versus stroma content, we performed univariable logistic regression and TRG variable was dichotomized in two groups, TRG 1 (complete response, pCR) versus TRG 2-5 (non-complete response). Our target population was a sample size of 80 evaluable patients, which would give 80% power to detect an increase in pCR from < 15% in stroma-high group to > 45% in stroma-low group, assuming a 50%/50% prevalence of stroma-high/-low. Univariable survival analyses were conducted with Cox's proportional hazards regression. Kaplan-Meier survival curves were compared with the log-rank test. Six-year relapse-free survival (RFS, considering relapse or death from any cause as events and censoring in the case of no event within six years) was used as endpoint. Multivariable models were not constructed given the small sample of this exploratory cohort. Final *P* values <0.05 were considered statistically significant.

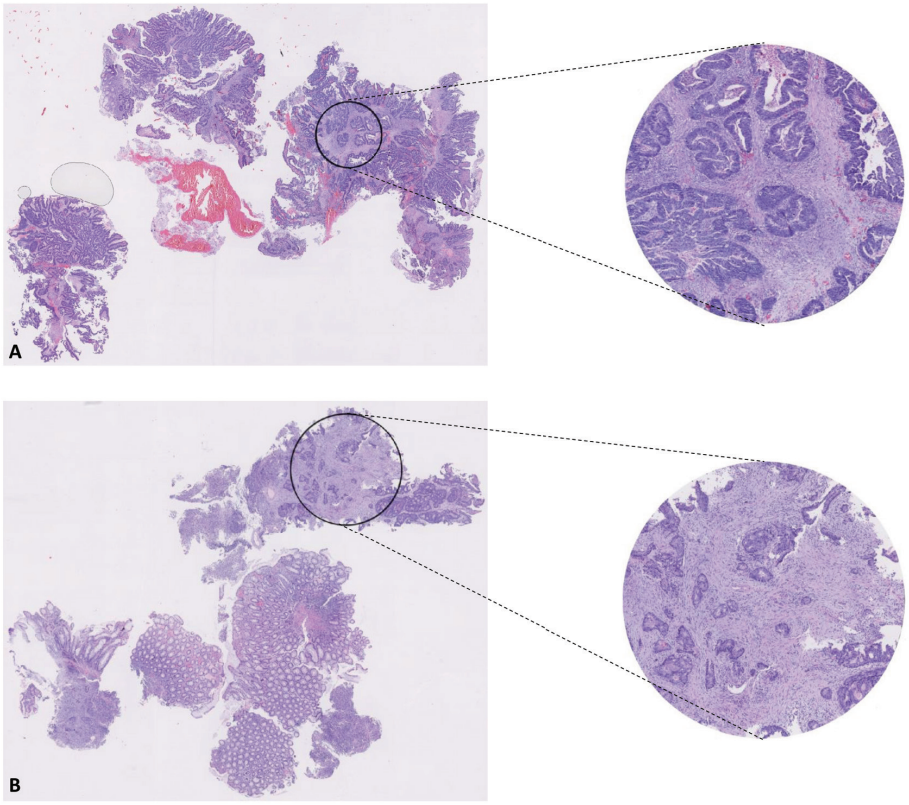


Figure. 1 Examples of H&E stained biopsy sections of rectal carcinoma, with on the left an overview of the biopsy with the annotated area magnified on the right. A) stroma-low, B) stroma-high. In both A) and B) the annotated area is 3.46 mm² in size.

Results

Patients

The initial cohort consisted of 82 stage II-III rectal cancer patients. Six patients were excluded because of diagnosis of metastatic disease during neoadjuvant therapy or participation in clinical trials with novel chemotherapy regimens, leaving 76 patients for downstream analyses. Median age was 69 years (range 46-87) at the start of nCRT, 58% (*N* = 44) were men and most patients (87%, *N* = 66) had clinical stage III disease. All patients completed radiotherapy as intended, however 3 patients (4%) stopped chemotherapy early because of toxicity. Median time between nCRT and surgery was 2.4 months (range 0.6-4.1). Clinico-pathological data of patients are shown in table 1. There were no significant differences between the two TSR groups.

Table 1. Patient, tumor and treatment characteristics, stratified by TSR.

	Total ***N*** **= 76 (%)**	**Stroma-low** ***N*** **= 37 (%)**	**Stroma-high** ***N*** **= 39 (%)**	***P*-value**
Gender				
Male	44 (58)	25 (68)	19 (49)	0.11
Female	32 (42)	12 (32)	20 (51)	
Median age years [range]	68.6 [45.5-86.8]	68 [48-86]	67 [45-87]	0.37
Median length tumor[a] (cm) [range]	2.0 [0.2-16.0]	2.2 [0.3-4.0]	2.0 [0.2-16.0]	0.52
Histology[b]				
Adenocarcinoma	52 (68)	22 (59)	30 (77)	0.21
Mucinous adenocarcinoma	6 (8)	3 (8)	3 (8)	
No tumor	18 (24)	12 (32)	6 (15)	
cT status				
cT2	15 (20)	10 (27)	5 (13)	0.24
cT3	47 (62)	22 (59)	25 (64)	
cT4	14 (18)	5 (14)	9 (23)	
cN status				
cN0	9 (12)	6 (16)	3 (8)	0.55
cN1	36 (47)	17 (46)	19 (49)	
cN2	29 (38)	13 (35)	16 (41)	
cN3	1 (1)	1 (3)	0 (0)	
Missing	1 (1)	0 (0)	1 (3)	
cTNM				
II	9 (12)	6 (16)	3 (8)	0.37
III	66 (87)	31 (84)	35 (90)	
Missing	1 (1)	0 (0)	1 (3)	
Differentiation grade[b]				
Well	24 (32)	9 (24)	15 (38)	0.25
Moderate	27 (36)	14 (38)	13 (33)	
Poor	6 (8)	2 (5)	4 (10)	
Not applicable	18 (24)	12 (32)	6 (15)	
Missing	1 (1)	0 (0)	1 (3)	
Therapy				
RT + Capecitabine/Oxaliplatin	7 (9)	2 (5)	5 (13)	0.43
RT + Capecitabine	69 (91)	35 (95)	34 (87)	
Pre CEA				
≤ 3.5 ng/ml	45 (59)	20 (54)	25 (64)	0.48
> 3.5 ng/ml	31 (41)	17 (46)	14 (36)	
Post CEA				
≤ 3.5 ng/ml	64 (84)	30 (81)	34 (87)	0.75
> 3.5 ng/ml	11 (15)	6 (16)	5 (13)	
Missing	1 (1)	1 (3)	0 (0)	
Median time between nCRT and surgery (months)[range]	2.4 [0.6-4.1]	2.6 [1.4-4.1]	2.3 [0.6-3.7]	0.12

[a]Tumor length was determined by MRI after neoadjuvant treatment.

[b]Differentiation grade and histology were determined on surgical resection specimen.

Abbreviations: TSR tumor-stroma ratio, TNM tumor-node-metastasis, RT radiotherapy, CEA carcinoembryonic antigen, nCRT neoadjuvant chemoradiotherapy.

Tumor regression grade

The pathological response assessment revealed 18 patients (24%) to have achieved pCR (TRG 1), whereas 12 (16%) cases reached almost complete response (TRG 2). The remaining 46 cases showed less than substantial to no tumor regression (TRG 3, N = 35; TRG 4, N = 8 and TRG 5, N = 3, respectively)(Table 2). Out of 18 patients who achieved a pCR (TRG 1), 2 (11%) had disease recurrence, as compared to 16 (28%) out of 58 patients without pCR (TRG2-5).

Table 2. Distribution of TRG categories versus TSR categories.

	Pathologic complete responders	**Non-responders**					
	TRG 1	TRG 2	TRG 3	TRG 4	TRG 5	**TRG 2-5**	Total
Stroma-low	**12 (32%)**	5	17	3	0	**25 (68%)**	37
Stroma-high	**6 (15%)**	7	18	5	3	**33 (85%)**	39
Total	18 (24%)	12	35	8	3	58 (76%)	76

Abbreviations: TRG tumor regression grade, TSR tumor-stroma ratio.

Tumor-stroma ratio

Out of 76 biopsies analyzed, 39 (51%) were categorized as stroma-high and 37 (49%) as stroma-low. A substantial inter-observer agreement was found for the assessment of the TSR (83% agreement, $\kappa = 0.67$). Discordant cases were re-assessed by the observers together and consensus was reached.

Predictive value of tumor-stroma ratio

In univariable logistic regression cT status was found to be a critical determinant for reaching pCR, whereas age was borderline significant. From 39 patients with a stroma-high biopsy, 6 (15%, 95%CI 6%-31%) had a pCR compared to 12 out of 37 (32%, 95%CI 19%-50%) of the stroma-low group (Fig. 2A). A non-significant difference was found for higher pCR numbers in stroma-low as compared to stroma-high group (OR 2.61, 95%CI 0.77-9.71, $P = 0.09$). None of the other variables were found to be of (potential) predictive value for pCR (Table 3).

Prognostic value of tumor-stroma ratio

Median follow-up of the entire cohort was 63 months (95%CI 59.8-67.2) and median RFS was not reached. Six-year RFS rate was 82.9% (95%CI 71.3% - 96.3%) in stroma-low patients and 52.9% (28.9% - 96.6%) in stroma-high population (HR = 0.46, 95%CI 0.17-2.24, $P = 0.10$)(Fig. 2B).

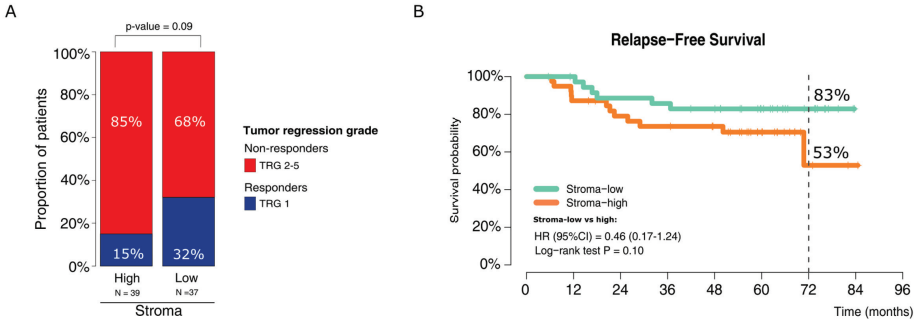


Figure. 2 A) The distribution of pathologic major responders within the stroma categories. The percentage of responders (in blue) versus non-responders (in red) within stroma-low and stroma-high categories, respectively. B) Kaplan-Meier survival curve for relapse free survival for stroma-low (green line) versus stroma-high (orange line).

Table 3. Univariable logistic regression analyses for having a complete response (TRG 1).

	Univariable analysis		
	OR	**95% CI**	***P*-value**
Gender			
Female	Ref		
Male	0.88	0.30-2.62	0.81
Age (years)	0.95	0.90-1.00	0.06
Length tumor (cm)	1.14	0.58-1.59	0.48
Pre-nCRT histology			
Adenocarcinoma	Ref		
Mucinous adenocarcinoma	0.91	0.13-4.24	0.91
cT status			
cT2	Ref		
cT3	0.21	0.06-0.71	0.01
cT4	0.07	0.00-0.47	0.02
cN status			
cN0	Ref		
cN1	0.57	0.11-3.17	0.49
cN2	0.52	0.10-3.02	0.44
cN3	0	0 -> 1000	0.99
cTNM			
II	Ref		
III	0.54	0.12-2.80	0.42
Therapy			
RT + Capecitabine/Oxaliplatin	Ref		
RT + Capecitabine	0.76	0.11-8.68	0.67
Pre CEA			
≤ 3.5 ng/ml	Ref		
> 3.5 ng/ml	0.33	0.07-1.23	0.99

Table 3. Continued

	Univariable analysis		
	OR	**95% CI**	***P*-value**
Post CEA			
≤ 3.5 ng/ml	Ref		
> 3.5 ng/ml	0.28	0.00-2.25	0.28
Tumor-stroma ratio			
Stroma-high	Ref		
Stroma-low	2.61	0.77-9.71	0.09

Abbreviations: TRG tumor regression grade, nCRT neoadjuvant chemoradiotherapy, TNM tumor-node-metastasis, RT radiotherapy, CEA carcinoembryonic antigen, OR odds ratio, CI confidence interval.

Discussion

Our results suggest that patients with high stroma tumors seem to be less likely to respond to nCRT compared to patients with tumors harboring low stroma content, which is linked to higher relapse rates with long-term follow-up. Eighty-five percent of the stroma-high patients did not have a response on nCRT, suggesting that novel treatment approaches are needed. Tumors with high stroma content might represent a group of lesions with an environment that is well armed against chemoradiation, or can even become resistant to therapy. The tumor stroma influences the aggressive behavior of cancer cells not only through cell-cell contact and auto- and paracrine signaling but also through mechanical pressure. Due to the abundant extracellular matrix and the high number of cancer-associated fibroblasts (CAFs), the tumor stroma forms a physical barrier around the tumor that increases the interstitial pressure and hypoxia in the tumor. Cancer cells respond to hypoxic conditions by up-regulating hypoxia-inducible factor 1α (HIF1α), a master transcription factor that activates a whole range of genes involved in angiogenesis, migration, metabolism, tumor invasion and metastasis [26]. Moreover, Lotti et al. showed chemotherapy-treated CAFs promoted tumor-initiating cells and tumor growth *in vivo* [27]. Similar results were found in endothelial cells able to induce chemo-resistance in CRC cells [28].

Different treatment strategies may have to be considered for stroma-high patients in order to achieve pCR. For instance, these tumors could be future candidates for therapies targeting activated oncogenic pathways (e.g. the TGF-β or PDGFR pathways), matrix remodeling, angiogenesis or CAFs [29-31].

In recent years, efforts have been made to find biomarkers which can predict the response to neoadjuvant therapy for rectal cancer patients. Multiple studies found pathological features and microenvironment signatures to be predictive of pCR [18, 32-36], whereas one study found no clinical and pathological variables to significantly associate with response to nCRT [37]. Furthermore, Van Stiphout et al. identified post-nCRT maximal radioactive isotype uptake (SUVmax) and relative change of SUVmax to be strong determinants of response [35, 36]. However, these are rather complex and expensive parameters, whereas the TSR is easy to assess with low costs and high reproducibility. In addition, microenvironmental features recently have been shown to be most critical determinants of patient outcome [17, 18].

Limitations of this study are the retrospective nature and the small sample size, underpowered for conclusive statistical analyses, which means that results should be interpreted with caution and validated in larger cohorts.

Another issue is the potential impact of the time-lag between the last nCRT dose and surgery influencing the rate of regression found in the surgical specimen. One might hypothesize that near-complete responders will further regress towards a pCR when surgery is delayed for 2-4 weeks more weeks [38].

While these results require validation in larger series, this study has shown that stromal infiltration could be an important marker to take into consideration when developing predictive models of response to neoadjuvant chemoradiotherapy in rectal cancer patients, besides stage at diagnosis and imaging techniques.

Conflict of interest

RD reported receiving honoraria for speaker activities and participation in advisory boards from Roche, Boehringer-Ingelheim, Ipsen, Amgen, Sanofi, Servier, Merck-Sharp Dome, and research grants from Merck and Pierre-Fabre.

RPL reported receiving research grant from AstraZeneca.

JC reported scientific consultancy role for Novartis, Pfizer, Ipsen, Bayer, Eisai, Sanofi, Advanced Accelerator Applications, Exelixis and Merck Serono.

All other authors declare no conflicts of interest.

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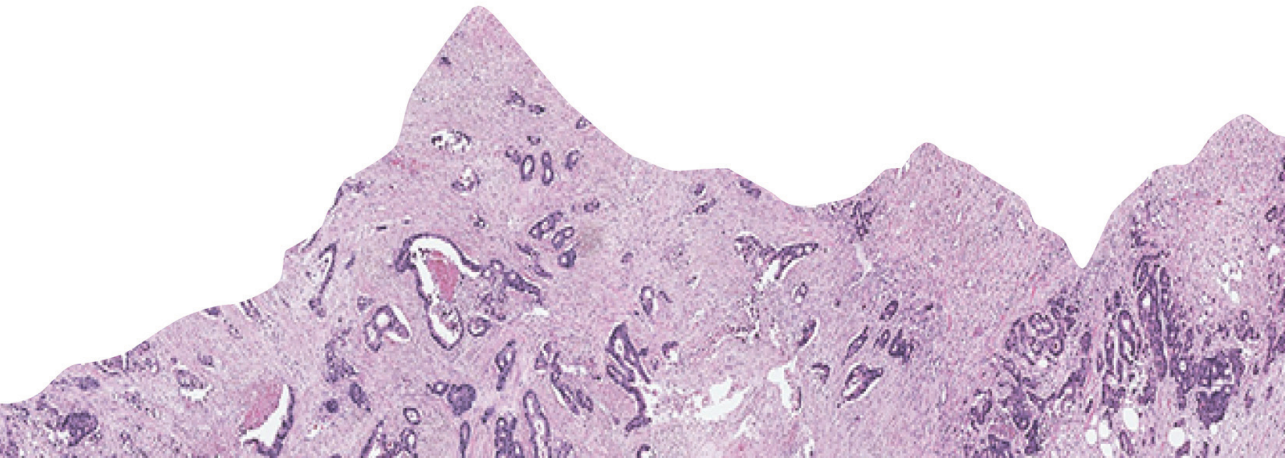

CHAPTER 8

The value of tumor-stroma ratio as predictor of pathologic response after neoadjuvant chemoradiotherapy in esophageal cancer

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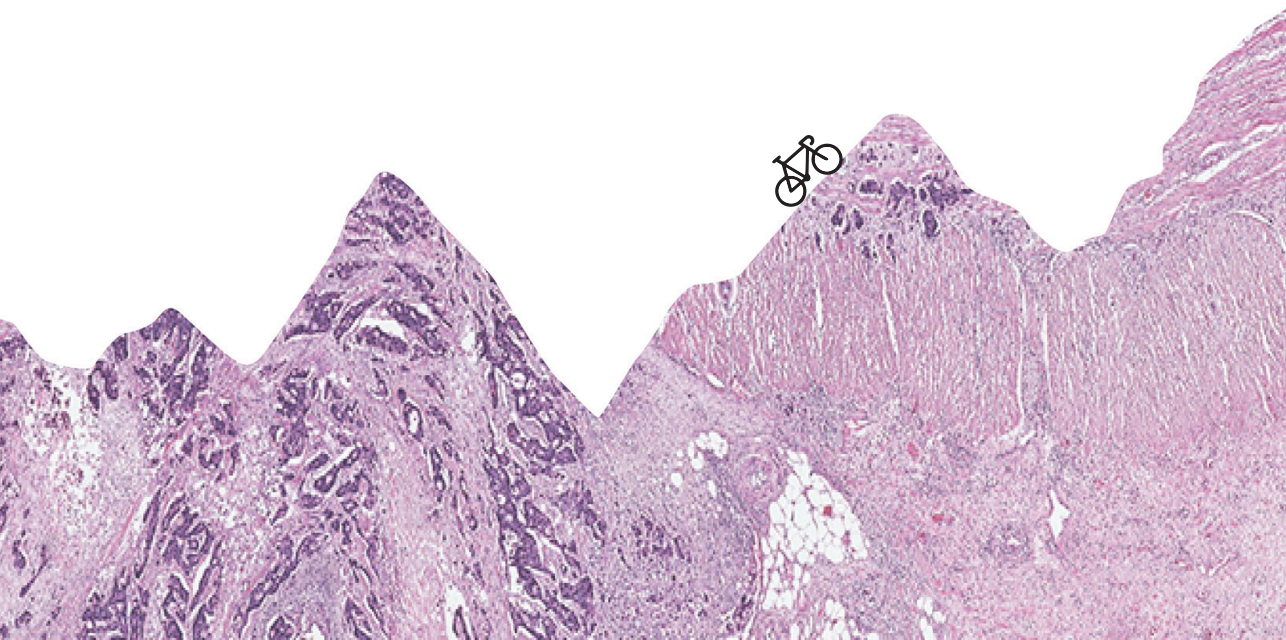

Abstract

Background and purpose With currently available techniques, the prediction of pathologic complete response after neoadjuvant chemoradiotherapy is insufficient. The tumor-stroma ratio (TSR) has proven to be a predictor of survival for several types of cancer, including esophageal. The aim of this study was to investigate the value of TSR in predicting pathologic response after neoadjuvant chemoradiotherapy in esophageal cancer patients.

Materials and Methods Patients with esophageal adenocarcinoma or squamous cell carcinoma who received neoadjuvant chemoradiotherapy followed by a resection were selected. Hematoxylin and eosin (H&E) stained sections of diagnostic biopsies were collected and TSR was independently assessed by two investigators. Patients were categorized in stroma-low (≤ 50% stroma) and stroma-high (> 50% stroma) groups for further analyses. The tumor regression grade (TRG) was assessed on H&E stained sections of the resected primary tumor to determine pathologic response.

Results A total of 94 patients were included in this study, of which 76 patients were categorized as stroma-low and 18 as stroma-high. Forty-two (45%) patients had a major pathologic response (TRG 1-2), whereas 52 (55%) were considered non-responders. After adjustment for gender, tumor type, cT-status and differentiation grade, patients with a stroma-high tumor showed a higher chance of no response compared to patients with a stroma-low tumor (OR 3.57, 95%CI 1.03-12.31, $P = 0.04$).

Conclusion TSR showed to have the potential to aid in the prediction of pathologic response in esophageal cancer patients receiving neoadjuvant chemoradiotherapy. Larger validation studies are necessary before implementing this method in daily practice.

Keywords

Esophageal cancer, neoadjuvant chemoradiotherapy, pathologic response, prediction, tumor-stroma ratio

Introduction

Esophageal cancer is the 9th most common cancer, affecting > 570.000 people each year worldwide, and the 6th most common cause of cancer related deaths [1]. Currently, the standard treatment for patients with resectable disease is neoadjuvant chemoradiotherapy (nCRT) followed by surgery. Since the addition of nCRT as part of esophageal cancer treatment, survival improved compared to patients who only underwent surgery [2, 3]. Treatment with nCRT leads to a pathologic complete response (pCR) in approximately 30% of patients, were another 30% of patients reach a near complete response [4]. It is debatable whether these patients should receive an additional resection or whether they should be followed up by an active wait-and-see procedure [5-8]. Achieving pCR proved to be associated with improved survival in patients with esophageal cancer [9]. In contrast, non-responders on nCRT have no survival benefit compared to primary surgery alone, but are still exposed to the potential side effects of nCRT [10-12]. Hence, it is important to define factors that predict whether or not a patient with esophageal cancer will benefit of nCRT.

Several imaging studies with endoscopic ultrasonography (EUS), computed tomography (CT) and ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) tried to assess the response to nCRT in patients with esophageal cancer. Unfortunately, for EUS and CT no predictive capacity could be found, whereas for FDG-PET the results were contradictory [7, 13, 14]. Moreover, a meta-analysis on the use of endoscopic biopsy and EUS for the detection of residual disease after nCRT, in order to use an organ-preserving approach, revealed both methods not suitable (yet) for withholding surgery [15]. Furthermore, there is an increase in the number of molecular and genetic studies aiming to identify markers that will predict the pathologic response after nCRT. These studies showed promising results, but need to be validated before implementation in clinical routine [16-18].

In the past decades, cancer research mainly focused on the malignant cell itself by understanding the role of tumor suppressor and oncogenic factors in the transformation to malignancy. Currently the stromal part of the tumor is subject of investigation. It is increasingly known that the malignant cell relies on the so-called tumor microenvironment (TME) and therefore does not act alone. Intratumoral stroma within the TME is variable and different cell-types like infiltrating immune cells, cancer-associated fibroblasts, endothelial cells and pericytes all play a role in supporting malignant transformation, invasion of the tumor and metastasis [19, 20]. Some studies have demonstrated that intratumoral stroma is associated with

reduced chemotherapy delivery [21] and increased chemotherapy resistance [22] and consequently could play a role in patient treatment outcome.

Furthermore, different studies found that the tumor-stroma ratio (TSR) is an independent predictor of survival in different types of carcinomas, for instance colon [23] and esophageal cancer [24, 25]. A high proportion of stroma is associated with poor clinical outcome. A study for assessment of TSR in esophageal biopsy specimens has been performed by Courrech Staal et al. [26], which showed that scoring TSR in biopsy specimens is representative and reproducible. The relationship between the proportion of tumor in diagnostic biopsies and the pathologic response has been studied in esophageal cancer patients, who received neoadjuvant chemotherapy followed by resection [27]. However, the relationship between TSR and pathologic response after concurrent chemotherapy and radiotherapy has, to our knowledge, not yet been investigated.

The aim of this current study was to evaluate the association of TSR in pre-treatment biopsies and the pathologic response after nCRT in esophageal cancer patients.

Materials and Methods

Patients and tissue material

The retrospective patient cohort consisted of consecutive patients with esophageal cancer, clinical stage I-III with adenocarcinoma or squamous cell carcinoma, who underwent neoadjuvant chemoradiotherapy followed by resection at the Leiden University Medical Centre (LUMC) between 2010 and 2016. The cohort was part of an existing study cohort available in the LUMC, which ended including patients at the end of 2016. Patients were diagnosed with an esophagogastroduodenoscopy and a biopsy for histological confirmation. All patients underwent external beam radiotherapy using a 3D conformal planning with a four-field box technique. A total dose of 41.4 Gy was given in 23 fractions of 1.8 Gy, 5 fractions per week. Concurrent chemotherapy consisted of 5 weekly administrations of Carboplatin (AUC 2) and Paclitaxel (50 mg/m2) [2]. Clinical data were retrospectively collected from the electronic patient files. Hematoxylin and eosin (H&E) stained pre-operative biopsies taken from the primary tumor were collected together with the related resection specimens from the Department of Pathology of the LUMC. In case of referred patients, the original biopsy slides were collected from regional hospitals using the Dutch Pathology Registry (PALGA) [28]. All tissue samples were coded and handled according to ethical standards ('Code for Proper Secondary Use of

Human Tissue', Dutch Federation of Medical Scientific Societies). This study was approved by the Medical Ethics Committee of the LUMC.

Histopathological procedure

For determining the TSR, 3 µm H&E-stained sections of biopsy specimens were microscopically analyzed using a 2.5 x or 5x objective to select the part with the largest amount of stroma. Then, with the 10x objective the image fields were scored for the percentage of stroma by increments of 10%. Tumor cells had to be present at 4 borders of the field of vision. When multiple sections per patient were available, all biopsies were assessed for TSR. The highest score was decisive for final stroma classification. All biopsies were independently assessed by two investigators (GvP, JK). After six weeks, one investigator (JK) assessed all samples a second time to determine intra-observer variation. A cut-off value of 50% stroma was used to categorize patients as stroma-low (≤ 50%) or stroma-high (>50%) as determined in earlier research to be most discriminative [29]. In case consensus could not be reached, a third observer (expert pathologist, AFS) was decisive.

The response to nCRT was assessed on the primary tumor resection specimens by a gastrointestinal pathologist using the tumor regression grade (TRG) defined by Mandard [30]. This classification is defined by 5 categories. TRG 1 is defined as complete regression with no residual cancer but only fibrosis through all layers of the esophageal wall and is called pathologic complete response (pCR). TRG 2 is characterized by scattered residual cancer cells or groups of cells within the fibrosis. TRG 3 shows an increase of residual cancer cells but fibrosis predominates. TRG 4 is characterized by residual cancer outgrowing the fibrosis. TRG 5 is defined by absence of any regressive changes. The TRG scores were taken from the clinical reports, however, they were all determined by the same, experienced pathologist (AFS).

Statistics

IBM SPSS version 25.0 (Statistical Package for the Social Sciences, Chicago, IL) was used for statistical analysis. Differences in categorical variables between patient, tumor and treatment characteristics for the TRG groups were analyzed using the Fisher's exact test or the Chi-square test. For continuous variables the Mann-Whitney test was used. Inter- and intra-observer variability was performed using the Cohen's Kappa coefficient (κ). TRG was dichotomized in TRG 1-2 (major responders) and TRG 3-5 (non-responders), as found to be of prognostic significance as well [31]. Uni- and multivariable logistic regression analyses were performed to investigate the relationship between TSR and other baseline factors for a major response. Factors known to

be predictive for pathologic response (gender, tumor type, cT-stage and differentiation grade) were added to a multivariable model [32, 33]. For multivariable analysis, missing cases for cT-stage (cTx, *N* =6) were imputed using the mode as default. A two-tailed *P* value ≤0.05 was considered significant in all analyses.

Results

Patient and tumor characteristics

The cohort consisted of 115 patients. Thirteen cases (11%) were excluded as invasive carcinoma within the biopsy could not be established with certainty. In 8 cases (7%) TSR could not be assessed due to insufficient quality of the tissue, leaving a total of 94 patients available for analysis. Median age was 64 years (range 25-82) at the start of nCRT, 76% (*N* = 71) were men and 80% (*N* = 75) of the tumors were adenocarcinoma. All patients completed radiotherapy as intended. However, 13 patients (14%) received <5 cycles of chemotherapy (Table 1).

Table 1. Patients, tumor and treatment characteristics, stratified by tumor regression grade (TRG).

	Total ***N* = 94 (%)**	**TRG 1-2** ***N* = 42 (%)**	**TRG 3-5** ***N* = 52 (%)**	***P*-value**
Gender				
Male	71 (76)	28 (67)	43 (83)	0.07
Female	23 (25)	14 (33)	9 (17)	
Median age (years)[range]	64 [25-82]	64 [39-74]	65 [25-82]	0.69
Weight loss at presentation				
None	29 (31)	18 (43)	11 (21)	0.06
≤ 10 %	42 (45)	14 (33)	28 (54)	
> 10%	23 (25)	10 (24)	13 (25)	
Alcohol consumption				
None or stopped	28 (30)	14 (33)	14 (27)	0.78
Yes	64 (68)	27 (64)	37 (71)	
Unknown	2 (2)	1 (2)	1 (2)	
Smoking				
Never or stopped	57 (61)	25 (60)	32 (62)	0.63
Yes	36 (38)	17 (41)	19 (37)	
Unknown	1 (1)	0 (0)	1 (2)	
Tumor location				
GEJ	11 (12)	3 (7)	8 (15)	0.46
Middle	9 (10)	4 (10)	5 (10)	
Low	74 (79)	35 (83)	39 (75)	
Median length tumor[a] (cm) [range]	5 [1-11]	5 [2-11]	6 [1-10]	0.53

Table 1. Continued.

	Total *N* = 94 (%)	TRG 1-2 *N* = 42 (%)	TRG 3-5 *N* = 52 (%)	*P*-value
Histology				
Adenocarcinoma	75 (80)	32 (76)	43 (83)	0.44
Squamous cell carcinoma	19 (20)	10 (24)	9 (17)	
Cycles of chemotherapy				
<5 cycles	13 (14)	4 (10)	9 (17)	0.28
5 cycles	81 (86)	38 (91)	43 (83)	
Median time interval between nCRT and surgery (days)[range]	44 [25-85]	43 [25-58]	47 [31-85]	0.12
cT status				
cT2	16 (17)	7 (20)	9 (6)	0.84
cT3	72 (77)	33 (74)	39 (89)	
cTx	6 (6)	2 (7)	4 (6)	
cN status				
cN0	23 (25)	8 (19)	15 (29)	0.48
cN1	42 (45)	19 (45)	23 (44)	
cN2	28 (30)	14 (33)	14 (27)	
cN3	1 (1)	1 (2)	0 (0)	
Differentiation grade				
Well/Moderate	44 (47)	20 (48)	24 (47)	0.98
Poor	50 (53)	22 (52)	28 (53)	

Abbreviations: GEJ: Gastro-esophageal junction

[a]Tumor length was determined by endoscopy. If tumor length by endoscopy was not reported, tumor length on CT scan was used instead.

Histopathology

A total of 142 H&E biopsy sections of 94 patients were available and evaluated. Seventy-six patients (81%) were categorized as stroma-low and 18 patients (19%) as stroma-high. Figure 1 shows examples of stroma-low and -high tumor biopsies. Intra-observer agreement was good (κ = 0.81, 93% agreement), whereas a substantial inter-observer agreement was found for the assessment of TSR (κ = 0.73, 91% agreement). In 5 out of 9 discrepant cases, consensus could not be reached and the pathologists' assessment was decisive.

The assessment of the pathological response revealed 28 cases (29.8%) to have a complete pathologic response (TRG 1) whereas 2 cases did not show any regressive changes at all (TRG 5). The other cases were categorized as TRG 2 (*N* = 14), TRG 3 (*N* = 31) and TRG 4 (*N* = 19), respectively. After dichotomization, 42 cases were classified as major pathologic responders (TRG 1-2), whereas 52 cases were considered non-responders (TRG 3-5). The distribution of TRG categories versus TSR classification is shown in table 2 and figure 2.

Table 2. Distribution of TRG categories versus TSR categories.

	Major pathologic responders			Non-responders				
	TRG 1	TRG 2	**TRG 1-2**	TRG 3	TRG 4	TRG 5	**TRG 3-5**	Total
Stroma-low	25	13	**38 (50%)**	23	13	2	**38 (50%)**	76
Stroma-high	3	1	**4 (22%)**	8	6	0	**14 (78%)**	18
Total	28	14	**42 (45%)**	31	19	2	**52 (55%)**	94

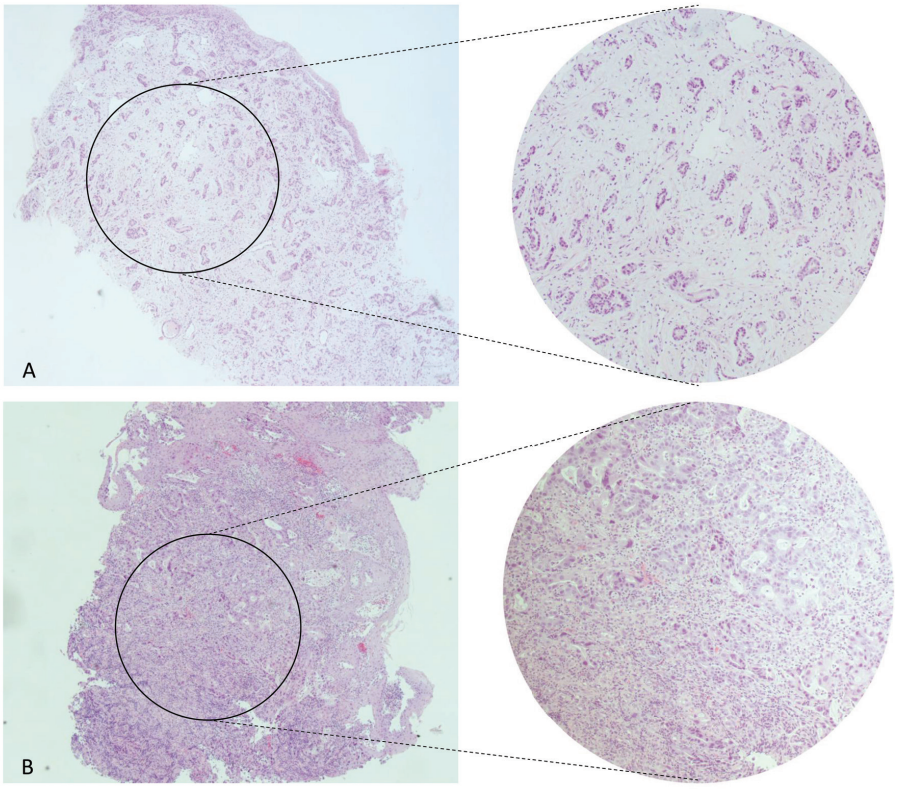

Figure 1. H&E stained biopsy sections of esophageal carcinoma. (A) represents a tumor with high stromal proliferation (stroma-high). As shown by the magnification on the right there is evident stromal proliferation between the tumor cells. (B) shows a tumor with few spots of stromal tissue (stroma-low). The magnification shows almost no stromal proliferation between tumor cells.

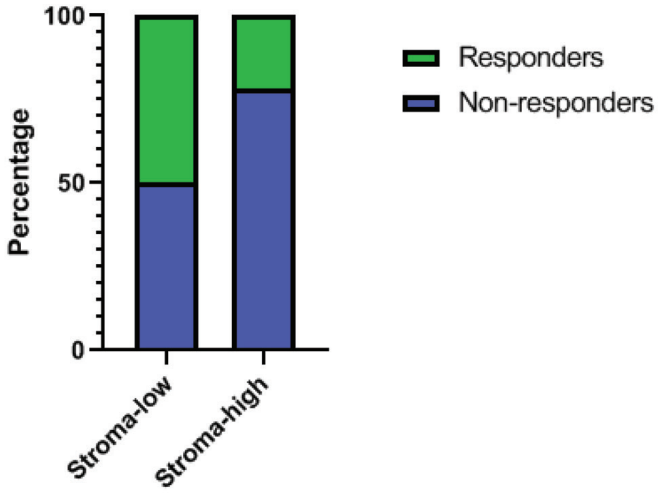

Figure 2. The distribution of pathologic major responders within the stroma categories. The percentage of responders (in green) versus non-responders (in blue) within stroma-low and stroma-high categories, respectively.

TSR and other predictive factors for pathologic response

No significant differences in baseline characteristics and possible predictors of pathologic response were seen between both TRG groups (Table 1). However, there was a significant difference for TSR between the group TRG 1-2 and the group TRG 3-5 (P = 0.033).

As shown in table 2, 78% (14/18) of the patients with a stroma-high tumor did not have a response to nCRT, whereas patients with a stroma-low tumor have only a 50% chance on a pathologic major response. In univariable analyses TRG 3-5 was used as reference category for all factors that potentially could influence pathologic response. Univariable analyses showed a significant higher chance for patients with a stroma-high tumor to have no response to nCRT (OR 3.50, 95%CI 1.06-11.61, P = 0.04). In multivariable analysis, after adjusting for gender, histology, differentiation grade and clinical T-stage, a stroma-high tumor remained an independent predictive factor for a higher chance of no response to nCRT (OR 3.57, 95%CI 1.03-12.31, P = 0.04) (Table 3).

Table 3. Uni- and multivariable logistic regression analyses for TRG group 3-5.

	Univariable analysis			Multivariable analysis		
	OR	**95% CI**	***P*-value**	**AOR**	**95% CI**	***P*-value**
Gender						
Male	Ref					
Female	2.39	0.91-6.26	0.08			
Age (years)	0.99	0.95-1.04	0.85			
Weight loss at presentation						
None	Ref					
≤ 10 %	0.31	0.11-0.82	0.02			
> 10%	0.47	0.15-1.43	0.18			
Alcohol consumption						
None or stopped	Ref					
Yes	0.73	0.30-1.78	0.49			
Smoking						
Never or stopped	Ref					
Yes	1.15	0.50-2.65	0.75			
Tumor location						
GEJ	Ref					
Middle	2.13	0.33-13.81	0.43			
Low	2.39	0.59-9.74	0.22			
Length tumor (cm)	0.97	0.81-1.17	0.78			
Histology						
Adenocarcinoma	Ref					
Squamous cell carcinoma	1.49	0.54-4.10	0.44			
Cycles of chemotherapy						
<5 cycles	Ref					
5 cycles	1.99	0.57-6.98	0.28			
cT status						
cT2	Ref					
cT3	1.05	0.35-3.09	0.93			
cN status						
cN0	Ref					
cN+	1.72	0.65-4.57	0.28			
Differentiation grade						
Well/Moderate	Ref					
Poor	0.94	0.42-2.13	0.89			
Tumor-stroma ratio						
Stroma-low	Ref			Ref		
Stroma-high	3.50	1.06-11.61	0.04	3.57	1.03-12.31	0.04

Abbreviations: OR: Odds ratio; CI: Confidence interval; TRG: Tumor regression grade; GEJ: Gastro-esophageal junction; AOR: Adjusted odds ratio.

Discussion

Our results show that patients with high stromal tumors have a significantly higher chance to not respond on nCRT (TRG 3-5) compared to patients with tumors with a low amount of stroma. Seventy-eight percent of the stroma-high patients did not have a response on nCRT. This suggests that assessment of TSR could fulfill a role in identifying patients that will or will not respond well to nCRT, next to currently used (imaging) methods, adding to the realization of personalized medicine. It could be possible that stroma-high tumors represent a group of tumors with an environment that is well armed against chemoradiation, or even become resistant to therapy [34]. This might indicate that, for obtaining a pathologic response in stroma-high tumors, it might be necessary to adjust the current therapy strategy. For instance, these tumors could be future candidates for therapies targeting the stromal compartment of the tumor, by targeting activated oncogenic pathways (e.g. the TGF-β or PDGFR pathway), angiogenesis (VEGF) or cancer associated fibroblasts [35]. Another option could be not to treat these patients with nCRT and continue with resection instead, thereby avoiding exposing the patients to the side effects of chemoradiation treatment.

There is evidence that the interaction between cancer cells and the TME can affect sensitivity of the cancer cells to chemotherapy [36] and radiotherapy [37]. However, the exact underlying mechanisms and interactions within the TME and their role in protection of cancer cells from eradicating therapy have to be further explored.

Several phase I and II studies are currently ongoing targeting different components of the TME of advanced esophageal carcinoma, e.g. angiogenesis, immune cells and stroma. However, as the TME has the paradoxical capacity to both promote and inhibit tumor growth and progression, effective intervention can be challenging [38].

Our results are in contrast with those of the study of Hale et al., who found a high proportion of tumor (PoT) (= stroma-low) in the diagnostic biopsy to be associated with no evidence of tumor regression (TRG 4 or 5) after neoadjuvant chemotherapy [27]. However, this relationship was only found when the PoT was analyzed as continuous variable. Furthermore, the assessment of PoT was performed with a different (semi-automated) method. In addition, the TRG was categorized into different categories compared to our study (TRG 1, 2, 3 / TRG 4, 5 versus TRG 1, 2 / TRG 3, 4, 5, respectively). Previous studies identified female gender, squamous cell carcinoma and cT1-2 stage as favorable factors in the prediction of complete pathologic response [32, 33]. However, in our current study none of these factors were significantly associated with pathologic tumor response grading. This might be explained by the smaller number of cases in our study.

We showed that assessment of TSR is simple and reliable as demonstrated by the substantial to good inter- and intra-observer agreement, allowing it to be easily implemented in routine pathology diagnostics.

A limitation of this study is the retrospective nature and the small sample size (N = 94) which means that results should be interpreted with caution. Furthermore, not all biopsy material suitable for diagnosing cancer, is suitable for assessment of TSR. Stroma has to be surrounded by tumor cells at four sides of the microscopic field in order to score TSR, which is not always possible with biopsy specimens. This might be solved by visually diminishing the field of vision and determine whether more stroma is present in comparison to tumor or *vice versa*. Still, approximately 11-18% of the biopsies are not suitable for TSR scoring (this study and [26]). Nevertheless, it seems that TSR predicts pathologic response after nCRT independently of other well-known factors.

In conclusion, this study shows that TSR might be an additional parameter in the prediction of pathologic response in esophageal cancer patients treated with nCRT. This relationship needs further exploration and validation in a larger population, preferably prospective, before implementing TSR as a novel predictor of pathologic response in daily practice.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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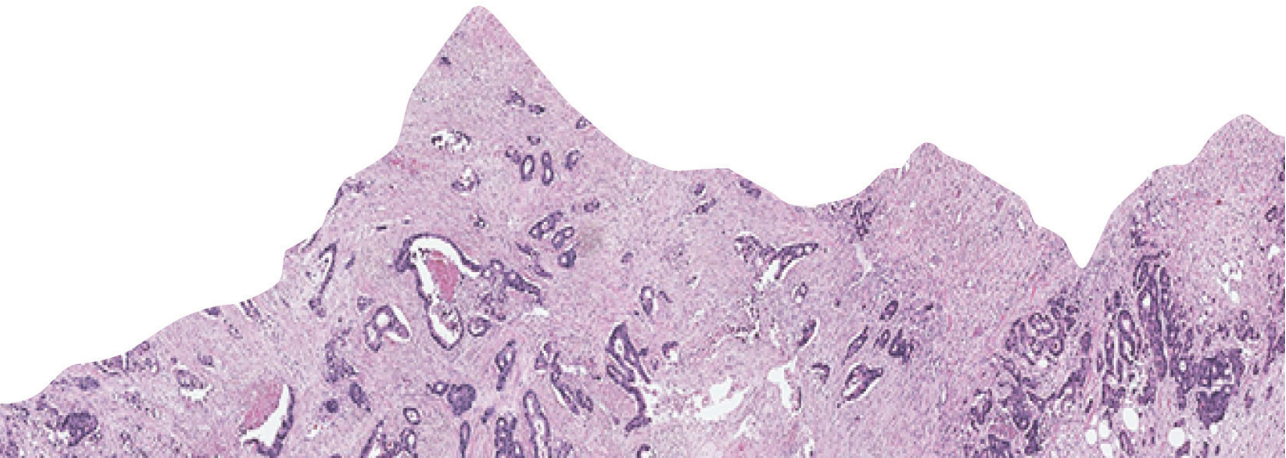
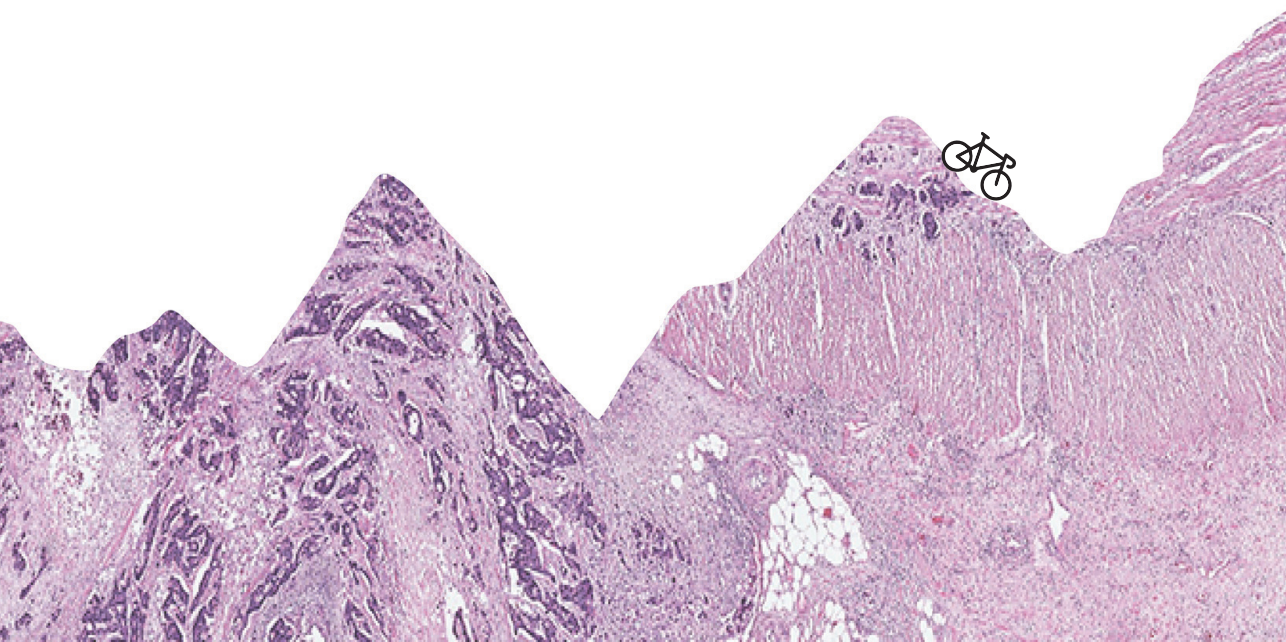


CHAPTER 9

Summary and discussion



In the last decade it has become more evident that the tumor microenvironment plays an important role in tumor progression and metastasis. The main subject of this thesis, the tumor-stroma ratio (TSR), reflects the tumor microenvironment by the percentage of stromal tissue that is present in a tumor. In the review presented in **Chapter 2** the cellular composition of the tumor stroma is described as well as the biological role of the tumor microenvironment in tumor progression and invasion. The tumor microenvironment consists of many different cell types, like cancer-associated fibroblasts, immune cells and endothelial cells which offer many opportunities in the development of targeted therapy or personalized medicine.

The TSR is assessed on routine hematoxylin and eosin (H&E) stained sections using conventional microscopy. In the detailed protocol as published in **Chapter 3** we describe how to score the TSR on colon cancer specimens. However, as the method has proven its robustness and usefulness for other types of solid epithelial cancers, for instance breast-, esophageal- and lung cancer, it can be applied to these tumors types as well. The protocol shows examples of correct and incorrect fields of vision for scoring, and recommendations are provided in case of difficulties. For instance, in case of uncertainty whether tissue is stromal tissue or muscle tissue fibers, it is recommended to perform an additional desmin staining. Although most studies validated the prognostic value of the TSR in various types of tumors, some studies failed to validate the prognostic value [1-3]. This was primarily caused by different interpretations of the TSR scoring method. The discrepancies in scoring methods show that it is essential to develop a standardized and uniform protocol for determining the TSR to be able to compare study outcomes.

In **Chapter 4** we investigated whether the analysis of the TSR in metastatic lymph nodes from patients with stage III colon cancer could add to prognosis. The importance of examining lymph nodes for treatment strategies is clearly recognized. In fact, examining less than 12 lymph nodes (in case of colorectal cancer) is considered a high-risk factor and patients will be treated accordingly. Even though the number of examined lymph nodes has increased over time, there is no evidence that this has led to an increase in higher-staged cancers [4]. This raises the question whether a different approach is necessary in the evaluation of lymph nodes when it comes to estimating prognosis. In our study, primary tumors and metastatic lymph nodes were assessed for the TSR. The amount of stroma within the lymph nodes appeared to be very heterogeneous, as in almost half of the cases the TSR category from the primary tumor was different from the TSR category of the lymph nodes. Results showed that combining the TSR of the primary tumor and the lymph nodes had an additional value with respect to disease free

survival. This might indicate that a different treatment approach is necessary for patients with stroma-high lymph nodes, regardless of the TSR of the primary tumor.
The heterogeneity found within the metastasizing process of the stroma based on visual evaluation of the TSR is in concordance with molecular studies which investigated the expression levels of various prognostic markers in colorectal cancer (CRC) and corresponding lymph node metastases [5-7]. Some of these markers showed heterogeneous expression patterns between the primary tumor and lymph node metastases. These differences in expression indicate that the primary tumor does not always reflect the situation in lymph node metastases, which might have important clinical implications [6].

The presence of lymph node metastases also plays an important role in treatment decision making in breast cancer patients. Guidelines state that involvement of lymph nodes is in itself no reason for adjuvant chemotherapy, as studies have shown that the presence of 1-3 positive lymph nodes does not necessarily lead to a worse prognosis compared to node-negative patients [8]. Similar to our work described in chapter 4, the possible additional value of the TSR in metastatic lymph nodes was investigated in 191 breast cancer patients. Results were comparable with our colon cancer cohort; an improved prediction of outcome and a more accurate selection of patients with a higher risk of disease recurrence [9]. Both these studies are summarized in **Chapter 5**.

The *BRAF V600E* mutation is one of the biomarkers currently used in clinical setting for CRC patients, next to *KRAS* and the microsatellite instability (MSI) status. It is primarily used in the metastatic setting, however, increasing evidence for the association of *BRAF* mutations and poor prognosis in stage II-III CRC might change this [10-12]. The results of our study described in **Chapter 6** suggest it would be of high interest to search for a *BRAF V600E* inhibitor to be used in the adjuvant setting in stage II-III patients for patients with a stroma-high tumor. In this study we analyzed 2368 patients for the TSR and combined this with the *BRAF V600E* mutation status. Survival analyses showed that patients with a stroma-high *BRAF V600E* mutated tumor had a significantly worse 5 year disease free survival compared to all other patient groups. More importantly, the *BRAF V600E* status did not affect survival within the stroma-low group, whereas it was of significant prognostic value within the stroma-high group. Therefore, *BRAF V600E* testing might be of interest for patients with a stroma-high tumor.

The prognostic value of the TSR is clearly recognized and validated. However, patients who receive neoadjuvant treatment are not eligible for TSR studies. Neoadjuvant treatment induces changes to the composition of the microenvironment, which makes it difficult to estimate the tumor-stroma ratio properly. As a consequence, patients with, for instance, rectal, esophageal and gastric cancer are usually excluded, as guidelines recommend these patients to be treated with chemo- and/or radiotherapy prior to surgery. A possibility to predict survival prognosis for these patients is the assessment of the TSR in diagnostic biopsies. It has been shown in esophageal cancer that assessing the TSR in biopsies could be used as an independent prognostic biomarker for survival, although biopsies do not always represent the most invasive part of the tumor [13].
One study investigated the prognostic value of the TSR in resection specimens of locally advanced colon cancer patients after neoadjuvant chemotherapy. The results indicate that the prognostic value of TSR remains present even after neoadjuvant treatment. However, this awaits validation in a larger cohort [14].

Assessing the TSR in biopsies might be a new approach to predict the response on neoadjuvant treatment. We investigated this possibility in rectal- (**Chapter 7**) and esophageal cancer patients (**Chapter 8**). The rectal cancer cohort consisted of 76 patients treated with neoadjuvant chemoradiotherapy (nCRT). Twenty-four percent of the patients were found to have a complete response on nCRT. Although not significant, more stroma-low patients reached a pathologic complete response compared to stroma-high patients.
Comparable results were found within the esophageal cancer cohort, which contained 94 patients receiving nCRT. Forty-two patients (45%) with a pathologic complete response or a near complete response (TRG 1 or 2) were considered major responders. Multivariate logistic regression analysis showed patients with a stroma-high biopsy were more likely to have no response to nCRT.
Although both studies consisted of a small number of cases and validation in larger series is necessary, these studies have shown that stromal infiltration is potentially an important marker to take into consideration when developing predictive models of response to neoadjuvant chemoradiotherapy in rectal- and esophageal cancer patients, next to currently used imaging techniques.

In addition, in light of an increasing number of studies into the so-called wait-and-see strategies [15, 16], our results might be of importance. In these studies regression after nCRT is determined with imaging techniques, and a decision is made to continue with surgery or to follow the patient

extensively. The length of the time period between the last neoadjuvant chemo- or radiation cycle and surgery will evidently influence the rate of regression found. One might hypothesize that near-complete responders will further regress towards a pathological complete response when surgery will take place 2-4 weeks later [17]. However, residual viable tumor cells might also start to proliferate again, leading to regrowth of the tumor. Furthermore, the question remains how accurate near-complete responders (<5% residual tumor cells) can be identified using imaging techniques.

Future perspectives

Prospective multicenter study

The ultimate goal for the assessment of the TSR is to make optimal use of its prognostic value and implement the method into worldwide daily routine diagnostics. After consulting the TNM Evaluation Committee (UICC) and the College of American Pathologists (CAP), they stated that the TSR has the potential to be included in the TNM staging algorithm but needs validation in a prospective cohort. Following this advice the UNITED study has been designed [18]. In this international multicenter study, which was approved in February 2018, the reproducibility of scoring the TSR is being investigated amongst pathologists, using an E-learning module. In parallel, stage II and III colon cancer patients are included in a prospective observational cohort to validate the prognostic value of the TSR.

TSR in biopsies

In addition to the applications of the TSR in biopsies described in this thesis and as summarized above, the assessment of TSR in biopsies offers other possibilities. As Fu et al. described in their study, the TSR in biopsies was predictive for the presence of lymph node metastasis in stage I-III CRC patients. The accuracy of correctly predicting metastatic LNs with TSR was higher compared to the prediction using clinical standard computed tomography. In addition, a subgroup analysis of patients diagnosed as cN0 revealed that patients categorized as high-risk (i.e. stroma-high) had a significantly greater probability of having LN metastasis. A proposed TSR-based nomogram yielded a favorable accuracy of 78% to actually identify patients with a high-risk of LN metastasis in this subgroup [19].

Furthermore, Park et al. suggests that assessment and staging of the tumor microenvironment (TME) of patients undergoing resection of stage I-III CRC is feasible using endoscopic biopsy specimens. They investigated the tumor microenvironment by analyzing CD3 and CD8

expression and TSR assessment, in both biopsies and resection specimens. Stroma-high biopsy predicted stroma-high in resected specimens, whereas high $CD3^+$ density in biopsies was associated with high $CD3^+$ density in whole tumor slides. In addition, both were associated with cancer specific survival. These results suggest that assessment of the TSR and other factors of the TME are comparable in biopsy and surgically resected specimens from patients with CRC, and biopsy-based assessment could allow for stratification prior to surgery or for therapy targeting the TME [20].

Automation

Several studies published the assessment of the TSR using semi-automated methods, for instance point-counting[2, 3, 21]. In 2015, Bianconi *et al.* showed the possibility to discriminate between tumor epithelial and stroma in colorectal cancer patients, with an accuracy of almost 97% using an automated image analysis system. However, this study, as well as other similar ones, was based on an image database that consisted of small parts of tissue samples instead of whole tumor slides [22-24]. Geessink et al. developed an algorithm for the automated analysis of the TSR within pre-set annotations in the most optimal area of tumor sections of rectal cancer patients [25]. The challenge remains to develop a fully automated software system using whole slide imaging where the algorithm selects the area with the highest amount of stroma within the tumor section and calculates the TSR. This will eventually lead to a standardized protocol with optimal reproducibility.

The main disadvantage of an automated scoring method is the potential increase of cost and time due to the acquirement of a slide scanner and software. However, more and more pathology laboratories change to a digitalized workflow and developing an automated scoring algorithm for TSR is therefore almost inevitable. In the meantime, to fill the gap between microscopic and automated TSR scoring, digital slides can easily be assessed for TSR using a circular annotation of 3.46 mm^2. This mimics the field of vision of a 100x magnification when using a conventional microscope [26].

Targeting the tumor microenvironment

As described in **chapter 2** of this thesis, stroma-high tumors are likely to represent a group of tumors with an environment that limits the access of therapeutic agents, causing reduced effect or even resistance to treatment [27, 28]. Treatment mechanisms discussed in literature mainly focus on targeting specific cell types within the stroma, like immune cells (e.g. macrophages, NK cells, neutrophils, T- and B-cells) and extracellular matrix components (e.g. collagen, glycoproteins, enzymes) [29, 30]. However, instead of targeting either epithelial cancer cells or stromal cells

directly, some strategies exploit stromal components for the therapeutic agent to be delivered at the tumor site or target cancer cell-stroma signaling interactions [31]. Nowadays it is believed that combination therapies targeting both cancer- and stromal cells can generate considerably better tumor responses than monotherapy. However, more knowledge regarding the optimal sequence and composition of combinations of stromal-targeting and cancer cell-targeting agents is necessary. In particular the order in which these agents are administered is important, as certain sequences can results in adverse effects [31].

BRAF inhibitors in stroma-high tumors

Patients with *BRAF-V600E* mutant (metastatic) CRC clearly have a poor prognosis, which is even worse in combination with the abundant presence of stroma. These patients do not respond well to standard adjuvant treatment strategies and new drugs, new drug combinations and new targets are urgently required for these cases.

BRAF is a downstream signaling protein in the epidermal growth factor receptor (EGFR)-mediated pathway. However, attempts to directly inhibit the active *BRAF* protein failed in metastatic CRC (mCRC) [32-34] due to the feedback reactivation of receptor tyrosine kinase signaling, suggesting a more complex process. A new generation of *BRAF* inhibitors is under investigation which could revolutionize the management of mCRC. However, even if monotherapy could produce better results, it still seems that combination regimens, either double or triple, are likely to work better [35-38]. Although these studies are conducted in metastatic disease, similar results are to be expected for patients with stage II or IIII disease.

Positron emission tomography (PET) imaging, image guided surgery (IGS) and theranostics

[^{18}F]Fluorodeoxyglucose (FDG)-PET and IGS are both useful imaging tools in the evolving management of patients with different types of carcinoma. FDG-PET enables the measurement and visualization of metabolic changes in cancer cells, resulting in the ability to distinguish viable tumor from scar tissue, in the detection of tumor foci at an earlier stage than possible by conventional anatomic imaging and in the measurement of alterations in tumor metabolism, indicative of tumor response to therapy. Nowadays, FDG-PET plays an important role in staging patients before surgical resection of recurrence and metastases, in the localization of recurrence in patients with an unexplained rise in serum carcinoembryonic antigen (in case of colorectal cancer) and in the assessment of residual masses after treatment [39].

With IGS, near-infrared fluorescence (NIRF) imaging is used to real-time visualize tumors during surgery using targeted fluorescent tracers [40]. Although successful intraoperative identification of various types of tumors has been demonstrated [41-43], the limited depth penetration of approximately 5-8 mm is an important drawback of NIRF imaging [44, 45]. Combining PET and NIRF imaging might therefore be a solution to monitor tumors both before and during surgery. Sibinga et al. showed the feasibility of using a single molecular imaging agent, ZW800F-cRGD-[89Zr]Zr-DFO, for serial PET imaging and NIRF-guided treatment of colorectal tumors [46]. Using a stromal marker as tracer might help in the recognition of aggressive tumors to treat these accordingly. Recently, a tracer labeled with fibroblast activation protein inhibitors (FAPI) was used to study the uptake and tumor-to-background ratio in 28 different tumor entities. FAP is overexpressed by CAFs of several tumor types and therefore useful to detect tumors with a high stromal content. The study showed that several epidemiologically important tumor types, like colorectal, breast, esophageal, lung, pancreatic, and head-and-neck cancer, had a remarkedly high uptake of ^{68}Ga-FAPI with low tumor-to-background ratios. These results may open new applications for noninvasive tumor characterization and staging examinations [47].

Another interesting field of medicine is theranostics, in which specific targets are labelled with a radioactive epitope. In this way it is possible to deliver a therapeutic dose of radiation to the patient. A specific diagnostic test shows a particular molecular target on a tumor or in the stroma, allowing a therapy agent to specifically target that receptor, rather than more broadly the disease. Another possibility is the use of theranostic nanomedicines. These nanoparticles can act as imaging agents, with drugs entrapped within or conjugated with therapeutic agents such as drugs, ligands or antibodies. This allows for simultaneous imaging and therapy [48].

Conclusion

The research presented in this thesis provides sufficient evidence that the TSR can be used to contribute to a better stratification for cancer patients. Thereby adding to the improvement of tailored personalized treatment; thus only treatment when necessary.

To make this parameter available for current patient diagnostics, the TSR has to be introduced as a new prognostic biomarker in the official guidelines of the TNM classification by the international UICC organization. Since the overwhelming evidence in literature, validating the prognostic value of the TSR in over 50 (inter)national studies in more than 10 different types of cancer, one would expect this to be just a formality. Unfortunately, this process can take several years. Moreover, pathologists have to be encouraged to believe in the strength of this

"non-sexy" simple parameter and include the scoring in their daily routine. This can be done without much effort, so why wait?

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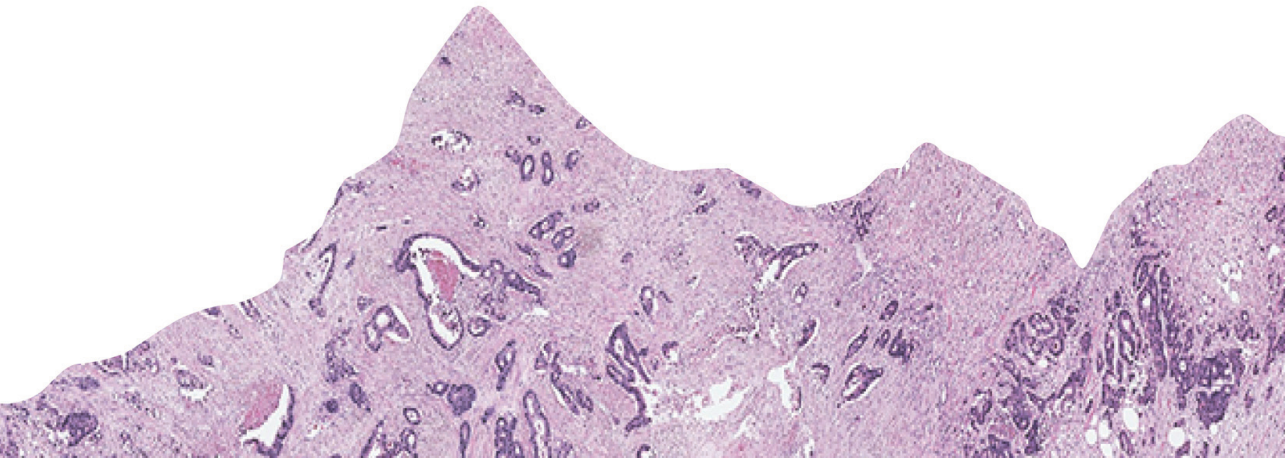


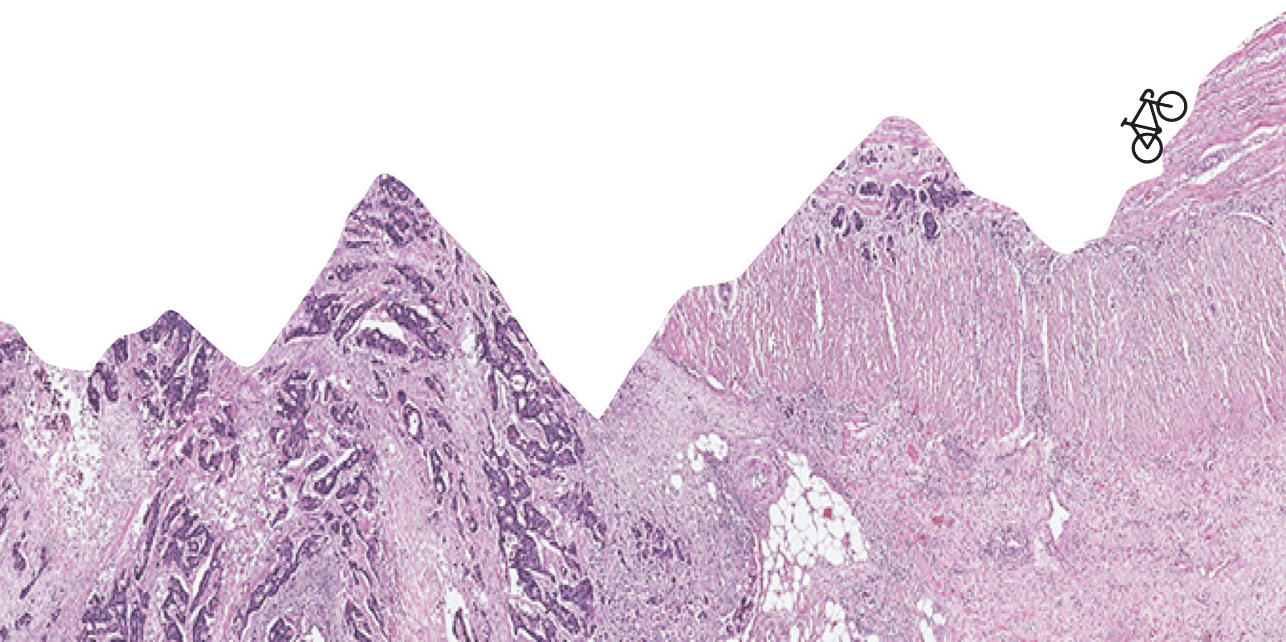
CHAPTER 10

Nederlandse samenvatting

List of Publications

Curriculum Vitae

Dankwoord



Nederlandse samenvatting

De kans op het overleven van kanker is de laatste jaren toegenomen, enerzijds door screeningsonderzoeken voor verschillende typen kanker (zoals bijvoorbeeld het bevolkingsonderzoek voor borst- en darmkanker), anderzijds door verbeterde behandelingen. Om deze trend in de toegenomen overleving door te zetten is het van belang het risico op terugkeer van de ziekte of de kans dat de patiënt zal overlijden aan de gevolgen van kanker te kunnen inschatten. Door een betere risicoschatting kunnen patiënten op maat behandeld worden, waardoor onder- en overbehandeling zoveel mogelijk kan worden voorkomen.

Het inschatten van de agressiviteit van een tumor gebeurt in de praktijk momenteel met behulp van het T (tumor), N (nodes; lymfeklieren), M (metastasen; uitzaaiingen) (TNM) classificatie systeem. Dit is voornamelijk gebaseerd op anatomische uitbreiding van de tumor.

De afgelopen jaren is duidelijk geworden dat de omgeving waarin de tumorcellen zich bevinden, ook wel tumor stroma genoemd, van belangrijke invloed is op de groei van de tumor en de ontwikkeling van metastasen. Tumor stroma bestaat uit verschillende componenten, zoals bindweefsel, fibroblasten, bloedvaten en cellen van het afweersysteem. Kankercellen en stromale cellen hebben een complexe interactie met elkaar die de ontwikkeling van kanker op verschillende manieren kan beïnvloeden. Ondanks deze vernieuwde inzichten zijn er tot op heden nog geen markers beschikbaar in de klinische praktijk voor de inschatting van de prognose die gericht zijn op het tumor stroma.

Dit proefschrift beschrijft de verschillende toepassingen van de tumor-stroma ratio (TSR). De TSR is de hoeveelheid stroma aanwezig in de tumor ten opzichte van de hoeveelheid tumor, uitgedrukt in een percentage. In **hoofdstuk 2** wordt een literatuuroverzicht gegeven over de samenstelling van het tumor stroma en de biologische rol die tumor stroma speelt in de ontwikkeling en progressie van dikke darmkanker. Doordat tumor stroma uit veel verschillende celtypes bestaat, biedt dit mogelijkheden voor het ontwikkelen van therapie gericht op specifiek celtypen (zgn. targeted therapie).

Hoofdstuk 3 beschrijft een gedetailleerd protocol voor het bepalen van de TSR in dikke darmkanker. De overvloed aan artikelen in de literatuur laat zien dat deze methode echter ook gebruikt kan worden voor andere typen kanker, zoals slokdarm-, borst- en longkanker. De TSR wordt microscopisch bepaald op een stukje tumorweefsel, dat in de diagnostiek al gebruikt wordt door de patholoog om het stadium en type van de tumor te bepalen. Het grote voordeel

van deze methode is dan ook dat het vrijwel geen aanvullende kosten met zich mee brengt en dat het een snelle en makkelijke methode is. Patiënten met veel stroma in het tumorweefsel (>50%, stroma-hoog) hebben een hogere kans op overlijden aan de ziekte, terugkeer van de ziekte of de ontwikkeling van metastasen in vergelijking met patiënten die weinig stroma in de tumor hebben (≤50%, stroma-laag).

Behalve het bepalen van de TSR in de primaire tumor hebben we ook onderzocht of de TSR in lymfeklieren met metastasen een aanvulling kan zijn op de prognose van patiënten met stadium III dikke darmkanker. Het onderzoeken van lymfeklieren op de aanwezigheid van metastasen is van groot belang voor de bepaling van de behandeling. Hoewel het aantal onderzochte lymfeklieren per patiënt is toegenomen, heeft dit niet geleid tot een toename van tumoren met een hoger stadium [1]. Hierdoor rijst de vraag of er meer informatie te halen is uit de weefselsamenstelling van de lymfekliermetastasen.

Hoofdstuk 4 beschrijft het onderzoek naar de TSR in de primaire tumor en de lymfeklieren van 102 patiënten met stadium III dikke darmkanker. Behalve dat de resultaten lieten zien dat het bepalen van de TSR in de lymfeklieren van toegevoegde waarde is voor het vaststellen van de prognose van de patiënt, viel vooral het verschil tussen de TSR van de primaire tumor en de TSR van de lymfeklieren op. Bijna de helft van de patiënten had een stroma-hoog tumor en stroma-laag lymfeklieren of andersom. Dit komt overeen met eerdere moleculaire studies naar de expressie van prognostische markers. Deze studies lieten zien dat er een verschil is in expressie tussen de primaire tumor en de lymfeklieren, wat belangrijk kan zijn voor de behandeling van de patiënt [2-4]. Behalve voor dikke darmkanker spelen metastasen in lymfeklieren ook een belangrijke rol voor het bepalen van de prognose van borstkankerpatiënten. Het onderzoek beschreven in hoofdstuk 4 is ook uitgevoerd in borstkankerpatiënten door Vangangelt et al. met vergelijkbare resultaten [5], en beide studies zijn samengevat in **hoofdstuk 5**.

De aanwezigheid van een specifieke mutatie in het *BRAF* gen (*BRAF V600E*) is één van de biomarkers die momenteel gebruikt wordt in de kliniek, al is dat nu vooral bij patiënten met gemetastaseerde dikke darmkanker. Er is echter steeds meer bewijs dat de aanwezigheid van deze mutatie ook voor een slechtere prognose zorgt bij patiënten met stadium II en III dikke darmkanker [6-8]. De resultaten van onze studie zoals beschreven in **hoofdstuk 6** onderbouwen dit. In deze studie vonden we dat vooral patiënten met een stroma-hoog tumor in combinatie met de *BRAF* mutatie een zeer slechte 5-jaars ziektevrije overleving hadden, slechter dan wanneer slechts één van beide markers ongunstig was, of elke andere combinatie van deze

markers. Opmerkelijk genoeg maakte de aan- of afwezigheid van de *BRAF* mutatie voor de patiënten met een stroma-laag tumor geen verschil. Dit wijst erop dat voor de patiënten met een stroma-hoog tumor het testen op een *BRAF* mutatie van belang kan zijn, zodat de behandelstrategie daarop aangepast kan worden.

De prognostische waarde van de TSR is inmiddels erkend en bewezen, ook door andere (inter) nationale studies. Echter, doordat pre-operatieve radio- en/of chemotherapie de samenstelling van het tumorweefsel verandert en de TSR daardoor niet goed te bepalen is, kunnen patiënten die voorbehandeld worden niet geïncludeerd worden in TSR studies. Deze behandeling wordt vooral gegeven bij rectum-, slokdarm- en maagkanker. Als alternatief zou voor deze patiënten het diagnostische biopt gebruikt kunnen worden om de TSR te bepalen, ook al is het biopt soms niet representatief voor de gehele tumor [9].

Een andere optie om de TSR van het biopt te gebruiken is om de reactie op pre-operatieve behandeling te voorspellen. In **hoofdstuk 7** en **hoofdstuk 8** hebben we deze optie onderzocht in respectievelijk rectum- en slokdarmkankerpatiënten die voorbehandeld werden met chemoradiatie. Beide studies lieten zien dat patiënten met een stroma-hoog tumor vaker niet reageerden op de voorbehandeling in vergelijking met patiënten met een stroma-laag tumor. Hoewel beide studies een relatief klein aantal patiënten bevatten, en er nog validatie in grotere patiëntgroepen nodig is, laten ze wel zien dat het stroma een belangrijke rol kan spelen in het ontwikkelen van voorspellingsmodellen voor de reactie op pre-operatieve behandeling, als aanvulling op de huidige gebruikte beeldvorming. Als patiënten met een stroma-hoog tumor slecht reageren op de voorbehandeling, dan zou overwogen kunnen worden deze patiënten niet voor te behandelen, maar meteen door te gaan met het operatief verwijderen van de tumor. Op die manier worden ze ook niet blootgesteld aan de bijwerkingen van radio- en/of chemotherapie.

Toekomstperspectieven

Het voornaamste doel van het onderzoek naar de TSR bepaling is het implementeren van de methode in de dagelijkse praktijk bij de internationale richtlijnen, naast het huidige TNM classificatie systeem. Hiermee kunnen hoog-risico patiënten geselecteerd worden die aanvullende, of aangepaste, therapie nodig hebben of laag-risico patiënten die misschien geen aanvullende therapie nodig hebben. Om dit te bereiken is er een internationale multicenter studie opgezet, waarin pathologen geïnstrueerd worden middels een e-learning hoe de TSR

te scoren en daarnaast worden stadium II en III dikke darmkankerpatiënten geïncludeerd om de prognostische waarde van de TSR te valideren in een prospectief cohort [10].

Behalve de al beschreven toepassingen van de TSR in biopten, zijn er nog andere klinische toepassingen voor de TSR van het biopt. Zo is het gebleken dat de TSR in biopten voorspellend is voor de aanwezigheid van lymfekliermetastasen [11]. Verder is aangetoond dat de TSR en expressie van andere factoren, zoals bijvoorbeeld immuuncellen, in het tumor stroma in biopten vergelijkbaar zijn met die in de primaire tumor. Dit zou betekenen dat de bepaling van de TSR en deze andere factoren in het biopt gebruikt kunnen worden voor het bepalen van de juiste behandeling [12].

Dat de juiste, gepersonaliseerde behandeling van groot belang is, blijkt uit de resultaten dat patiënten met een stroma-hoog tumor, zeker in combinatie met een *BRAF* mutatie een zeer slechte prognose hebben. Stroma-hoog tumoren lijken een omgeving te creëren waar (chemo) therapeutica niet of nauwelijks tot door kunnen dringen, waardoor er nauwelijks reactie op de therapie is of deze tumoren zelfs resistent kunnen worden [13, 14]. Voor deze patiënten zouden huidige therapieën moeten worden aangepast of nieuwe therapieën ontwikkeld. Hierbij wordt steeds vaker ingezet op het "targetten" van zowel specifieke tumorcellen als stromale cellen voor het beste effect [15].

De aanwezigheid van tumor stroma kan behalve voor diagnostiek en prognose ook een functie hebben in monitoring van de ziekte, zowel pre-operatief als na chirurgie. Door middel van *PET-CT scan imaging*, *image guided surgery* of *theranostics* kunnen tumoren gevisualiseerd worden voor, tijdens en na de behandeling. Met deze methoden worden cellen zichtbaar gemaakt door er een label/target aan te hangen. Dit kan niet alleen met tumorcellen, maar ook met stromale cellen, waarmee tegelijkertijd de agressiviteit van de tumor bepaald kan worden. In het geval van theranostics worden de gelabelde cellen zelfs direct behandeld door middel van radiotherapie. Het combineren van deze technieken levert de unieke situatie op dat het in kaart brengen van de tumor direct gecombineerd kan worden met therapie [16-19].

Tot slot is er momenteel een trend in de digitalisering van de diagnostische workflow van de pathologie. Dit leidt haast onvermijdelijk tot de automatisering van de bepaling van de TSR, zodat dit ook in de toekomst blijft passen binnen de dagelijkse routine. En hoewel het al mogelijk is om digitale beelden van tumorweefsel te analyseren voor TSR door gebruik te maken van circulaire annotaties, blijft het vooralsnog een uitdaging om dit volledig geautomatiseerd te

kunnen. Dit wordt voornamelijk veroorzaakt door variaties in de bewerking van het weefsel en de kleuring, wat betekent dat men ook hierbij niet ontkomt aan automatisering en standaardisering.

Samengevat laten de onderzoeken beschreven in dit proefschrift zien dat de TSR een sterke prognostische waarde heeft en dat er vele mogelijkheden zijn om de TSR/het tumor stroma te gebruiken in diagnostiek, monitoring en/of behandeling. Gezien het feit dat het bepalen van de TSR een snelle, makkelijke en goedkope methode is, kan het met weinig moeite in de dagelijkse praktijk geïmplementeerd worden!

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Curriculum vitae

Gabi van Pelt was born on September 15th 1980 in Gilze en Rijen, the Netherlands. After graduating from the Cambreur College in Dongen in 1999, she started with her Bachelor of Applied Science in Biology and Medical Laboratory Research at the University of Professional Education in Etten-Leur. During the last year of her study she did her internship at the laboratory of the department of Surgery at the Amsterdam Medical Center under supervision of prof. dr. Thomas van Gulik. After obtaining her bachelor degree in February 2003, she started working at the department of Hematology at the Leiden University Medical Center (LUMC) under supervision of prof. dr. Fred Falkenburg, in collaboration with the department of Surgical Oncology. After 2 years, in March 2005, she moved to the department of Surgical Oncology of the LUMC to work under the supervision of dr. Wilma Mesker and prof. dr. Rob Tollenaar. In December 2014 she started her official PhD training at the department of Surgical Oncology of the LUMC under supervision of prof. dr. Rob Tollenaar and the department of Pathology of the Radboud University Medical Center in Nijmegen under supervision of prof. dr. Han van Krieken. The results of her studies are presented in this thesis and have been published in international journals. On August 1st, Gabi started her new job as a senior molecular technician at Aqualab Zuid, Werkendam.

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