Applications of the tumor-stroma ratio: Towards clinical implementation

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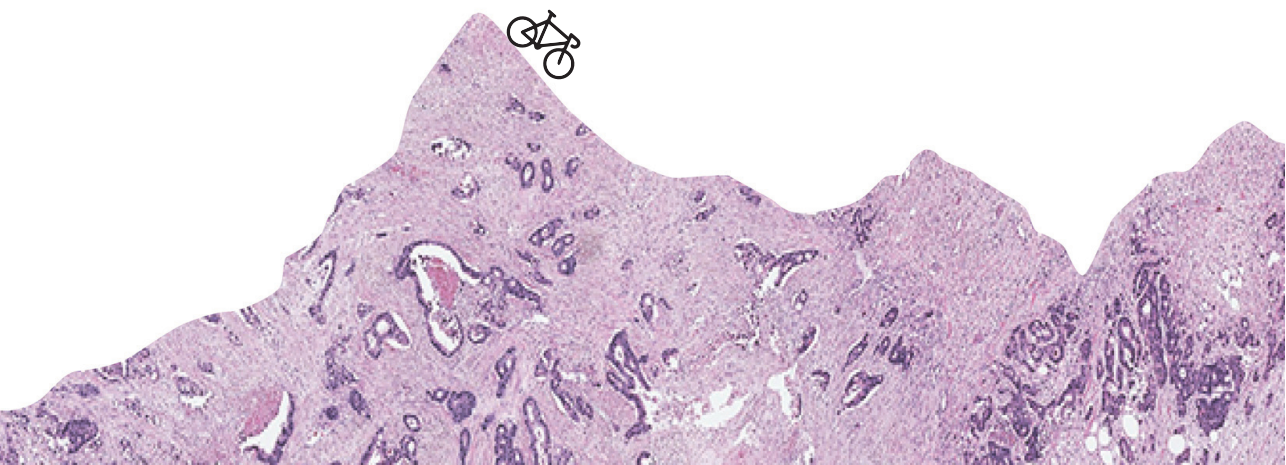

CHAPTER 2

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

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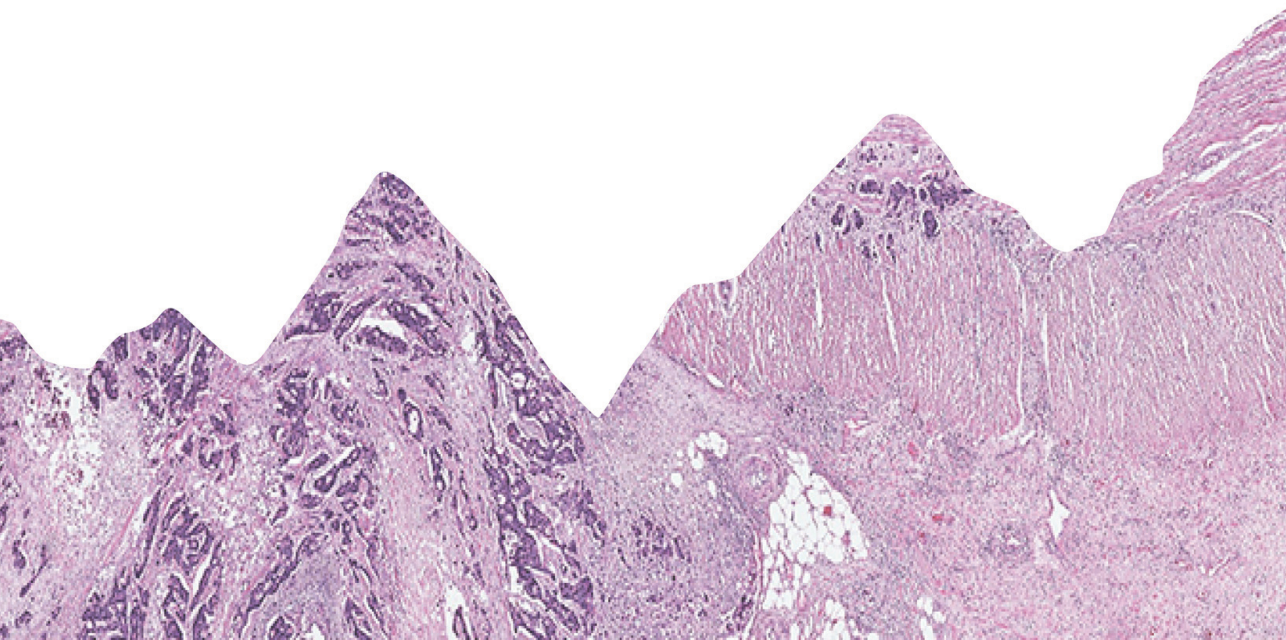

Abstract

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].

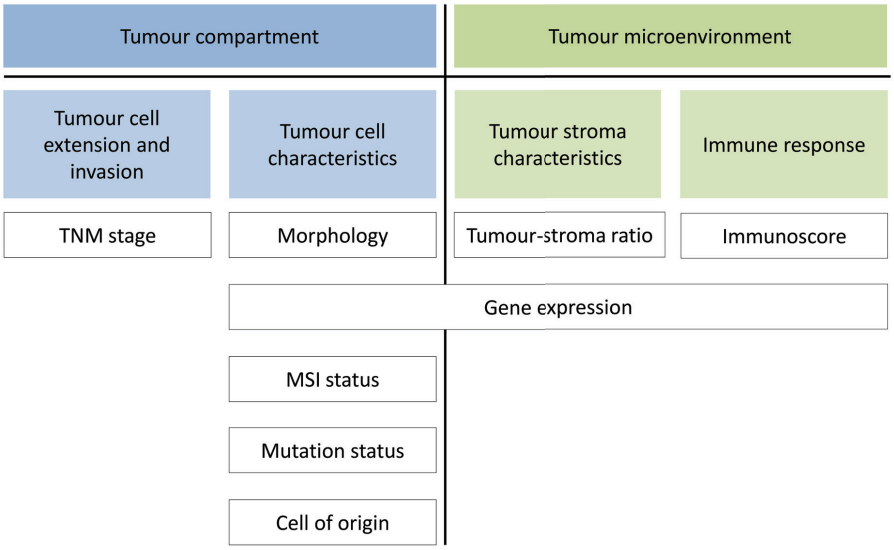

Figure 1. Distinct colorectal cancer classifications based on tumor compartment and tumor microenvironment.

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.

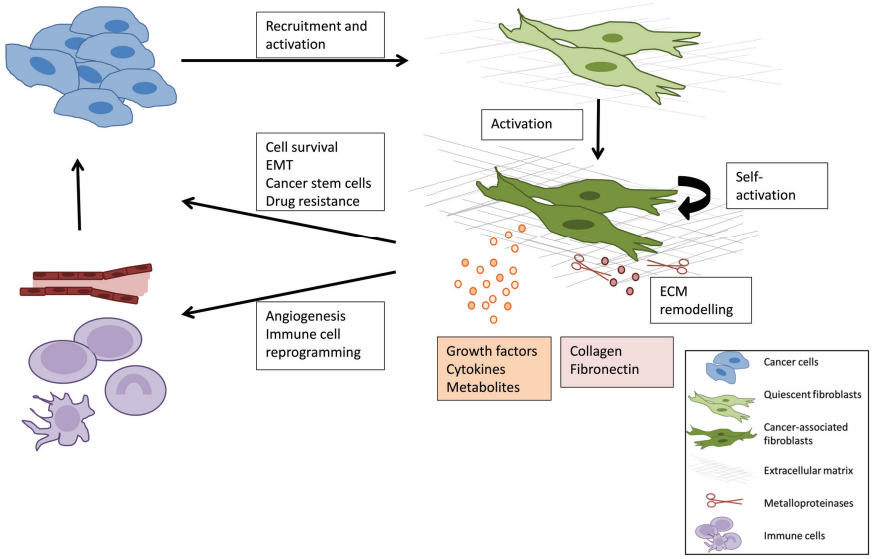

Figure 2. A simplistic scheme of the direct and indirect effect of cancer-associated fibroblasts (CAFs) on cancer cells. The activation of CAFs through transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF) induces angiogenesis, reprograms immune cells in the tumor microenvironment 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).

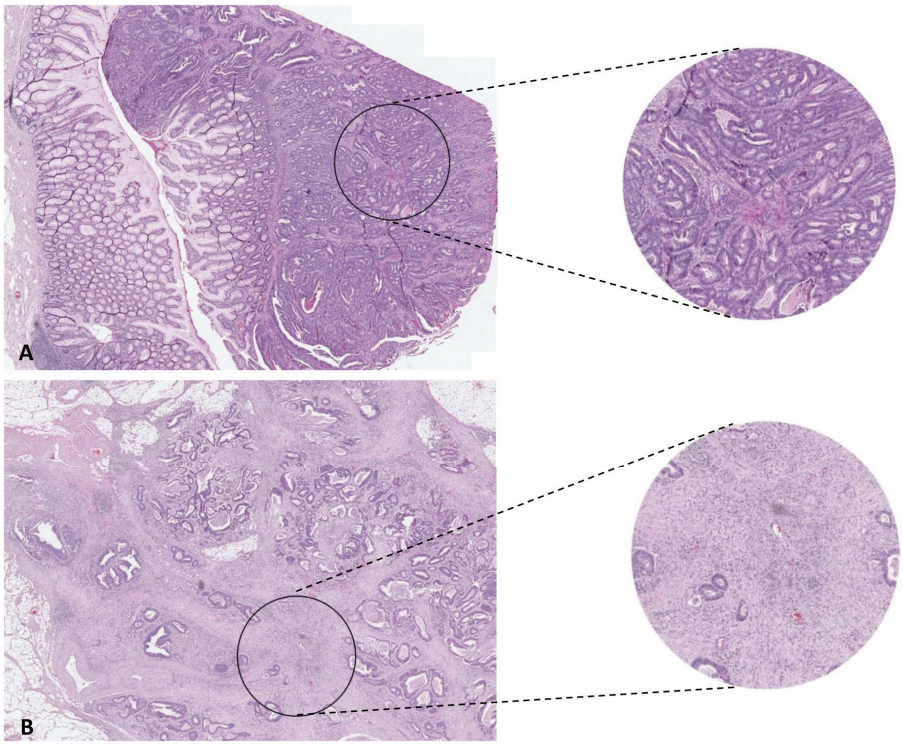

Figure 3. Examples of stroma-low (A; 20% stroma) and stroma-high (B; 90% stroma) hematoxylin and eosin (H&E) stained paraffin sections at the most invasive part of primary colon cancers.

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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