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REVIEW

The tumour–stroma ratio in colon cancer: the biological role and its prognostic impact

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The tumour–stroma ratio in colon cancer: the biological role and its prognostic impact

The tumour 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 tumour microenvironment plays an active role in tumour behaviour. By interacting with cancer cells, it influences disease progression and the metastatic capacity of the tumour. Tumours with a high amount of stroma correspond to poor patient prognosis. The tumour–stroma ratio (TSR) is a strong independent prognostic tool in colon cancer and provides additional value to the current clinically used tumour–node–metastasis classification. The TSR is assessed on

conventional haematoxylin and eosin-stained paraffin sections at the invasive front of the tumour. Here we review studies demonstrating the prognostic significance of the TSR in solid epithelial tumours with a focus on colon cancer. Moreover, the biological role of the tumour microenvironment during tumour progression and invasion will be discussed, as well as the attempts to target the tumour 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, tumour microenvironment, tumour–stroma ratio

Introduction

The tumour–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 tumour 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 than 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 a need for additional prognostic and/or predictive markers.⁵ Additional biomarkers have been proposed based on tumour cell characteristics, including tumour cell morphology, molecular pathways, genetic mutations, cell of origin and gene expression (see below), as well as the tumour 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

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therefore desirable. The tumour–stroma ratio (TSR), also referred to as the tumour–stroma percentage, is assessed on conventional haematoxylin and eosin (H&E)-stained paraffin sections at the invasive front of the tumour 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 tumour 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. Tumours 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}

The tumour 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 tumour stroma and are involved in tumour progression and

invasion. Stromal cells supply the tumour with growth factors, cytokines and metabolites and stimulate blood vessel formation (Figure 2). In this way the tumour stroma contributes to tumorigenesis and induction of EMT in cancer cells.²⁷ This explains why a tumour with a high stromal content reflects a pro-metastatic 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 understood completely.

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

Methodology of TSR

The TSR is evaluated based on routine 5- μ m thick H&E sections using conventional microscopy. The

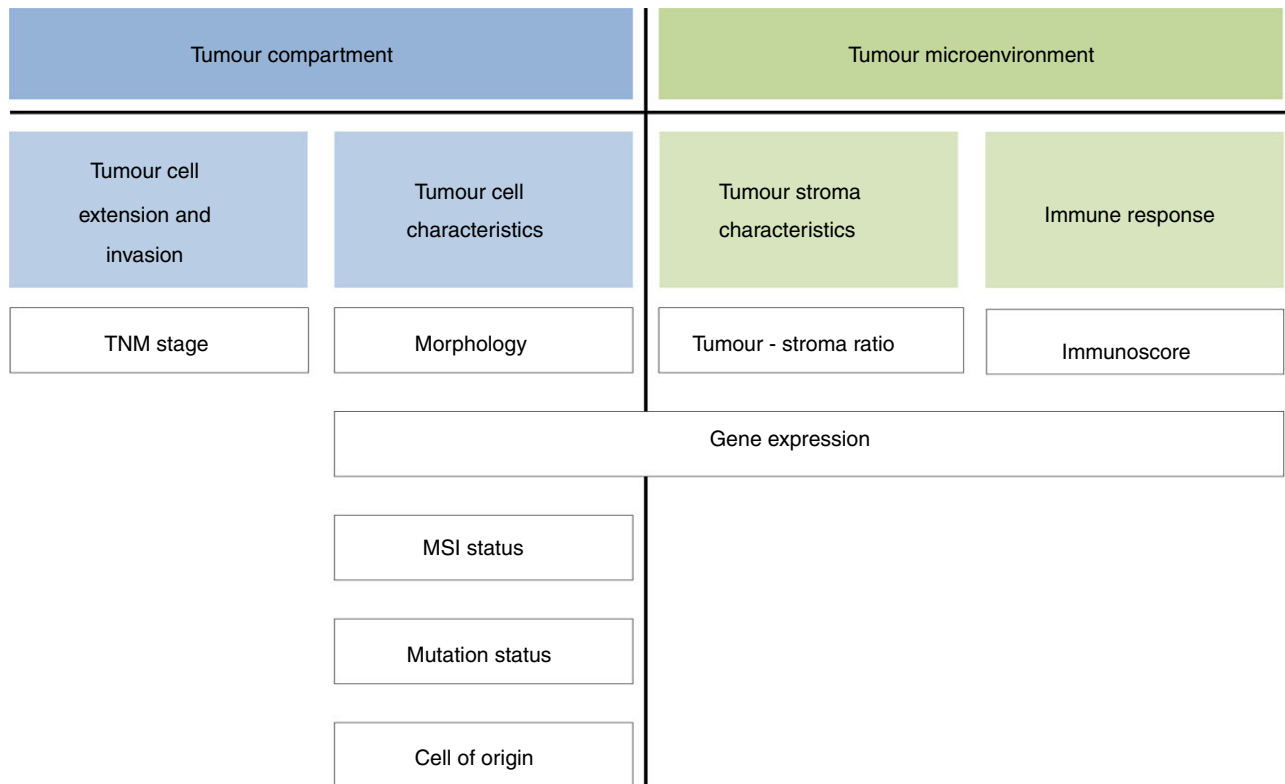


Figure 1. Distinct colorectal cancer classifications based on tumour compartment and tumour microenvironment.

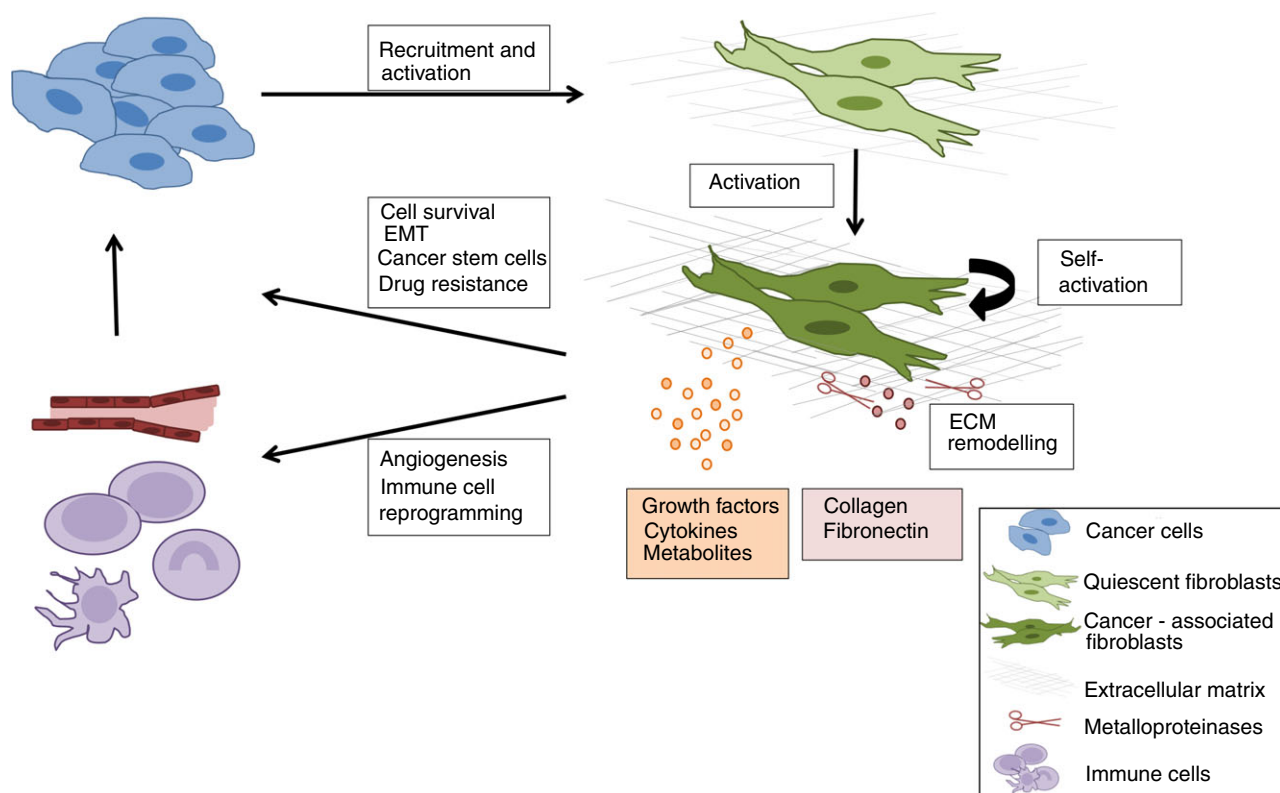


Figure 2. A simplistic scheme of the direct and indirect effect of cancer-associated fibroblasts (CAFs) on cancer cells. The activation of CAFs through transforming growth factor (TGF)- β and platelet-derived growth factor (PDGF) growth factors induce angiogenesis and reprogramme immune cells in the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

intratumoural stroma formation is assessed at the invasive part of the tumour, which is most determinative for tumour progression. This was decided in a study of colon cancers in which multiple H&E slides from different areas of the tumour were available for scoring. Heterogeneity in the percentage of stroma was observed throughout the tumour and the highest stroma percentages were observed in the tumour 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 tumour 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 $\times 2.5$ or $\times 5$ objective. Using the $\times 10$ objective, image fields are scored in increments of 10%. Tumour 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 ($\leq 50\%$)

patients.⁸ Using these criteria, scoring of the TSR is relatively easy, resulting in a low interobserver variation in different published validation studies (Table 1).^{7–12,30}

The TSR is estimated adequately in resection specimens of patients operated for a primary epithelial tumour, including mucinous tumours. 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 tumour.^{31–34} Analysing the TSR in biopsies to assess the prognostic value of the patient is an alternative for patients pretreated with chemo- and/or radiotherapy (see below).

TSR, 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

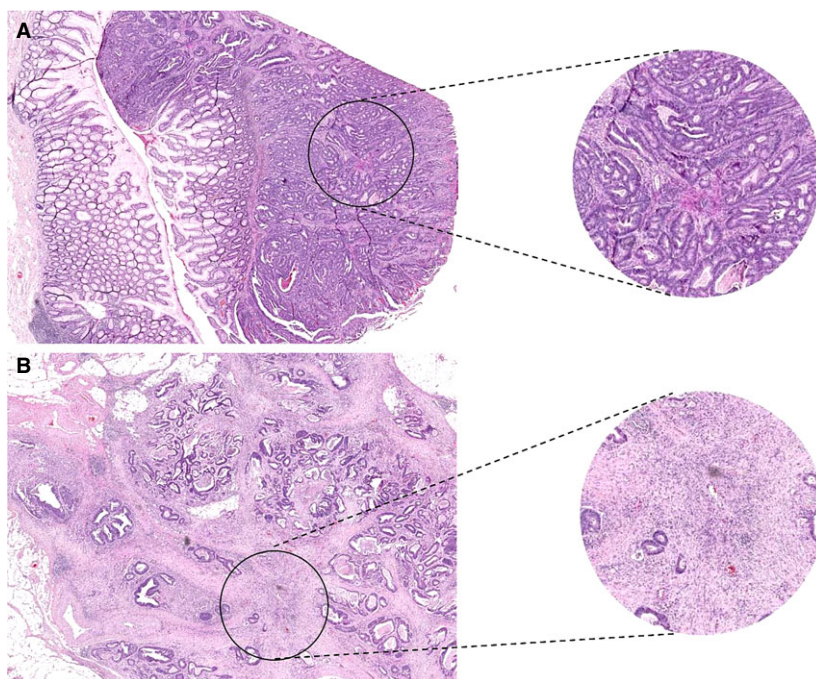


Figure 3. Examples of stroma-low (A, 20% stroma) and stroma-high (B, 90% stroma) haematoxylin and eosin-stained paraffin sections at the most invasive part of primary colon cancers.

Table 1. Characteristics of tumour stroma studies in colorectal cancer

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

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

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

et al. developed the TSR for patients with stages I–III disease, and found that patients with tumours 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 by 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 tumour at the luminal surface. Although a different method compared to the TSR, they found a comparable cut-off value of 47%, leading to similar results¹² (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 investigating only colon cancer patients (from caecum to sigmoid colon).

The adverse prognostic impact of high tumour 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 tumour stage, the number of lymph nodes examined (<10), poor tumour differentiation, presence of lymphatic, vascular and/or perineural invasion and perforation of the bowel wall. The study by 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 tumour stroma content. Further research should assess the effectiveness of adjuvant therapy in stroma-high patients.

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 proved 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 been shown recently 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 tumour ($P = 0.007$).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 tumour 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 oesophageal cancer, for instance, TSR score assessed on biopsies was an independent prognostic factor for survival, in line with the TSR in primary tumours.³⁶ The TSR scores of the primary tumour and the matching presurgical biopsy correlated in 81% of the cases. The remaining discrepant scores were stroma-high primary tumours 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 tumour stroma in colon cancer

THE TUMOUR MICROENVIRONMENT FORMATION

A high stromal content is a reflection of the highly activated interaction between tumour and stromal cells. During tumour 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 analysing transcriptomic data have reported that the activation level of CAFs present in the tumour showed prognostic value in colorectal cancer.^{26,41,42}

The TGF- β signalling pathway is considered a central player during tumour progression. The pathway exerts a dual role: its activation can function as a tumour suppressor by inducing apoptosis in normal cells and early stage cancers and can later promote tumorigenesis. The paradox that high levels of TGF- β 1 correlate with poor prognosis can be explained partially by the fact that the tumour stroma remains highly responsive to the growth factor. TGF- β -activated CAFs secrete a range of growth factors that support tumour growth and induce a mesenchymal phenotype in cancer cells.³⁷

THE ROLE OF THE TUMOUR MICROENVIRONMENT IN TUMOUR PROGRESSION

Various mechanisms have been proposed to explain how the tumour microenvironment contributes to tumour progression, tumour invasion and metastasis, for instance by: (i) impacting the proliferation and survival of cancer cells, (ii) increasing their stem-like properties and favouring EMT,^{27,38,43} (iii) rewiring the tumour metabolism⁴⁰ 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 tumour growth, invasion and metastasis compared to co-injection of cancer cells with normal fibroblasts.^{44,45}

The tumour stroma provides a nourishing environment that maintains cancer stem cells (CSCs) in a tumour. 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 tumour 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 tumour. These results suggest that CAFs foster stemness of cancer cells.²⁷ Tumours 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 tumour 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 reprogramming of CAFs. CAFs rely upon 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 tumour stroma can impact the aggressive behaviour of cancer cells not only through cell–cell contact and auto- and paracrine signalling but also through mechanical pressure. Due to the abundant ECM and the high number of CAFs, the tumour stroma forms a physical barrier around the tumour that increases the interstitial pressure and hypoxia in the tumour. Cancer cells respond to hypoxic conditions through the up-regulation of hypoxia-inducible factor 1 α , a master transcription factor that activates a whole range of genes involved in angiogenesis, migration, metabolism, tumour invasion and metastasis.⁴⁸

TARGETING THE STROMAL COMPARTMENT

While tumour cells have been the main therapeutic target in the past, different components of the tumour microenvironment, such as immune cells and angiogenesis, have been targeted recently. Based on the understanding of the tumour stroma, oncogenic pathways activated in the tumour 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 tumours. 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 signalling 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 signalling pathway is the PDGFR pathway which can be targeted by the imatinib anti-cancer drug. The ongoing ImPACCT clinical trial investigates the efficacy of the drug in patients with colon cancer characterised as CMS4, described in Ubink *et al.*⁵¹

Therapeutically targeting CAFs can also promote anti-tumour response, and it could be used in combination with standard therapy in order to target both CAFs and cancer cells. For instance, sibtuzumab 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 tumour microenvironment exerts an important influence on therapy response. Previous preclinical and clinical studies showed that tumours with high stromal content become resistant to therapy. Lotti *et al.* demonstrated that chemotherapy-treated CAFs promoted tumour-initiating cells and

tumour 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 tumours 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 a poor response to radiotherapy.²⁶

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

TSR in solid epithelial tumours

The prognostic value of the TSR reaches further than colon cancer; it is also observed in a range of other different solid epithelial tumours.^{13–24} Recently, an elaborated meta-analysis was conducted on 14 studies with 4238 patients to study the TSR on prognosis in solid tumours. The authors identified that stroma-high tumours were associated with worse overall survival and disease-free survival in colon cancer, breast cancer, ovarian cancer, non-small-cell lung cancer, nasopharyngeal cancer, oesophageal cancer and hepatocellular cancer.⁵⁸ However, two papers 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 investigated further. 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 tumour types emphasizes the robustness of the method.

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 characterise colorectal tumours 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 tumours

Table 2. Characteristics of tumour 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 <i>et al.</i> , 2010	93	I–IV	Oesophageal	K = 0.84
De Kruijf <i>et al.</i> , 2011	574	I–III	Breast	K = 0.85
Moorman <i>et al.</i> , 2012	124	I–III	Breast (triple-negative)	K = 0.74
Dekker <i>et al.</i> , 2013	403	I–III	Breast	K = 0.80
Wang <i>et al.</i> , 2013	95	I–III	Oesophageal	K = 0.84
Gujam <i>et al.</i> , 2014	361	I–III	Breast	K = 0.83
Liu <i>et al.</i> , 2014	184	I–II	Cervical	K = 0.81
Zhang <i>et al.</i> , 2014	93	I–IV	Nasopharyngeal	K = 0.85
Lv <i>et al.</i> , 2015	300	I–IV	Liver	K = 0.87
Pongsuvareeyakul <i>et al.</i> , 2015	131	I–II	Cervical	K = 0.78
Li <i>et al.</i> , 2017	51	II–IV	Gallbladder	K = 0.85

should be treated accordingly. However, there is as yet no information how stroma-high tumours will respond to adjuvant therapy.

Potential prognostic markers as the Immunoscore,⁶⁰ tumour budding⁶¹ and the CMS classification⁶ 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 tumour biology, including the interaction with the tumour 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 centres.

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 tumour 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 tumour 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, digitalization of the pathology workflow asks for automated scoring of the TSR. Therefore, the automation of the method is almost inevitable.

PROSPECTIVE MULTICENTRE 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 multicentre 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 tumour progression and metastasis. The TSR probably reflects this interaction. CAFs constitute the most abundant cell type in the tumour stroma, and this cell population releases a cascade of growth factors promoting tumorigenesis. The tumour 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 into the complexity between the cancer cells and its microenvironment may provide novel tumour stroma-targeted therapy and understand drug resistance.

Given the current understanding of the tumour stroma, colon cancer should not be categorized based solely on tumour cell characteristics, but also according to the tumour microenvironment. The TSR has been proved 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 effort in current routine diagnostics of the pathologist.

Conflicts of interest

The authors declare no conflicts of interest.

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