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Tumour Budding/T-cell infiltrates in Colorectal Cancer: Proposal of a Novel Combined Score

Running title: Tumour buds and T-cells in colorectal cancer

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Abstract (word count 248, max. 250)

Aims: The TNM classification system is used for prognostication purposes and to guide patient management. However, in colorectal cancer (CRC), additional markers are needed to stratify prognostic subgroups. From large bodies of research, two promising markers have emerged: Tumour budding and T-cell host response (CD3, CD8 and CD45RO infiltrates). However, attempts to combine these two parameters have been sparse. The aim of this study was to perform an assessment of potential protagonists that could be used in a combined score (Budding/T-cell Score, BTS).

Methods and Results: This descriptive, retrospective study was performed on a multi-punch tissue microarray containing material from 345 patients with Stage I-IV CRC. Areas from tumour centre,

front and microenvironment were stained for Pancytokeratin/CD3, Pancytokeratin/CD8 and Pancytokeratin/CD45RO. Tumour buds were scored manually and T-cell infiltrates digitally using open-source software (QuPath). Tumour buds, T-cell counts and combined BTS were associated with clinico-pathological features and overall survival (OS).

A higher combined BTS score (Buds/CD8, tumour centre) performed better than budding or CD8/CD3 alone in predicting nodal metastases ($p<0.0001$, OR 1.466, 95%CI: 1.115-1.928). Only higher BTS (Buds/CD3) was significantly associated with poorer OS on multivariate analysis ($p= 0.012$, HR 1.218, 95%CI: 1.044-1.419).

Conclusions: Although CD8+/CD3+ T-cells are predictive of tumour biology in CRC, we found a combined BTS to be stronger in predicting survival and certain features with high clinical relevance, such as nodal metastases, in comparison to budding or T-cells alone. Further studies combining T-cell infiltrates and tumour budding are necessary to optimize risk assessment of CRC.

Keywords: Tumour budding, colorectal cancer, host response, Immunoscore

Introduction

The TNM staging system remains the main classification system used to determine prognosis and therapy in cancer patients. However, as the TNM classification in colorectal cancer (CRC) is quite heterogeneous in different stages [1, 2], additional parameters have been proposed to complement this system. Here, the concepts of tumour budding [3, 4] and Immunoscore [5, 6] have emerged as particularly promising candidates.

Many studies have investigated tumour budding, defined as single tumour cells and small clusters of four or less tumour cells as a robust predictor of lymph node and distant metastasis, relapse and poorer outcome [7-9]. These strong associations can be exploited in specific clinical scenarios [10]. A recent proposal of standardized assessment of tumour budding (according to the International Tumor Budding Consensus Conference 2016, ITBCC) [10] has led to its integration in the CRC checklist of the College of American Pathologists [11], and widespread reporting of this feature can be expected in the future. From a biological perspective, tumour budding may represent the morphological correlate of epithelial-mesenchymal-transition as a key step in progression and invasion of solid cancers [12-14] .

Immune cells have also been intensively studied and are now recognized as an essential component of the tumour microenvironment [15]. Indeed, certain tumours, most notably microsatellite-instable (MSI-high) CRC, are associated with a dense T-cell lymphocytic infiltrate, less tumour budding and better prognosis [16-18]. Meanwhile, many works have led to the recognition of three lymphocyte markers: CD3+, CD8+ and CD45R0+ [19-21] and the concept of Immunoscore [22, 6]. A validation study demonstrating Immunoscore to be a reliable predictor of recurrence risk has recently been published [5].

To date, most investigations of tumour budding and T-cell infiltrates in CRC have been independent from each other. Introducing the concept of the 'attacker-defender model', a previous study examining a combined CD8+T-cell/tumour budding index demonstrated superior prognostic information than either marker alone [23]. Nevertheless, a systematic analysis of potential protagonists of the attacker-defender model has not yet been conducted. The aim of this study was to assess tumour budding in relationship to the T-cell infiltrate focusing on CD3+, CD8+ and CD45R0+ subpopulations (Budding/T-cell Score, BTS) in different tumour areas and to determine which elements lead to the best correlation with aggressive tumour biology and patient outcome.

Materials and Methods

Patient cohort

A retrospective collective of 345 primary colorectal cancer patients treated by resection at the University Hospital of Bern between 2002 and 2014 was used for this study. Cases were re-reviewed (A.L. and H.D.) based on the TNM 7th edition (review performed prior to the publication of the 8th edition). Assessed features (Table 1) include histological subtype, tumour location, pT, pN, pM and TNM stage (defined as pT, pN and either pM or cM at the time of diagnosis), grade, lymphatic invasion, venous invasion, perineural invasion, Klintrup-Mäkinen score [24], tumour budding scored by the ITBCC recommendations [10] and by immunohistochemical pankeratin stains ('overall tumour budding, OTB') [25]. Importantly, tumour budding had previously been assessed by both of these methods on whole tissue slides on these cases and was significantly associated with poorer survival [25, 26]. Status of mismatch repair (MMR) proteins was assessed by immunohistochemistry of MLH1, MSH2, PMS2 and MSH6 as previously described [27] and classified as mismatch-repair deficient (nuclear loss of at least one marker) and proficient. Information on therapy and overall survival were obtained for all patients (mean and median follow-up time, 47.8 and 40.7 months, respectively). Information on synchronous or metachronous distant metastases was available for 324 patients (cM0/cM1 in Table 1). No patients received preoperative therapy. The use of patient material was approved by the ethics commission of the canton of Bern (KEK 2017-01803, October 24, 2017).

Next Generation Tissue Microarray construction

This study was performed on a tissue microarray using the next generation tissue microarray (ngTMA®) approach [28]. Annotations made from areas containing tumour tissue were selected in the highest budding areas. For each patient, three punches from different histological regions were investigated: tumour centre, tumour front, and tumour microenvironment (areas at the tumor/host interface containing tumor buds as well as non-tumoural cells) defined as previously described [29] (n=9 spots per patient, core diameter 0.6mm for all tumour areas).

Double immunohistochemistry for CD8/pankeratin, CD3/ pankeratin and CD45R0/ pankeratin

Three double immunostains were performed, namely the pankeratin cocktail AE1/AE3 to highlight tumour cells and CD8, CD3 or CD45RO, respectively. Double immunostains ngTMA blocks containing cores from tumour centre, front and microenvironment using the BOND-RX Automated System (Leica Microsystems GmbH, Newcastle, United Kingdom). ngTMA blocks were cut at 4 µm, deparaffinised and pre-treated with the Epitope Retrieval Solution 2 (Citrate-buffer pH 8.8) at 100 C° for 20min. After wash steps, peroxidase blocking was carried out for 4 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). Tissues were again washed, then incubated with primary antibody against AE1/AE3 with the dilution 1:200 (DakoCytomation, Glostrup, Denmark) for 30min. Subsequently, tissues were incubated with polymer for 15 min and then with DAB-Chromogen for 10min (Bond Polymer AP Red Detection Kit DS9305, Leica Microsystems GmbH). After washing, incubation was carried out with anti-CD8 (DakoCytomation, Glostrup, Denmark), anti-CD3 (Abcam, Cambridge) and anti-CD45RO (Abcam, Cambridge) with the dilutions CD8 1 :100, CD3 1 :400, CD45RO 1 :5000 for 30 min (reduced to 15 min in optimizing the CD45RO protocol) followed by application of AEC-substrate for 10 min and counterstaining with haematoxylin for 5 min.

Evaluation of tumour budding cells and T-cell infiltrates on digital slides

Analysis of tumour buds and T-cell infiltrates on the double immunostains was performed on scanned slides (P250, 3DHistech, Budapest, Hungary) using the open source software QuPath [30].

As algorithms for reliable automated digital assessment of tumour budding are still under development, buds were scored manually using an online tool 'Scorenado' especially developed for TMA scoring [31], on dearrayed spots. Tumour budding was defined as an isolated tumour cell or a cluster of four or less tumour cells irrespective of location. Punches containing only stroma (no tumour) were excluded from further evaluation and therefore have no BTS scores.

For assessment of T-cell infiltrates with QuPath, automatically detected TMA spots underwent manual quality control and correction. Tissue areas were detected automatically and manual quality control was performed to remove artefacts. Stroma areas were defined by subtracting tumour areas from their parent tissue areas. Cell detection was performed within stroma areas and the detected cells subsequently classified as 'T-cells' and 'other' using a threshold classifier based on eosin staining. The procedure of digital assessment of T-cell infiltrates is depicted in Fig. 1. All

scripts used for image analysis in QuPath are available under https://github.com/TranslationalResearchUnit/CRC-BTS.

Calculation of Budding/T-cell scores (BTS)

To calculate the density of T-cells, the digital count was divided by the corresponding stroma area. Tumour bud cell density was calculated by dividing the number of buds counted on Scorenado divided by the corresponding stroma area. The Budding/T-cell score (BTS) was defined as the number of tumour buds divided by the number of lymphocytes. The BTS was first calculated for each tissue spot individually. The BTS on a patient level was calculated for each combination of tumour area type and T-cell type as the mean of all BTSs on tissue spot level of the corresponding combination. As division by zero is not possible, the count of all tumour buds (numerator) and immune cells (denominator) was increased by +1.

Statistical analysis

The association of the BTS scores with clinico-pathological features was obtained using the Wilcoxon rank sum test for binary features, the Kruskal-Wallis test for ordinal features, and the Spearman test for continuous features. All p-values (Supplementary file S2) were two-sided and considered significant when $p < 0.05$. These analyses were carried out using the SciPy library (version 1.1.0) in Python 3.5.1. For survival analysis a univariate Cox regression and a multivariate Cox regression (including T-stage, N-stage, cM stage and postoperative therapy) was performed, using the lifelines library (0.13.0) for Python 3.5.1. Hazard ratios (HR) and 95%CI were obtained to determine the effect of each feature.

Results

T-cell/budding count alone versus BTS and clinico-pathological features

The distribution of all scores in different tumour areas is shown in Supplementary File S1. Associations between T-cell infiltrates, tumour budding scores and a combined BTS score are depicted in Fig. 3, with exact p-values in Supplementary File S2. Comparing the T-cell infiltrates, the number of significant statistical associations with clinico-pathological features for CD3 and CD8 were markedly higher than CD45RO both as single parameters and in a combined BTS score. Focusing on CD3 and CD8, infiltrates assessed in the tumour centre stood out with high

associations with lower pT-stage (p<0.001, both), lower pN-stage (p=0.0085 and p<0.001, respectively), cM-stage (p<0.001 and p=0.0088), higher pTNM stage (p<0.001, both), absence of lymphatic invasion (p=0.0024 and p=0.0003) and blood vessel invasion (p=0.0106 and p=0.009), higher KM-Score (p<0.001, both), and lower tumour budding counts as assessed by the ITBCC method (p=0.0005 and p=0.0042).

Tumour budding showed consistent significant associations in virtually all areas (centre, front, tumour microenvironment) with aggressive tumour biology, with higher pT-stage (p=0.0125, p=0.0418 and p=0.0062), pN-stage (p=0.0002, p=0.0005 and p<0.001), TNM-stage (p=0.0011, p=0.0025 and p<0.001), lymphatic invasion, blood vessel invasion and perineural invasion. Importantly, correlations between tumour budding assessed on the TMA punches and by ITBCC and OTB methods were both highly significant (p<0.001, all areas)

For CD3 and CD8 BTS from the tumour centre, higher scores were associated with higher pT-stage (p= p<0.001, both), higher pN-stage (p=0.004 and p<0.001, cM-stage (p=0.0007 and p=0.0015), pTNM-stage (p<0.001, both), lymphatic invasion (p<0.001, both), venous invasion (p=0.001, p=0.006), perineural invasion (p=0.0032 and p=0.0003), lower Klintrup-Mäkinen score (p=0.0042 and p=0.0002), OTB (p=0.0162 and p=0.0162) and ITBCC scores (p<0.001, both).

BTS vs. immune cell/budding count alone as a predictor of lymph node metastasis and survival

In the context of clinically relevant endpoints (lymph node metastasis and overall survival), associations with T-cell infiltrates, tumour buds and BTS scores were assessed.

On univariate analysis, higher CD8 infiltrates in the tumour centre were associated with better OS (p=0.0002), followed by CD3 in the tumour centre (p=0.0016). The BTS showed centre and TME CD3 BTS as well as centre CD8 BTS to be associated with poorer survival (p=0.0437, 0.0266, and 0,0214 respectively). Budding on the TMA spots as a sole parameter was not significant for OS in this cohort. However, in multivariate analysis including T-stage, N-stage, cM stage and postoperative therapy, only higher centre CD3 BTS scores were significantly associated with poorer OS (p= 0.012, HR 1.218, 95%CI: 1.044-1.419).

For predicting nodal metastases, only centre CD8 BTS scores were significant for predicting nodal metastases (p<0.0001, OR 1.466, 95%CI: 1.115-1.928).

Discussion

This study is the first analysis to explicitly examine T-cell populations and tumour budding cells in colorectal cancer as a combined Budding/T-cell Score in different tumour areas. Here, although T-cell infiltrates and tumour budding were both strongly associated with clinico-pathological features, a combined score was superior to either marker alone, especially for predicting nodal metastases and survival.

T-cell infiltrates in solid cancers have been the focus of many studies, and the recently validated Immunoscore has been demonstrated to provide valuable information on recurrence risk in non-metastatic colon cancer [5]. However, Immunoscore may not completely reflect the biology of the tumour itself. Here, we study how the 'attacker-defender model' might enhance the estimation of tumour biology compared to tumour budding or T-cell infiltrates as separate parameters. For instance, it would be conceivable that additional quantification of T-cell infiltrates could counteract the effect of tumour budding. This may prove useful in clinical scenarios where tumour budding can be implemented to guide patient management, such as estimating the risk of nodal metastases in endoscopically resected pT1 CRC [9, 32] and in stage II CRC [33, 34] [10].

Our group has previously demonstrated superior prediction of survival when using a combined index of tumour buds and T-cells. For instance, a CD8/tumour budding index was shown to increase the prognostic effect of tumour budding [23]. Similarly, a combined assessment of CD8, FOXP3 and CD68 positive immune cells and tumour buds was also described to improve stratification of patients into prognostic risk groups [35]. Even histomorphological assessment of tumour-infiltrating lymphocytes and tumour buds may increase the impact of tumour budding alone, as recently suggested [36].

Taking into account insights on the T-cell landscape of CRC, we focus on several well-established T-cell protagonists (CD3, CD8 and CD45RO) in different areas of the tumour. Interestingly, the most robust parameters in our assessment are CD3 and CD8 BTS scores originating from the tumour centre. Although traditionally assessed at the tumour front [10], tumour budding within the tumour centre (intratumoural budding, ITB) is well-documented in the literature and strongly

correlated with budding seen at the tumour front [25, 37, 38]. The fact that budding can be seen in the centre of a tumour strongly supports that dissemination does not occur only in regions rigidly defined as 'tumour front' and 'tumour microenvironment'[39]. Such areas of infiltrative tumour growth may also be highly convoluted and therefore also captured in regions corresponding to our 'tumour centre' punches. Finally, technical aspects such as levelling through TMA cores may have additionally contributed to a higher consistency of scores derived from the tumour centre. Nonetheless, practical implications of this finding include potential application of the BTS score in pre-operative rectal biopsies, where additional information on tumour spread may influence the decision to administer neoadjuvant therapy [40, 41].

From the T-cell counts, CD3 and CD8 counts were superior compared to CD45RO, which was also reflected in the BTS scores. Though regulatory T-cells have been implicated as an important player in the immune cell infiltrate in CRC [6, 42, 43], inconsistencies and background positivity in immunohistochemical stains have previously been reported [6, 44], leading to the exclusion of CD45RO from Immunoscore [6]. The diminished performance of CD45RO-based scores here is most likely also due to this phenomenon despite efforts to improve the quality of the immunostain, indicating that this marker may not be suitable for quantification performed by visual scoring or digital assessment.

Our proposal of the BTS aims to reflect the 'attacker' and 'defender' as aggressive and protective forces in CRC. However, further studies are required to validate whether these opposite markers can indeed be united in a combined score. Although our study was performed on a multi-punch TMA including several punches from different tumour areas, due to the nature of this approach evaluated tissue originated from pre-selected areas. This would account for the lack of association between tumour buds scored on the TMA spots and survival, as tumour budding assessed on whole tissue slides from this collective was associated with poorer overall survival (by both OTB and ITBCC methods[25, 26]). Validation studies may be performed on whole tissue slides to optimize the selection of regions most suitable for BTS assessment.

One of the novel aspects of this study is the assessment of T-cell infiltrates using open-source software. The implementation of such methods will likely be able to replace cumbersome manual assessment in the near future. As digital scoring of tumour buds can also be anticipated, a fully

automated BTS-algorithm could be developed. The BTS is not meant to replace tumour budding and Immunoscore as robust biomarkers in CRC, but to further underline and reflect their importance alongside other pathological parameters. Potential applications of the BTS include clinical scenarios where a precise estimation of tumour biology is required to guide patient management, such as in endoscopically resected pT1 tumours, stage II CRC and pre-operative rectal biopsies. Our results warrant further study and development of the BTS in CRC.

Author Contributions and Acknowledgements

HD and LC performed manual scoring, ME and SR conducted digital analyses. AL and HD designed the study. ME and IZ performed statistical analysis. HD, ME and LC drafted the manuscript. AL, AB, SR and IZ critically reviewed the manuscript.

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Tables

Feature		Freq N (%) or Mean, median
Gender (n=345)	Male	209 (60.6)
	Female	136 (39.4)
Patient age (n=345)	Mean, median	69.5, 70.9
Histological subtype (n=326)	Adenocarcinoma	283 (86.8)
	Mucinous	37 (11.3)
	Other	6 (1.8)
Tumour location (n=320)	Left-sided	142 (44.4)
	Right-sided	118 (36.9)
	Rectum	60 (18.8)
pT (n=342)	pT1-2	59 (17.3)

Table 1: Patient characteristics (n=345)

	pT3-4	283 (82.2)
pN (n=342)	pN0	160 (46.7)
	pN1-2	182 (53.3)
Number of examined lymph nodes (n=338)	Mean, median	24.2, 21.0
Distant metastases (n=324)	cM0	238 (73.5)
	cM1	86 (26.5)
p/cTNM-Stage (n=339)	Stage I	37 (10.9)
	Stage II	126 (37.2)
	Stage III	120 (35.4)
	Stage IV	56 (16.5)
Tumour grade (n=332)	G1-2	259 (78.0)
	G3	73 (22.0)
% expanding tumour border (n=302)	Mean, median	45.9, 50.0
Number of buds ITBCC (n=106)	Mean, median	8.1, 6.0
OTB mean (n=186)	Mean, median	11.2, 8.0
Lymphatic invasion (n=310)	L0	98 (31.6)
	L1	212 (68.4)
Venous invasion (n=293)	V0	141 (48.1)
	V1-2	152 (51.9)
Perineural invasion (n=300)	Pn0	247 (80.7)
	Pn1	59 (19.3)
Klintrup-Mäkinen (n=305)	0	25 (8.2)
	1	129 (42.3)
	2	119 (39.0)
	3	32 (10.5)
Postoperative therapy (n= 284)	No	196 (69.0)
	Yes	88 (31.0)
MMR status (n=190)	Proficient	164 (86.3)
	Deficient	26 (13.7)

Abbreviations: ITBCC: International Tumor Budding Consensus Conference; OTB : Overall tumor budding; MMR : Mismatch repair

Figure Legends

Fig. 1: Low (A-C) and high (D-F) BTS scores for each double immunostain. A+D: CD3/Pankeratin, B+E: CD8/Pankeratin, C+F: CD45RO/Pankeratin (images all taken between 15-20x). Selected tumour buds marked by asterisks, selected T-cells marked by arrows (A-F). The CD45RO/Pankeratin immunostain shows extensive non-specific staining which was still present after protocol optimization.

Fig. 2: Digital analysis of TMA spots (0.6 mm in diameter) and assessment of T-cell infiltrates. a) Double immunostain (CD8(red)/Pankeratin(brown)) before and b) after tissue separation. 'Stroma' was defined as the non-epithelial, non-T-cell area (nuclei annotated in green).

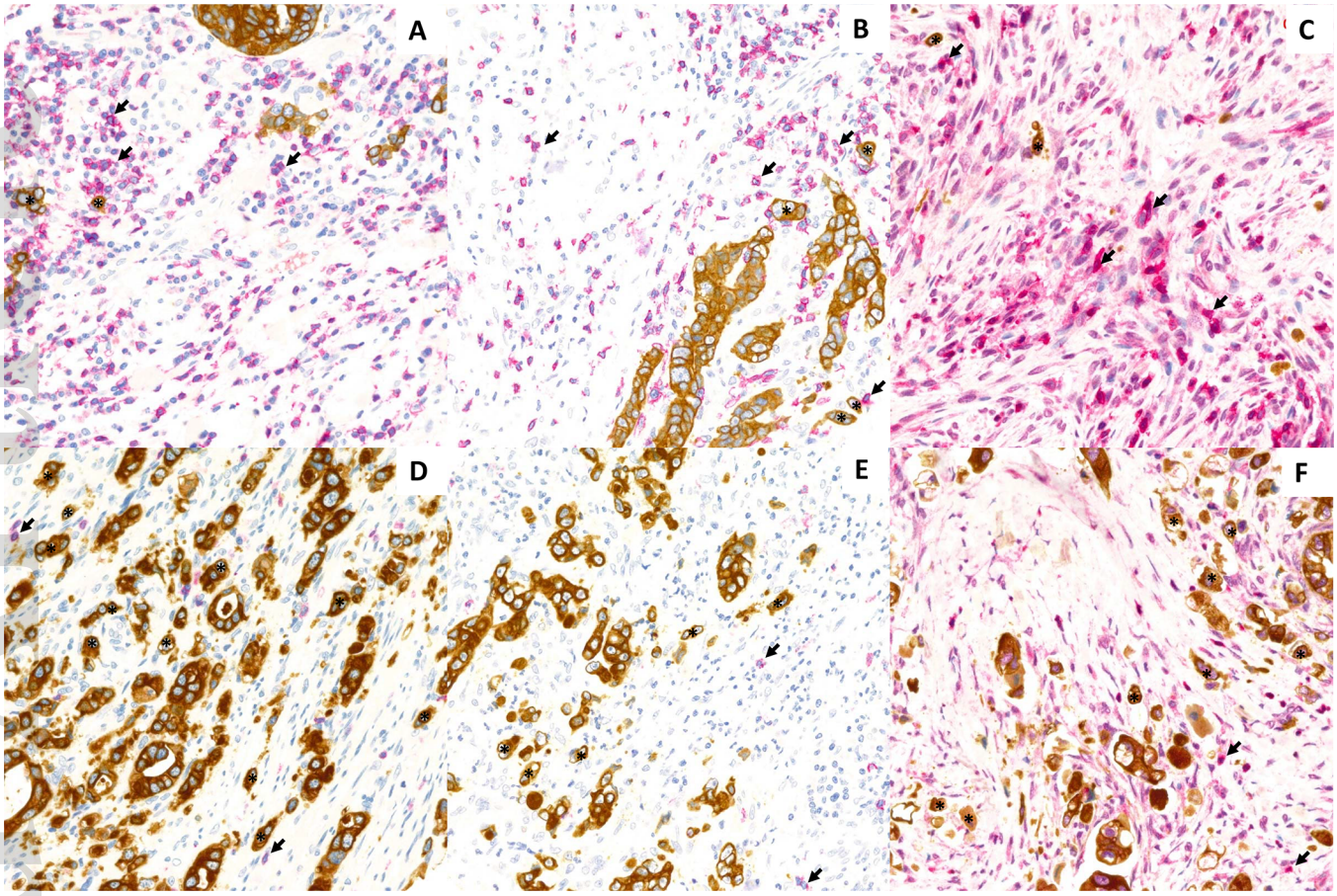
Fig. 3: Plot of associations between T-cell infiltrates, tumour buds and Budding/T-cell scores with clinico-pathological features and overall survival. Increased numbers of T-cells are associated with

more favourable tumour biology, whereas increased numbers of tumour buds and higher Budding/T-cell scores are associated with aggressive tumour biology. Abbreviations: TME: tumour microenviroment, BTS: Budding/T-cell scores, KM-Score: Klintrup-Mäkinen score, OTB: overall tumour budding, ITBCC: International tumour budding consensus conference, OS: overall survival

Supplementary Files:

S1: Distribution of T-cell and tumour bud counts and BTS scores in different tumour areas

S2: Depiction of Fig. 3 with exact p-values



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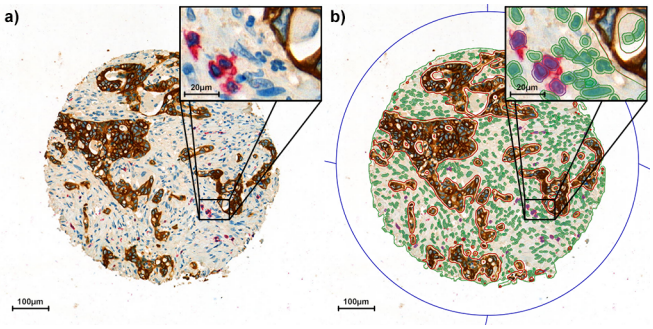

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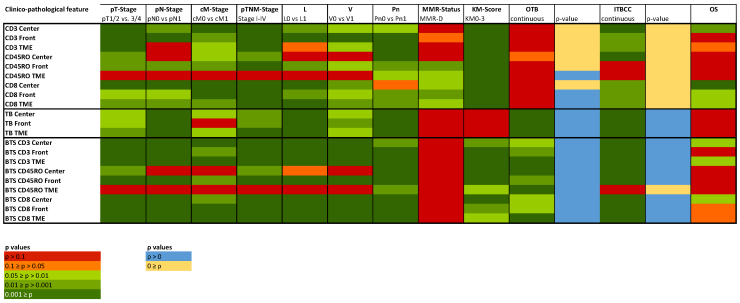

his_14006_f3.tif