Molecular mechanisms of tumour budding and its association with microenvironment in colorectal cancer.

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Running Title: Tumour budding and its relationship with tumour signalling and microenvironment

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Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide. Poor survival of CRC associated with development of tumour metastasis led to investigation of the potential biomarkers to predict outcome in CRC patients. Tumour budding (TB) is a well-known prognostic marker as well as an independent predictor of disease metastasis and poor survival. Therefore, it has been suggested that TB status is included in routine clinicopathological factors for risk assessment in CRC. In contrast to a vast majority of studies regarding the prognostic power of TB, there is no clear evidence pertaining to the underlying molecular mechanism driving this phenotype, or an understanding of TBs relationship with the tumour microenvironment (TMA). The aim of this review is to presents a comprehensive review of tumour cell signalling pathways and the crosstalk with immune cells that could drive TB formation in CRC.

Background

Colorectal cancer (CRC) is the third most diagnosed cancer and the second most lethal malignancy worldwide (1). A high level of heterogeneity is a feature of CRC which may play a major part in tumour metastasis (2). Targeting the mechanism of metastatic disease could lead to its prevention, however, only a few metastasis-specific markers are available to identify patients with the risk of disease recurrence (3)**.** Consequently, novel prognostic markers are required to either predict treatment outcomes or target tumour growth signalling, thereby preventing tumour progression and improving survival in CRC (4). Tumour budding (TB) is defined as a single cell or small cluster of up to 4 cells at the invasive front of a tumour and is well established as being associated with a poor prognostic and metastatic behaviour in CRC (5). According to the international tumour budding consensus conference (ITBCC) 2016, TB is an independent prognostic marker and should be included in regular clinical CRC reports (6).

There is an abundance of evidence supporting the role of EMT, a reversible cellular phenotype that enables tumour migration and progression (7), in promoting TB. However, this process alone cannot explain the budding phenotype (8, 9). Many studies, therefore, suggest pathways related to a TB phenotype either through EMT, or non-EMT related signalling (10, 11), however, no clear evidence has been found. In addition to tumour signalling, the role of tumour microenvironment (TMA) has also been shown to have an important impact on CRC patients' survival (12-14). The recent approval of immunotherapy suggests a promising immune target treatment (15). The communication between tumour cells and the tumour microenvironment (TME) has been reported as an important factor in cancer development, metastasis, and clinical treatment response (16-18). However, in addition to the association between TB and several types of immune cells in CRC, its importance remains to be fully elucidated.

The crosstalk between TME and the tumour could lead to cell signalling and promote tumour progression and metastasis possibly via interactions between inflammatory infiltration and TB (19). There are many signalling pathways involved in tumour metastasis and progression in CRC (12, 13), however, few studies have examined the association between tumour signalling pathways, the tumour microenvironment, and the presence of TB. With international agreement on the definition of TB, this provides the opportunity to examine the signalling pathways and tumour microenvironment associated with TB in CRC. Such information will provide the basis for understanding the pathways underlying the development of TB and potential treatment in patients with CRC.

Pathways related to tumour budding

Transforming growth factor-beta (TGF-β)

TGF-β is a multifunctional cytokine that has a multiple role in both tumour suppressor and pro-oncogenic functions. However, in tumour development, TGF-β activation favours cancer survival, progression, and metastasis (eg. EMT and immune invasion) (20, 21). SMAD activities are known to mediate TGF-β and therefore promote EMT by decreasing the expression of E-cadherin and increasing transcriptional repressors such as SNAIL, ZEB1 and TWIST (21). The TGF-β pathway can also be modulated by SMAD-independent signalling, such as Ras-MAPK and PI3K-AKT, which also modulate EMT in tumour cells and other cellular activities including proliferation and apoptosis (21). The relationship between TB and factors associated with TGF-β activation is complex and is often contradictory. There are a limited number of studies investigating the relationship between TB and transforming growth factor-beta (TGF-β) signalling (Table1).

SMAD-dependent pathway

Mothers against decapentaplegic homolog 4 (SMAD4)

The relationship between *SMAD4* expression and TB in CRC is more qualitative observations than quantitative measurements. According to Oyanagi et al., the alteration of *SMAD4* was significantly correlated with invasive front pathological markers and poor prognosis in patients with CRC. However, there was no significant association between TB and *SMAD4* mutations in CRC patients (22).

Mammary serine protease inhibitor (Maspin)

Maspin is a human protein encoded by the *SERPINB5* gene. The regulation of maspin through the integrated pathway of TGFβ/Snail and TNF**α**/NFκB has been reported in CRC (23). However, the prognostic role of maspin is paradoxical and only a few reports have investigated its relationship with TB (23, 24). Markl et al. reported a highly significant association between TB and nuclear maspin expression in stage II&III CRC (24). A recent study, using cDNA analysis of gene expression, showed that *SERPINB5* was upregulated in TB compared to the stromal area (23). Even though a prognostic role for *SERPINB5* in the database from The Cancer Genome Atlas (TCGA) was not found, Li and colleagues suggested that high expression of *SERPINB5* quantified from TB at the invasive area was a poor prognostic factor in CRC patients. However, this study is limited as only three cases were selected to perform the gene expression profiles studies on.

SMAD-independent pathway

Raf-1 Kinase Inhibitor Protein (RKIP)

RKIP is known to inhibit Raf/MEK/ERK signalling and control EMT activity in CRC (25). Decreased expression of RKIP was found in budding cells, and loss of RKIP at the invasive front was observed in tumours with high budding phenotype (25). The results indicated a role for RKIP in predicting advanced features such as TB, metastatic disease and vascular invasion in stage III CRC. However, Koelzer and colleagues found that, although RKIP expression significantly predicts survival in CRC, no data demonstrated its prognostic role for survival in association with budding cells.

Caudal Type Homeobox 2 (CDX2)

CDX2 is regulated through downstream signalling of the Raf/MEK/ERK pathway and functions as a tumour suppressor. The loss of CDX2 expression has been reported in advance CRC and is associated with a patient's poor survival (26). According to Slik et al., there was no correlation between loss of CDX2 and budding phenotype. However, patients with microsatellite stability (MSS) tumours, who have negative CDX2 expression, progressed to metastatic disease whereas no invasion was found in high microsatellite instability (MSI-H) tumours. However, the study evaluated only in stage II CRC TMAs, further investigation in full CRC sections across all stages may verify a role for this protein in the tumour, particularly with TB phenotype.

MicroRNA-21 (miR-21)

Studies have shown that microRNAs are involved in EMT by forming double negative feedback loops with EMT-inducing transcription factors. The expression of miRNA by TGF- β was reported in EMT modulation as well as the tumour microenvironment (27). A recent study purposed that co-expression of miR-21 and TNF- α in budding cells could benefit CRC treatment and warrants attention for further research (28). In-situ hybridisation (ISH) demonstrated the presence of miR-21 in all stage III CRC (n=22) (29). A similar trend was observed by Moller et al., albeit only in 7 cases (28). These results did not report any significant association with budding cells, however, miR-21 was frequently found in budding cells in both studies and warrants further investigation.

Wingless-related integration site (Wnt) signalling

Deregulation of Wnt signalling impacts various biological processes such as cell proliferation, differentiation, tissue regeneration and tumorigenesis (30). 90% of CRC cases have been reported to have a disruption in Wnt signalling and this accounts for 60-80% of mutations in CRCs regulated through this pathway (31). The crucial role for Wnt signalling in tumour metastasis is to promote EMT activity (32). The mechanism of tumour metastasis in budding phenotype, regarding Wnt signalling, could enhance the biological knowledge underlying budding cells in CRC. Several studies have reported that some members of the Wnt signalling pathway may induce TB in CRC(Table1).

β-catenin

A few studies have investigated the correlation between TB and β-catenin, however, strong evidence to support the relationship with β-catenin and TB has not yet been established. According to El-Gendi and Al-Gendi, high TB is associated with increased nuclear β-catenin (33). The study, however, utilised small cohorts (n= 44). The expression of nuclear β-catenin was investigated in budding cells and was significantly expressed in MSS budding cells compared to MSI budding cells tumours (34). Similar results, reported by Martensson et al., also showed a significant correlation between the mean nuclear β-catenin expression and its expression in TB at the invasive front, tumour type and growth pattern in CRC (35).

Laminin-5 gamma 2 chain (LAMC2)

The increased expression of laminin-5$\boldsymbol{\gamma}$2, another β-catenin target gene, was significantly associated with high TB phenotype. According to Knudsen et al., thirty-one cases (53%) showed a frequently high level of laminin-5$\boldsymbol{\gamma}$2 in budding cells (34). Similar results from Zhou et al. demonstrated that laminin-5$\boldsymbol{\gamma}$2 expression was higher in TB cells than the tumour cores, and that patients with high laminin-5$\boldsymbol{\gamma}$2 expression had a poor survival outcome (36). Another study performing multivariate analysis, demonstrated that laminin-5$\boldsymbol{\gamma}$2 expression was considered as an independent prognostic marker in high TB CRC (37). Furthermore, positive expression of C4.4A, a glycolipid-anchored membrane protein that has been hypothesised to bind to laminin-5$\boldsymbol{\gamma}$2, was reported to be significantly higher in budding cells compared to C4.4A negative cells in pT1 CRC. This indicates the prognostic role of C4.4 in early-stage CRC with a high budding profile (38). Oshiro and colleagues also showed, albeit without evaluating the TB profile, that positive expression of C4.4A significantly associated with loss of E-cadherin expression while nuclear β-catenin was increased at the invasive front when compared to the main tumour. The results suggest the mutual interaction between the regulation of C4.4A and β-catenin, which may prompt the possible invasive mechanism of TB in CRC (38).

Interestingly, cleaved laminin-5$\boldsymbol{\gamma}$2 was also observed in a high Abl Interactor 1(Abi1) expressing cell line. Abi1 expression has been reported in tumour migration and invasion through the activation of Ras signalling in CRC (39). Abi1 expression was higher in colorectal adenomas and carcinomas compared to the healthy tissue and in cells with mutated KRAS compared to cells with wild-type KRAS (39). More importantly, overexpression of Abi1 is significantly associated with a high TB phenotype at the invasive front of CRC tumours (39).

Erythropoietin-producing human hepatocellular (Eph) receptors

Eph receptors make up the largest group of receptor tyrosine kinases. The role of Eph receptors varies in CRC and the underlying mechanism behind Eph activity is unknown. In this review, albeit a statistical analysis with TB, a significant reduction of EphB3 as well as E-cadherin, an EMT-related biomarker, in budding compared to non-budding cells was observed, suggesting an

anti-tumour function with the concomitant role of EphB3 and EMT activities in CRC**.** Induced EphB3 in a CRC cell line also suggested that EphB3 suppression might contribute to the downregulation of E-cadherin, indicating the aggressive behaviour of TB in CRC (40).

Leucine-rich repeating-containing G-protein coupled receptor 5 (LGR5)

LGR5 is a target gene of β-catenin/Wnt signalling and one of the well-known stem cells markers. It has been reported to be upregulated in various types of tumours including CRC. Expression of LGR5 has been reported in the invasive area of the tumour (n=99) (41). The results showed a significant association between LGR5 and TB as well as TNM stages and other adverse clinical features such as tumour invasion and lymph node metastasis in CRC (41).

Programmed cell death

Loss of apoptotic control allows damaged cells to survive, and as a consequence, accumulate mutations, leading to tumour development and invasion of organs (42). In cancer metastasis, an increase in apoptosis could prevent cells from invading the body, however, tumours can sometimes become resistance to cell death which allows them to thrive (43). An apoptotic-related protein has been discovered and is currently under study with a view to developing a small targeted molecule against its activity in cancer development (44). Studies have reported a correlation between cell death activities and TB in CRC, though the mechanism remains to be elucidated (Table1).

According to Dawson et al., an absence of caspase 3, an essential protein for the apoptotic scenario, was observed in most of the buds (45). A decreased apoptotic rate inTB compared to the tumour centre and the tumour front in CRC were also reported (45). Regarding the expression of caspase 3 in budding cells, Dawson and colleagues suggested that TB could represent hypo-apoptotic cells and may develop resistance to programmed cell death, therefore, detach from the extracellular matrix leading to metastases (45). The authors then investigated the correlation between TB and the tyrosine kinase receptor B (TrkB), a promotor of EMT and anoikis-like apoptotic tolerance (AAT), in CRC patients (46). Unsurprisingly, the results showed a significant association between high TB and overexpression of membranous/cytoplasmic TrkB in budding cells compared to the main tumour. Moreover, the positive membranous TrkB inversely corresponded with proliferation and apoptotic rate in TB. Dawson and colleagues, therefore, confirmed their previous results that budding cells are not representative of proliferating cells, and may instead develop anoikis resistance evading the apoptotic process to promote invasion and progression in CRC.

Stem cell, cell cycle and cell proliferation

The self-renewal ability of stem cells can promote tumour development and reconstruction, and therefore, induce invasion and cancer metastasis (47). Overexpression of CD44, a widely used

cancer stem cell (CSC) marker, is a poor prognostic factor and reflects metastatic disease in CRC (48). In addition to the relationship between TB and CD44, a correlation of CD44 with poor prognosis and significantly higher expression of CD44 in tumours with high TB in CRC was observed (49). The same trend was also found with other CSC biomarkers in the same study; Notch1 and ALDH1. Furthermore, the study suggested that TB may reduce cell cycle activity by having low expression of the mitotic marker, PHH3, in tumours with high TB; however, this observation was not statistically significant (50). Cell proliferation markers can be used to identify aggressive types of cancer cells, which may lead to tumour progression and metastasis. Dawson et al. observed a negative association of tumour cell proliferation, as assessed by Ki67, in budding cells (45). However, van Wyk et al. showed no association between budding phenotype and KI67 proliferation index in CRC patients, though expression of KI67 was significantly associated with cancer-specific survival (CSS) (51).

Mesenchymal-epithelial transition (MET) signalling

Metastasis Associated in Colon Cancer 1(MACC1)

MACC1 regulates tumour invasion through activation of c-MET and is a downstream target for HGF signalling in CRC patients (52). MACC1 expression at the tumour invasive front was significantly associated with high TB. MACC1 positive buds were also found in CRC patients with more advanced stages and nodal metastasis, indicating that MACC1 is a marker for poor prognosis and has a positive relationship with TB (52). However, further study is required to confirm this finding.

The relationship between tumour budding and tumour microenvironment

In addition to signal transduction, the tumour microenvironment (TME) also influences tumour progression and impact patients' survival (16, 17). TMEs enriched with T lymphocytes are associated with a good prognosis in CRC (12-14, 18). However, the relationship between immune cells and TB has not yet been fully investigated.

Tumour infiltrating lymphocytes (TILs)

TILs

Dawson et al. and van Wyk et al. reported an inverse relationship between inflammatory and budding cells, using Klintrup–Mäkinen (KM) scores (51, 53). In addition, a high density of TILs at metastatic sites was shown associated with better overall survival (OS) among patients with CRC (54). A recent study, albeit in a low number of cases (n=107), indicated no relationship between TILs and TB in patients with CRC (55). However, increased TILs showed a correlation with the number of proliferating cells and the number of TILs negatively correlated with CRC stage (I-IV).

Combined TB and TILs markers

Recently, studies have demonstrated that the combined score between TILs and TB is a strong predictive marker for patient survival and other adverse features in CRC. A high TB/CD3 ratio showed a significant association with poorer OS (56). A similar trend was demonstrated by Gonzalez et al., the combined TILs and TB scores were shown to stratify the prognostic groups in CRC patients. The authors reported a worse recurrence-free survival (RFS) in tumours with high TB and no TILs compared to cases with TILs, the results were reversed in cases with TILs and low TB (57).

Three studies from Lang-Schwarz et al. showed a significant association between combined TILs/TB scores and favourable OS in CRC patients (58-60). The 2018 study demonstrated that cases without systemic metastases significantly present with low TB and high TILs, whereas cases with metastatic disease had higher TB with low TILs (58). Interestingly, in 2019, the team further investigated the prognostic role of TB and TILs in WHO low-grade CRC. A combined score of TILs, TB and gland formation was performed to separate a large group of low-grade CRCs into subgroups. Unsurprisingly, the results showed a significant outcome in each of the subgroups. However, when multivariate analysis was performed, no correlation was found regarding the association between scores of TILs/TB and survival (59). Furthermore, the most recent article from Lang-Schwarz and colleagues, published in 2021, showed the benefit of chemotherapy choices when using TILs/TB combined scores for treatment classification. The study collected samples before treatment in stage II and III CRC, and found that in stage II CRC, tumours with high TB and high TILs showed a difference in overall survival compared to high TB low TILs, however, the findings were not statistically significant. In stage III patients, cases with high TB and low TILs tended to benefit from adjuvant treatment, however, no treatment benefit was seen in patients with low TB and low TILs (60).

T cells

One of the well-known prognostic lymphocyte markers is number of T cells. Three studies reported the association between TB and T cells lymphocytes in CRC (Table2). Interestingly, tumours with low TB showed a significantly higher number of cytotoxic T cells (CD8+) at the invasive front than in the main tumour (61). An inverse correlation between high density of CD3+ T cells and low TB at the invasive front using automated analysis in two different cohort studies was reported (62). High CD3+ and CD8+ T cells at the invasive area were significantly correlated with decreased number of TB and showed favourable clinicopathological features. Furthermore, the authors also reported that an increased number of lymphocytes near TBs significantly predicted DFS for stage II CRC (62). Moreover, the relationship between multiple lymphocytes (CD3+, CD4+, CD8+, CD45RO+ and FOXP3) and TB using multiplex immunofluorescence showed that T cells densities (CD3+ CD8+ CD45RO+) inversely correlated with the number of TB in a large CRC cohort (N=915) and low TB with high T cells showed higher CSS than high TB with low T cells (63). Considering

this evidence, with a budding phenotype, these results may suggest that cytotoxic T cells play an important role in anti-tumorigenesis and may suppress tumour micro-invasion in high budding CRC. Further studies are required to confirm this hypothesis.

Macrophages

A negative association between TB and macrophages is emerging. Koelzer et al. reported a significant increase of CD68+ cells in tumours with low TB, a high number of CD68+ predicted longer overall survival in CRC (64). Moreover, when combining two different types of macrophages, low CD68+/CD163+ ratio showed a favourable outcome in stage II CRC patients (65). Additionally, the statistical correlation between increased CD14 cells, another macrophage marker, and the number of TB in CRC, especially in tumours with the high budding was also reported (55). The authors also showed that CD14+ was positively correlated with other lymphocytes such as CD3+ and CD4+ in the stroma area.

Other immune markers

Cancer-associated fibroblasts (CAFs) modulate cancer metastasis through remodelling of the extracellular matrix (ECM) as well as promoting angiogenesis and growth factors in the tumour microenvironment. It has been suggested that immature fibroblasts could lead to infiltrating tumour growth and TB, however, no significant association was found in the study (66). According to Korehisa et al., PD-L1 was expressed in both cancer cells and the surrounding myeloid cells. This correlated with poor prognostic parameters and high TB and was even more significant in MSI high cases (67). In addition, loss of major histocompatibility complex I (MHC-I) has been reported to be significantly associated with poor outcome in CRC (68). The study showed that MHC-I expression at the invasive front is strongly associated with low TB. Furthermore, expression of both MHC-I and CD8 significantly correlated with low TB and favoured survival when compared to double negative cases in CRC (68).

Discussion

The purpose of this study is to provide a comprehensive review of markers that relate to the TB phenotype (Figure2), and additionally, the relationship with the tumour microenvironment to understand the mechanism underlying the development of TB in CRC. Until now, no signalling pathways have been consistently associated with bud formation. Perhaps the most promising biomarker, identified by the current review is the association between TB and β-catenin. The translocation of β-catenin from the cytoplasm to the nucleus and activation of downstream signalling results in EMT activation which may trigger tumour cell progression in CRC (69). The study from Martensson et al. also demonstrated the association between nuclear β-catenin expressed in budding cells and p53 oncogene accumulation, thus representing an adverse phenotype of TB. Higher expression of nuclear β-catenin has been observed in MSS when compared to MSI budding cells as reported by Knudsen et al., indicating an aggressive type of MSS that is correlated with high numbers of TB, which is relevant to our recent systematic review and meta-analysis (70). This review also reported a positive correlation between an increased expression of laminin-5$\boldsymbol{\gamma}$2 and TB. The interaction between integrinβ1 and laminin-5$\boldsymbol{\gamma}$2 was reported as it may represent an important factor in promoting budding in CRC cell lines (36). These results, therefore, add to the body of evidence in support of the potential prognostic power of laminin-5$\boldsymbol{\gamma}$2 in CRC (71). However, these results need further validation to confirm the hypothesis.

Additionally, it is likely that TB have low apoptotic activity and could develop resistance to cell death signalling indicating an advanced phenotype of TB which can invade organs by the suppression of cell death signalling in CRC. However, two studies suggested an opposing way by which budding cells may increase apoptosis activity indicated by a significant decrease in anti-apoptotic Bcl-2 expression in tumours with high-grade budding (72, 73). However, these studies were conducted in TMAs, therefore, tumour heterogeneity and expression of some markers may be lost. Although cancer stem cells (CSC) have been characterised in CRC, to date they have not been identified in tumour buds (74). An increase in apoptosis, proliferation and CSC activity may, in part, be responsible for the aggressive characteristics of advanced disease. All these signalling pathways could play a role in budding formation. Spatial transcriptomic / proteomic profiling of budding cells may help elucidate the potential pathway that may explain the aggressive phenotype of TB in CRC.

In this review, the majority of studies observed an inverse correlation between TB and TILs, where increased T cell lymphocyte infiltration was associated with higher survival in CRC patients. This may indicate an ability to evade the host immune system in high TB CRC via ~~a~~ crosstalk between tumour cells and the microenvironment. In addition to T cell lymphocytes, macrophages may also be important in TB. Infiltrating CD68+ tumour-associated macrophages (TAMs) were reported to be a favourable prognostic marker in CRC (75). It is, however, important to note that TAMs not only

exhibit anti-tumour activity but can also promote tumour growth due to their different polarisation forms (76). According to Trumpi el al., co-culture of patient's derived colon spheres with M2 macrophages promoted budding cells, suggesting a comprehensive role for the tumour microenvironment in CRC metastasis disease (77). The role of macrophages, especially the polarity subtypes, should be considered and warrants future investigation.

An investigation utilising spatial profile techniques to look at the characteristics of TB in CRC and the interaction between immune cells and TB may provide a wealth of information (78). There are a few reports looking particularly at the budding area in the invasive front, using laser captured technology. and analysis identified some genes of interest which may be associated with budding phenotype in CRC (23, 79). The limitation of these studies is that only a few cases were selected (N=3 and 8 respectively), therefore, further investigation in a larger number of studies is crucial to understand the molecular profile of budding cells in CRC. In addition, two studies, recently published by Nearchou et al., utilised multiplex immunofluorescent and automated analysis to evaluate multiple protein expression in immune cells and compared this expression with clinical features including TB in CRC (62, 65). These may lead to a future approach of combined immunotherapies, as co-expression of lymphocyte markers were predominantly observed (56, 65, 80). An investigation into immune cell expression using advanced multiplex staining in full CRC sections could be performed to enhance the understanding of how these immune cells interact with each other and any association with TB in CRC.

There are some limitations to this review with regards to the study design. Firstly, there are a variety of methodology used to assess TB, and there is still an argument with whether IHC with pan-cytokeratin staining could improve the quantification of TB and should be used instead of standard H&E (81-83). However, most studies in this review evaluated TB phenotype in H&E with some studies utilising the IHC method. Furthermore, with regards to the assessment of the immune profile, a variety of TILs evaluation methods were found among studies. In this review, two studies utilised KM scores for TILs assessment (51, 53). KM scores showed reproducibility and promising prognostic value for peritumoral inflammation in CRC (84). It is considered a predictive factor for lymphocyte and inflammatory infiltrating cells in CRC, and perhaps the assessment of TILs using KM scores may reduce the variability among studies. Lastly, the spatial expression of specific protein and immune cells investigated in whole full sections might be an appropriate model to represent an overview of TB characteristics and its microenvironment at the invasive area of CRC specimens.

Conclusion

The prognostic role of TB is widely accepted, however, the molecular mechanism and its relationship with the tumour microenvironment are still questionable. A comprehensive investigation of how tumour biological signalling and the tumour microenvironment association with TB in CRC is crucial. The possible tumour development-related signalling was consistently reported according to budding phenotype. These signalling pathways may contribute to TB development and tumour metastasis in CRC. The spatial transcriptomic and proteomic analysis of tumours with a budding phenotype could improve our understanding of why TB is associated with poor survival in CRC. Furthermore, an emerging knowledge of the spatial profiling of tumour cells as well as their interaction with the microenvironment is crucial to expand the knowledge underlying TB development and its relationship with immune cells infiltration. Multiplex phenotyping is a promising technology that may provide information on the interaction between the tumour and its surrounding immune cells. Understanding the possible signalling pathways and immune microenvironment of TB is promising, thus, could lead to the enhancement of treatment options in patients with CRC in the future.

Compliance with ethical standards

Conflict of interest: All authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors. All analyses are based on previously published papers. Therefore, no ethical approval and patient consent are requiring

Availability of data and materials: Data sharing not applicable to this article as no datasets were generated or analysed during the current study

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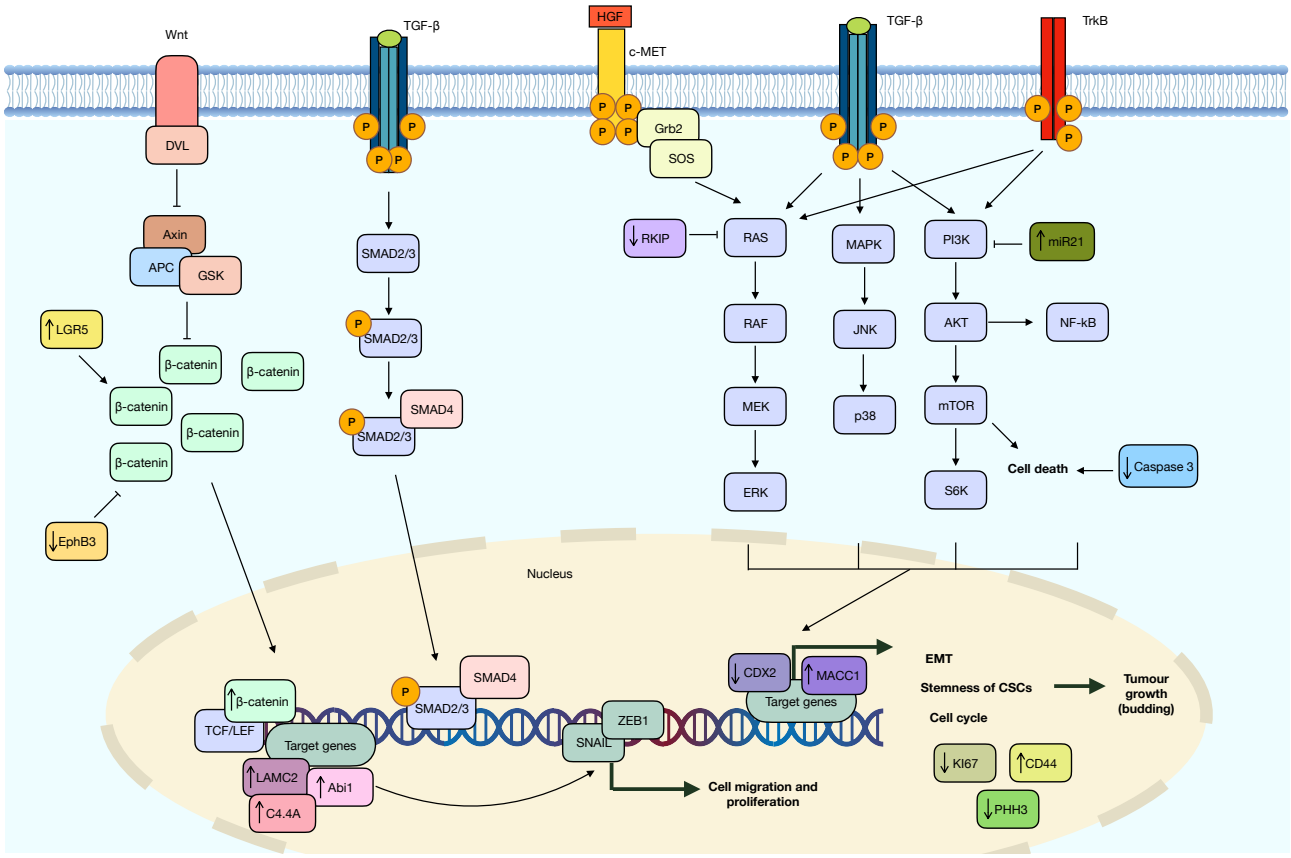

Figure 1 Number of biological processes related to EMT, cancer stemm cells (CSC) and cell cycle through the signalling cascades which may associated with budding phenotype in CRC. TGF-β SMAD-dependent and independent signalling. TGF-β ligands regulated phosphorylated SMAD2/3 protein to form a complex with SMAD4 and independent downstream components such as Ras, MAPK and PI3K signalling. In Wnt signalling, DVL is activated and induces the suppression of GSK. Subsequently, stabilized β-catenin translocates from cytoplasm into the nucleus and binds to TCF leading to the transcription of target genes. Hepatocyte growth factor (HGF) binding c-Met activates signalling cascades including Ras signalling. MUC1, a glycoprotein that regulates c-Src signalling induced multiple downstream targets through Ras signalling. Activation of tyrosine receptor kinase B (TrkB) triggers phosphoinositide 3-kinases (PI3K), AKT and Ras signalling.

Table 1 Studies investigated the correlation between tumour budding and target signalling in colorectal cancer

Author/Year	Country	N	Stages	Staining	Cut-off	Magnification	Details
TGF-β							
SMAD4							
Oyanagi, H., et al. (2019)	Japan	201	I-IV	H&E	ITBCC criteria*	20X	*SMAD4* alteration significantly associated with T (p=0.027), N (p=0.037) and M category (p=0.028). The correlation was found with poor prognosis for relapse-free and OS (p=0.047; p=0.022 respectively).
Maspin							
Markl, B., et al. (2010).	Germany	153	I/II	maspin	10 buds	20X	Maspin cytoplasmic expression significantly associated with high tumour grade (p<0.01) while nuclear expression associated with TB (p<0.001).
Li, H., et al. (2017).	China	50	NA	NA[a]	10 buds	10X	Higher expression of *SERPINB5* (p=0.008) in tumour buds showed poorer prognosis in CRC patients.
RKIP							
Koelzer, V. H., et al. (2013).	Switzerland	220	III	Cytokeratin	10 buds	40X	Decrease expression of RKIP predicted metastasis disease (p=0.00307), vascular invasion (p=0.0506), loss of E-cadherin (p<0.001) and TB (p=0.0002). Loss RKIP reported more common in pMMR tumour (p=0.0191).
CDX2							
Slik, K., et al. (2019)	Sweden	209	II	H&E	7 buds	20X	Loss CDX2 associated with shorter DFS in MSS patient (p<0.001). No significant correlation found between CDX2 and TB (p=0.313).
MicroRNA							
Knudsen, K. N., et al. (2018).	Denmark	58	II&III	Cytokeratin	10 buds	20X	All miR-21 positive budding cells were stage III CRC with lymph node metastasis.
Moller, T., et al. (2019).	Denmark	7	NA	H&E	ITBCC criteria*	20X	Expression of miR21 co-expressed with TNF-α was frequently found in TB.
Wnt							
β-catenin							
Martensson, A., et al. (2007).	Sweden	77	Duke stages	B-catenin	10 buds	NA	β-catenin expression significantly associated with nuclear expression in tumour buds at the invasive margin (p<0.001). Nuclear B-catenin expression in TB also

							associated with tumour type (p=0.03) and growth pattern (p=0.02).
El-Gendi, S. and A. Al-Gendi (2011).	Egypt	142	NA	H&E /Cytokeratin	10 buds	20X	Nuclear β-catenin significantly higher in budding area at the invasive front compared to tumour center (p=0.001).
Knudsen, K. N., et al. (2017).	Denmark	58	II&III	Cytokeratin	10 buds	40X	Increase nuclear β-catenin in TB significantly found in MSS compared to MSI TB (p<0.001).
Laminin5γ2							
Shinto, E., et al. (2006).	Canada	73	NA	Cytokeratin	10 buds	20X	Reduce expression of laminin5γ2 reported in TB with dMMR tumour.
Knudsen, K. N., et al. (2017).	Denmark	58	II&III	Cytokeratin	10 buds	40X	High laminin 5γ2 expression frequently reported in TB, however, there was no significant difference between MSI and MSS tumour.
Zhou, B., et al. (2020).	China	176	I-IV	H&E /Cytokeratin	NA[a]	NA[a]	Expression of laminin 5γ2 in TB significantly higher than tumour center (p<0.01). A positive correlation found between TB and TNM stage (p<0.0001)), pathological grade (p=0.0404) in CRC. Patients with high number of TB had short survival time (p<0.0001).
C4.4							
Oshiro, R., et al. (2012).	Japan	213	I-IV	H&E	10 buds	20X	Positive C4.4A showed larger budding cells than negative C4.4A in both early and advance CRC stage.
Abi1							
Steinestel, K., et al. (2014).	Germany	56	I-IV	H&E	10 buds	25X	Overexpression of Abi1 associated with high TB (p=0.001) and infiltrating phenotype.
EphB3							
Jang, B. G., et al. (2020).	Republic of Korea	11	I-IV	EphB3	NA	NA	Expression of EphB3 significantly lower in budding cells compared to non-budding cells (p<0.001). Patients with high EphB3 expression showed a better clinical outcome in both OR (p = 0.007) and RFS (p < 0.001).
LGR5							
Sadek, S. A., et al. (2020).	Egypt	92	I-IV	H&E /Cytokeratin	10 buds	20X	LGR5 significantly associated with histological grade (p=0.01), lymph node metastasis (p=0.002), vascular invasion (p=0.02), TNM stage

							(p=0.000), dukes stage (p=0.000), TILs (p=0.03) and TB in both H&E and cytokeratin staining (p=0.003 and 0.001 respectively).
Programme cell death							
Caspase 3							
Dawson, H., et al. (2014).	Switzerland	188	I-IV	Cytokeratin	10 buds	40X	Caspase 3 expression lower in tumour buds compared to tumour centre and tumour front (p<0.001).
TrkB							
Dawson, H., et al. (2015).	Switzerland	211	I-IV	Cytokeratin	10 buds	40X	Overexpression of membranous and cytoplasmic TrkB in buds higher than main tumour (p<0.001), larger tumours (p=0.0236) and KRAS mutation (p<0.001). TrkB expression was significant, independent adverse prognostic factor (p=0.033; OR=1.79 (95% CI =1.05-3.05)).
Stem cells							
CD44							
Mohamed, S. Y., et al. (2019).	Egypt	70	I-IV	H&E	10 buds	20X	Positive CD44 highly correlated with high-grade TB (p<0.001), lymphovascular invasion (p<0.001), lymphocytic infiltrate (p<0.001), lymph node metastasis (p<0.001), AJCC stage (p<0.001).
Cell cycle/ proliferation							
PHH3							
Hacking, S., et al. (2020)	USA	234	I-IV	H&E	10 buds	20X	PHH3 expression paradoxically associated with TB. Moreover, patients with intact nuclear expression of MLH1, gene encode MMR, are likely to have higher TB when compared with patient with loss MLH1 expression.
KI67							
Dawson, H., et al. (2014).	Switzerland	188	I-IV	Cytokeratin	10 buds	40X	KI67 is absent in most tumour buds, however, KI67 positive buds was detrimental to survival in univariate (p=0.0352) and multivariate (p=0.0355) analysis.
van Wyk, H. C., et al. (2019).	UK	952	I-IV	H&E	10 buds	20X	No significant association found between TB grade and KI67 expression (p=0.285).
MET							
MACC1							

Koelzer, V. H., et al. (2015).	Switzerland	187	I-IV	Cytokeratin	10 buds	40X	Overexpression of MACC1 found in 55% of budding cells. Expression of MACC1 highly expressed in tumour front compared to tumour core (p=0.0012). High MACC1 expression predicted TB, pT and pN-stages, venous and lymphatic invasion and poor survival (p<0.05).

H&E = Haematoxylin and Eosin; OS = Overall Survival; DFS= Disease Free Survival; MSS = Microsatellite stable; pMMR= proficient mismatch repair; dMMR= deficient mismatch repair; AJCC stage = The American Joint Committee on Cancer Stage; TB = Tumour Budding; NA[a]= laser capture microdissection (LCM); NA = no information; *BD1: 0-4; BD2: 5-9; and BD3: ≥10

Table 2 Studies investigated the correlation between tumour immune microenvironment and TB.

Author/Year	Country	N	Stages	Staining	Cut-off	Magnification	Details
TILs							
Dawson, H., et al. (2019).	Switzerland	379	I-IV	H&E	ITBCC criteria*	20X	Decrease KM grade associated with tumour budding in either BD1,2,3 (p=0.206) or BD1+2 vs 3 (p=0.0048).
van Wyk, H. C., et al. (2019).	UK	952	I-IV	H&E	10 buds	20X	Weak KM grade significantly associated with high budding phenotype (p<0.001).
Chatzopoulos, K., et al. (2021)	Greece	170	I-IV	H&E	ITBCC criteria*	20X	High TILs in metastasis area showed the predictive favourable in patients OS (p=0.044). High number of TILs associated with overall mutational burden (p=0.0058), MMR genes (p=0.0069), RAS gene (p=0.0043) and less number of TB (p=0.050).
Zadka, L., et al. (2021).	Poland	94	I-IV	H&E	ITBCC criteria*	20X	There were no significant association between TB and TILs, however, increase TILs correlated with degree of CRC proliferation. Negative correlation was found between CRC stage (I-IV) and number of TILs.
Combined scores (TILs+TB)							
Dawson, H., et al. (2020).	Switzerland	345	I-IV	Cytokeratin	ITBCC criteria*	20X	Combined budding with CD8/CD3 performed better prognosis than budding or CD8/CD3 alone in predicting nodal metastasis (p<0.001, OR = 1.466, 95% CI=1.115-1.928). Increase buds/CD3 ratio significantly associated with poorer OS (p=0.012, hazard ratio = 1.218, 95% confidence interval = 1.044-1.419.

Gonzalez, I. A., et al. (2021).	USA	372	I-III	H&E	ITBCC criteria*	20X	Combined score of TILs and low TB associated with better RFS compared to cases without TILs and with intermediate/high TB (HR = 0.214, 95% CI = 0.109-0.421).
Lang-Schwarz, C., et al. (2018)	Germany	501	I-IV	H&E	ITBCC criteria*	20X	Combination of TILs and TB revealed highly significant different in OS between groups (p=0.001). Low TB/high TILs had longest survival, followed by high TB/high TILs and low TB/low TILs. HighTB/ low TILs group showed the worst OS. The score also significantly associated with disease metastasis during follow up (p=0.022).
Lang-Schwarz, C., et al. (2019).	Germany	576	I-IV	H&E	ITBCC criteria*	20X	Combined score of TB, TILs and gland formation stratified subgroup of CRC patients. Low score correlated with low tumour stages (p<0.001), less lymphatic vessel invasion (p<0.001) and less venous invasion (p<0.001).
Lang-Schwarz, C., et al. (2021).	Germany	205	II-III	H&E	ITBCC criteria*	20X	TB and TILs combination score could benefit for patient in II and III colon cancer in adjuvant treatment decisions. The significant different in OS between stage II colon cancer patient with low TB/high TILs and high budding/low TILs- group (mean OS: 153.17 vs 105.77 months, 95%CI: 141.16-165.18 vs 136.06-155.94, respectively, p=0.001). The same trend showed in patient with stage III between (low budding/high TILs-group and the high budding/low TILs-group (mean OS: 125.40 vs 89.34 months, 95%CI: 104.09-146.72 vs 62.46-116.22, respectively, p=0.036).
T cells							
Jang, T. J. (2013).	Republic of Korea	123	NA	E-cadherin	10 buds	NA	The ratio of CD8+ T cells in both tumour center and invasive area were correlated with gender, node metastasis and tumour budding (p<0.05).
Nearchou, I. P., et al. (2019).	UK	170	II	Cytokeratin	NA[b]	NA[b]	High tumour buds immunospatial index (TBISI) was associated with reduced DSS (p<0.001).
Fujiyoshi, K., et al. (2020).	USA	915	I-IV	H&E	ITBCC criteria*	20X	The number of tumour budding cells inversely correlated with CD3+CD8+ density (p<0.001; OR= 0.5; 95% CI 0.35-0.70) and CD3+CD8+CD45RO+ (p<0.001; OR= 0.44; 95% CI 0.31-0.63).
Macrophage							

Koelzer, V. H., et al. (2016).	Switzerland	205	I-IV	Cytokeratin	10 buds	40X	High number of CD68+ associated with less tumour budding ($p<0.01$).
Nearchou, I. P., et al. (2020).	UK	230	II	Cytokeratin	NA[b]	NA[b]	Weak correlation between TB and CD68+ CD163- ($r=0.15$). CD68+ CD163- density was correlated with CD3+ at the invasive area ($r=0.33$).
Zadka, L., et al. (2021).	Poland	94	I-IV	H&E	ITBCC criteria*	20X	The number of CD14+ cells associated with tumour budding ($p=0.0324$). In stroll area, CD14+ significantly associated CD3+ ($p=0.033$), CD4+ ($p<0.001$) but not with CD8+ ($p=0.68$) and CD45+ ($p=0.38$).
PD-L1							
Korehisa, S., et al. (2018).	Japan	499	I-IV	H&E	ITBCC criteria*	20X	PD-L1 expression level significantly correlated with adverse CRC features; poor differentiation, lymphatic & vascular invasion, early stage and high budding phenotype ($p<0.05$).
MHC-1							
Koelzer, V. H., et al. (2015).	Switzerland	220	I-IV	Cytokeratin	10 buds	40X	Positive MHC-1 at the invasive area predicted tumour with low budding ($p<0.001$). Triple-positive stain of MHC-1/CD8/TIA1 can predict early T-stage ($p=0.003$), absence of lymph node metastasis ($p=0.035$), lymphatic ($p=0.012$) and venous invasion ($p=0.006$). Loss MHC-1 frequently observed in KRAS-mutated, CD8+ CRC ($p=0.023$).
Fibroblast							
Shin, N., et al. (2019).	Republic of Korea	151	I-IV	H&E	10 buds	40X	TB was observed in almost half of both mature and immature fibroblast in the stroma area.

H&E = haematoxylin and eosin; NA[b] = automate analysis; r = Pearson test; OR= odd ratio; OS = Overall Survival; DSS = Disease Specific Survival;RFS = Recurrence-Free Survival; TILs = Tumour Infiltrating Lymphocytes; KM = Klintrup-Mäkinen; TB = Tumour Budding

*BD1: 0-4; BD2: 5-9; and BD3: ≥10