Systematic review of tumour budding and association with common mutations in patients with colorectal cancer

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Running Title: Tumour budding and its association with CRC mutations

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Highlights

- Meta-analysis evaluated the relationship between mutated *KRAS/BRAF* or MSI tumour and tumour budding in colorectal cancer patients
- 17 potential studies were included in this review
- Mutated *KRAS* and MSS/pMMR tumour significantly associated with high-grade budding phenotype

Abstract

Introduction: Despite a well-known prognostic role in colorectal cancer, the genomic profiling of tumour budding remains to be elucidated. We aim to review the association of common mutations with tumour budding.

Methods: A systematic review of studies relating to tumour budding and genetic mutation in CRC was performed. The relationship between mutational status and tumour budding was evaluated using meta-analysis.

Results: A total of 6153 patients from 17 articles were included. According to the meta-analysis, high-grade tumour budding was significantly associated with *KRAS* mutation (OR =1.52, 95%CI: 1.13-2.02, P=0.005) and MSS/pMMR (OR = 2.06, 95%CI: 1.42-2.97, P=0.0001).

Conclusion: The significant association between high-grade tumour budding and mutated *KRAS* or MSS/pMMR may suggest a role of these mutations in the development of the tumour budding phenotype and be useful for stratifying patient outcome in CRC.

Keywords: Tumour budding, *KRAF*/*BRAF* mutation, MSS/pMMR tumour, Systematic Review, Colorectal Cancer.

1. Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer and the second most lethal malignancy worldwide. In 2020, nearly 2 million new CRC cases were diagnosed and almost 1 million deaths from CRC were estimated (1). CRC is a biologically heterogeneous disease comprising of many genetic alterations, which are thought to initiate the early development of the adenoma. Mutations then accumulate in the adenoma and subsequently drive transformation into a carcinoma (2). Tumour-Nodes-Metastasis (TNM) staging is a useful tool for staging CRC patients and selecting them for a specific treatment, however, many patients experience variable outcomes within the same TNM stage due to the disease's heterogeneity (3). Biomarker discovery is an ongoing area of interest within CRC research. There is an increasing demand to identify molecular biomarker and improve patient's risk stratification for a better treatment decision for CRC patients, and some biomarkers are already used in current clinical studies (4, 5). However, studies are still required to validate future markers which could potentially improve the outcome for CRC patients (5).

Among the histopathological biomarkers studied to date, tumour budding (TB), the presence of a single cell or small cluster of up to 4 cells at the tumour invasive front, is perhaps the most promising prognostic marker in CRC as well as several other solid tumour types (6). According to the International Tumour Budding Consensus Conference (ITBCC) in 2016 (7), TB should be considered an independent prognostic marker and included in the pathological reporting of CRC to aid clinical decision making. The prognostic role of TB is well-established with a large body of evidence to support the significance of TB and its correlation with metastasis, recurrence and poor prognosis (8-10). Despite the increased interest in risk stratification of CRC by TB, the mechanisms underlying the budding phenotype are still unclear. One hypothesis is that TB could represent the Epithelial-Mesenchymal Transition (EMT), a reversible cellular process that transforms epithelial cells into mesenchymal cells, a key regulatory step in tumour progression and metastasis (11-13).

Therefore, it is of interest that many studies have reported a possible correlation of TB with genetic mutation in CRC, however, the results appear contradictory. Three biomarkers that are most frequently proposed are mutations in *KRAS* (Kristen rat sarcoma virus) and *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) as well as Microsatellite Instability (MSI). Approximately 70-85% of CRC develop through the Chromosomal instability (CIN) pathway and is characterised by mutations in *APC*, *KRAS* and the tumour suppressor *TP53* (14). Mutations in *KRAS* result in constitutive activation of the RAS-RAF-MEK-ERK pathway which regulates cell growth, differentiation, proliferation, and survival (15). Approximately 40% of CRC cases have *KRAS* mutations with codons 12,13 or 61, the most commonly mutated sites, and less frequently in codons 63, 117, 118 and 146 (16). RAS signalling has reported to be involved in the initiation of EMT in CRC leading to tumour invasion and metastasis (17). In addition to RAS signalling activation, mutated *BRAF,* a serine/threonine protein kinase, have been reported in about 10% of CRC patients with metastasis (18). The most frequently mutated site on the *BRAF* gene is at codon 600 within exon 15 (V600E). Although clinical data about *BRAF* mutations are limited, the available data suggests *BRAF* status as a promising prognosis marker in CRC (19).

Another important type of genetic alteration in CRC is MSI, which is observed in the early stages of adenoma development and also through progression towards malignancy (20). MSI tumours account for 15% of CRC and are known to arise through dysfunction of DNA mismatch repair (MMR). Deficiency in MMR (dMMR) leads to the accumulation of a high number of mutations, resulting in a hypermutated phenotype or MSI tumours (21). Tumours can be divided into three different types based on microsatellite status: MSI-high (MSI-H), MSI-low (MSI-L) and Microsatellite stable (MSS). Unlike MSI, MSS tumours are found in approximately 85% of CRC patients and are proficient in MMR (pMMR) (22). High levels of MSI are classified as MSI-H/dMMR and MSI-L tumours share similar molecular features to MSS/pMMR tumours, such as the loss of heterozygosity (LOH) of genes mutation and the relatively high degree of chromosomal instability (23-25)

The aim of this systematic review is to establish which genetic mutations are consistently associated with TB and, therefore, build a genetic profile for further work relating to a budding phenotype in patients with CRC.

2. Materials & Methods

2.1 Search strategy

An online literature search was performed between 19th April 2021 and 26th May 2021 to assess the role of tumour budding in colorectal cancer. The published literatures in the PubMed and Web of Science databases were filtered using the following keywords “tumour budding” or “tumor budding”, “Colorectal cancer” or “CRC” and “*KRAS*”, “*BRAF*”, “MSS”, “MSI”. The titles and abstracts of selected publications were used to determine the relevance of all searched publications which were carefully reviewed afterwards.

2.2 Inclusion and exclusion criteria

To be eligible for inclusion in the present review, the following criteria were used; (1) The manuscript examined the association of tumour budding with *KRAS*/*BRAF*, microsatellite status in patients with colorectal cancer, (2) The manuscript provided data sufficient to estimate odd ratios (ORs); (3). Only English language studies were included.

The following articles were excluded: (1) reviews, abstract, opinion and cases reports; (2) studies that collected data from treated CRC patients who had undergone radical or chemotherapy treatment before the collection, to eliminate factors that can induce a TB phenotype in CRC; (3) non-human studies; (4) in vitro studies

2.3 Data extraction

To reduce the bias and to improve the reliability, 3 reviewers (PH, JE, JQ) checked all relevant studies independently. Afterwards, the full texts were independently read and checked carefully. Data on the following characteristics were also extracted from each study: first author, year of publication, sample size, tumour budding status and mutational data. Finally, 17 articles were considered eligible for inclusion in this systematic review.

2.4 Statistical analysis

All statistical tests were performed using Review Manager 5.4 software. The association between TB and mutational genes evaluated by odd ratio (OR) with 95% confidence intervals (CIs). The number of cases with high and low TB were obtained directly from the paper or

calculated using the parameters provided in the manuscript. I^2 test was used to measure heterogeneity between each paper. I^2 <50% indicated no heterogenicity between studies as the random-effects model was used. P <0.05 was considered statistically significant

3. Results

3.1 Study Selection and Characteristic

The search revealed a total of 87 publications from the databases and subsequently 57 articles were excluded after review of titles and abstracts. The full text was evaluated for the remaining 30 papers. After review of the 30 papers, an additional three relevant studies were identified by manual references search, and 16 studies were excluded for the following reasons: four papers were reviews, five studies lack sufficient information, one was non-English, one was an animal study, and five studies did not exclude treated samples. Ultimately, 17 studies were included in the meta-analysis (Figure 1).

A total of 6153 patients were included for analysis, with study groups ranging from 80 - 952 patients. Eleven studies included less than 300 patients while the other six studies investigate more than 300 patients. The studies were published between 2003 and 2021. Most studies included patients with a variety of TNM stages (26-36) while two studies did not provide staging information (37, 38), three studies include stage I-III (39-41) and one study investigated stage II only (42). The studies overlapped according to the mutational status. Eleven studies reported the association between TB and *KRAS/BRAF* mutation and 13 studies examined the correlation between TB with microsatellite status in CRC.

3.2 Definitions of tumour budding

There was a lack of standardisation of TB assessment between studies. Fifteen studies assessed the budding phenotype in full sections whereas one study quantified TB in constructed Tissue Microarrays (TMAs). Thirteen studies utilised haematoxylin and eosin (H&E) staining, and cytokeratin staining were applied in four studies. The definition of TB was defined as an isolated cancer cell or cluster of cells (four studies), up to 5 cells (four studies), less than five cells (six studies) and up to 4 cells (three studies) at the invasive front of the tumour.

In addition to the stratification of budding status, three studies used ROC curves (38, 39, 41) and one study used median scores (27). Other studies identified high TB if there were >1 budding foci (42), >5 budding foci (29, 32, 37), >6 budding foci (26, 40), >10 budding foci (28, 30, 32). Four studies, conduct after 2018, quantified the number of buds as low, medium, and high according to the ITBCC 2016 criteria (34-36, 43).

3.3 Tumour budding and *KRAS*/*BRAF* mutation status in CRC

KRAS mutation

Nine studies were evaluated in which 3216 patients were included (Table 1). High and low budding was defined by either differently generated cut-off point by ROC curves (38, 39), median (27), 5 buds (29), 6 buds (40), 10 buds (28, 30, 32) or ITBCC 2016 criteria (35). The total events, according to the meta-analysis, showed a significant correlation between high-grade TB and *KRAS* mutation (OR =1.52, 95%CI: 1.13-2.02, P=0.005) (Figure 2). Moderate heterogeneity was detected using the random-effect model, with I^2= 58% (Figure 2). It is noted that two studies from Lugli et al. and Zlobec et al. included only CRC stage I-III (39, 40), and one study conducted in 2007 by Zlobec et al. did not report the CRC stage (38).

BRAF mutation

A total of 2735 patients were investigated for association of TB and *BRAF* mutation (Table1). Budding phenotype were classified as high and low using median (27), 5 buds (29, 31), 6 buds (40), 10 buds (28) and ITBCC 2016 criteria (35, 41) as a cut-off point. The results showed that, in relation to *BRAF* mutation, there was no significant association between high-grade TB and mutated *BRAF* with OR 1.11 (95% CI: 0.66-1.89, P=0.69) and substantial heterogeneity was shown across the studies (I^2= 63%) (Figure 3).

3.4 The association between tumour budding and microsatellite status

Thirteen potential studies (3935 patients) qualified for the meta-analysis to assess the potential link between TB and microsatellite status in CRC. The detailed characteristics of these studies are shown in Table 2. High and low budding were defined using ROC curves (39, 41), 1 bud (42), 5 buds (31, 37), 6 buds (26, 40), 10 buds (28, 30) and ITBCC 2016 criteria (33-36). Lugli et al. used a constructed TMA with areas representative of intense TB, as determined from the corresponding slides (26), while others used full CRC sections. Most studies reported stage I-IV CRC, four studies reported stage I-III (39-42), and one study from Jass et al. did not report tumour stage (37). However, despite this variety, the meta-analysis showed a statistically significant association between high-grade TB and MSS/pMMR status (OR = 2.06, 95%CI: 1.42-2.97,

P=0.0001), and the heterogeneity across studies was substantial in the random-effect model (I^2= 66%) (Figure 4).

4. Discussion

Metastasis is the major cause of CRC related death with multiple factors including genetic mutations and dysregulation of signalling pathways modulating the metastatic route in patients (44, 45). *KRAS/BRAF* mutations lead to the aberrant activation of the MEK–ERK pathway causing tumour development, progression and drug resistance in CRC (46). It is becoming clear that mutated *KRAS* and *BRAF* are involved in metastatic CRC and are associated with a worse outcome in CRC (47, 48). The systematic study from Popat et al. also demonstrated that patients with MSI CRC showed better survival outcomes when compared to MSS (49). CRC patients who exhibit *KRAS* or *BRAF* mutation and MSS/pMMR tumour represented the poorest prognosis group (50, 51). Despite the widely accepted of TB as a marker of poor prognosis which strongly predicts disease recurrence and metastatic progression in CRC (10, 43), the correlation of these mutations with TB is not fully understood.

The results presented in our meta-analysis showed a significant correlation between high-grade TB, mutated *KRAS* and MSS/pMMR tumour, suggesting a predictive role of genetic alterations in the high-grade budding phenotype in CRC. There was no significant association between TB and *BRAF* mutation. This is in line with some studies that report mutations in *KRAS* and *BRAF* are mutually exclusive in CRC (52, 53). When *BRAF* and *KRAS* mutations co-occur, it is possible that *KRAS* is the driver mutation, and that TB acquires *BRAF* at a later stage. If this was the case, then *KRAS* but not *BRAF* would be found in early-stage CRC and may lead to better treatment options in patients with CRC. Therefore, further prospective TB work, including transcriptomic and proteomic profiles of high- and low-grade budding phenotypes, is required to tease out these relationships in patients with early-stage CRC.

The present systematic review has several limitations. First, this systematic review protocol should be registered to an approved international database to avoid the duplicated works. However, the authors have carefully reviewed all the publications regarding the genetic alterations associated with TB. To our knowledge, none of them demonstrated the meta-analysis of TB with CRC mutations. Secondly, there are only a small number of studies that investigated a correlation between other CRC mutations and TB, therefore, this meta-analysis focused only on *KRAS*, *BRAF* and microsatellite status where there was a relatively larger number of studies. Another caveat was that there was no uniform methodology of TB assessment across all the studies. Most of the eligible

studies in the present review assessed TB phenotype using standard H&E section staining. Yamadera et al. compared the use of cytokeratin immunohistochemical staining for TB quantification with standard H&E staining, as high levels of inflammation can sometimes obscure TB; despite promising results with the cytokeratin staining, further studies are required (54). Moreover, it should be noted that there are differences in budding assessment due to the lack of standard criteria. The different cut-off points used to determine high and low budding adds a further level of complexity when comparing different studies and might result in contradictory findings. To address this, the ITBCC 2016 has published an agreed standard criteria for the assessment of TB (7). In this review, however, 13 studies were conducted before 2017, therefore the standard criteria were not applied, and this should be taken into consideration. A comprehensive study that investigates the relationship of TB, assessed using the ITBCC 2016 criteria, with gene mutations and cell signalling should be conducted.

5. Conclusion

The present meta-analysis revealed an association between common mutations in CRC and TB. Although a variety of budding assessments were used, a consistent association between TB and *KRAS,* as well as TB and MSS/pMMR mutations, were found. Local tumour aggressiveness may depend on the complex interplay of multiple tumour-specific aberrations that occur not only on a genetic level but also at the mRNA and protein levels and this warrants further study. Such studies could potentially lead to a clearer understanding of the mechanism behind TB in CRC.

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

Acknowledgements We thank Dr Gerard (University of Glasgow) for comments and edited language on the manuscript.

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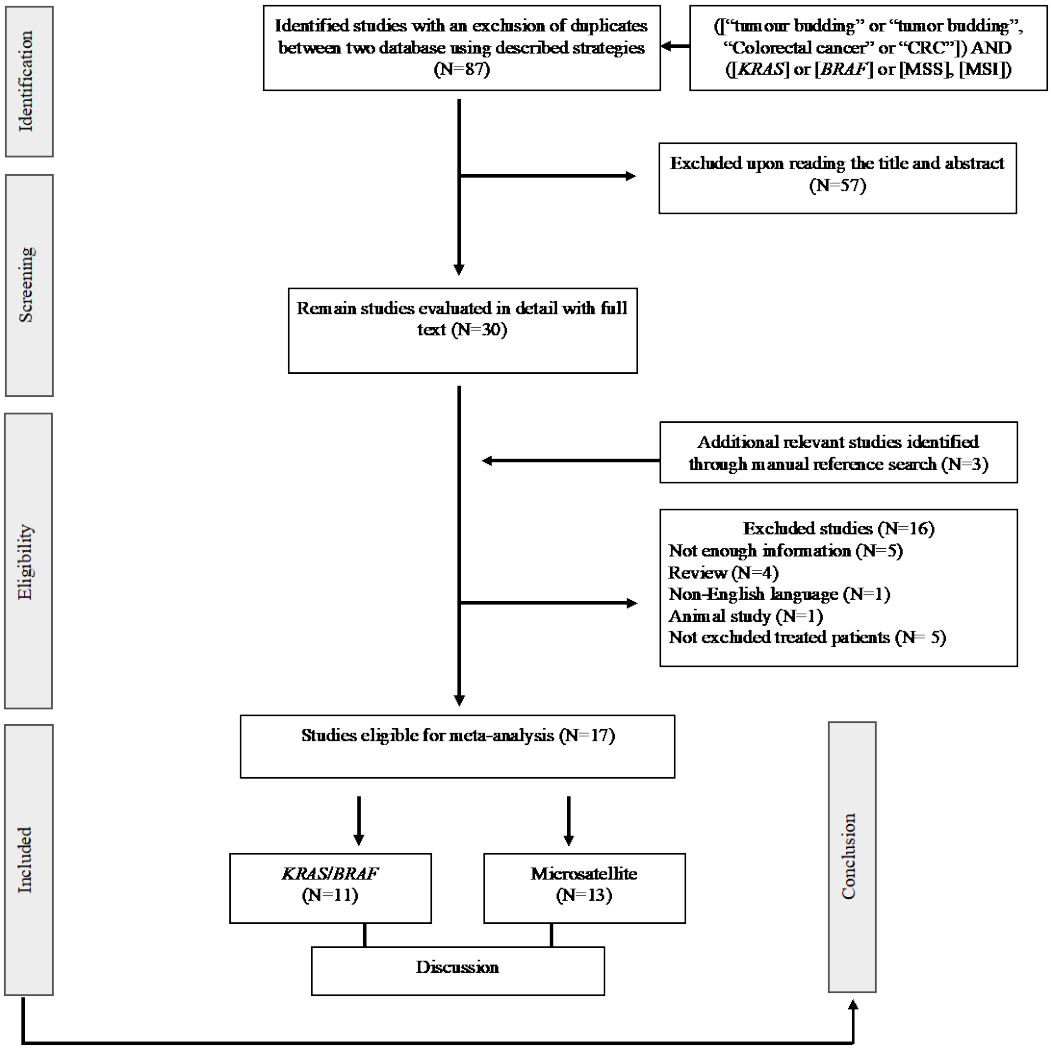

Figure 1 Consorted diagram for the selection of studies. A total of 17 studies were selected to determine the association between tumour budding and mutations in CRC.

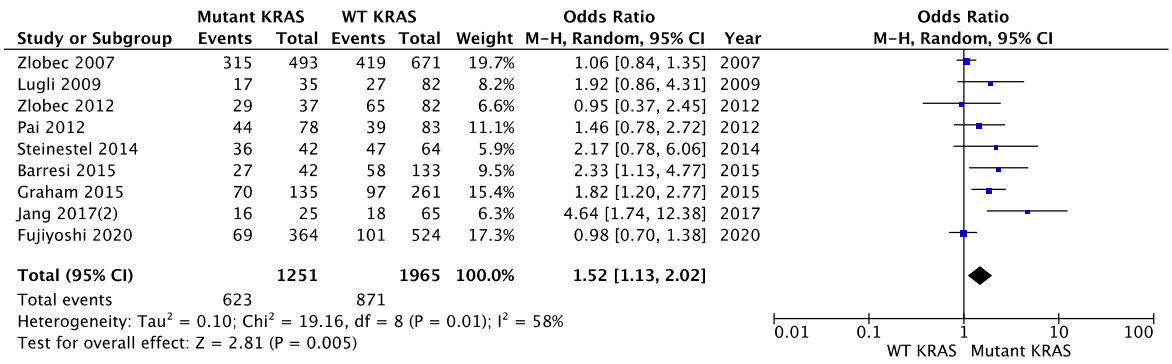

Figure 2 The association between tumour budding and *KRAS* mutation in colorectal cancer patient.

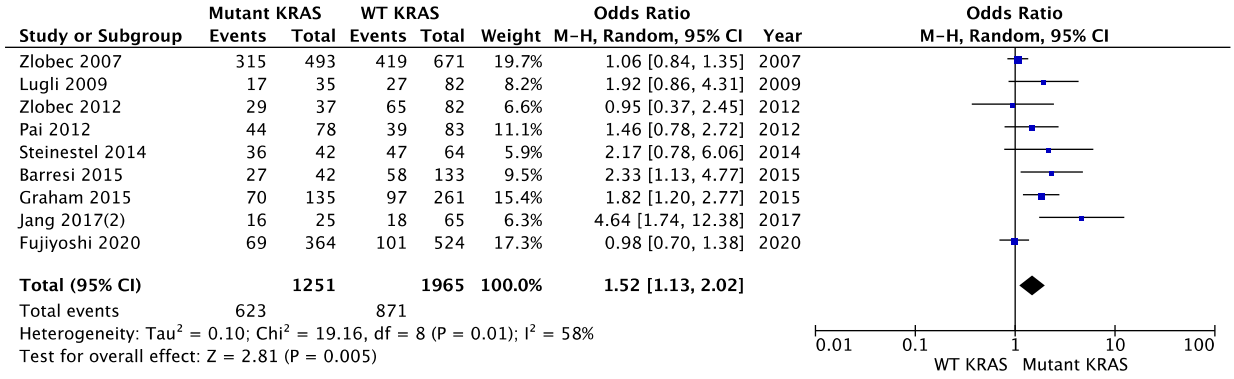

Figure 3 The association between tumour budding and *BRAF*mutation in colorectal cancer patient.

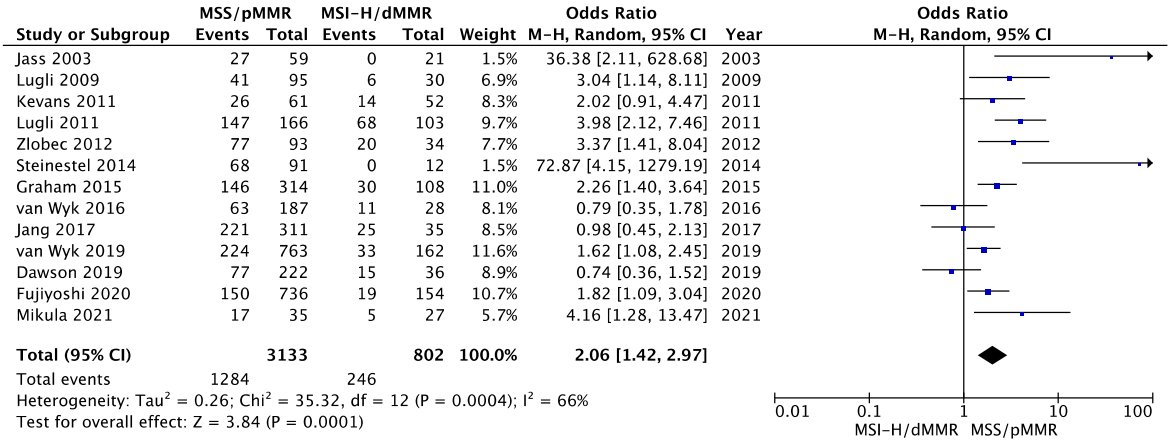

Figure 4 The association between tumour budding and microsatellite status in colorectal cancer patient.

Table legends:

Table 1 Studies investigated the correlation between tumour budding and *KRAS*/*BRAF* mutation in colorectal cancer.

Author/Year	Country	*N*	Mutation	Stages	Staining	Cut-off	Magnification
Zlobec, I., et al. (2007).	Canada	1164	*KRAS*	NA	H&E	ROC analysis	NA
Lugli, A., et al. (2009)	Switzerland	279	*KRAS*	I-III	Cytokeratin	ROC analysis	40X
Pai, R. K., et al. (2012).	USA	181	*KRAS* and *BRAF*	I-IV	H&E	median	20X
Zlobec, I., et al. (2012).	Switzerland	127	*KRAS* and *BRAF*	I-III	Cytokeratin	6 buds	40X
Steinestel, K., et al. (2014).	Germany	117	*KRAS* and *BRAF*	I-IV	Cytokeratin	10 buds	20X
Barresi, V., et al (2015).	Italy	175	*KRAS* and *BRAF*	I-IV	H&E	5 buds	20X
Graham, R. P., et al.(2015)	USA	553	*KRAS*	I-IV	H&E	10 buds	20X
Jang, S., et al. (2017).	Republic of Korea	90	*KRAS*	I-IV	H&E	10 buds	20X
Jang, M. H., et al. (2017).	Republic of Korea	349	*BRAF*	I-IV	H&E	5 buds	20X
van Wyk, H. C., et al. (2019)	UK	952	*BRAF*	I-IV	H&E	ITBCC criteria*	20X
Fujiyoshi, K., et al. (2020).	USA	915	*KRAS* and *BRAF*	I-IV	H&E	ITBCC criteria*	20X

H&E; Haematoxylin and Eosin
**ITBCC*; International Budding Consensus Conference (BD1: 0-4; BD2: 5-9; and BD3: ≥10)

Table 2 Study characteristic and access of tumour budding in relation with microsatellite status in colorectal cancer.

Author/Year	Country	*N*	Subtype	Stages	Staining	Cut-off	Magnification
Jass, J. R., et al. (2003)	Canada	80	MSI/MSS	NA	H&E	5 buds	40X
Lugli, A., et al. (2009)	Switzerland	279	MSI/MSS	I-III	Cytokeratin	ROC analysis	40X
Kevans, D., et al. (2011).	Ireland	122	MSI/MSS	II	H&E	1 bud	20X
Lugli, A., et al. (2011).	Switzerland	289	dMMR/ pMMR	I-IV	Cytokeratin	6 buds	40X
Zlobec, I., et al. (2012).	Switzerland	127	MSI/MSS	I-III	Cytokeratin	6 buds	40X
Steinestel, K., et al. (2014).	Germany	117	dMMR/ pMMR	I-IV	Cytokeratin	10 buds	20X
Graham, R. P., et al. (2015)	USA	553	MSI/MSS	I-IV	H&E	10 buds	20X
van Wyk, H. C., et al. (2016).	UK	303	dMMR/ pMMR	I-III	H&E	ROC analysis	20X
Jang, M. H., et al. (2017).	Republic of Korea	349	MSI/MSS	I-IV	H&E	5 buds	20X
Dawson, H., et al. (2019).	Switzerland	376	dMMR/ pMMR	I-IV	H&E	ITBCC criteria*	20X
van Wyk, H. C., et al. (2019)	UK	952	dMMR/ pMMR	I-IV	H&E	ITBCC criteria*	20X
Fujiyoshi, K., et al. (2020).	USA	915	MSI/MSS	I-IV	H&E	ITBCC criteria*	20X
Mikula, M., et al. (2021).	USA	81	dMMR/ pMMR	I-IV	H&E	ITBCC criteria*	20X

H&E; Haematoxylin and Eosin

**ITBCC*; International Budding Consensus Conference (BD1: 0-4; BD2: 5-9; and BD3: ≥10)