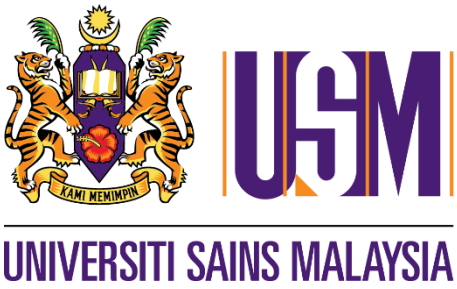
PERITUMOURAL BUDDING IN COLORECTAL CARCINOMA, ITS ASSOCIATED FACTORS AND THE POTENTIAL AS AN INDEPENDENT HISTOPATHOLOGICAL PARAMETER PREDICTOR

DR DAHZIELA BINTI HAJI YUNUS

Dissertation Submitted in Partial Fulfilment of The Requirement For the Degree of Master of Pathology (Anatomic Pathology)



UNIVERSITI SAINS MALAYSIA

2023

ACKNOWLEDGEMENT

First, praises and thanks to Allah, the Almighty, for his blessings throughout my research work to complete the thesis. I owe a deep sense of gratitude to my supervisor and co-supervisors, Dr Seoparjoo Azmel Mohd Isa and Dr Nur Asyilla Che Jalil from Pathology Department, Universiti Sains Malaysia for providing endless guidance throughout this work. Their constant counsel, scholarly commentary and scientific approach have greatly aided me to accomplish this task. A special thanks go out to Dr Suhaily Mohd Hairon from Community Medicine Department for guiding me on the statistical analysis. It was a real honour and privilege to work under their direction. My family, especially my parents Yunus Masir and Dg Bongsu Abdullah are much appreciated for their love, understanding, prayers, and wavering support. In addition, I want to thank all my colleagues and batchmates at Universiti Sains Malaysia, thank you for their encouragement and support, which have strengthened me throughout the journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AJCC : American Joint Committee on Cancer

BD : Budding

BRAF : B-type Raf

CAP : College of American Pathologists

CEA : Carcinoembryonic

CRC : Colorectal cancer

CRM : Circumferential resection margin

EMT : Epithelial-mesenchymal transition

FN : Field number

H&E : Haematoxylin and eosin

HPE : Histopathological examination

H-PTB : High-risk peritumoural budding

HUSM : Hospital Universiti Sains Malaysia

IDEA : International Duration Evaluation of Adjuvant

iFOBT : Immunological Faecal Occult Blood Test

ITB : Intratumoural budding

ITBCC : International Tumour Budding Consensus Conference

KRAS : Kirsten rat sarcoma

LIS : Lab information system

L-PTB : Low-risk peritumoural budding

LVI : Lymphovascular invasion

MOH : Ministry of health

MSI : Microsatellite instability

NCCN : National Comprehensive Cancer Network

NRAS : Neuroblastoma rat sarcoma

PDC : Poorly differentiated cluster

PNI : Perineural invasion

PTB : Peritumoural budding

RCPA : Royal college of Pathologists of Australasia

SD : Standard deviation

SPSS : Statistical Package for Social Sciences

TB : Tumour budding

TME : Tumour microenvironment

TNM : Tumour-node-metastasis

USM : Universiti Sains Malaysia

WHO : World Health Organization

ABSTRAK

Latar Belakang: Sepanjang tiga dekad yang lalu, tunas tumor (TT) telah menjadi faktor prognosis tambahan yang mantap dalam kanser usus besar kerana ia diketahui kesan buruk kepada pesakit. Matlamat utama kajian ini adalah untuk menilai TT dalam kanser usus besar dan faktor-faktor yang berkaitan dengannya. Penilaian ini mungkin bermanfaat untuk stratifikasi pesakit pasca operasi secara serentak mengukuhkan nilai TT dan pelaksanaan laporan patologi rutin.

Kaedah: Kajian retrospektif keratan rentas ke atas kanser usus besar primer yang telah dibuang melalui pembedahan. Kajian ini meneliti 162 slaid arkib pewarnaan haematoxylin dan eosin secara mikroskopik. TT telah dinilai dan dikategorikan kepada tunas berisiko rendah dan berisiko tinggi. Perkaitan antara TT dengan pemboleh ubah klinikopatologinya dianalisis secara statistik dengan melakukan model regresi logistik menggunakan SPSS.

Keputusan: TT diperhatikan dalam kebanyakan pesakit (87.7%). Tunas berisiko tinggi dilihat dalam 41 kes (25.3%) manakala tunas berisiko rendah adalah dalam 121 kes (74.7%). Pada regresi logistik ringkas, terdapat perkaitan yang signifikan bagi tunas tumor berisiko tinggi dengan ciri klinikopatologi yang buruk termasuk tahap CEA yang tidak normal, peringkat TNM yang lebih tinggi (III, IV), peringkat pN yang lebih tinggi, serangan limfa dan vaskular, serangan saraf, kanser sekunder dalam nodus limfa dan perulangan kanser (semua ciri mempunyai nilai $P < 0.05$). Regresi logistik berbilang menunjukkan hanya tahap CEA yang tidak normal, peringkat TNM III, serangan saraf dan perulangan kanser dikekalkan dan secara statistik ketara kepada ramalan, $p < 0.05$. Model ini dikelaskan dengan betul dalam 79% kes dan ujian Hosmer Lemeshow adalah 0.246.

Kesimpulan: Penilaian TT adalah perlu dan boleh digunakan sebagai parameter histopatologi prognostik yang bermanfaat untuk pesakit dari segi susulan yang berkesan dan kemoterapi tambahan selepas pembedahan.

ABSTRACT

***Background*:** Throughout the past three decades, tumour budding has become a well-established additional prognostic factor in colorectal carcinoma as it's known adverse effects on patients. The primary aim of this study is to evaluate peritumoural budding (PTB) in colorectal cancer and its associated factors. This evaluation may be beneficial for post-operative patient stratification simultaneously cementing the tumour budding values and its routine pathological report implementation.

***Methods*:** A cross-sectional retrospective study on surgically resected primary colorectal cancer. This study examined 162 haematoxylin and eosin-stained archived slides microscopically. PTB was evaluated and categorized into low-risk and high-risk budding. The association between PTB with its clinicopathological variables were statistically analysed by performing logistic regression models using SPSS.

***Results*:** PTB was observed in most patients (87.7%). High-risk budding was seen in 41 cases (25.3%) meanwhile low-risk budding was in 121 cases (74.7%). On univariable logistic regression, there is a significant association of high-risk tumour budding with adverse clinicopathological characteristics including abnormal CEA level, late TNM stage (III, IV), higher pN stage, lymphovascular invasion, perineural invasion, lymph node metastasis and recurrence (all variables have P-value <0.05). Multivariable logistic regression shows only abnormal CEA level, TNM stage III, perineural invasion and recurrence were retained and statistically significantly to the prediction, $p < 0.05$. The model is correctly classified in 79% of cases with the Hosmer Lemeshow test: 0.246.

***Conclusion*:** PTB evaluation is necessary and applicable as a prognostic histopathological parameter which is beneficial for patients in terms of efficacious follow-up and decision of postoperative adjuvant chemotherapy.

Keywords: Colorectal carcinoma, tumour budding, high-risk budding, recurrence, prognosis.

CHAPTER 1: INTRODUCTION

1.1 Epidemiology of colorectal cancer

Worldwide, an estimated 19.3 million new cancer cases and 10.0 million deaths due to cancer occurred in 2020. More than 1.9 million (11.4%) new cases of colorectal cancer (CRC) and 935,000 (10.0%) deaths are estimated to occur in 2020. Generally, colorectal cancer ranks third in terms of incidence but second in terms of mortality. It is the third most common cancer in men and the second most common cancer in women. The highest rate of colon cancer occurred in European regions, Australia/New Zealand, and Northern America, with Hungary and Norway ranking first in men and women, respectively. Malaysia is part of Southeast Asia, which ranks third in Asia-Pacific for colon cancer and third for rectal cancer (1).

In Malaysia, 48 639 new colorectal cases and 29 530 deaths were reported in 2020. Colorectal cancer is the second most common cancer in both sexes, with an incidence rate of 19.6 per 100,000 people and a fatality rate of 13.5 per 100,000 people. Males had 1.18 times higher age-adjusted incidence rate of colorectal cancer than females (2). Colorectal cancer prevalence rises with age in both males and females. For both sexes, Chinese ethnicity has the highest incidence rate. According to the National Cancer Registry, between 2008 to 2013, the mean age for colorectal cancer was 61.6 years (standard deviation of 12.7). Colorectal cancer staging was mostly detected at a late stage (III and IV), accounting for more than 70% of cases in both sexes (3–5).

1.2 An overview of colorectal cancer

Based on the 5th edition of the WHO classification of digestive system tumours, colorectal cancer is defined as a malignant epithelial tumour originating in the large bowel and showing glandular or mucinous differentiation The majority of CRCs are found on the left side of the colon and rectum. Commonly, the patients presented with altered bowel habits, anaemia, haematochezia, abdominal pain, and weight loss (6).

Many risk factors have been implicated in the pathogenesis of colorectal cancer. Any person with a family history of colorectal cancer has a significantly higher risk (6). A personal history of having cancer or a history of colon polyps, inflammatory bowel diseases, diabetes mellitus, or cholecystectomy also increased the risk of CRC. Lifestyle also has an important role, such as a dietary pattern that includes a high intake of red or processed meat, a low intake of fibre, fruit and vegetables, and a low intake of calcium, vitamin D and dairy products. In lifestyle also, increase body fatness, sedentary life, smoking, and alcohol intake are much involved in the risk of CRC development. Other least factors associated with colorectal cancer are age, gender, race, gut microbiota, and socioeconomic factors (6–8).

According to Malaysia Registry, there are 22.3% of CRC patients had diabetes mellitus, 6.4% of patients with positive family history and commonly arises from the left side, specifically the rectum and the rectosigmoid as the most common sites (4). In the United States of America, CRC screening is mostly opportunistic, with some organised screening (6). Malaysia has an opportunistic screening programme using an immunological Faecal Occult Blood Test (iFOBT) followed by a colonoscopy (9).

A colonoscopy can help detect CRC precursor lesions such as a suspicious mass with central ulceration or a raised edge. A biopsy will be taken and sent for histopathological examination. It was offered to asymptomatic men and women aged 50–75 and was initiated in 2014 by the Malaysia Ministry of Health (MOH) with 2-year screening intervals. In 2020, a total of 598 health clinics under the MOH provided the service. The number of people screened by health clinics each year was less than 1% of the total eligible population in the country. Out of those screened, only 60% of positive iFOBT cases referred for colonoscopy underwent the procedure (9,10).

For the treatment approach, the first line of treatment for CRC is surgical resection. Polypectomy, endoscopic mucosal resection, or submucosal resection can also be performed for the therapeutic removal of the early lesion. Adjuvant chemotherapy post-resection or neoadjuvant chemotherapy and radiotherapy may also be given (6).

1.3 Colorectal cancer prognostic and predictive biomarkers

CRC is primarily adenocarcinoma (90%) and has several histological subtypes that can be differentiated according to their clinical and molecular characterization. It can be graded based on gland formation: low-grade (well to moderately differentiated) and high-grade (poorly differentiated). Some important histological features also need to be included in the reporting of CRC as they have value for prognostication and prediction, which are involved in CRC management (6).

Some of the few important prognostic factors are carcinoembryonic (CEA) level, tumour regression score, circumferential resection margin (CRM), lymphovascular invasion (LVI), perineural invasion (PNI), microsatellite instability (MSI), Kirsten rat sarcoma (KRAS), neuroblastoma rat sarcoma (NRAS), and B-type Raf (BRAF) mutation

status (11). According to clinical practice guidelines for colorectal cancer in Malaysia, some prognostic factors concerning 5-year survival in surgically resected CRC are local invasion, the total number of lymph nodes retrieved, the total number of lymph node metastasis, extramural vascular invasion, peritoneal involvement, tumour perforation, the distance of invasion beyond the muscularis propria, and CRM involvement (12). In addition, the prognostic factors for local recurrence in rectal carcinoma are as follows: location below the peritoneal reflection and completeness of the plane of mesorecta excision, histologic grade of regression after preoperative therapy, CRM, and distal resection margin (12).

The clinical pathological stage is still the most significant independent prognostic factor in CRC, whether classified as the Astler and Coller system or the Tumour-node-metastasis (TNM) system for patient stratification. However, given the heterogeneity of the disease in most cancers, optimization of treatment is based on the outcome approach, which also gives insight to the clinician about the biology and history of the disease (11). A few additional prognostic biomarkers may have a role in CRC, and one of them is the presence of tumour budding, which has recently been incorporated into Western protocols for CRC reporting such as the College of American Pathologists (CAP) and Royal College of Pathologists of Australasia (RCPA) protocol for the handling of the colorectal specimen. The guidelines recommended reporting on tumour budding, especially in pT1 and stage II CRC (13,14).

1.4 Overview of tumour budding in colorectal cancer

Tumour budding is a histological manifestation of initiating invasion and metastasis features at the tumour-invasive front in the tumour microenvironment (TME) (15) (Figure

1.1). It represents the CRC epithelial-mesenchymal transition (EMT), in which the cells gain invasiveness and migratory capacity as a result of basement membrane loss and poorly developed or absent desmosomes or junctional complexes (16–18). Cells that undergo EMT also exhibit vigorous invasion, metastasis, and chemoresistance (19).

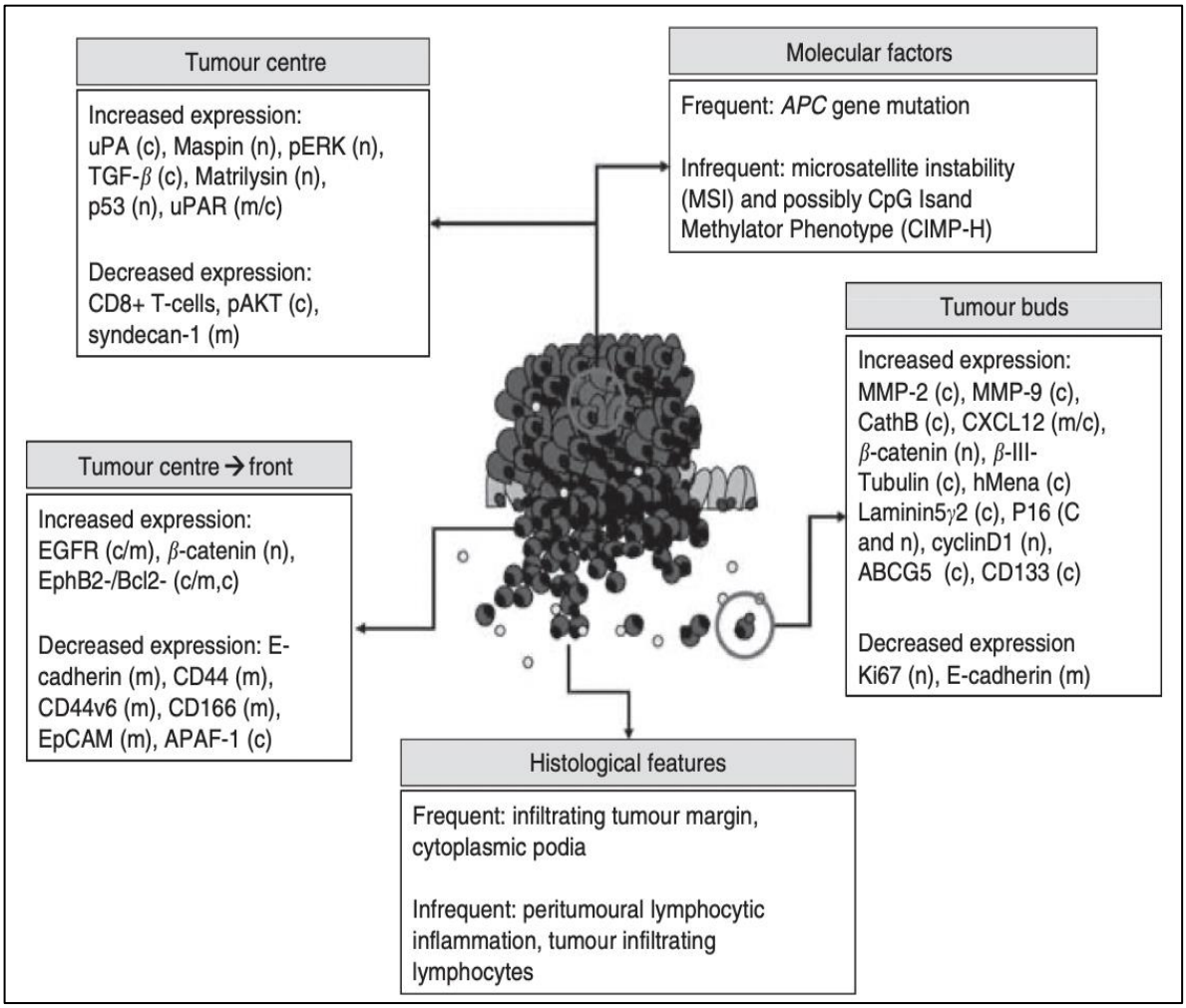


Figure 1.1: An overview of the histomorphology and molecular features of the tumour centre, invasive front, and tumour buds in CRC. Reproduce from Tumour budding in colorectal cancer: Molecular rationale for clinical translation (15).

In 2016, the International Tumour Budding Consensus Conference (ITBCC) was held to standardise tumour budding reporting. In this conference, tumour budding is defined as a single tumour cell or a cell cluster consisting of four tumour cells or less at the invasive margin of CRC. Tumour budding is further stratified into peritumoral budding (PTB, tumour buds at the tumour front) and intratumoral budding (ITB, tumour buds in the tumour centre). As four tumour cells are the most widely used cut-off for tumour budding, and this cut-off distinguishes tumour budding from the novel histopathological parameter 'poorly differentiated cluster (PDC)', which is defined as five or more cells (20). ITBCC was also able to achieve a consensus of ten statements, as depicted as follows (20):

1. Tumour budding is defined as a single tumour cell or a cell cluster consisting of four tumour cells or fewer.
2. Tumour budding is an independent predictor of lymph node metastases in pT1 colorectal cancer.
3. Tumour budding is an independent predictor of survival in stage II colorectal cancer.
4. Tumour budding should be considered along with other clinicopathological features in a multidisciplinary setting.
5. Tumour budding is counted on H&E.
6. Intratumorally budding exists in colorectal cancer and has been shown to be related to lymph node metastasis.

7. Tumour budding is assessed in one hotspot (in a field measuring 0.785 mm^2) at the invasive front.
8. A three-tier system should be used along with the budding count to facilitate risk stratification in colorectal cancer.
9. Tumour budding and tumour grade are not the same.
10. Tumour budding should be included in guidelines/protocols for colorectal cancer reporting.

Initially, tumour budding was reported by Japanese researchers. First was Hase *et al*., who defined tumour budding as a cluster of less than 5 cancer cells and graded them into two groups: none/ mild, moderate/ severe at the invasive front (21). Then in 2002, Ueno *et al.* stated that tumour budding foci are an isolated single cancer cell or a cluster composed of fewer than 5 cancer cells; a count of 0 to 9 per field was considered to be low grade, and a count of 10 or more was regarded as high grade (22). Meanwhile, Nakamura *et al*. stated that tumour budding is defined as small clusters of undifferentiated cancer cells or microtubular cancer nests at the invasive margin, and further classified into 3 categories as follows: mild, less than 1/3 of the entire invasive margin; moderate, 1/3 to 2/3 of the entire invasive margin; marked, more than 2/3 of the entire invasive margin (23) (Figure 1.2).

All of the studies mentioned above used only the haematoxylin and eosin (H&E) staining method; however, a few studies are also using additional cytokeratin immunohistochemistry to identify tumour budding (24,25). Despite using cytokeratin for easy detection of tumour budding, it did not affect the 5-year survival of the patient nor improve interobserver agreement (26). However, cytokeratin stain is recommended for use whenever possible (24–27).

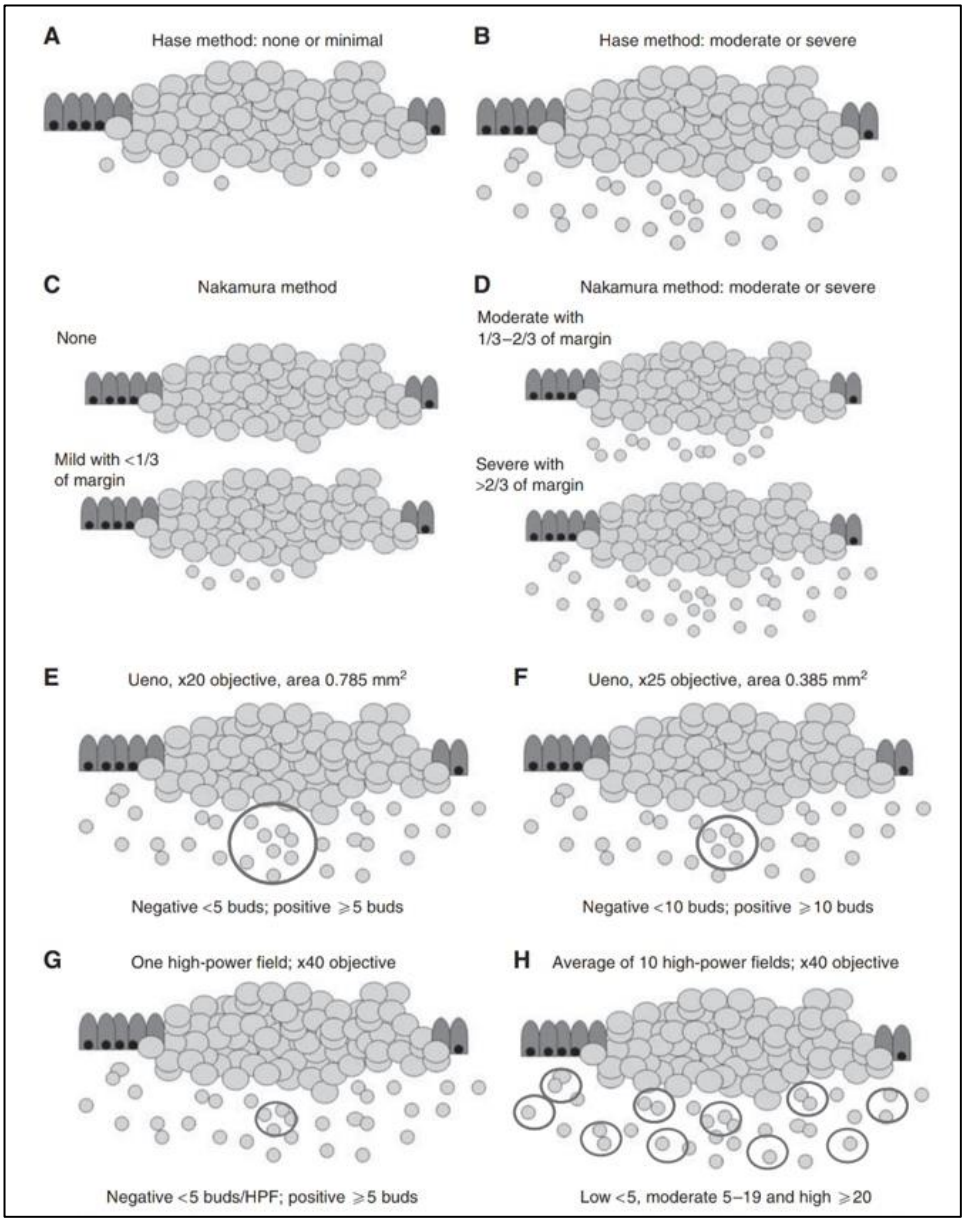


Figure 1.2: Proposed tumour budding scoring systems according to Hase *et al*. (A, B), Nakamura *et al*. (C, D), Ueno *et al*. (E, F), one high-power field (G) and 10 high-power

fields (H) average. Reproduce from Tumour budding: a promising parameter in colorectal cancer (23).

1.5 Role of tumour budding as a biomarker in colorectal cancer

Tumour budding has a role in emerging prognostic factors in CRC. Multiple studies have shown that tumour budding has clinical significance in CRC as an independent risk factor associated with an adverse outcome (20). Tumour budding in colorectal cancer has an impact, according to a 2016 review of systematic reviews and meta-analyses, as it is strongly predictive of lymph node metastases, recurrence, and cancer death at 5 years (28). However, due to the lack of standardisation of tumour budding reporting, it has not yet been incorporated into the TNM staging of CRC. This part includes the disagreement in its definition, identification, and method of reporting. Another difficulty is introducing tumour budding into clinical practice of which method to use for all stages of CRC since different studies used different methods for specific stages (28).

It has been decades, and tumour budding is already well-established and known as a predictor of lymph node metastasis, recurrence, and a poor survival rate (28). One of the earliest studies in 1989 by Morodomi *et al.* in Kurume, Japan, stated that budding was concentrated mainly in the actively invasive region of cancer and that if budding was observed, the possibility of lymph node metastasis should be considered in rectal cancers (29). Meanwhile, Hase *et al.* revealed that rectal adenocarcinoma with marked budding has a high incidence of recurrence and a low five-year survival rate (21).

Subsequently, multiple cross-sectional studies were done to substantiate tumour budding as an independent prognostic factor, and these were summarised in a systematic review in 2015, which stated that "tumour budding should therefore be considered a promising and strong prognostic factor in colorectal cancer, thus its routine implementation will depend on a selected, internationally accepted assessment system" (30). In addition, tumour budding is also associated with other histopathological factors known to have a worse prognosis, such as higher TNM stage, higher tumour grade, depth of invasion, infiltrating tumour border, the presence of lymphovascular, perineural invasion, recurrence, and distant metastasis (21,29,31–36)

The tumour budding's clinical significance was applied to a variety of clinical scenarios, including early-stage CRC, stage II CRC, and intratumorally budding in the preoperative biopsy. These scenarios were mentioned in the ITBCC and systematic review as they have potentially aided in the management of CRC patients (20,30). In CRC with pT1, the presence of tumour budding has a predictive value for the pathological risk of LNM. Hence, in patients with endoscopically resected pT1 CRC, further surgical resection is beneficial (37).

According to the National Comprehensive Cancer Network (NCCN) guideline for colon cancer 2018, high-risk features in stage II only include positive or close surgical margin, unknown surgical margin, high grade, angiolymphatic invasion, perineural invasion, lymph nodes less than 12, bowel obstruction, and localised perforation (38). According to Koelzer *et al.,* the presence of high-grade tumour budding (more than 10 clusters) has an additional high-risk factor CRC in stage II, and the patient may require

adjuvant therapy post-resection (39). As a result, tumour budding could be included as a high-risk feature and a predictor for treatment management.

Lastly, tumour budding in preoperative biopsy may have prognostic value, as stated by Zlobec *et al*., in which "tumour budding can be assessed in CRC patients' preoperative biopsy as it is useful, reproducible, and predicts node and distant metastasis" (40). AC Roger *et al*. confirmed that “intra-tumoral budding is a marker of poor prognosis and poor response to neoadjuvant chemoradiotherapy; if confirmed, intratumoral budding may be an indication to avoid neoadjuvant chemoradiotherapy in its present form” (41). As a result, this will help pick out which patients may not benefit from or qualify for neoadjuvant therapy.

1.6 Tumour budding and its future potential in colorectal cancer.

Nowadays, treatment has become more specific at the biomolecular level; anti-budding targeted therapy might be explored, developed in the future and directed at the CRC tumour microenvironment (42). Tumour budding can also be integrated into digital pathology for more objective quantification (43). With the help of artificial intelligence, this technology will help lessen the burden of human workload and significantly increase the sensitivity and specificity of tumour budding identification.

Generally, tumour budding is a strong prognostic factor, and it has an impact on CRC management. It should be added as one of the additional prognostic factors in TNM staging other than lymphovascular involvement, MSI, KRAS, NRAS, and BRAF mutation. Furthermore, more prospective clinical trials are needed to validate ITBCC recommendations and the role of tumour budding in preoperative biopsies. From a local

perspective, a larger sample size and multicentre participation from different states will be the next further step.

Therefore, the current study also may pave the way or initiate tumour budding reporting in local settings. Tumour budding has been well established, but not yet practised routinely in Malaysia. Besides, it might help raise awareness for the local pathologist to get more experience in tumour budding reporting and for oncologists to better understand its clinical impact. Plus, the surgical team may also benefit from tumour budding reporting and use it as part of CRC management. Thus, tumour budding can be incorporated into Malaysian CRC management guidelines as an additional prognostic factor.

CHAPTER 2: OBJECTIVES OF STUDY

2.1 General objectives

To determine the proportion and associated factors of peritumoural budding among CRC cases in the HUSM.

2.2 Specific objectives

1. To study the proportion of peritumoural budding among CRC cases in the HUSM.
2. To determine factors associated with peritumoural budding among CRC cases in HUSM.

CHAPTER 3: MANUSCRIPT

3.1 TITLE PAGE

TYPE OF MANUSCRIPT: Original article

Peritumoural Budding in Colorectal Carcinoma, Its Associated Factors and The Potential as An Independent Histopathological Parameter Predictor

Dahziela YUNUS[1,3,4], Suhaily MOHD HAIRON[2,3], Nur Asyilla CHE JALIL[1,3], Seoparjoo Azmel MOHD ISA[1,3*]

[1]*Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, health campus, 16150 Kubang Kerian, Kelantan, Malaysia,* [2]*Department of Community Medicine, School of Medical Sciences, Universiti Sains Malaysia, health campus,16150 Kubang Kerian, Kelantan, Malaysia,* [3]*Hospital Universiti Sains Malaysia, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia,* [4]*Hospital Queen Elizabeth, Karung Berkunci No 2029, 88586 Kota Kinabalu, Sabah, Malaysia.*

***Correspondence Author**

Seoparjoo Azmel Mohd Isa (MD USM)

Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia

Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia.

Email: seoparjooazmel@usm.my

Tel: +609-7673000

Fax: +609-7653370

3.2 ABSTRACT

Background: Throughout the past three decades, tumour budding has become a well-established additional prognostic factor in colorectal carcinoma as of its known adverse effects. The primary aim of this study is to evaluate peritumoural budding (PTB) in colorectal cancer and its associated factors. This evaluation may be beneficial for post-operative patient stratification while simultaneously cementing the tumour budding values and its routine pathological report implementation.

Methods: A cross-sectional retrospective study on resected primary colorectal cancer which examined 162 archived slides stained with haematoxylin and eosin under a microscope. PTB was evaluated and classified as low-risk and high-risk budding. The association between PTB and its clinicopathological variables were statistically analysed with logistic regression models using SPSS.

Results: PTB was observed in most patients (87.7%). High-risk budding was seen in 41 cases (25.3%), while low-risk budding was in 121 cases (74.7%). On univariable logistic regression, there is a significant association between high-risk tumour budding with adverse clinicopathological characteristics including higher CEA level, late TNM stage (III, IV), higher pN stage, lymphovascular invasion, perineural invasion, lymph node metastasis

and recurrence (all variables have P-value <0.05). Multivariable logistic regression shows only higher CEA level, TNM stage III, perineural invasion and recurrence were retained and statistically significantly to the prediction, $p < 0.05$. The model is correctly classified in 79% of cases with the Hosmer Lemeshow test: 0.246.

Conclusion: In conclusion, PTB alone is significantly associated with multiple adverse prognostic factors across all TNM stages, as validated in our study. It deserves special attention as a microscopic biomarker in CRC, and it is about time to take notice.

Keywords: Colorectal carcinoma, tumour budding, high-risk budding, recurrence, prognosis.

3.3 INTRODUCTION

Worldwide, more than 1.9 million (11.4%) new cases of colorectal cancer (CRC) and 935,000 (10.0%) deaths are estimated to occur in 2020. Generally, colorectal cancer ranks third in terms of incidence but second in terms of mortality. It is the third most common male cancer and the second most common female cancer (1).

In Malaysia, CRC is the second leading in incidence (19.6 per 100 000 population) and the third leading in mortality (13.5 per 100 000 population) for both sexes. The age-adjusted incidence rate of colorectal cancer was 1.18 times higher among males than females. The proportion of CRC is increasing with age for both males and females (2). Chinese ethnicity has the highest incidence rate for both sexes. According to the National Cancer Registry, between 2008 to 2013, the mean age for CRC was 61.6 years. CRC staging was mostly detected at a late stage (III and IV), accounting for more than 70% of cases in both sexes (3).

The tumour-node-metastasis (TNM) system is widely used for essential prognostication in CRC patients and is based on the histopathological evaluation of resected specimens (4). However, given the biological heterogeneity of individual tumours, it has flaws such as different behaviour despite the same final TNM stage, for example, some stage II tumours may recur despite curative surgery and controversy over several revisions of the TNM system that lack validation (5). Consequently, this prompted

researchers to explore alternative pathology features that are feasible, easily seen under a light microscope, and inexpensive so that they can be used as outcome predictors. These include angiogenesis, tumour-infiltrating lymphocytes and tumour budding (TB) which are commonly seen in the tumour microenvironment (TME) and play a role in CRC carcinogenesis (5).

Throughout the past three decades, tumour budding has been recognised as an emerging prognostic biomarker for CRC and in the last decade for others across various solid malignancies, as the more tumour buds, the worse the clinical outcome for the patients (6). Morphologically, it is primarily located in the stroma near the invasion margin and consists of single tumour cells or small clusters of four tumour cells that are detached from the main tumour either at the tumour centre (intra-tumoral bud, ITB) or tumour front (peritumoural bud, PTB) (7). At the biomolecular level, tumour budding represents a partial epithelial-mesenchymal transition (EMT) by showing co-expression of a loss of epithelial markers and a gain of mesenchymal markers as compared to the main tumour mass, which is regarded as the histological manifestation of the same and provides the basis of cell migration for tumour invasion and metastasis (8).

Based on an overview of a systematic review and meta-analysis in 2016, tumour budding in CRC is strongly predictive of lymph node metastases, recurrence, and cancer-related death (9). The analysis shows CRC patients with tumour budding which is significantly associated with positive lymph node infiltration, are more likely to have tumour recurrence and cancer-related death at five years. Despite its clinical implications, tumour budding has not yet been incorporated into the TNM staging of CRC; this is partly because of disagreements in its definition, and methods of identification, and also because

it is difficult to apply to all stages of CRC in clinical practice as previous studies used different methods for a specific stage of CRC (9).

Currently, tumour budding assessment is based on ITBCC guideline recommendations, which resulted in ten consensus statements during the meeting, with the primary goal being to reach an agreement on an international, evidence-based standardised scoring system for tumour budding in colorectal cancer (7). During the meeting, the conference covered tumour budding definition, a standardised method of assessment, a three-tiered budding grading system, and its application in two different scenarios. As for the budding grading system, it was recommended for a three-tiered classification with cut-offs as follows; BD1: low (0-4 buds), BD2: intermediate (5-9 buds), or BD3: high (10 or more buds). Meanwhile, the two scenarios are: first, tumour budding is an independent predictor of lymph node metastasis in endoscopically pT1 stage CRC where BD2 and BD3 are high-risk and second, tumour budding is an independent predictor of recurrence or survival in stage II CRC where BD3 is high-risk (7).

Since the ITBCC guideline was published, it has been validated where high tumour budding, ten or more buds shows significant association with TNM stage, competent mismatch repair (MMR), venous invasion, and reduced cancer-specific survival (10). The study used a simpler two-tiered budding grading system to facilitate risk stratification in CRC. Recently, tumour budding has been included as an additional prognostic factor in the WHO Classification of Tumours, Digestive System Tumours (11) and College of American Pathologists protocol and guideline (12).

There is little information about tumour budding in Malaysia, with only a few published studies. It also was not incorporated in the Malaysian colorectal guideline (13).

Therefore, this study aimed to find out the proportion of tumour budding at the invasive front, which is the PTB in different stages (I-IV) of CRC patients followed by identifying its associated clinicopathological factors.

3.4 MATERIALS AND METHODS

Samples selection

This is a single-centred cross-sectional retrospective study conducted in the Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan, Malaysia from the year 2021 to 2022. The clinicopathological data were retrieved from the Pathology Laboratory archive, Laboratory Information System (LIS) and medical record archive at the HUSM. The ethical prerequisite for all aspects of the study was granted by the Human Research Ethics Committee (HREC), Universiti Sains Malaysia on 28 June 2021 with JEPeM Code USM/JEPeM/21030218.

Following JEpeM approval, a total of 162 primary CRC (adenocarcinoma, non-otherwise specified) cases confirmed by histopathological examination (HPE) on resected specimens were selected from January 2013 through December 2020 (inclusion criteria). Prior neoadjuvant chemotherapy and a diagnosis based on colonic biopsies are exclusionary criteria. Cases such as neuroendocrine tumour or cancer and histological subtypes of adenocarcinoma such as mucinous adenocarcinoma, signet-ring cell carcinoma, and adenosquamous carcinoma were also excluded. When a medical record was missing, or unavailable relevant slides were also excluded.

The selected cases were searched via the institutional LIS using the "colorectal adenocarcinoma" keyword. Glass slides and/or pathology reports were retrieved and used to assess for pathologic features such as tumour diameter, laterality, border, grade, lymphovascular invasion, perineural invasion, lymph node metastasis and final TNM staging using the AJCC 8th edition (4). The clinical data for each case is then obtained from the medical record. The acquisition of sociodemographic data such as age, gender, smoking, and family history of cancer, as well as the serum preoperative carcinoembryonic (CEA) level, recurrence, and distant metastasis, were gathered from the patient folder at the medical record archive. Subsequently, all the data were combined, and for the purpose of the study, PTB was assessed separately and blinded from all the clinicopathological data.

Peritumoural budding (PTB) assessment.

When all the glass slides are available, peritumoural budding is assessed by two investigators blinded to any of the patient's clinical and other histologic review data using an Olympus BX53 microscope with an eyepiece field number (FN) diameter of 22. To assess peritumoural budding, International Tumour Budding Consensus Conference (ITBCC) recommendations were followed, in which tumour budding is defined as a single tumour cell or a cell cluster consisting of 4 tumour cells or less at the invasive margin specifically at the tumour front (peritumoural budding, PTB) (7).

Haematoxylin and eosin stained (H&E) slide with the highest density of budding were chosen and scanned under low power (10X objective) for ten individual fields to identify the hotspot that had the greatest degree of budding (Figure 3.1A). Then PTB was counted in the chosen hotspot under medium power (20X objective). The counted PTB was then divided by the normalisation factor of 1.210 to determine the absolute PTB count per

0.785mm^2. The budding category was then reported as BD1: low (0-4 buds), BD2: intermediate (5-9 buds), or BD3: high (10 or more buds). Following the proposed two-tiered classification, BD1 and BD2 are considered low-risk PTB (L-PTB), and BD3 is considered high-risk PTB (H-PTB) for a resected specimen for easy risk stratification of the patient (10) (Figure 3.1 B-D). Any discrepancy in reporting between the investigators was resolved via slide review and discussion at a multiheaded microscope to achieve a consensus report of the PTB count.

Statistical analysis

All the tabulated data was entered and analysed using SPSS version 26. Descriptive analysis was used for the frequency of categorical variables and the mean with standard deviation distribution for numerical variables. The association between PTB and its clinicopathological variables were statistically analysed with a binomial logistic regression model. The level of significance in this study was set as P-value <0.05. Significant adverse clinicopathological variables identified in univariate analysis were then included in subsequent multivariate analysis using the backward elimination method. The selection of the logistic models was based on the classification table, Hosmer-Lemeshow test, and receiver operating characteristic curve.

3.5 RESULT

The proportion of peritumoural budding in colorectal cancer

Among the 162 CRC patients included in this study, the proportion of PTB is shown in Table I. There are 142 (87.7%) positive for PTB, while only 20 (12.3%) are negative for PTB. As per the 3-tiered classification of tumour bud categories, BD1 was the highest, at about 83 (51%). Then followed BD2 and BD3, which were 38 (23.5%) and 41 (25.3%), respectively. Meanwhile, as per the 2-tiered classification, the majority of PTB was L-PTB, which is 121 (74.7%), and only 41 patients have H-PTB, which is only 41 (25.3%) (Table 1).

Clinical and histopathological features in colorectal cancer

Of the 162 patients, 85 (52.5%) were male and 77 (47.5%) were female. The mean age was 60.7 years (SD: ± 13). 49 (30.2%) patients were smokers, and 113 (69.8%) patients were non-smokers. A family history of cancer was documented in 137 (84.6%) of the patients and was negative in 25 (15.4%) of them. The mean diameter of the tumour is 55.3 mm (SD: ± 23.6). Most of the tumours were in the left colon, which was about 136 (84%). CEA levels preoperatively are mostly higher, with a mean of 112.6 g/L (SD: ± 320.7) (Table 2).

The majority of patients presented at TNM stages II and III were 58 (35.8%) and 72 (44.4%), respectively. The CRC in our study shows a mainly irregular border (94.4%) with a grade 2 tumour grade (92.6%). More than half of patients have an invasion beyond the muscularis propria, in the pT stage, which is pT3 (58%). Furthermore, 83 (51.2%) patients have the pN0 stage, followed by pN1 (29%) and pN2 (19.8%). Histological features were documented, such as perineural invasion in 17 (89.5%) (Figure 3.1 A) and lymphovascular invasion in 50 (30.9%) (Figure 3.1 B). The metastatic lymph node was seen in 79 (48.8%) (Figure 3.1 C) of the patients. Meanwhile, recurrence disease was documented in only 24 (14.8%) and distant metastatic disease in only 58 (35.8%) patients (Table 2).

Association of peritumoural budding and its associated factors

On simple logistic regression analysis, there is an association between H-PTB and its adverse clinicopathological features, including a higher CEA level, a late TNM stage (III, IV), a higher pN stage, the presence of lymphovascular invasion, the presence of perineural invasion, the presence of lymph node metastasis, and recurrence with significant differences of P-value <0.05 (Table 2). There are no significant differences in the peritumoural budding category with regard to sociodemographic, tumour diameter, tumour site, tumour location, tumour border, tumour grade, pT stage and distant metastasis.

Subsequently, on multiple logistic regression analysis, only a higher CEA level, late TNM stage III, the presence of perineural invasion and recurrence were significantly associated with high-risk peritumoural budding which is statistically to the prediction of a P-value <0.05 (Table 3). This model used the backward elimination method with no interference and multicollinearity. The model is statistically best fit, in which the Hosmer Lemeshow test shows the P-value is not significant (P-value: 0.246), correctly classified in

79% of cases in the classification table and the Area under the ROC curve is 0.875 (Table 3).

3.6 DISCUSSION

Our study is the second of its kind in Malaysia on the evaluation of PTB in colorectal cancer and its associated factors. The first study was done at the International Islamic University Malaysia and had a similar proportion reported as BD1 48.8%, BD2 27.2%, and BD3 24% (n = 129) (14). The PTB occurs in 80.7% of our patients, which also corresponds to Zlobec *et al*., and the same with the three-tiered system (BD 51.2%, BD2 23.5%, and BD3 25.3%) (15). Meanwhile, the proportion of L-PTB and H-PTB in this study is also comparable with the previous study that adopted the two-tiered system to make risk stratification of patients easier, which was reported as 70.9% and 21.2%, respectively (10).

The sociodemographic traits of CRC patients in our study were comparable to those in the national registry. The average age upon diagnosis was more than 60 in both situations (61.6 and 60.7). The gender-specific CRC percentage also showed similar results, with a male preponderance of over 50%. Each patient's smoking history did not differ appreciably. In comparison to this study, the registry found a lower family history of cancer, at just 9%. Regarding the left-side colon preference and its advanced development, the observations are likewise consistently the same (16).