ORIGINAL ARTICLE

Prognostic Value of Tumor Budding in Hepatocellular Carcinoma Patients: A Clinicopathologic Study

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Background: Hepatocellular carcinoma (HCC) is the most common primary liver cancer, characterized by evidence of hepatocytic differentiation. Tumor budding, defined by the presence of isolated single cancer cells or clusters of up to four cancer cells at the invasive front, has been regarded as an adverse prognostic factor for several solid tumors. It is a histologic finding that can be evaluated on hematoxylin and eosin (H&E)-stained slides. Data on its prognostic value in HCC is limited.

Objective: To assess the prognostic value of tumor budding in resected HCC patients at a tertiary hospital in Thailand.

Materials and Methods: A 6-year retrospective analysis of resected HCC was conducted. Tumor budding was evaluated following the recommendations provided by the International Tumor Budding Consensus Conference. To assess the quantity of tumor budding, the "hotspot" area method was used. Immunohistochemistry was used as an adjunct in morphologically confusing cases. Retrieved cases were classified into low-budding HCC (0 to 4 buds) and high-budding HCC (5 buds or more). Tumor budding status was evaluated with other prognostic parameters such as vascular invasion and multifocality.

Results: Fifty-eight cases of resected HCC, including 54 conventional HCC, three steatohepatitic HCC, and one macrotrabecular-massive HCC, were retrieved. Tumor budding could be identified in fibrocollagenous areas within 1 mm from the invasive front of HCC. High-grade tumor budding was associated with a higher rate of vascular invasion, multifocal tumors, and increased 3-year disease-specific mortality. Common histologic features resulting in over- and under-estimation of tumor budding were bile ductular proliferation in five cases (5.6%) and small amount of fibrocollagenous stroma in three cases (5.2%), respectively.

Conclusion: Tumor budding is an adverse prognostic factor in resected HCC. Although tumor budding can be evaluated on H&E-stained slides using immunohistochemistry as an adjunct, pathologists should be aware of several histologic that mimic and the limitations of assessing such a parameter.

Keywords: Tumor budding; Histopathology; Hepatocellular carcinoma; Prognosis

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Hepatocellular carcinoma (HCC) is the most common primary liver cancer, characterized by evidence of hepatocytic differentiation. Over the last three decades, the prevalence of HCC has been increasing, and similar trends are projected through 2030[1]. According to the World Health Organization (WHO), HCC is the fifth most common type of cancer worldwide and the second leading cause of cancer-related death[2]. More than 90% of HCC cases are associated with chronic liver diseases. Regardless of the cause, cirrhosis is the most significant risk factor for HCC[3,4]. Most HCCs show the radiological hallmark as arterial enhancement and delayed washout. They can be accurately diagnosed by computed tomography or magnetic resonance imaging[5]. Surgical resection or liver transplantation is the preferred treatment modality for early-stage HCC, with a 5-year survival rate of 70% to 80%[3,4].

Tumor budding, defined by the presence of isolated single cancer cells or clusters of up to four cancer cells at the invasive front, has been regarded as an adverse prognostic factor for several solid tumors[6,7]. It is a histologic parameter that can be evaluated on hematoxylin and eosin (H&E)-stained slides. However, data on the prognostic value of tumor budding and other histologic features resulting in false estimation of tumor budding in HCC are limited. The present study aimed to assess the prognostic value of tumor budding and histologic

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features that could result in false estimation of tumor budding in resected HCC patients at a tertiary hospital in Thailand.

Materials and Methods

Study population

Archive data between March 2015 and March 2021 were drawn from the Division of Pathology in Thammasat University Hospital using a hospital software system search tool. Surgically treated patients with histologically confirmed HCC were recruited. All paraffin blocks and H&E-stained slides of resected HCC were retrieved. Clinical data were obtained from the electronic medical record. Non-surgically treated cases, cases treated prior to resection such as ablation and embolization, recurrent HCC, and cases without available paraffin blocks were excluded.

All procedures performed in the present study were approved by the Human Research Ethics Committee of Thammasat University (Medicine) in accordance with the 1964 Helsinki declaration and its later amendments (COA No. 283/2021). Formal written informed consent was not required due to retrospective study.

Morphologic examination

Tumor budding is characterized by single cells that form clusters of up to four cells near the tumor's invasive front. When examining samples, the tumor area was first screened at a low magnification to identify areas with the greatest amount of tumor budding. Then, the number of buds in an area measuring 0.785 mm^2, from a single field of view at a total magnification of 200, was counted. Specimens with fewer than five buds were classified as low-grade tumor budding. In contrast, those with five or more buds were classified as high-grade tumor budding. To assess the quantity of tumor budding, the "hotspot" area method, which was regarded as the most helpful way for evaluating tumor budding in colorectal tumors, was used[8,9]. Tumor budding status was evaluated with other prognostic parameters, including vascular invasion and multifocality.

Immunohistochemical study

In cases with equivocal histologic features such as HCC with dense inflammation at the invasive front, immunohistochemistry for hepatocyte paraffin 1 (HepPar1), arginase 1 (ARG1), and glypican 3 (GPC3) were used as an adjunct to assess the invasive front and to evaluate if tumor buds were present. The scoring was done on H&E.

Table 1. Baseline characteristics of patients

Characteristics	All patients (n=58)	Bud-positive (n=29)	Bud-negative (n=29)
Age (years); mean±SD	60.7±9.9	61.9±10.2	59.9±10.4
Sex; n (%)			
Male	44 (75.9)	23 (79.3)	21 (72.4)
Female	14 (24.1)	6 (20.7)	8 (27.6)
Chronic liver diseases; n (%)			
Chronic hepatitis B	8 (13.8)	5 (17.2)	3 (10.3)
Chronic hepatitis C	7 (12.0)	4 (13.8)	3 (10.3)
HBV cirrhosis	16 (27.6)	7 (24.1)	9 (31.0)
HCV cirrhosis	5 (8.6)	2 (6.9)	3 (10.3)
Alcoholic cirrhosis	8 (13.8)	4 (13.8)	4 (13.8)
Cryptogenic cirrhosis	2 (3.4)	1 (3.4)	1 (3.4)
NASH	1 (1.7)	1 (3.4)	0 (0.0)
None	11 (19.0)	5 (17.2)	6 (20.7)
Median AFP; n (%)			
<400 ng/mL	44 (75.9)	20 (69.0)	24 (82.8)
≥400 ng/mL	14 (24.1)	9 (31.0)	5 (17.2)

HBV=hepatitis B virus; HCV=hepatitis C virus; NASH=non-alcoholic steatohepatitis; AFP=alpha-fetoprotein

Statistical analysis

Baseline characteristics were presented by number and percentage for categorical data, mean ± standard deviation (SD) for age, and median and interquartile range (IQR) for tumor size. Chi-square test was used to examine the significance of the association (contingency) between tumor budding and other prognostic parameters.

Results

Baseline characteristics of patients

Fifty-eight cases of resected HCC were retrieved, including 29 bud-positive HCC and 29 bud-negative HCC. Most patients had chronic liver diseases and elevated plasma alpha-fetoprotein (AFP) levels. The baseline characteristics of these patients are detailed in Table 1. There was no observed difference in these baseline characteristics between the bud-positive and bud-negative groups.

Morphologic assessment of tumor budding

A thorough morphological evaluation of resected HCC was performed. There were 54 conventional HCC, three steatohepatitic HCC, and one macrotrabecular-massive HCC. Tumor budding could be identified in fibrocollagenous areas within

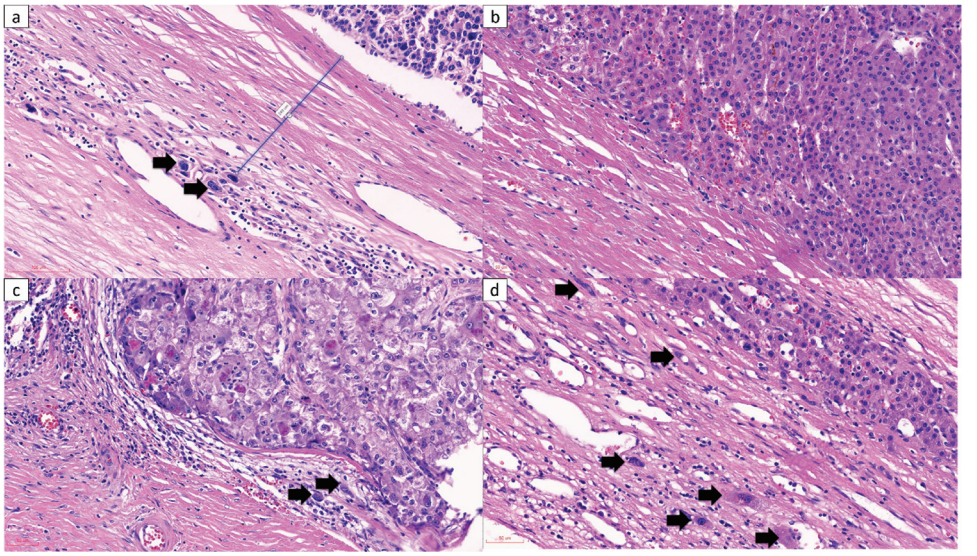

Figure 1. Morphological assessment of tumor budding in resected HCC. (a) Tumor budding is detected within 1 mm from the invasive front of HCC. Note a few isolated cells with nuclear pleomorphism (arrows). (b) Bud-negative HCC is characterized by the absence of tumor budding at the invasive front. Of note, connective tissue elements and mononuclear inflammatory cells should not be interpreted as tumor budding. (c) Low-budding HCC shows fewer than five buds at a 200x magnification (arrows). (d) High-budding HCC contains five or more buds (arrows).

1 mm from the invasive front of HCC. It was characterized by an isolated cell or small clusters of cells with nuclear pleomorphism (Figure 1a). Connective tissue elements and mononuclear inflammatory cells at the invasive front were not considered tumor budding (Figure 1b). Low-budding HCC exhibited fewer than five buds (Figure 1c), while high-budding HCC contained five or more buds (Figure 1d). In well or moderately differentiated HCC, nuclear pleomorphism and the presence of cytoplasmic bile pigment were helpful clues to identify tumor budding.

Immunohistochemical assessment of tumor budding

Immunostaining for HepPar1, ARG1, and GPC3 were performed in two cases of resected HCC, in which the traditional morphologic evaluation on H&E-stained slides showed an invasive front with dense mononuclear inflammatory infiltrate (Figure 2a). It was challenging to determine if these isolated mononuclear cells were tumor budding or mononuclear inflammatory cells, or reactive fibroblasts. HepPar1 and ARG1 stains were helpful for identifying hepatocytes (Figure 2b). However, inhomogeneous staining could occur (Figure 2c). Therefore, the scoring was done on H&E. Due to focal immunoreactivity, GPC3 was not helpful for tumor budding assessment (Figure 2d).

Table 2. Baseline characteristics of resected hepatocellular carcinoma

Caveats	Number of cases (%)
Bile ductular proliferation	5 (8.6)
Foreign body granulomas	1 (1.7)
Hemosiderin pigment	2 (3.4)
Artifactually dislodged tumor cells	2 (3.4)
Partial absence of fibrocollagenous stroma	3 (5.2)
Peliosis-like changes	1 (1.7)
Clusters of five to ten neoplastic cells	2 (3.4)

Level of significance: $p<0.05$

Prognostic implication of tumor budding in resected HCC

Baseline characteristics of resected HCC are shown in Table 2. Abundant tumor buds were observed in a case of macrotrabecular-massive HCC. Three cases of steatohepatitic HCC showed a few tumor buds. High-grade tumor budding was associated with a higher rate of vascular invasion, multifocal tumors, and 3-year disease-specific mortality ($p<0.05$).

Histologic features resulting in false estimation of tumor budding

Due to a variety of tumor and non-tumor interfaces, histologic features that might lead to false estimation of tumor budding were detected. Of note,

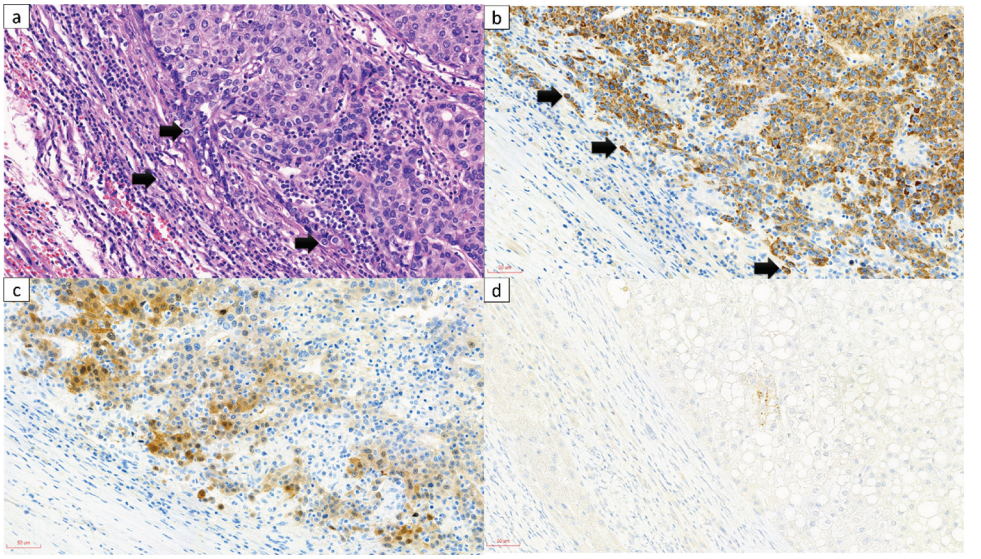

Figure 2. Immunohistochemistry for tumor budding evaluation. (a) It is challenging to identify tumor budding in HCC in which the invasive front is infiltrated by several mononuclear inflammatory cells (i.e., isolated tumor cells versus histiocytes; arrows). (b) HepPar1 is helpful to identify isolated neoplastic hepatocytes (arrows). (c) The tumor cells show inhomogeneous immunoreactivity with ARG1. Therefore, pathologists should rely on H&E-stained slides for grading tumor budding and use immunostain as an adjunct in difficult cases. (d) Due to focal and weak immunoreactivity, GPC3 is not helpful for tumor budding assessment.

Table 3. The number of cases with histologic features that could result in false estimation of tumor budding

Tumor characteristics	Bud-positive (n=29)		Bud-negative (n=29)	p-value
	Low-budding (n=18)	High-budding (n=11)		
Histologic grade; n (%)				
G1 (well differentiated)	1 (5.6)	0 (0.0)	1 (3.4)	LB=HB=NB
G2 (moderately differentiated)	15 (83.3)	9 (81.8)	26 (89.7)	LB=HB=NB
G3 (poorly differentiated)	2 (1.1)	2 (18.2)	2 (6.9)	LB=HB=NB
Tumor focality; n (%)				
Unifocal	15 (83.3)	7 (63.6)	27 (93.1)	HB<NB (p=0.04), PB=NB, LB=HB
Multifocal	3 (16.7)	4 (36.4)	2 (6.9)	HB>NB (p=0.04), PB=NB, LB=HB
Tumor size (cm), median (IQR)	4.7 (2.3)	4 (2.7)	5 (4.2)	N/A
Vascular invasion; n (%)	15 (83.3)	6 (54.5)	3 (10.3)	PB>NB and HB>NB (p<0.01), LB=HB
pT staging; n (%)				
pT1a	1 (5.6)	1 (9.1)	4 (13.8)	LB=HB=NB
pT1b	4 (22.2)	4 (36.4)	15 (51.8)	LB=HB=NB
pT2	9 (50.0)	5 (45.4)	5 (17.2)	PB>NB (p=0.02), LB=HB=NB
pT3	3 (16.6)	1 (9.1)	5 (17.2)	LB=HB=NB
pT4	1 (5.6)	0 (0.0)	0 (0.0)	LB=HB=NB
Liver cirrhosis; n (%)	10 (55.6)	6 (54.5)	17 (58.7)	LB=HB=NB
1-year disease-specific mortality; n (%)	3 (16.7)	3 (27.3)	2 (6.9)	LB=HB=NB
3-year disease-specific mortality; n (%)	3 (16.7)	5 (45.5)	3 (10.3)	HB>NB (p=0.03), PB=NB, LB=HB

these histologic findings were common in the present study cohorts. The number of cases with histologic features potentially resulting in false estimation of tumor budding are shown in Table 3.

Histologic mimickers of tumor budding could be present at the invasive front of HCC. Bile ductular proliferation, composed of benign bile ductules with slightly enlarged, rounded nuclei, was common at the invasive front (Figure 3a). Multinucleate giant cells in foreign body granulomas showing enlarged nuclei might resemble tumor budding (Figure 3b). Hemosiderin pigment could be present in neoplastic

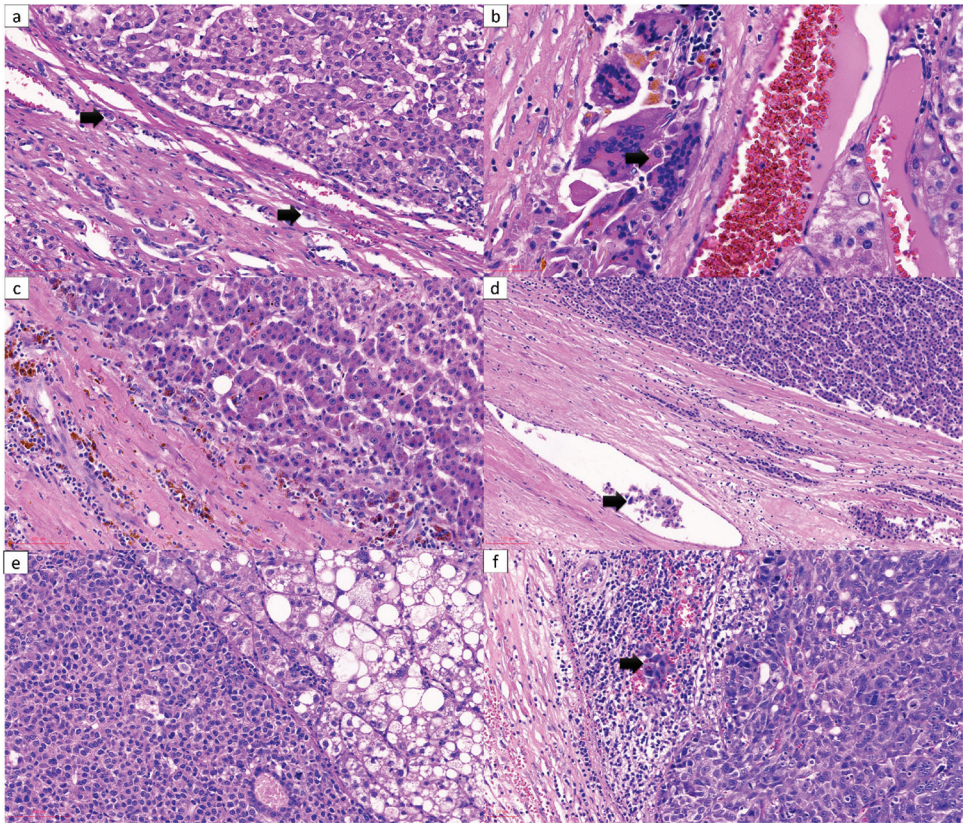

Figure 3. Histologic mimickers and limitations of tumor budding. Several histologic mimickers of tumor budding exist. (a) Bile ductular proliferation is composed of several bile ductules. Some of them are lined by a few biliary epithelial cells (arrows). (b) Multinucleate giant cells result from fusion of activated macrophages at the invasive front (arrow) should not be overcalled tumor budding. (c) Hemosiderin pigment could be present in neoplastic hepatocytes, macrophages, or fibrocollagenous stroma. Therefore, the presence of brownish pigment at the invasive front is not equivalent to the presence of tumor budding. (d) Artifactually dislodged tumor cells in lymphovascular spaces at the invasive front are not regarded as tumor budding. (e) It is impossible to evaluate tumor budding in HCC where the cancer cells are adjacent to non-neoplastic hepatocytes (arrow). (f) Clusters of five to ten neoplastic cells near the tumor's invasive front are not qualified as tumor budding.

hepatocytes, macrophages, or fibrocollagenous stroma (Figure 3c). Therefore, a presence of brownish pigment in either macrophages or fibrocollagenous tissue at the invasive front was not equivalent to a presence of tumor budding. Artifactually dislodged tumor cells in lymphovascular spaces at the invasive front were occasionally noted (Figure 3d). Although this finding was not regarded as lymphovascular invasion, the presence of small clusters of cancer cells should not be overinterpreted as tumor budding.

There were limitations in assessing tumor budding in HCC. Although it was straightforward to look for tumor budding in fibrocollagenous stroma, it was impossible to detect such a histologic feature in areas where the cancer cells were adjacent to non-neoplastic hepatocytes (Figure 3e). This occurrence was noted in three cases of steatohepatitic HCC in the present series. Regarding these cases, tumor budding could only be evaluated in a few small areas where fibrocollagenous stroma was presented. Peliosis-like changes, characterized by dilated sinusoids filled with red blood cells, were rarely present at the invasive front, leading to difficulties in assessing tumor budding. Clusters of five to ten neoplastic cells near the tumor's invasive front were occasionally observed (Figure 3f). Although they were not qualified for tumor budding, these clusters might have prognostic implications.

Discussion

The present study revealed the prognostic significance of tumor budding in HCC. High-grade tumor budding is associated with a higher rate of vascular invasion, multifocal tumors, and 3-year disease-specific mortality. Although tumor budding could be evaluated on routine H&E-stained slides,

there were caveats to evaluating such a histologic feature in routine pathology practice. These factors could potentially lead to the underestimation of tumor budding in HCC.

Although tumor budding has been recently introduced into the mainstream pathology literature, it was first described in a Japanese literature by Imai in the 1950s. At that time, it was hypothesized that the presence of sprouting at the invasive edge of carcinomas indicated a more rapid tumor growth rate[10]. Tumor buds are biological components of the tumor microenvironment and are associated with the epithelial-mesenchymal transition, which is characterized by cytoskeletal rearrangements, increased cell motility and invasion, increased proteolytic activity in cells, and gene expression reprogramming[11,12]. Tumor budding is a well-established independent prognostic factor in colorectal cancer. High-grade tumor budding is also associated with KRAS mutation and microsatellite stable tumors[13]. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) were first provided in 2016[8] with an improvement in a subsequent Delphi consensus study(9). A semi-automated approach built within an open-source software for tumor budding assessment in colorectal cancer was proposed. Such an innovative method is strongly correlated with the established one[14].

Data on the prognostic value of tumor budding in HCC is limited. At the time of writing, there have been only two studies regarding the prognostic implications of tumor budding in HCC. One study is from the west. The other is from the east. Similar to the present study, both former studies showed that tumor budding is associated with an adverse prognosis in surgically treated patients[15,16]. Furthermore, the eastern study showed that the extent of tumor budding is also an independent adverse prognostic factor for resected HCC[15]. However, the western study showed no significant differences in mortality between negative budding and positive budding groups in patients undergoing nonsurgical treatment such as local ablation, transarterial treatment, or palliative treatment[16].

The role of immunohistochemistry for assessing tumor budding is controversial. Studies revealed that the addition of cytokeratin immunohistochemistry is not superior to conventional analysis using only H&E slides[17,18]. However, studies reported that immunohistochemistry is helpful for tumor budding evaluation. One study showed that immuno-histochemical analysis of tumor budding outperforms H&E staining[19]. Another study recommended using pan-cytokeratin staining to assess tumor budding in confusing cases[20]. The other study revealed that although both tumor budding on H&E staining and tumor budding on cytokeratin staining were associated with lymph node metastasis, tumor budding on immunostaining was found to be more associated with lymph node metastasis[21]. Regarding the present study, it is recommended that immunohistochemistry for hepatocytic differentiation, including HepPar1 and ARG1, may be helpful as an adjunct for assessing tumor budding, particularly in poorly differentiated and HCC with a dense mononuclear inflammatory infiltrate at the invasive front.

Tumor budding is a prognostic feature that can be found in other solid tumors, including head and neck cancers, esophageal cancers, lung cancers, breast cancer, gastric cancer, pancreaticobiliary cancers, muscle-invasive bladder cancer, and endometrial cancer[22-25]. This histologic parameter is also an adverse prognostic factor for intrahepatic cholangiocarcinoma and colorectal liver metastasis[26-28]. The number of tumor buds is associated with an unfavorable clinical result. The Union for International Cancer Control (UICC), the American Joint Committee on Cancer (AJCC), and the WHO have all released cancer classification standards that include tumor budding as a prognostic feature. According to the latest College of American Pathologists (CAP) Cancer Reporting Protocols, the number of tumor buds, per 'hotspot' field, and tumor bud score are included as optional histologic parameters for resection specimens from patients with primary carcinoma of the colon and rectum[25]. Systematic reporting of tumor budding for other malignancies is not described in other cancer reporting protocols endorsed by CAP. Regardless of these standards, previous studies using several grading systems for tumor budding revealed the extent that tumor budding was associated with a worse prognosis[29].

Although the recommendations provided by the ITBCC stated that assessing tumor budding is straightforward and can be done on H&E, this may not be true for evaluating such a histologic parameter in HCC. HCC is a malignant neoplasm showing hepatocytic differentiation that arises in the liver parenchyma. Unlike other solid tumors, the invasive front of HCC is adjacent to the liver parenchyma, which is composed of hepatocytes and bile ducts. The present study showed that tumor budding could

only be evaluated at the invasive front, where HCC is separated from its non-neoplastic counterpart by fibrocollagenous tissue. In the setting of HCC with an infiltrative border and a partial absence of fibrocollagenous stroma, tumor budding cannot be evaluated in some areas. This can result in an underestimation of tumor budding in some cases. The steatohepatitic variant of HCC, in particular, may be difficult to evaluate for tumor budding. However, this should not be overemphasized since the present cohorts consisted of few cases. In liver biopsy samples, the interpretation of tumor budding in HCC should be avoided since it is difficult to determine whether the fibrocollagenous stroma corresponds with the invasive front or intratumoral fibrosis. Moreover, the small number of biopsy samples may not represent the entire tumor.

The present study has limitations affecting its conclusiveness and generalizability. The small sample size of 58 resected HCC cases limits the power to detect nuanced prognostic differences, suggesting a need for a larger cohort for more robust results. The retrospective design introduces potential biases, as selection was based on the availability of specific clinical materials, not representing the broader HCC patient population, and limits control over confounding variables, especially concerning the prognostic implications of tumor budding. The present study's focus on a single tertiary hospital in Thailand further narrows its applicability, as findings might not extend to different demographics or healthcare systems. A more diverse, multi-institutional sample would enhance its relevance. Additionally, the exclusive focus on tumor budding as a prognostic marker, while neglecting other well-established factors like tumor stage or liver function, offers an incomplete prognostic picture. Lastly, the absence of an assessment of interobserver variability in tumor budding raises concerns about the consistency and reliability of these findings.

The present study showed that tumor budding is an adverse prognostic factor in resected HCC. Although tumor budding can be evaluated on H&E-stained slides using immunohistochemistry as an adjunct, pathologists should be aware of several histologic that mimic and the limitations of assessing such a parameter.

What is already known on this topic?

Tumor budding, defined by the presence of isolated single cancer cell or clusters of up to four cancer cells at the invasive front, is a histologic parameter that can be evaluated on H&E-stained slides. It has been regarded as an adverse prognostic factor for several solid tumors.

What does this study add?

Tumor budding was an adverse prognostic factor in resected HCC. High-grade tumor budding was associated with a higher rate of vascular invasion, multifocal tumors, and increased 3-year disease-specific mortality. Common histologic features resulting in over- and under-estimation of tumor budding were bile ductular proliferation and small amount of fibrocollagenous stroma, respectively.

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Conflicts of interest

The authors declare no conflict of interest.

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