Review Article

Narrative review: research progress of tumor budding in gastrointestinal tumor

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Abstract: Tumor budding is a histological phenomenon observed in various cancers, which means that single malignant cells and/or small malignant cell clusters can be observed in the tumor stroma. In several retrospective studies, it has been found that tumor budding is related to the poor prognosis of tumors. Tumor budding has been included in the guidelines for the treatment of colorectal cancer in 2017. However, significant heterogeneity in its accurate definition, evaluation methods, and patient stratification need to be addressed. The mechanism of tumor budding remains unclear. Tumor budding was considered to be the manifestation of tumor epithelial-mesenchymal transition (EMT) in the early stage, but it was considered to be the manifestation of "partial EMT" in recent years. Some researchers have tried to explain budding by molecular pathways, however, it is still not clear the important genetic mutations in tumor budding as so far. In colorectal cancer, tumor budding provides an important guide for tumor staging and treatment in clinical practice. For other gastrointestinal tumor except colorectal cancer, there have been some studies on the tumor budding, but further research is needed to evaluate its significance. This review aims to summarize the history, current status, controversy, and the latest research in gastrointestinal tumors of tumor budding.

Keywords: Tumor budding; gastrointestinal tumor; partial epithelial-mesenchymal transition (partial EMT)

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Introduction

Tumor budding, also known as "tumor sprouting", is a histological phenomenon encountered in all kinds of cancers, whereby single malignant cells and/or small malignant cell clusters in the front stroma of the tumor. However, significant heterogeneity in its accurate definition, evaluation methods, and patient stratification need to be addressed. The mechanism of tumor budding is not clear. Tumor budding is considered to be the transition from mirror epithelium to mesenchymal, which is related to the poor prognosis of cancer. In recent years, the research from protein level to gene level has been improved, which puts forward another possibility. Here we review the history of tumor budding, the controversy over the evaluation of tumor budding, the different explanations of the causes of tumor budding, and the progress of its application in clinical practice. We present the following article in accordance with the Narrative Review reporting checklist (available at http://dx.doi.org/10.21037/dmr-20-113).

The history of tumor budding

The phenomenon of tumor budding was first studied by Fukuoka Igaku in 1954 when he was observing gastric cancer cells (1). He found that there was "sprouting" at the edge of the invasion of gastric cancer. There were undifferentiated clusters of malignant cells in the

invasive stroma of gastric cancer, which were mainly (but not completely) located in front of the tumor invasion. Then, in the 1950–1960s, other Japanese scholars found this phenomenon also existed in pathological sections of tongue, throat, breast, stomach, colon, rectum, and cervical cancer. In the 1980s, when Gabbert and his colleagues experimentally-induced colon cancers in mice treated with dimethylhydrazine-dihydrochloride, they found an obvious invasive front of the lesions displayed striking disorganization at the tumor architecture level which helps to mobilize cancer cells from major tumor masses (2). In the 1990s, Hase analyzed the pathology of surgical samples of colorectal cancer and found that poorly differentiated malignant cells moved outward from the invasive front alone or in groups, and the aggregated cells formed a large cell mass, similar to the anterior edge (3). Immunohistochemistry suggested that E-cadherin was down-regulated, accompanied by nuclear translocation of β-catenin and loss of cell polarity. They defined this phenomenon as "tumor budding". This definition has triggered some criticism related to terminology. Some people think that “budding” means that the tumor cell population is still connected to the main tumor, but this is not the case observed in 2D sections. Bronsert and his colleagues show that budding is not a static snapshot, but a dynamic process through the 3D reconstruction of 2D serial sections of various tumor types, through which the tumor extends many finger-like processes (4). Each process contains many cells, which are separated from the main tumor mass in the form of small cell clusters at a later point in time. If you see such extension buds on a 2D section, it may give the wrong impression that the cell mass has been isolated from the main tumor mass. In short, 2D sections show only small cell masses that seem to have lost contact with major masses, while serial sections and 3D reconstruction provide a real picture that the tumor is not separated from the bud.

Early studies have suggested a significant positive correlation between “tumor budding” and poor prognosis of colorectal cancer (5-year survival rates: 22% and 71%, respectively), but this feature has progressed slowly in routine reports (5). Part of the reason is that the methods reported in the literature to evaluate budding are different. But the 2017 AJCC American Society of Pathologists’ guidelines for colorectal cancer reporting explicitly identified tumor budding as an optional reporting field and recommended reporting in all stage I and II cases. The meeting reached a consensus on the assessment of tumor budding and provided us with a standardized reporting tool for colorectal cancer sprouting. Tumor budding is also increasingly reported as a useful pathological prognostic feature of other gastrointestinal cancers, including esophageal squamous carcinoma and adenocarcinoma, gastrointestinal adenocarcinoma, pancreatic ductal adenocarcinoma and ampullary adenocarcinoma (6-10).

The present situation of the research on tumor budding

The evaluation criteria of “tumor budding”

Because “tumor budding” is affected by the subjective observation of pathologists, many scholars have put forward different evaluation methods. These differences of evaluation are mainly focused on the staining, counting, and "intra-tumor budding" of tumor budding. It is now widely used to evaluate intra-tumoral and peritumoral tumors using H&E or pan-cytokeratin staining. Both stainings have advantages and disadvantages. Pan-cytokeratin contributes to visual counting, causing more real tumor buds to be detected, and the nucleus needs to be observed to classify cells as buds, but the nucleus can be masked by strong cytokeratin staining, which can lead to overestimation of non-budding objects, such as cell fragments. This leads to variability between observers, especially at the single object level. H&E is a standard staining method that can be performed in all pathological laboratories, but it is difficult to identify inflammatory areas around the tumor, and it is difficult to distinguish between tumor budding and activated fibroblasts. Two staining methods were evaluated in a group of 50 patients with colorectal cancer, the overall repeatability was good, although cytokeratin staining increased the number of tumor sprouts (partly single cells in the detected glands destroyed by inflammatory cells), it did not increase the consistency between observers (11).

The counting of tumor sprouting is also controversial. Some scholars have proposed that the evaluation of “tumor sprouting” should be counted in an area that shows the maximum sprouting. Ueno in a study on the pathological prognostic characteristics of PT1 colorectal cancer, defined tumor budding as a single cell or <5 cell clusters, counted the maximum number of buds in the budding area with a visual field of ×250 (0.385 mm^2) and then divided the patients into negative buds (<10 buds) and positive buds (≥10 buds), which was called “hot spot method” (12). In a subsequent study, the team further improved the counting method by counting buds in an area of ×200 (0.785 mm^2), with five buds as the dividing line (13). Karamitopoulou

outlined another popular method of budding assessment, which evaluates the entire invasion frontier, using an average count of 5–10 areas of the budding count, also known as the "average method" (14). In their study of pancreatic ductal adenocarcinoma, cytokeratin staining was used to highlight buds and average bud numbers on 10 high-power fields of (HPF). In a multicenter study on the repeatability of tumor germination, the hot spot method and the average method had similar inter-observer variability (kappa 0.35 *vs.* 0.25) (15).

Whether "peritumoral" and "intra-tumoral" tumor budding should be evaluated, or both is also controversial. The concept of intra-tumoral budding was introduced in 2011 (16). Intra-tumoral budding was defined as a cluster of single cells or less than 5 tumor cells surrounded by stroma. The budding at the frontier of tumor invasion in malignant polyps or PT1 colorectal cancer is usually clear, but it will be difficult and poorly reproducible to pathologically describe the invasive frontiers of pancreatic ductal adenocarcinomas in biopsies or ductal adenocarcinoma of the pancreas. Giger found that intra-tumoral budding during the biopsy and peritumoral germination during resection had similar repeatability (k=0.65 *vs.* k=0.68) and significant correlation. This study provides help for clinicians to evaluate "tumor budding" in biopsies because biopsies rarely sample the invasive edge, so it is possible to increase tumor-related information and change treatment options for patients before operation. but a larger sample control analysis is needed. Because of the diversity of evaluation criteria for "tumor budding", the standardized method selected at the 2017 AJCC consensus meeting on Colorectal Cancer sprouting report: according to hematoxylin-eosin staining, the consensus definition of tumor budding is as follows: a single cell or a cluster of <5 cells, counting a ×200 field of vision in the maximum germination area ("hot spot method"). Then the patients were divided into low grade (0–4 buds), medium (5–9 buds), and high grade (≥10 buds) (17). To minimize variability in the selection of maximum budding areas, they recommend that at least 10 HPF be screened at the invasive frontier. Although it is believed that intra-tumoral sprouting may also be a useful prognostic factor, it is suggested that more research is needed before it is incorporated into routine practice. At present, these are recommendations for colorectal cancer. however, the best way to evaluate tumor germination, especially the best cut-off value, may vary in other parts of the gastrointestinal tract.

The mechanism of tumor budding

The cause of tumor budding is not clear. The study of tumor budding is mostly based on immunohistochemical observation in early research. The membrane localization of the key cell-cell adhesion molecule E-cadherin in epithelial cells of tumor budding is relatively low, and the loss of E-cadherin membrane localization is a feature of epithelial-mesenchymal transition (EMT). High-grade tumor budding shows strong and uniform nuclear β-catenin staining and associated loss of membrane E-cadherin expression, which is consistent with the EMT study. Therefore, the tumor budding is considered to be "EMT-like" (18). Many studies have found that from the tumor center to the tumor frontier, several markers mainly related to cell adhesion are heterogeneously expressed in tumor tissue and tumor budding. E-cadherin, CD44, EpCAM, and CD166 are expressed in the medial membrane of tumor tissue, while is missing in tumor budding (19-22). High-grade tumor budding is associated with increased expression of protein markers (such as u-PA and u-PAR), matrix lysin, or matrix metalloproteinases (MMPs) that are closely related to ECM degradation. In lung cancer, the level of β-catenin decreased in tumor budding (23). In invasive ductal breast cancer, the E-cadherin membrane localization of budding cell population was lower than that of central tumor cell, while the cytoplasmic level of vimentin was higher than that of central tumor cell (24). In tongue squamous cancer, the membrane localization of E-cadherin in the tumor budding was less than that in the main mass, while vimentin was positive in the tumor budding, but not in the main mass. High sprouting was related to the decrease of membrane localization of E-cadherin and the increase of vimentin (25). Although there is an obvious relationship between tumor budding and migration and invasion, paradoxically, tumor budding seems to experience a low proliferation rate, with a decrease in the expression of proliferation marker Ki67, accompanied by an increase in the expression of cell cycle arrest mediators cyclinD1 and p16, which validates the "go-or-grow" dichotomy hypothesis.

However, another view is that although tumor budding show down-regulation of E-cadherin, they do not share other co-regulatory changes of EMT, and there is insufficient evidence to associate tumor sprouting with complete EMT (pure mesenchymal phenotype). The cytoplasmic projection similar to flake liposome extends from a single cell in the tumor budding observed in the early stage. However, slice artifacts cannot be ruled out,

which makes the discovery of individual cells ambiguous. In 2014, Bronsert and colleagues found no evidence of single-cell migration in their studies of different tumor types (colorectal cancer, PDAC, lung adenocarcinoma, and invasive breast ductal carcinoma), so the invasion of cancer cells mainly (if not all) depends on collective cell migration rather than individual cell migration, which indicates that some epithelial cell-to-cell adhesions persist. These findings suggest that not all cells become pure mesenchymal, and some tumor cells still express epithelial cells. Interestingly, this expression has also occurred in the process of embryonic cell migration, which is called partial EMT. Partial EMT reflects the epithelial plasticity of epithelial cells, that is, EMT and MET are not all or no response, but a switch between pure epithelial and pure mesenchymal phenotypes (26). What we have observed is a multistate process of one or more intermediate phenotypes from pure epithelial to pure mesenchymal.

Part of the phenomenon of EMT has attracted widespread attention in recent years, and researchers have investigated the expression of epithelial and interstitial markers in different cell lines, patient-derived xenografts, and primary cancer. In the breast, pancreas, kidney, lung, colorectal, and ovarian cancer cell lines, these two markers were co-expressed in the same cell, suggesting the existence of EMT hybridization. *In vitro*, hybrid phenotypes are associated with increased invasion and migration. Similarly, epithelial and interstitial markers are co-expressed in human primary cancers, such as breast cancer, colorectal, esophageal, lung, and pancreatic cancer, and are significantly associated with poor prognosis. Carcinosarcoma is a rare tumor that contains epithelial and mesenchymal portions of clonal origin in the same tumor, representing an example of spontaneous EMT observed in primary human cancers from different organs. Sidharth V Puram identified the partial/mixed EMT regimen in 18 patients with head and neck squamous cell carcinoma (HNSCC) by analyzing 6,000 single-cell transcriptional groups (including 5 pairs of primary tumors and lymph node metastasis), which was defined as the incomplete activation of EMT transcription factor (TF) (27). Interestingly, they found that some of the EMT cells were spatially located at the leading edge of the tumor. This seems to confirm the possibility of EMT in the budding part of the tumor. In the study of PDAC, compared with the tumor center, the membrane localization of E-cadherin and β-catenin in tumor buds was lower (at the cell population level). However, few tumors showed vimentin-positive in tumor budding, which indicated that most of the EMT in patients with PDAC was partial EMT (28). The sprouting of skin squamous carcinoma shows an increasing level of laminin-5γ2, which is an extracellular matrix glycoprotein expressed at the front of collective migration cells during wound healing, suggesting the possibility of partial EMT (29). Although the tumor budding of oral squamous carcinoma significantly up-regulates fibronectin, only a small number of tumor samples of oral squamous carcinoma cells undergo a complete "cadherin conversion" from E-cadherin to N-cadherin (30). Compared with the major tumor, the sprouting of lung cancer did not express ZEB141, the main regulator of EMT. Meyer found that pan-cytokeratin (epithelial marker) and vimentin (interstitial marker) were both positive in colorectal cancer tumor budding (31). In summary, these conclusions suggest that tumor budding does not have a completely stable mesenchymal phenotype; on the contrary, they are partial manifestations of EMT.

In recent years, with the maturity of sequencing technology, the gene sequencing of tumor budding has been realized, which provides an important tool to unravel the mechanism of tumor budding. In 2015, Jensen used laser capture microdissection, RNA sequencing, and miRNA-qPCR array to detect oral squamous carcinoma tumor budding. Compared with the cells in the central part of the tumor, budding cells showed unique gene expression characteristics, including factors involved in epithelial-mesenchymal transformation and activated transforming growth factor signal TGF-β, up-regulated expression of TFs ZEB1 and Prrx1, while decreased expression of mesenchymal-epithelial TFs (such as OVOL1) (32). Subsequently, Linde used the same method to sequence the tumor sprouts and tumor masses of colon cancer and found that 296 genes were differentially expressed (33). Compared with the tumor blocks, the tumor budding is characterized by phenotypic transformation, which is related to the acquisition of migration characteristics and the reduction of cell proliferation. In addition, other studies have shown that tumor budding is associated with increased expression of hypothetical stem cell markers. Tumor budding expresses high levels of stem cell markers, such as CD133 and aldehyde dehydrogenase 1 (ALDH1), indicating that tumor budding has cancer stem cell-like characteristics. Budding tumor cells expressing ALDH1 are positively correlated with increased invasiveness and poor prognosis (34). A number of studies on the interaction between EMT and stem cells have shown that some EMT phenotypic cells may be more like stem cells than pure epithelial cells or

pure mesenchymal cells, supporting the tumor budding is partial EMT (35-37). Therefore, the tumor budding at the edge of invasion can be considered as the realization of the proposed "transitional tumor stem cells".

The tumor budding has attracted much attention in recent years for its correlation with poor prognosis. Few studies aim to its molecular mechanism because of lacking vitro model. it is generally believed that tumor budding is tumor clusters detached from tumor bulk. When and how this happen is still unknown, the phenomenon of EMT or partly EMT is a picture of this process. Because tumor budding take placed in three dimensions and Influenced by the surrounding microenvironment, the molecular mechanism is completely different from what we find in two dimensions. What we know of potential mechanisms of tumor budding is still too little. But we believe with the application of new technology, mechanisms of tumor budding will be clearly expounded in the near future.

Clinical application

For colorectal cancer, tumor budding has become an independent prognostic factor. Moreover, tumor budding has other guiding values for the choice of treatment for patients with colorectal cancer.

Tumor budding and colorectal cancer stage

First of all, for stage I colon cancer, tumor budding guides patients to benefit from oncological resection after diagnosis of primary tumors growing into the submucosa (PT1). In 2004, Ueno investigated a set of clinicopathological parameters, including tumor location, tumor diameter, macroscopic tumor morphology (stemless and pedicled), tumor grade, vascular invasion, tumor germination, and width and depth of submucosal infiltration (13). The study concluded that the lack of some features, including high tumor grade, vascular infiltration, budding, and extensive submucosal infiltration, may tend to be followed up. Similar results were obtained from a meta-analysis of 17 studies and 3,782 PT1CRC in 2013 by Bosch *et al.* (38). The guidelines, therefore, indicate that tumor budding is an independent predictor of lymph node metastasis in patients with PT1 colorectal cancer, and therefore strongly recommend that tumor budding be used in conjunction with other histopathological predictors of lymph node metastasis (such as poor differentiation, lymphatic vascular infiltration, and submucosal invasion depth/level) in patients with PT1 colorectal cancer to evaluate participation in treatment decision-making.

Second, the budding of stage II colorectal cancer may indicate that adjuvant therapy should be considered to improve survival. The latest management and treatment guidelines of the European Medical Oncology Society (ESMO) recommend that patients with low-risk stage II colorectal cancer be followed up, while patients with high-risk factors should consider fluorouracil adjuvant therapy, such as T4 (the tumor has grown to all layers of the colon and attaches or invades other structures and organs), the number of lymph nodes examined is less than 12, perforated or obstructed, grade 3, no microsatellite instability (MSI) and tumor budding (39). This recommendation is supported by the 2019 classification of digestive system tumors of the World Health Organization (WHO), which reports tumor budding with perineural infiltration, intramural and extramural vascular infiltration, lymphatic infiltration, and tumor deposition as risk factors, with an OR of 4.51 (95% CI, 2.55–7.99). Multivariate analysis showed that tumor budding had an independent effect on DFS. The study concluded that stage II colon cancer should routinely report tumor budding.

Third, the effectiveness of tumor budding in patients with stage III cancer has not been deeply evaluated. However, since adjuvant therapy is standardized, whether tumor budding can predict the response of this subgroup to chemotherapy remains to be studied. Rogers found that among the 89 patients with locally advanced rectal cancer, 18 (20%) showed budding in the tumor biopsy before treatment, and the patients with budding in the tumor did not show grade 1 tumor regression or complete pathological remission after neoadjuvant therapy (40). Intratumoral budding indicates the adverse pathological reaction to neoadjuvant radiotherapy and chemotherapy. Patients who may not have a complete pathological response to neoadjuvant therapy must be upgraded accordingly.

Fourth, in patients with stage IV colorectal cancer, the presence of tumor budding within or around metastasis (IMB and PMB, respectively) in liver metastasis may be a supporting marker for stratification of patients with different treatment options. The ESMO consensus guidelines for the treatment of patients with metastatic colorectal cancer emphasize the importance of multidisciplinary treatment, including oncology, surgery, radiology, and pathology. The most commonly used molecular markers in clinical practice are RAS, BRAF, and MSI status. The histopathological features of clinical treatment are the size of metastasis, the percentage of fibrosis and necrosis, the status of resection,

and the degree of tumor degeneration (41). Several studies have shown that, tissue growth patterns such as connective tissue proliferation, push and metastasis have the potential to predict prognosis (42,43). Similar to primary tumors, tumor budding can be detected in the presence of intra-metastatic or premetastatic tumor budding (IMB and PMB, respectively) in colorectal cancer liver metastases (CRLM). Therefore, tumor budding may also be an important factor in the disease progression of patients with stage IV colorectal cancer, but compared with primary tumors, the detection of tumor budding in liver metastasis is still a major challenge.

Tumor budding and molecular typing of colon cancer

In addition, there is also a certain tendency for tumor budding in the molecular typing of colon cancer. CMS1 subtype is characterized by the defect of the DNA mismatch repair system, which is consistent with MSI. In addition, they are characterized by strong immune penetration and strong activation of immune escape pathways. Tumor budding is rarely found in patients with MSI tumors. One possible explanation is that inherent immune cell infiltration leads to the destruction of tumor budding. CMS4 subtype showed obvious activation of transforming growth factor-β pathway and Wnt- signal pathway, as well as markers of EMT and angiogenesis. This subtype showed the worst overall survival rate, the worst 5-year survival rate, and the relapse-free survival rate of all subtypes. A study also found that the frequency of budding of this type of tumor was significantly higher than that of other types. Interestingly, in this study, the researchers also found that the overall expression of the tumor was CMS2, while the tumor budding tissue was CMS4. Then, in 2018, Casasent *et al*. assured the genome copy number of a single tumor cell by topographic single-cell sequencing (TSCS) in the study of breast ductal carcinoma *in situ*, indicating that most mutations and copy number distortions evolved in the ducts before the invasion (44). These results support the polyclonal invasion model, in which one or more clones are transferred from ducts to adjacent tissues, resulting in the establishment of invasive cancer.

Tumor budding and gastric cancer

With tumor budding as an optional reporting field in the 2017 AJCC American Society of Pathologists' Colorectal Cancer reporting guidelines, a growing body of evidence also points to tumor sprouting in gastric and pancreatic cancer, emphasizing the effectiveness of this standardized scoring method (45,46). The study of tumor budding in gastric cancer is less than that in colon cancer. A meta-analysis in 1961 analyzed seven cohorts containing data from 2,178 patients; high-level budding was positively correlated with high tumor stage and low differentiation (47). In the comprehensive analysis of 1,833 patients, high-grade tumor budding was significantly correlated with lymphatic invasion and lymph node metastasis, and with a poor 5-year overall survival rate. These results were confirmed in the subgroup analysis of intestinal type, but not in diffuse type. In the evaluation of tumor budding during radical gastrectomy in 621 patients with early submucosal gastric cancer, it was also found that high-grade tumor germination was a predictor of lymph node metastasis (48).

Discussion

Although a consensus has been reached in summarizing tumor budding in colorectal cancer, there is still great variability in evaluating budding for inexperienced pathologists. An artificial intelligence-assisted scoring system for judging tumor budding can save time and help to improve repeatability. Although tumor budding is also a very important prognostic marker in many kinds of tumors, it should be noted that the definition and scoring system of tumor budding may be different according to different tumor types, which requires further research to reach a consensus.

At present, the study on the mechanism of tumor budding still can not fully explain the formation of tumor budding. High-throughput multi-sample genomics research is helpful to further clarify the causes of tumor budding. The study of potential targets expressed in tumor budding may provide a promising method for anti-budding therapy to specifically target tumor cells that seem to be responsible for local and distant metastasis, thus improving the survival rate of tumors.

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