

Original Article

Hypoxia induced HIF-1 α expression promotes angiogenesis, tumor budding cell survival and cell proliferation arrest in high-grade tumor budding colorectal carcinomas

Monika Vyas, Natalie Patel, Arvindhan Nagarajan, Narendra Wajapeyee, Dhanpat Jain, Xuchen Zhang

Department of Pathology, Yale University School of Medicine, New Haven, CT

Received July 21, 2016; Accepted July 22, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: Tumor budding in colorectal cancer is a hallmark of unfavorable tumor biology correlating with advanced tumor stage, lymphovascular invasion, metastasis and cancer-specific death. Hypoxia, one of the most important microenvironmental changes in solid tumors, has been found to activate HIF-1 α , the key actor of the intracellular hypoxia pathway, and subsequently promotes tumor progression/metastasis. The aim of the study is to investigate the association of microenvironment hypoxia and HIF-1 α activation in tumor budding and their impact on colorectal cancer biology. Our data revealed that HIF-1 α was strongly expressed in tumor budding cells at the invasive front of tumor and its expression was significantly increased in HCT116 colon cancer cells after hypoxia exposure. The microvessel density was significantly increased at the invasive front of high-grade tumor budding group than the low-grade tumor budding group. Phospho-AKT, a cell survival serine/threonine protein kinase, was strongly expressed in tumor budding cells and HCT116 colon cancer cells after hypoxia exposure. The proliferation index (Ki-67) of tumor budding cells is significantly low as compared to the tumor cells from the main tumor. The hypoxic HCT116 colon cancer cells showed increased cell cycle arrest as compared to those in normoxic condition. Our data demonstrated that tumor budding cells in hypoxic microenvironment and HCT116 colon cancer cells under hypoxia exposure show increased expression of HIF-1 α , and HIF-1 α activation may be a key factor in tumor cell survival, angiogenesis and therapy resistance and hence confers aggressive behavior of colorectal cancers with high-grade tumor budding.

Keywords: Tumor budding, colorectal carcinoma, hypoxia, hypoxia-induced factor-1 α

Introduction

Tumor budding is defined as the presence of single tumor cells or small clusters of up to 5 tumor cells at the invasive front of colorectal cancer [1]. High-grade tumor budding in colorectal cancer is an unfavorable histologic feature that correlates with advanced tumor stage, lymphovascular invasion, and metastasis [2-5]. Tumor budding has emerged in recent years as a promising prognostic indicator in colorectal cancer [2, 3, 6, 7] as well as in other malignancies [8-14].

The physiological mechanism of tumor budding and how it translates into increased vascular and lymph node metastasis is unknown. Tumor budding initiation is generally considered to be

the histologic reflection of epithelial-mesenchymal transition (EMT), which involves the conversion of epithelial cells into migratory and invasive cells. EMT has been mainly investigated *in vitro*, and data on EMT in human samples remain sparse, with the exception of some immunohistochemical studies on tumor budding [15, 16]. Recent studies of human colorectal cancer by microarray analysis or immunohistochemistry did not reveal increase of EMT-related molecules in tumor front budding cells as compared to tumor center [17, 18]. Therefore, the relationship between tumor budding and EMT remains controversial.

Hypoxia, one of the most important micro-environmental changes in solid tumors, has been shown to activate hypoxia-induced factor-1 α

High-grade tumor budding in colorectal carcinomas

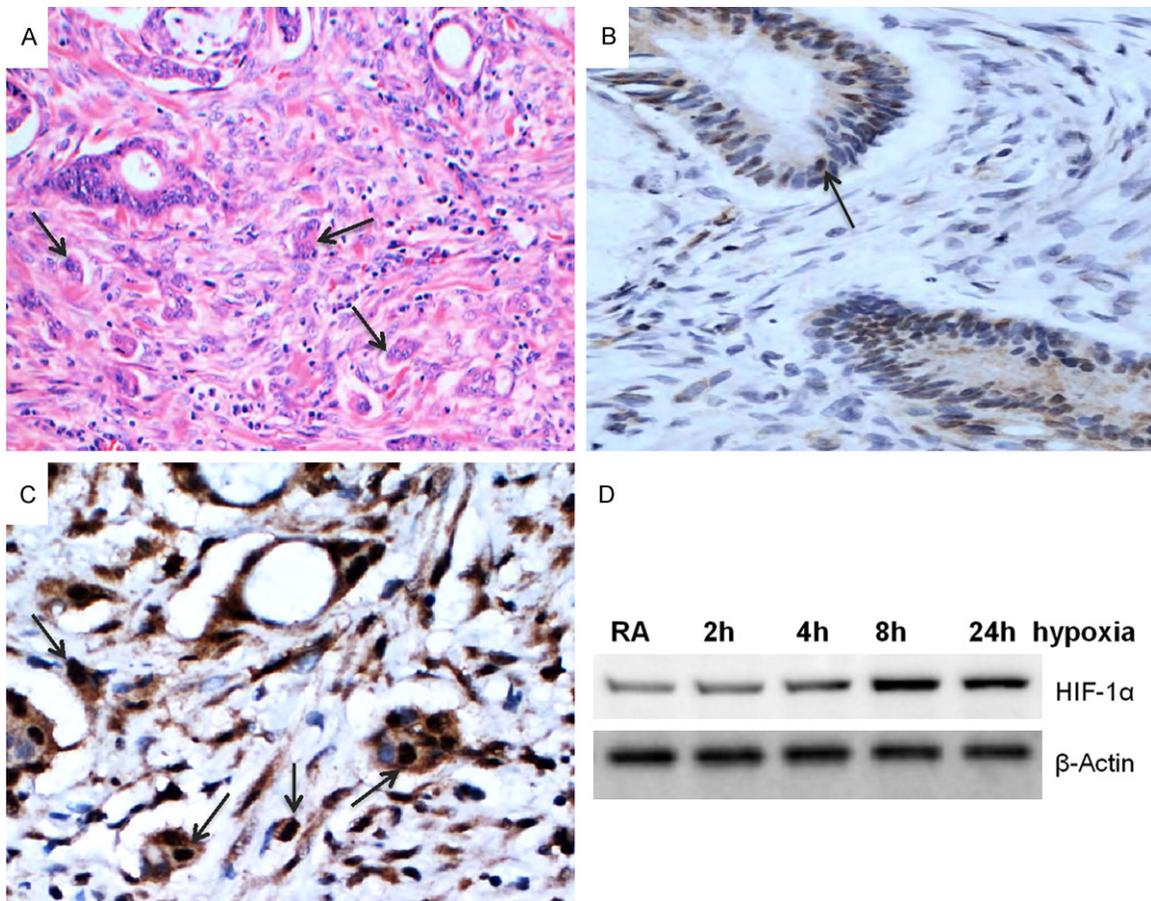


Figure 1: HIF-1 α expression. A. Representative section shows high-grade tumor budding (arrows) (H&E, 400X). B. Colorectal cancer cells from the main tumor show rare HIF-1 α expression (arrow) (Immunohistochemistry, 400X). C. Tumor budding cells at the invasive front show increased expression of HIF-1 α (arrows) indicating a hypoxic micro-environment of tumor invasive front (Immunohistochemistry, 400X). D. HCT116 colon cancer cells were exposed to hypoxia (5% O₂) in different time courses and expression of HIF-1 α was assessed by western blot. Similar to tumor budding cells in colorectal cancer, HIF-1 α expression is significantly increased in cultured HCT116 colon cancer cells after hypoxia exposure (RA, room air control).

(HIF-1 α), a key player in the intracellular hypoxia pathway, and promotes tumor progression/metastasis and induces radio- and chemotherapy resistance [19, 20]. However, the association of microenvironment hypoxia and HIF-1 α activation in tumor budding and their impact on colorectal cancer biology remains unclear.

Materials and methods

Tumor budding assessment and immunohistochemical stains

The study was approved by appropriate institutional review board. Ten cases of CRC with high-grade (≥ 10 buds in a 20X objective field) and 9 cases without tumor budding were included in

this study. The initial evaluation to classify the cases as high tumor budding versus no tumor budding was performed on H&E stained sections. Single to clusters of up to 5 tumor cells were considered as a tumor bud. Immunohistochemical stain for pancytokeratin AE1/3 (Chemicon, 1:4000) was performed to highlight/confirm the tumor budding seen in H&E stained slides (**Figure 1A**). The count was performed on the invasive tumor front based on H&E stained sections. Similar to pancytokeratin AE1/3, immunohistochemical stains for HIF-1 α (Abcam, 1:100), Ki-67 (Dako, 1:300), phospho-Akt (Thr³⁰⁸) (Cell Signaling, 1:100), and CD31 (JC70A, neat, Dako) were performed on representative sections of colorectal cancers with and without high-grade tumor budding.

High-grade tumor budding in colorectal carcinomas

Tumor cell proliferation

Budding tumor cell proliferation status was evaluated by Ki-67 immunohistochemical stain. For analysis of tumor cell proliferation index (Ki-67), 3 high power fields (400X) of tumor budding cells and tumor cells of the main tumor were counted and the proliferation index was calculated as percentage of Ki-67 positive cells out of total cells.

Microvessel density evaluation

Immunohistochemical stain for CD31 was used for microvessel staining [21]. For calculation of microvessel density (MVD), thin walled vessels with well-formed lumina, highlighted by CD31 staining, were considered as microvessels. Three high power field (400X) areas with highest microvessel density of tumor front with or without high-grade tumor budding were chosen for calculation. The MVD count was documented as number of microvessels/mm².

Cell culture and hypoxia exposure

The HCT116 colon cancer cells (ATCC) were cultured in DMEM/F12 media supplemented with 10% FCS, 100 units/mL penicillin G, and 100 µg/mL streptomycin at 37°C under air/5% CO₂. The cells were used when approaching 90% confluence on P60 cell culture dishes. For hypoxia, a model previously established, cells were exposed to 95% N₂, 5% CO₂ in a sealed modular chamber (Billup-Rothberg) with continuous monitoring and adjustment to maintain <0.5% O₂ (BioSpherix) for different time courses, as described previously [22].

Cell cycle analysis

The HCT116 colon cancer cells were collected at the different time points and stained with propidium iodide as previously described [23]. Cell cycle distributions were determined by fluorescence-activated cell sorter (FACS, BD Biosciences) analysis. Quantitation of the fraction of cells in different cell cycle phases was done using FlowJo software. The numbers of cells in G1 (2n DNA), S phase (>2n and <4n DNA), and G2/M (4n DNA) were quantified, and the percent fraction of cells in each phase was calculated.

Western blot analysis

As described previously [24], protein was extracted from HCT116 colon cancer cells, elec-

trotransferred, and immunoblotted with HIF-1α or phospho-Akt (Thr³⁰⁸) antibody (Cell Signaling). Detection was performed with a Phototope-horseradish peroxidase Western detection system (Cell Signaling). Equivalent sample loading was confirmed by stripping membranes with blot restore membrane rejuvenation solution (Thermo Scientific) and reprobed with anti-β-actin or Akt antibody.

Statistics

Data were expressed as means ± SE and statistically significant difference was accepted at P < 0.05.

Results

Tumor budding cells and in vitro hypoxia exposed colon cancer cells show increased expression of HIF-1α

Hypoxia is a common feature in most solid human tumors. Hypoxiainducible factor1α (HIF1α), a regulatory transcription factor, plays a crucial role for tumor cells in responding to lower oxygen in their resident microenvironment. Increased HIF-1α induced by hypoxia can commit the adaptive changes in gene expressions of tumor cells. HIF-mediated gene expression regulates many critical aspects of tumor biology, including cell survival, metabolic programming, angiogenesis, metastasis, and therapy resistance [25, 26]. However, the role of hypoxia and associated HIF-1α expression in tumor budding in colorectal cancers are still unclear. We first investigated if there is increased HIF-1α expression in tumor buds comparing to that of tumor cells from the main tumor. Tumor budding cells show increased expression of HIF-1α comparing to tumor cells from the main tumor (**Figure 1B** and **1C**). Next, we investigated if there is increased HIF-1α expression in HCT116 human colon cancer cells in hypoxic environment. The HCT116 human colon cancer cells were exposed to hypoxia in different time courses and expression of HIF-1α was assessed by western blot. Similar to tumor budding cells in colorectal carcinomas, HIF-1α expression is significantly increased in cultured colon carcinoma cells after hypoxia exposure comparing to cultured control cells in room air (**Figure 1D**). These data indicate that hypoxia induces HIF-1α expression and tumor budding cells exist in a hypoxic microenvironment in the tumor front.

High-grade tumor budding in colorectal carcinomas

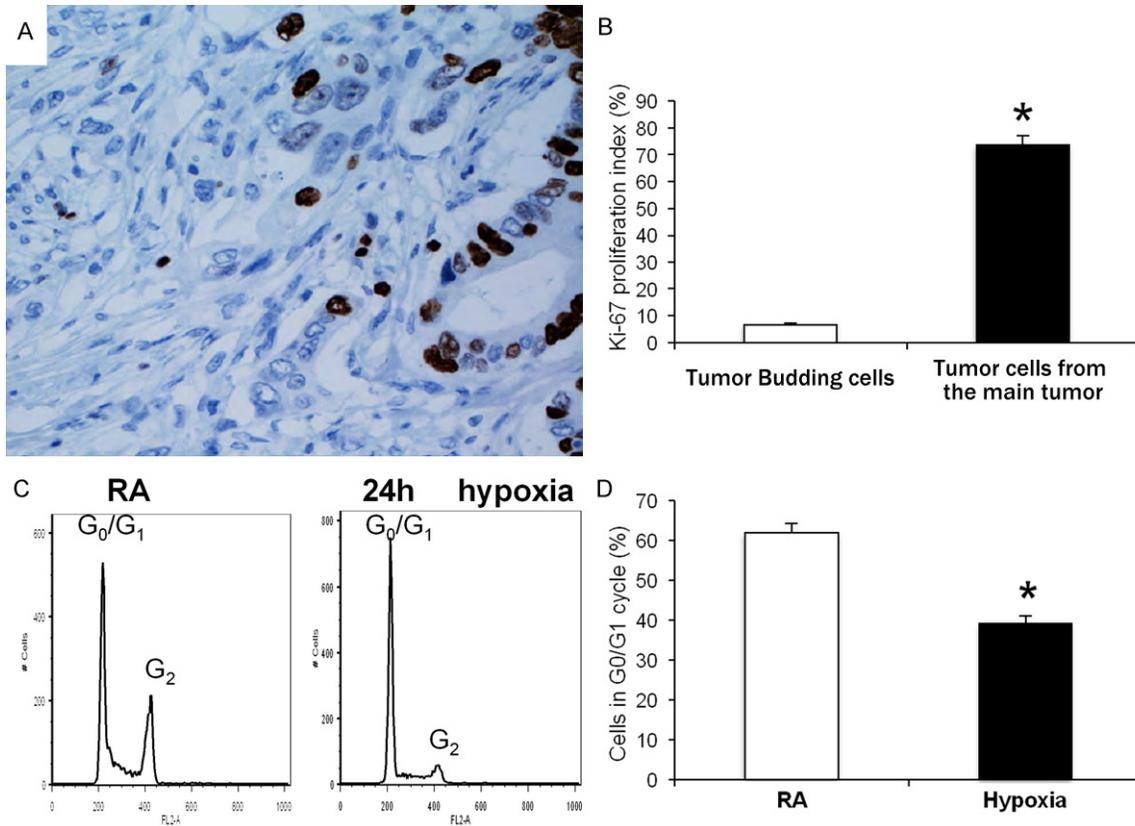


Figure 2. Cell cycle arrest. A. Tumor budding cells show a significantly decreased proliferation index as compared to adjacent tumor gland epithelial cells assessed by Ki-67 immunohistochemical stain (Immunohistochemistry, 400X). B. Graphical quantitation of Ki-67-positive cells in tumor budding cells and tumor cells from adjacent tumor glands. Data are expressed as mean \pm S.E. * $p=0.001$, compared with high-grade tumor budding group. C. Cultured HCT116 colon cancer cells showed significant increase of cell cycle arrest (G₀/G₁ phase) after 24 h hypoxia exposure comparing to that of room air (RA) cells. D. Graphical quantitation of G₀/G₁ cycle of HCT116 colon cancer cells with or without hypoxia. Data are expressed as mean \pm S.E. * $p=0.003$, compared with RA.

Tumor budding cells and in vitro hypoxia exposed colon cancer cells show proliferation arrest

It is well accepted that high proliferation index is associated with tumor progression, lymphovascular invasion and metastasis. Tumor budding in colorectal cancer correlates with advanced tumor stage, lymphovascular invasion and metastasis. We investigated if there is increased proliferation index of tumor budding cells by counting Ki-67 positive cells. The expression of Ki-67 is strongly associated with tumor cell proliferation and growth, and is widely used in routine pathological investigation as a proliferation marker. To our surprise, tumor budding cells in colorectal cancers show significantly decreased proliferation index compared to adjacent tumor gland epithelial cells assessed by Ki-67 immunohistochemistry ($6.7\% \pm 2.0$ vs. $73.7\% \pm 9.4$, $p=0.001$) (**Figure 2A** and

2B). Studies have shown that hypoxia inhibits cell proliferation via HIF-1 α [27]. Next we investigated if hypoxia inhibits cell proliferation in vitro. Cultured HCT116 colon cancer cells showed significant increase of cell cycle arrest (G₀/G₁ phase) after 24 h hypoxia exposure as compared to that of room air control cells ($61.90\% \pm 2.45$ vs. $39.36\% \pm 1.73$, $p=0.003$) (**Figure 2C** and **2D**). These data indicate that tumor budding cells have a low proliferation index. Tumor hypoxia microenvironment may play an important role in maintaining the low proliferation status of tumor budding cells in colorectal cancers.

Tumor budding cells and in vitro hypoxia exposed colon cancer cells show increased expression of p-Akt

Phospho-Akt, a Serine/Threonine protein kinase, activated by phosphorylation at Thr³⁰⁸ or

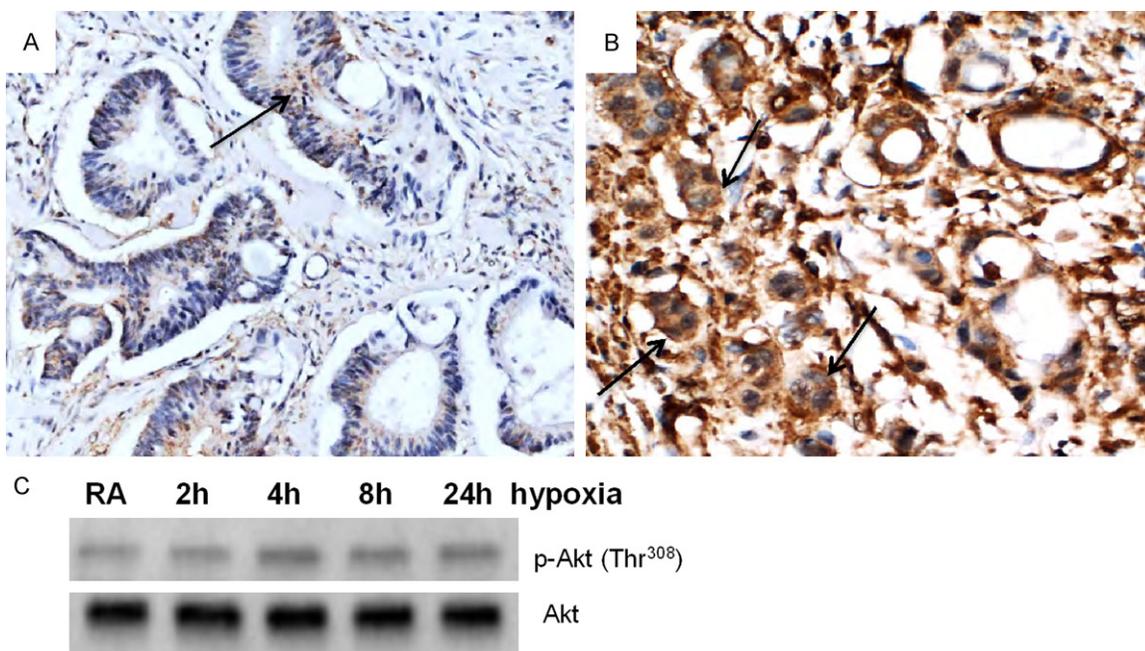


Figure 3. Phospho-Akt expression. A. Colorectal cancer cells from the main tumor show focal and weak p-Akt Thr³⁰⁸ expression (arrow) (Immunohistochemistry, 400X). B. Tumor budding cells at the invasive front show strong p-Akt Thr³⁰⁸ expression (arrows) (Immunohistochemistry, 400X). C. HCT116 colon cancer cells were exposed to hypoxia in different time courses and expression of p-Akt Thr³⁰⁸ was assessed by western blot. Expression of p-Akt Thr³⁰⁸ is significantly increased in cultured colon carcinoma cells after hypoxia exposure.

Ser⁴⁷³, plays a critical role in promoting cell survival in different cell types by inhibition of apoptosis [28]. Studies have shown cell survival in hypoxic condition is mediated through activation of HIF-1 α /Akt pathway [29]. We, thus, assessed whether hypoxia affects Akt activation. We first investigated if there is increased Akt activation in tumor budding cells compared to that of tumor cells from the main tumor. Tumor budding cells show strong phospho-Akt (p-Akt) Thr³⁰⁸ expression by immunohistochemistry (**Figure 3A** and **3B**). We then exposed the HCT116 human colon cancer cells in hypoxia at different time courses and observed a strong Akt activation (p-Akt Thr³⁰⁸) after a 4 h exposure compared to room air control cells. Total Akt was unaffected (**Figure 3C**). These data indicate that tumor hypoxia microenvironment activates survival molecule, p-Akt, that maintains tumor budding cell survival in a hypoxia condition.

Significant high microvessel density at the tumor invasive front in high-grade tumor budding colorectal cancers

The activation of HIF-1 α in hypoxic microenvironment plays important role in angiogenesis.

We have observed increased expression of HIF-1 α in tumor budding cells, thus we tried to investigate if there is increased angiogenesis in tumor invasive front with high-grade tumor budding cells. Angiogenesis was evaluated by microvessel density using CD31 immunohistochemistry at the tumor invasive front. Colorectal cancers with high-grade tumor budding show significantly increased microvessel density as compared to that of control group (114.95 ± 36.01 vs. 58.43 ± 15.84 , $p=0.015$) (**Figure 4A-C**). These data indicate that tumor hypoxia microenvironment induces HIF-1 α expression in the tumor budding cell and stimulates angiogenesis at the tumor invasive front, by which the tumor budding cells obtain nutrients and gain access to newly formed lymphovascular channel leading to lymphovascular invasion and metastasis.

Discussion

The concept of tumor budding first appeared in the 1950s in Japanese literature described by Imai, who postulated that the presence of ‘sprouting’ at the invasive edge of carcinomas reflected a more active tumor growth [30]. After this early description, the concept of “sprout-

High-grade tumor budding in colorectal carcinomas

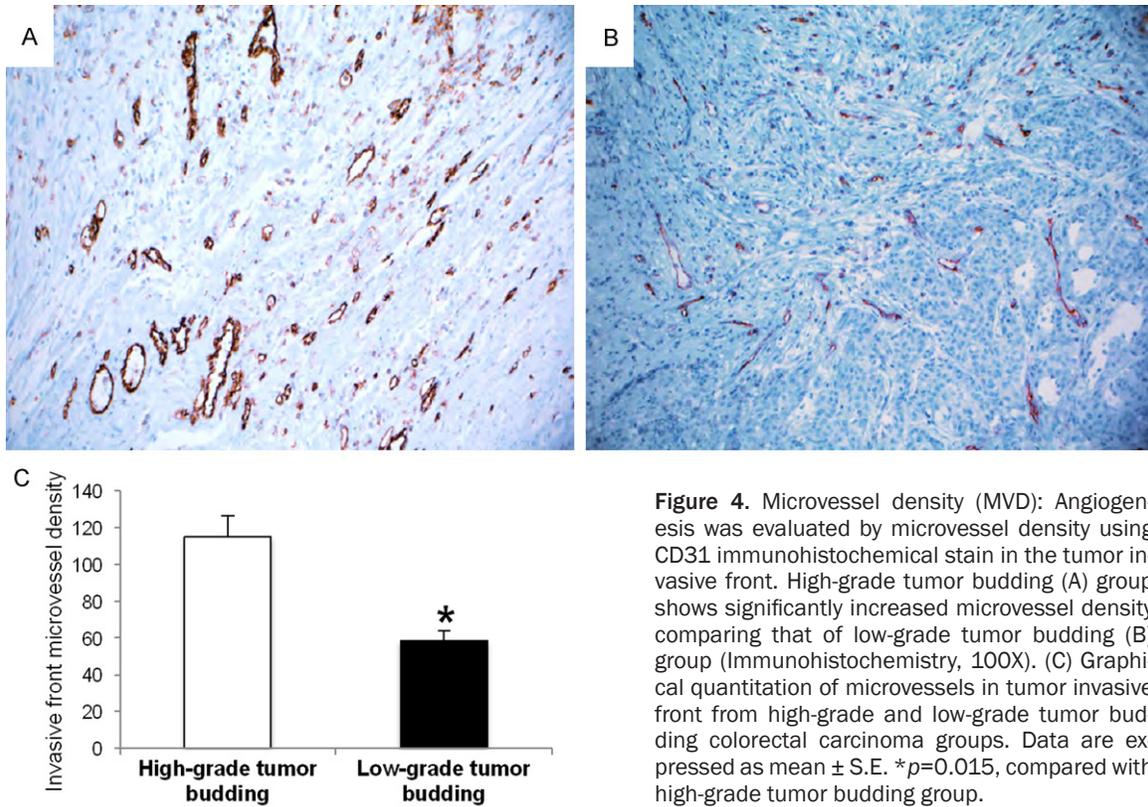


Figure 4. Microvessel density (MVD): Angiogenesis was evaluated by microvessel density using CD31 immunohistochemical stain in the tumor invasive front. High-grade tumor budding (A) group shows significantly increased microvessel density comparing that of low-grade tumor budding (B) group (Immunohistochemistry, 100X). (C) Graphical quantitation of microvessels in tumor invasive front from high-grade and low-grade tumor budding colorectal carcinoma groups. Data are expressed as mean \pm S.E. * $p=0.015$, compared with high-grade tumor budding group.

ing” remained dormant, until Morodomi et al. described “tumor budding” in terms of microtubular cancer nests and undifferentiated cancer cells in 1989. They reported an important role of these tumor budding cells in predicting the presence of lymphovascular invasion and lymph node metastasis in advanced rectal cancer [31]. In 1993, Hase et al. determined the potential prognostic value of tumor budding, which they described as small clusters of undifferentiated cancer cells in the invasive front of the colorectal cancers [32]. In 2002, Ueno et al. first introduced a more quantitative definition: tumor budding as isolated single cancer cells and clusters composed of fewer than 5 cancer cells [33]. Though immunohistochemistry for cytokeratin has been used to facilitate the assessment of the degree of budding, especially for pathologist who is not experienced or does not have enough confidence in the assessment of budding [34], studies have shown that tumor budding detection by immunohistochemical staining is not superior to hematoxylin and eosin staining for predicting lymph node metastasis in colorectal cancer [35]. Tumor budding has been significantly associated with lymphatic invasion, vascular

invasion, tumor recurrence and metastases, as well as worse overall and disease-free survival [5, 7, 16]. Therefore, the presence of tumor budding at the invasive front of primary tumor may provide an early warning of the subsequent aggressive behavior of the tumor.

It is well known that hypoxia microenvironment in solid tumors promotes tumor progression/metastasis and induces radio- and chemotherapy resistance [19, 20]. Tumors with more hypoxic areas have a more aggressive behavior. This adverse behavior has been attributed to increased expression of HIF-1 α , which has a role in regulating the pathways mediating angiogenesis, glycolysis, metabolic reprogramming, apoptosis and cell cycle arrest [36]. Inhibition of HIF-1 α has been proposed as a therapeutic target in cancer [37, 38]. In our current study, we demonstrated HIF-1 α activation in colon cell line by exposure to hypoxia. Similarly, tumor budding cells showed increased expression of HIF-1 α indicating tumor budding cells are exposed to a hypoxic microenvironment in the tissue. Our current study also demonstrated that a significant increase of microvessel density is present at tumor invasive front in

High-grade tumor budding in colorectal carcinomas

colorectal cancers with high-grade tumor budding. One of the important roles of HIF-1 α is to stimulate the proliferation and migration of endothelial cells and angiogenesis, by increased expression of vascular endothelial growth factor (VEGF). The increased microvessel density, at tumor invasive front where the tumor budding cells are present, can not only provide oxygen and nutrient to maintain the budding tumor cell survival, but also provide the opportunity of lymphovascular invasion. Other studies have shown that tumor budding cells are often found adjacent to areas with newly formed microvessels [39, 40]. We also observed the same phenomenon in our current study.

Another interesting phenomenon occurring in tumor budding cells is the reduced proliferation index detected by Ki-67 immunohistochemical stain. Similarly, cultured colon cancer cells *in vitro* also showed cell cycle arrest after hypoxia exposure. These data indicate that the hypoxic microenvironment may contribute to the decreased proliferation index of tumor budding cells. The decreased proliferation index may facilitate tumor budding cell survival by reducing energy consumption. The cell arrest in G0/G1 cycle may also be associated with insensitivity and resistance to neoadjuvant therapy. Akt is an anti-apoptotic pro-survival kinase that is implicated in the molecular pathogenesis of many malignancies [41]. Studies have shown cell survival in hypoxia condition is mediated through activation of HIF-1 α /Akt pathway [29]. In our current study, we identified increased expression of active form Akt, p-Akt, in the tumor budding cells as compared to the carcinoma cells in the main tumor. These findings indicate that the tumor budding cells are even less likely to undergo apoptosis than the carcinoma cells from the main tumor, which confers a greater survival advantage to the tumor budding cells which are in a cell cycle arrest/low proliferation stage.

In conclusion, our results may suggest that the hypoxic microenvironment is associated with tumor budding and increased angiogenesis. Increased microvessel density may facilitate lymphovascular invasion and metastasis. Increased expression of p-AKT can promote cell survival in a low proliferation state in the hypoxic microenvironment, and hence confer resistance to neoadjuvant therapy. HIF-1 α activation

may be a key factor in tumor budding cell survival, angiogenesis and neoadjuvant therapy resistance, and hence confers aggressive behavior in colorectal cancers with high-grade tumor budding.

Address correspondence to: Dr. Xuchen Zhang, Department of Pathology, Yale University School of Medicine. 310 Cedar Street. PO Box 208023, New Haven, CT 06520, United States. Tel: +1-203-7856010; Fax: +1-203-7372922; E-mail: Xuchen.zhang@yale.edu

References

- [1] Prall F. Tumour budding in colorectal carcinoma. *Histopathology* 2007; 50: 151-162.
- [2] Lugli A, Karamitopoulou E and Zlobec I. Tumour budding: a promising parameter in colorectal cancer. *Br J Cancer* 2012; 106: 1713-1717.
- [3] van Wyk HC, Park J, Roxburgh C, Horgan P, Foulis A and McMillan DC. The role of tumour budding in predicting survival in patients with primary operable colorectal cancer: a systematic review. *Cancer Treat Rev* 2015; 41: 151-159.
- [4] Petrelli F, Pezzica E, Cabiddu M, Coinu A, Borronovo K, Ghilardi M, Lonati V, Corti D and Barni S. Tumour Budding and Survival in Stage II Colorectal Cancer: a Systematic Review and Pooled Analysis. *J Gastrointest Cancer* 2015; 46:212-218.
- [5] Graham RP, Vierkant RA, Tillmans LS, Wang AH, Laird PW, Weisenberger DJ, Lynch CF, French AJ, Slager SL, Raissian Y, Garcia JJ, Kerr SE, Eun Lee H, Thibodeau SN, Cerhan JR, Limburg PJ and Smyrk TC. Tumor Budding in Colorectal Carcinoma: Confirmation of Prognostic Significance and Histologic Cutoff in a Population-based Cohort. *Am J Surg Pathol* 2015; 39: 1340-1346.
- [6] Betge J, Kornprat P, Pollheimer MJ, Lindtner RA, Schlemmer A, Rehak P, Vieth M and Langner C. Tumor budding is an independent predictor of outcome in AJCC/UICC stage II colorectal cancer. *Ann Surg Oncol* 2012; 19: 3706-3712.
- [7] Petrelli F, Pezzica E, Cabiddu M, Coinu A, Borronovo K, Ghilardi M, Lonati V, Corti D and Barni S. Tumour Budding and Survival in Stage II Colorectal Cancer: a Systematic Review and Pooled Analysis. *J Gastrointest Cancer* 2015; 46: 212-218.
- [8] Karamitopoulou E. Role of epithelial-mesenchymal transition in pancreatic ductal adenocarcinoma: is tumor budding the missing link? *Front Oncol* 2013; 3: 221.
- [9] Nakagawa Y, Ohira M, Kubo N, Yamashita Y, Sakurai K, Toyokawa T, Tanaka H, Muguruma

High-grade tumor budding in colorectal carcinomas

- K, Shibutani M, Yamazoe S, Kimura K, Nagahara H, Amano R, Ohtani H, Yashiro M, Maeda K and Hirakawa K. Tumor budding and E-cadherin expression are useful predictors of nodal involvement in T1 esophageal squamous cell carcinoma. *Anticancer Res* 2013; 33: 5023-5029.
- [10] Gulluoglu M, Yegen G, Ozluk Y, Keskin M, Dogan S, Gundogdu G, Onder S and Balik E. Tumor Budding Is Independently Predictive for Lymph Node Involvement in Early Gastric Cancer. *Int J Surg Pathol* 2015; 23: 349-358.
- [11] Kadota K, Yeh YC, Villena-Vargas J, Cherkassky L, Drill EN, Sima CS, Jones DR, Travis WD and Adusumilli PS. Tumor Budding Correlates With the Protumor Immune Microenvironment and Is an Independent Prognostic Factor for Recurrence of Stage I Lung Adenocarcinoma. *Chest* 2015; 148: 711-721.
- [12] O'Connor K, Li-Chang HH, Kalloger SE, Peixoto RD, Webber DL, Owen DA, Driman DK, Kirsch R, Serra S, Scudamore CH, Renouf DJ and Schaeffer DF. Tumor budding is an independent adverse prognostic factor in pancreatic ductal adenocarcinoma. *Am J Surg Pathol* 2015; 39: 472-478.
- [13] Salhia B, Trippel M, Pfaltz K, Cihoric N, Grogg A, Ladrach C, Zlobec I and Tapia C. High tumor budding stratifies breast cancer with metastatic properties. *Breast Cancer Res Treat* 2015; 150: 363-371.
- [14] Seki M, Sano T, Yokoo S and Oyama T. Histologic assessment of tumor budding in preoperative biopsies to predict nodal metastasis in squamous cell carcinoma of the tongue and floor of the mouth. *Head Neck* 2016; 38 Suppl 1: E1582-1590.
- [15] Li H, Xu F, Li S, Zhong A, Meng X and Lai M. The tumor microenvironment: An irreplaceable element of tumor budding and epithelial-mesenchymal transition-mediated cancer metastasis. *Cell Adh Migr* 2016; 10: 434-46.
- [16] De Smedt L, Palmans S and Sagaert X. Tumour budding in colorectal cancer: what do we know and what can we do? *Virchows Arch* 2016; 468: 397-408.
- [17] Kobayashi T, Masaki T, Nozaki E, Sugiyama M, Nagashima F, Furuse J, Onishi H, Watanabe T and Ohkura Y. Microarray analysis of gene expression at the tumor Front of colon cancer. *Anticancer Res* 2015; 35: 6577-6581.
- [18] Righi A, Sarotto I, Casorzo L, Cavalchini S, Frangipane E and Risio M. Tumour budding is associated with hypoxia at the advancing front of colorectal cancer. *Histopathology* 2015; 66: 982-990.
- [19] Kuwai T, Kitadai Y, Tanaka S, Onogawa S, Matsutani N, Kaio E, Ito M and Chayama K. Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. *Int J Cancer* 2003; 105: 176-181.
- [20] Rohwer N and Cramer T. Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways. *Drug Resist Updat* 2011; 14: 191-201.
- [21] Saponaro C, Malfettone A, Ranieri G, Danza K, Simone G, Paradiso A and Mangia A. VEGF, HIF-1alpha expression and MVD as an angiogenic network in familial breast cancer. *PLoS One* 2013; 8: e53070.
- [22] Zhang X, Shan P, Jiang D, Noble PW, Abraham NG, Kappas A and Lee PJ. Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. *J Biol Chem* 2004; 279: 10677-10684.
- [23] Nagarajan A, Dogra SK, Liu AY, Green MR and Wajapeyee N. PEA15 regulates the DNA damage-induced cell cycle checkpoint and oncogene-directed transformation. *Mol Cell Biol* 2014; 34: 2264-2282.
- [24] Zhang X, Shan P, Homer R, Zhang Y, Petrache I, Mannam P and Lee PJ. Cathepsin E promotes pulmonary emphysema via mitochondrial fission. *Am J Pathol* 2014; 184: 2730-2741.
- [25] LaGory EL and Giaccia AJ. The ever-expanding role of HIF in tumour and stromal biology. *Nat Cell Biol* 2016; 18: 356-365.
- [26] Balamurugan K. HIF-1 at the crossroads of hypoxia, inflammation, and cancer. *Int J Cancer* 2016; 138: 1058-1066.
- [27] Kumar S and Vaidya M. Hypoxia inhibits mesenchymal stem cell proliferation through HIF1alpha-dependent regulation of P27. *Mol Cell Biochem* 2016; 415: 29-38.
- [28] Sasaki T and Kuniyasu H. Significance of AKT in gastric cancer (Review). *Int J Oncol* 2014; 45: 2187-2192.
- [29] Inukai M, Hara A, Yasui Y, Kumabe T, Matsuoto T and Saegusa M. Hypoxia-mediated cancer stem cells in pseudopalisades with activation of hypoxia-inducible factor-1alpha/Akt axis in glioblastoma. *Hum Pathol* 2015; 46: 1496-1505.
- [30] Imai T. The growth of human carcinoma. A morphological analysis. *Fukuoka Igaku Zasshi* 1954; 45: 13-43.
- [31] Morodomi T, Isomoto H, Shirouzu K, Kakegawa K, Irie K and Morimatsu M. An index for estimating the probability of lymph node metastasis in rectal cancers. Lymph node metastasis and the histopathology of actively invasive regions of cancer. *Cancer* 1989; 63: 539-543.
- [32] Hase K, Shatney C, Johnson D, Trollope M and Vierra M. Prognostic value of tumor "budding" in patients with colorectal cancer. *Dis Colon Rectum* 1993; 36: 627-635.

High-grade tumor budding in colorectal carcinomas

- [33] Ueno H, Murphy J, Jass JR, Mochizuki H and Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* 2002; 40: 127-132.
- [34] Kai K, Aishima S, Aoki S, Takase Y, Uchihashi K, Masuda M, Nishijima-Matsunobu A, Yamamoto M, Ide K, Nakayama A, Yamasaki M and Toda S. Cytokeratin immunohistochemistry improves interobserver variability between unskilled pathologists in the evaluation of tumor budding in T1 colorectal cancer. *Pathol Int* 2016; 66: 75-82.
- [35] Okamura T, Shimada Y, Nogami H, Kameyama H, Kobayashi T, Kosugi S, Wakai T and Ajioka Y. Tumor Budding Detection by Immunohistochemical Staining is Not Superior to Hematoxylin and Eosin Staining for Predicting Lymph Node Metastasis in pT1 Colorectal Cancer. *Dis Colon Rectum* 2016; 59: 396-402.
- [36] O'Donnell JL, Joyce MR, Shannon AM, Harmey J, Geraghty J and Bouchier-Hayes D. Oncological implications of hypoxia inducible factor-1alpha (HIF-1alpha) expression. *Cancer Treat Rev* 2006; 32: 407-416.
- [37] Tang CM and Yu J. Hypoxia-inducible factor-1 as a therapeutic target in cancer. *J Gastroenterol Hepatol* 2013; 28: 401-405.
- [38] Masoud GN, Wang J, Chen J, Miller D and Li W. Design, synthesis and biological evaluation of novel HIF1alpha inhibitors. *Anticancer Res* 2015; 35: 3849-3859.
- [39] Morodomi T. Clinicopathological studies of advanced rectal cancers--prediction of the degree of lymph node metastasis from histopathological finding of pre-operative biopsy specimens. *Nihon Geka Gakkai Zasshi* 1988; 89: 352-364.
- [40] Ohtsuki K, Koyama F, Tamura T, Enomoto Y, Fujii H, Mukogawa T, Nakagawa T, Uchimoto K, Nakamura S, Nonomura A and Nakajima Y. Prognostic value of immunohistochemical analysis of tumor budding in colorectal carcinoma. *Anticancer Res* 2008; 28: 1831-1836.
- [41] Roy HK, Olusola BF, Clemens DL, Karolski WJ, Ratashak A, Lynch HT and Smyrk TC. AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. *Carcinogenesis* 2002; 23: 201-205.