



Elevated ZBTB7A expression in the tumor invasive front correlates with more tumor budding formation in gastric adenocarcinoma

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Received: 23 June 2020 / Accepted: 5 September 2020 / Published online: 23 September 2020
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Abstract

Purpose Tumor budding (TB) is reported to predict nodal involvement and recurrence in multiple human malignancies. However, it is not clear how TB forms. The purpose of this study is to find markers related to TB formation in gastric cancer and to investigate the underlying mechanisms.

Methods TB was scored on hematoxylin–eosin staining slides in 122 gastric cancer cases. Immunostaining score of CREB1, GAGE12I, CTNND1, KIF26B and ZBTB7A both at the invasive front and in the center of the tumor were assigned to each case. Spearman's correlation with the TB score was performed to find the TB-related markers. In vitro study and RNA-seq using gastric cancer cell lines were done to unveil the mechanisms.

Results TB could predict lymph node metastasis and is negatively associated with overall survival of the patients. The expression of ZBTB7A in the invasive front, rather than the other four markers, was much higher than that in the tumor center and was positively correlated with TB score. ZBTB7A could enhance migration and invasion of gastric cancer cells in vitro. RNA-seq data followed by RT-qPCR and western blot verification demonstrated the activation of EGFR-MAPK-ERK and PI3K-AKT-mTOR pathways and increased expression of EMT related markers upon ZBTB7A over-expression.

Conclusion Higher ZBTB7A expression in the tumor margin may contribute to the dissociation of tumor cells from the tumor mass to form TB by initiating EMT via EGFR-MEK-ERK and PI3K-AKT-mTOR pathway.

Keywords Tumor budding · Gastric carcinoma · ZBTB7A · Epithelial-mesenchymal transition · Metastasis

Introduction

Gastric cancer is one of the most lethal malignancies worldwide (Bray et al. 2018). Patients with early-stage gastric cancer usually have mild or no symptoms. Diagnosis is often made until advanced tumor stages. Although careful tumor staging, lymph node examination for a trace of metastasis

could help selecting appropriate treatment and predicting post-surgery outcome of the patients, tumor recurrence is still the main obstacle in cancer therapy.

Tumor budding (TB) is defined as “the presence of clusters of up to five tumor cells in the tumor invasive front appears detached from the main tumor mass” (Berg and Schaeffer 2018; Ueno et al. 2002; Wang et al. 2009). Studies have shown that in colorectal cancer increased TB is associated with lymph node involvement, distant metastasis, and tumor recurrence and is an independent prognostic factor. The College of American Pathologists guidelines for colorectal carcinoma reporting have included TB as an optional reporting field and recommend to report TB in all stage I and stage II cases. The predictive value of TB in patients' survival and recurrence is not unique to colorectal carcinoma, and has been addressed in many other gastrointestinal carcinomas such as esophageal squamous carcinoma, pancreatic cancer and gastric adenocarcinoma (GAC) (Berg and Schaeffer 2018). Studies evaluating TB in gastric cancer found that TB is an independent risk factor for lymph

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00432-020-03388-3>) contains supplementary material, which is available to authorized users.

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node involvement in pT1a and pT1b patients (Gulluoglu et al. 2015) and higher tumor budding scores is associated with unfavorable prognosis (Che et al. 2017). However, the prognostic relevance of TB is limited to the intestinal type of GAC, whereas in diffused type TB scoring is hardly feasible and has no prognostic value (Kemi et al. 2019; Olsen et al. 2017).

There are very few reports on molecular markers that could drive the tumor to bud. Studies have shown that the extracellular matrix components, Laminin5 γ 2, could promote tumor budding formation via activated YAP1 by interacting with integrin β 1 in colorectal cancer. Low expression of miR200c in the budding area but not the tumor center is reported to be associated with tumor budding probably via elevated ZEB (Martinez-Ciarpaglini et al. 2019; Zhou et al. 2019). Identifying genes that drive the tumor cells to dissociate from the tumor body may provide a new idea in interpreting the mechanism that tumor cells utilized to spread over the body.

Previously, we reported several genes (GAGE12I, CTNND1, Kif26B, CREB1 and ZBTB7A Shi et al. 2019, 2015; Wang et al. 2015; Xing et al. 2015; Zhang et al. 2017)) that play oncogenic roles in gastric cancer progression and are closely related to prognosis. In this study, we systematically analyzed the immunohistochemical staining pattern of these genes and correlated the staining score of each gene with TB count. We found that ZBTB7A are differentially expressed between the center and the margin (invasive front) of the tumor mass and that marginal ZBTB7A expression is positively correlated with TB count. The purpose of this study was to investigate the relationship between ZBTB7A expression and tumor budding formation. Also, we carried out a functional experiment using gastric cancer cell lines attempting to unveil the role and mechanism of ZBTB7A in promoting cell migration and invasion.

Materials and methods

Patients and specimens

A total of 122 GAC specimens from the year 2004–2007 from Qilu Hospital of Shandong University were included while signet ring cell carcinoma and mucinous carcinoma were excluded. Pathological features of the HE slides were assessed by two pathologists independently. Pathological classification and tumor staging were performed according to the criteria established by the World Health Organization (WHO) (2010) and UICC/AJCC TNM classification (the 7th edition). In addition, Lauren's classification (intestinal and diffused) was also used. The patients were followed up until cancer-related death or last known to be alive. The study was

approved by the Ethics Committee of Shandong University, China.

Tumor budding evaluation

Tumor bud scores were evaluated on H&E slides using methods described in the study on colorectal adenocarcinoma with minor modification (Ueno et al. 2002). Briefly, a tumor bud was defined as a single cell or clusters of less than 5 detached tumor cells in the tumor invasive front. Two slides from each case, 5 areas with the highest density of tumor buds (hot spot) from each slide were identified and selected for analysis at low power (40 \times) microscopic field. One 200 \times field from each of the hot spot (total of 10 fields) was assessed, and the number of tumor buds in each field was counted. TB score is recorded as the median of these counts.

Immunohistochemical staining and scoring

Immunohistochemical staining for GAGE12I, CTNND1, Kif26B, CREB1 and ZBTB7A were performed previously. Antibodies used were: Anti-GAGE12I and anti-ZBTB7A polyclonal antibody from Proteintech (Wuhan, China) diluted at 1:50; anti-CREB1 (1:350 dilution), anti-CTNND1 (1:350 dilution) and anti-Kif26B (1:200 dilution) polyclonal antibodies from Abcam (Cambridge, UK). Staining inside the tumor mass and in the tumor margin were evaluated and scored respectively. Tumor margin was defined as 0.5 mm from the invasive front. The staining intensity was graded into 0 (negative staining), 1 (weak), 2 (moderate), and 3 (strong). The immunostaining score was calculated by multiplying intensity by area%.

Cell culture and transfection

Human gastric cancer cell line MKN45 and BGC823 were purchased from the Chinese Academy of Medical Sciences and cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a humidified cell incubator with 5% CO₂.

Expression vector of ZBTB7A (pEnter-ZBTB7A, NM_015898) and control vector (pEnter-NC) was purchased from Vigene Biosciences (Vigene, USA). Small interfering RNA targeting ZBTB7A and scrambled negative control were synthesized by GenePharma (GenePharma, Shanghai). The targeted sequence of siRNA is 5'-CGCAGAAGGUGGAGAAGAATT-3' (Knockdown efficacies see Supplementary Fig. 1); Transfection of expression vector and siRNA was performed as described previously (Liu et al. 2018). The expression vector of ZBTB7A and siRNA were transfected with Lipofectamine 2000 (Invitrogen) or X-tremeGENE

transfection reagent (Roche) following the instructions from the manufacturers.

Migration and invasion assay

Cell migration and invasion assay was performed using Transwell chambers with 8 μ m pore size (Corning, New York, USA) as previously described (Shi et al. 2015). For invasion assay, transwell chambers were precoated with the Matrigel matrix (BD, USA).

Western blot

Whole-cell lysate was extracted using RIPA buffer (Beyotime Biotechnology, China). Primary and secondary antibodies used in this study are GAPDH, ZBTB7A, PIK3CA, EGFR and mTOR (all from Proteintech, Wuhan, 1:500 dilution); AKT1/2/3 and pAKT (from Abways, China, 1:500 dilution); MEK1/2, pMEK1/2, ERK1/2, pERK1/2, E-cadherin, N-cadherin, Vimentin and Snail (all from CST, 1:1000 dilution).

Real-time quantitative PCR

Total RNA was isolated using Trizol reagent (TaKaRa). Real-time quantitative PCR (RT-qPCR) was performed using primers shown in Supplementary Table 1. The relative expression of target mRNA to the reference mRNA was assessed using the $2^{-\Delta\Delta C_t}$ method. Each sample was analyzed in triplicates.

RNA sequencing

Total RNA from BGC823 cells transfected with ZBTB7A siRNA or scrambled negative control were sent to Annoroad (Beijing, China) for transcriptome sequencing on Illumina platform. Genes with a fold-change above 1.5 or under 0.66 were considered as differentially expressed genes (DEGs). Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) online tool (<https://david.abcc.ncifcrf.gov/>).

Statistical analysis

Statistical analysis was performed using Graphpad Prism 7 and SPSS 20. The χ^2 test was used to assess the association between TB score and the clinical characteristics. Receiver operating characteristic (ROC) curves were drawn, and area under the curve (AUC) was calculated to evaluate TB score in predicting lymph node involvement. Overall survival time (OS) was the period from the date of surgery until the date of

cancer-related death or the date at which the patient was last known to be alive. Patients who died from cancer-unrelated causes were excluded from this study. Survival curves were plotted using Kaplan–Meier method and compared using the log-rank test in a univariate analysis. Correlation between immunostaining score and TB score were calculated using Spearman correlation. All the tests were two-tailed and $P < 0.05$ was considered statistically significant.

Results

Clinical significance of TB score in predicting lymph node metastasis and patients' survival

TB score was assigned to each case as described above. TB scores in patients with lymph node metastasis (LNM; mean \pm SEM: 114 ± 19.54 , $n = 81$) were much higher than those without LNM (mean \pm SEM: 45.46 ± 22.42 , $n = 41$; Fig. 1a). Receiver operating characteristic (ROC) curve was drawn to evaluate the predictive accuracy of TB score to distinguish cases with LNM from those without LNM. The area under curve (AUC) was 0.7435 ($P < 0.0001$), indicating that TB score is a good predictor for LNM (Fig. 1b). ROC curve analysis also determined the optimal cut-off value of TB score to be 5. Sixty-nine patients (57%) showed high-grade tumor budding (> 5 tumor buds per HPF). Overall survival of patients with high-grade TB was much shorter than patients with low-grade TB (median survival of 23 months vs. 72 months; hazard ratio [HR]: 1.901, 95% confidence interval [CI]: 1.168–3.196, $P = 0.0098$; Fig. 1c).

χ^2 test was performed to assess the association between TB score and clinicopathological characteristics (Table 1). High-grade TB score was associated with diffused/mixed histology ($P < 0.0001$), poor differentiation ($P < 0.0001$), LNM ($P < 0.0001$), higher pathological stage ($P = 0.007$) and tend to indicate distant metastasis ($P = 0.0806$).

There are studies indicating that diffused/mixed type GAC, tending to metastasize at earlier stage and with poor prognosis, has high-grade TB score by nature and that TB score is a valuable LNM predictor in intestinal-type GAC only (Kemi et al. 2019; Olsen et al. 2017). Thus, we analyzed the association between TB score and LNM in 71 intestinal-type GACs. For intestinal GACs, patients with LNM had much higher TB score than those without LNM (mean \pm SEM: 7.989 ± 1.422 , $n = 44$ vs. 3.879 ± 1.044 , $n = 33$; Fig. 1d). ROC curve showed that TB score could effectively differentiate patients with and without LNM (AUC = 0.6659, $P = 0.0168$, Fig. 1e). However, the optimal cut-off value of TB score in intestinal GAC was 1 other than 5 in the whole cohort. As a result, we divided the intestinal GAC patients into low- and high-grade TB subgroups using the cut-off value 1. Patients with high-grade TB score had

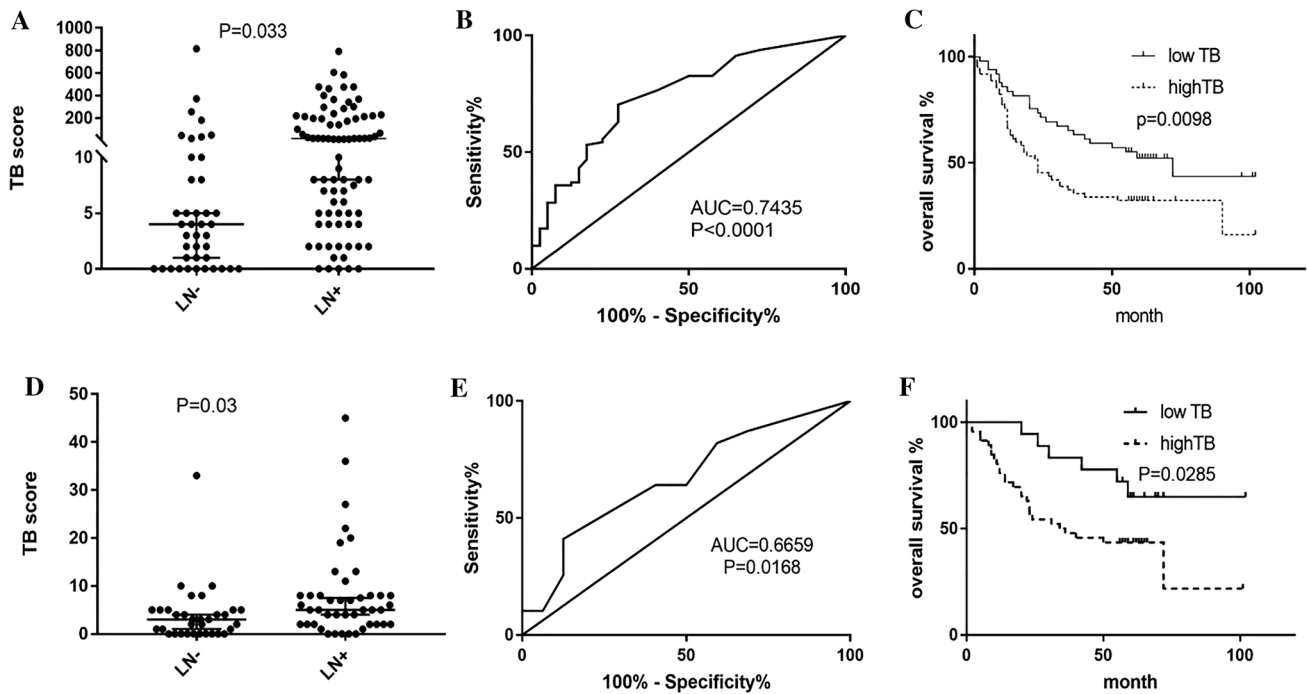


Fig. 1 Predictive role of TB score in nodal involvement and overall survival. **a, b, c** Total cohort ($n=122$) were analyzed. **d, e, f** Intestinal type GACs ($n=77$) were analyzed. **a, d** TB score in patients with lymph node metastasis (LN+) was much higher than that in patients without lymph node metastasis (LN-). Non-paired student's

t test was used to analyze the difference between the two groups. **b, e** ROC curve showed that TB score could well separate patients with and without lymph node metastasis. **c, f** Survival was analyzed by the Kaplan–Meier method and compared by log-rank tests. Patients with higher TB score had a significantly poorer overall survival period

significantly shorter OS (median survival of 35 months vs. 59 months; HR: 2.558, 95% CI: 1.258–5.202, $P=0.0285$; Fig. 1f).

ZBTB7A expression was positively associated with TB score

Previously we have reported the role of GAGE7B, CTNND1, Kif26B, CREB1 and ZBTB7A in gastric cancer progression. Immunostaining was performed to assess the expression of these genes in gastric cancer tissues. In this study, we reassessed the expression of each gene inside the tumor mass and tumor margin respectively. Center score (inside the tumor mass) and margin score were assigned to each case (as described in materials and methods) and were correlated with corresponding TB score respectively. Correlation were performed in intestinal and diffused type GAC subgroups respectively. Neither central nor marginal expression of CREB1 (Supplementary Fig. 2A–D), KIF26B (Supplementary Fig. 2E–H), GAGE12I (Supplementary Fig. 2I–L) and CTNND1 (Supplementary Fig. 3) were significantly correlated with TB scores in both subgroups. Studies have shown that CTNND1 cellular localization is important as for its role in tumor progression (Xing et al. 2015), so membrane staining score and cytoplasm staining score were assigned to

each case and correlated with TB scores respectively (Supplementary Fig. 3A–D, E–H).

Among the five genes, marginal ZBTB7A expression in intestinal-type GACs was found to be positively correlated with TB score ($P=0.0156$, $r=0.3403$, 95% CI: 0.06–0.5708, $n=50$, Fig. 2a), while central ZBTB7A tended to have a positive correlation with TB score but of no statistical significance ($P=0.1337$, $r=0.215$, 95% CI: -0.076 – 0.472 , $n=50$, Fig. 2b). Cases with a ZBTB7A score greater than median tend to have greater TB score both in the tumor edge or within the tumor mass ($P=0.04$ in the tumor margin, $P=0.03$ in the tumor center, Fig. 2c, d). Neither marginal nor central ZBTB7A expression showed any correlation with TB scores in diffused type ($n=12$, Fig. 2e, f) GACs.

Association of TB score and higher ZBTB7A expression in the tumor margin

In our previous study we have demonstrated that higher ZBTB7A expression was associated with more advanced tumor stage, positive LNM, diffused type of Lauren's classification and unfavorable prognosis (Shi et al. 2015). In the present study, it was interesting to find that the ZBTB7A immunostaining score were much higher in the tumor edge than that inside the tumor body in a portion of the intestinal

Table 1 Patients Clinicopathologic characteristics of gastric cancers associated with TB

Variables	Patients (122)		Tumor budding		
	Number	%	Low	High	<i>P</i> value
Gender					
Male	101	83	41	60	0.55
Female	21	17	10	11	
Age (years)					
≤ 60	60	49	22	38	0.26
> 60	62	51	29	33	
Tumor size (mm)					
≤ 40	56	46	27	29	0.33
> 40	66	54	26	40	
Lauren's classification					
Intestinal type	71	58	51	20	< 0.0001****
Diffuse/mixed type	51	42	2	49	
Differentiation					
Well/moderately differentiated	58	48	39	19	< 0.0001****
Poorly differentiated	64	52	14	50	
Lymphnode metastasis					
Negative	41	34	29	12	< 0.0001****
Positive	81	66	24	57	
Distant metastasis					
Negative	96	79	46	51	0.08
Positive	26	21	7	18	
Tumor staging					
T1/T2	21	17	11	10	0.36
T3/T4	101	83	42	59	
Pathological stage					
Stage I + II	50	41	29	21	0.007***
Stage III + IV	72	59	24	48	

P*<0.05, *P*<0.01, ****P*<0.001, *****P*<0.0001

GAC cohort (*n* = 18, Fig. 3a–g). We defined this group of patients who have a stronger ZBTB7A staining in the tumor margin than the tumor center as “higher marginal ZBTB7A +” (*n* = 18) and patients with a ZBTB7A expression in the margin equal to or less than the tumor center as “higher marginal ZBTB7A -” (*n* = 30). Patients who were higher margin ZBTB7A + tend to have more tumor buds in the tumor edge (Fig. 3h) and have unfavorable prognosis (Fig. 3i).

ZBTB7A promotes migration and invasion of tumor cells via PI3K-AKT-mTOR and MAPK-ERK pathway

To explore the mechanisms how ZBTB7A promotes TB formation, we studied the function of ZBTB7A in cultured gastric cell lines. ZBTB7A was over-expressed using an expression plasmid or knocked down by small interfering RNAs in BGC823 and MKN45 cells. Transwell assay showed that in both cell lines, ZBTB7A knock-down could inhibit tumor

cell migration and invasion while ZBTB7A over-expression increased migrated and invaded cells (Fig. 4a–d).

We subsequently asked how ZBTB7A promoted cell motility. RNA-seq were performed using BGC823 cells that transfected with scrambled negative control siRNA or siRNA targeting ZBTB7A respectively. 2059 genes were down-regulated and 558 genes were up-regulated by 1.5-fold upon ZBTB7A knockdown. To obtain a better understanding of the differentially expressed genes, KEGG pathway enrichment analysis was performed. EGFR signaling pathway together with mTOR pathway and MAPK pathway were among the most significantly enriched cancer-related pathways (Fig. 4e). Because the signals of EGFR pathway were transduced mainly through mTOR and MAPK pathway, the enriched EGFR pathway is probably an up-stream event of the latter two pathways. RT-qPCR were performed to verify the RNA-seq data of selected mRNAs. We found that mRNA levels of mTOR pathway-related genes (PIK3CA, AKT3 and mTOR) and MAPK related genes (EGFR and KRAS) were greatly

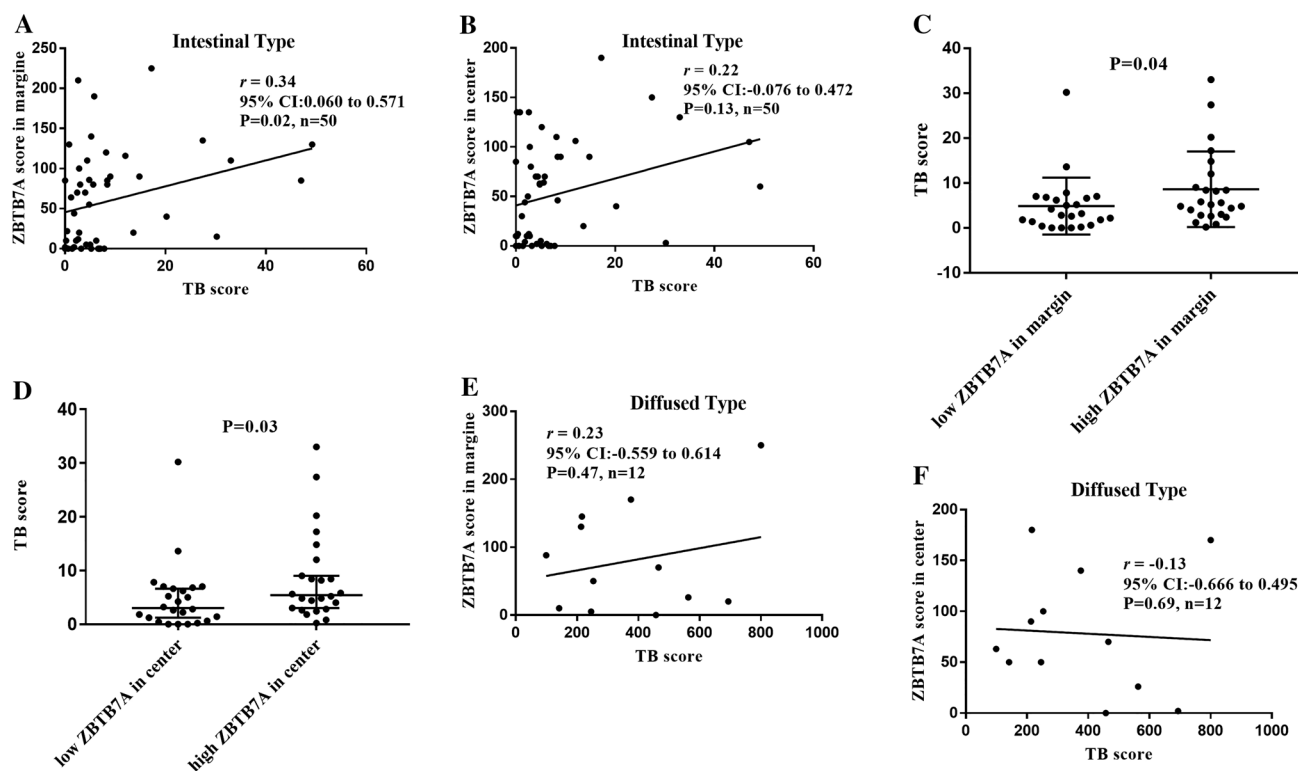


Fig. 2 Correlation of TB score and ZBTB7A expression score. **a**, **b** In the intestinal type GAC subgroup, ZBTB7A expression in the tumor margin (**a**) but not inside the tumor (**b**) is positively correlated with TB score (spearman, $r=0.34$, $P=0.02$). **c**, **d** Cases with a ZBTB7A score greater than median tend to have greater TB score

both in the tumor edge (**c**) and within the tumor mass (**d**). Non-paired student's t test was used to analyze the difference between the two groups. Neither marginal (**e**) nor central (**f**) ZBTB7A expression showed any correlation with TB scores in diffused type GACs

decreased upon ZBTB7A knock-down (Fig. 4f, g). Western blotting confirmed the up-regulation of these genes by ZBTB7A overexpression and down-regulation by ZBTB7A knockdown (Fig. 4h–l). Of note, although mRNA levels were not changed significantly, detected by RT-qPCR or RNA-seq, the phosphorylated form of MEK1/2 and ERK1/2 proteins were greatly increased or decreased upon ZBTB7A manipulation.

It is thought that tumor budding is the result of epithelial-mesenchymal transition (EMT), and both the PI3K/AKT/mTOR and MAPK/ERK pathways are classical oncogenic pathways and the main regulator of EMT. As a result, we examined the expression of EMT markers following ZBTB7A over-expression or knock-down by western blot. Results showed that ZBTB7A could increase the expression of N-cadherin, Vimentin and Snail but decreased E-cadherin expression (Fig. 4m–o).

Taken together, our data indicated that ZBTB7A promoted tumor budding formation by enhancing EMT via PI3K-AKT-mTOR and MAPK-ERK signaling pathways (Fig. 4p).

Discussion

Tumor budding is a well-established prognostic feature in colorectal carcinoma, and it is gaining increasing attention in GAC. GAC tumor budding was first described by Gabbert et al. (1992) as cell dissociation in the tumor invasive front and was predictive of decreased survival independently. In the study by Gulluoglu et al. (2015) that included only early-stage GAC, tumor budding was the only factor significantly and independently related to LNM in pT1a patients. There are studies showing that tumor budding is associated with nodal involvement, recurrence and survival in intestinal-type GAC rather than diffused type GAC (Che et al. 2017; Kemi et al. 2019; Olsen et al. 2017). In our study, we showed that patients with LNM tend to have greater TB scores than those without LNM which is in accordance with previously studies. We determine the cut-off value of TB score by ROC curve rather than assigning to a certain commonly used value (1, 5 or 10). Patients with higher TB scores have poorer overall

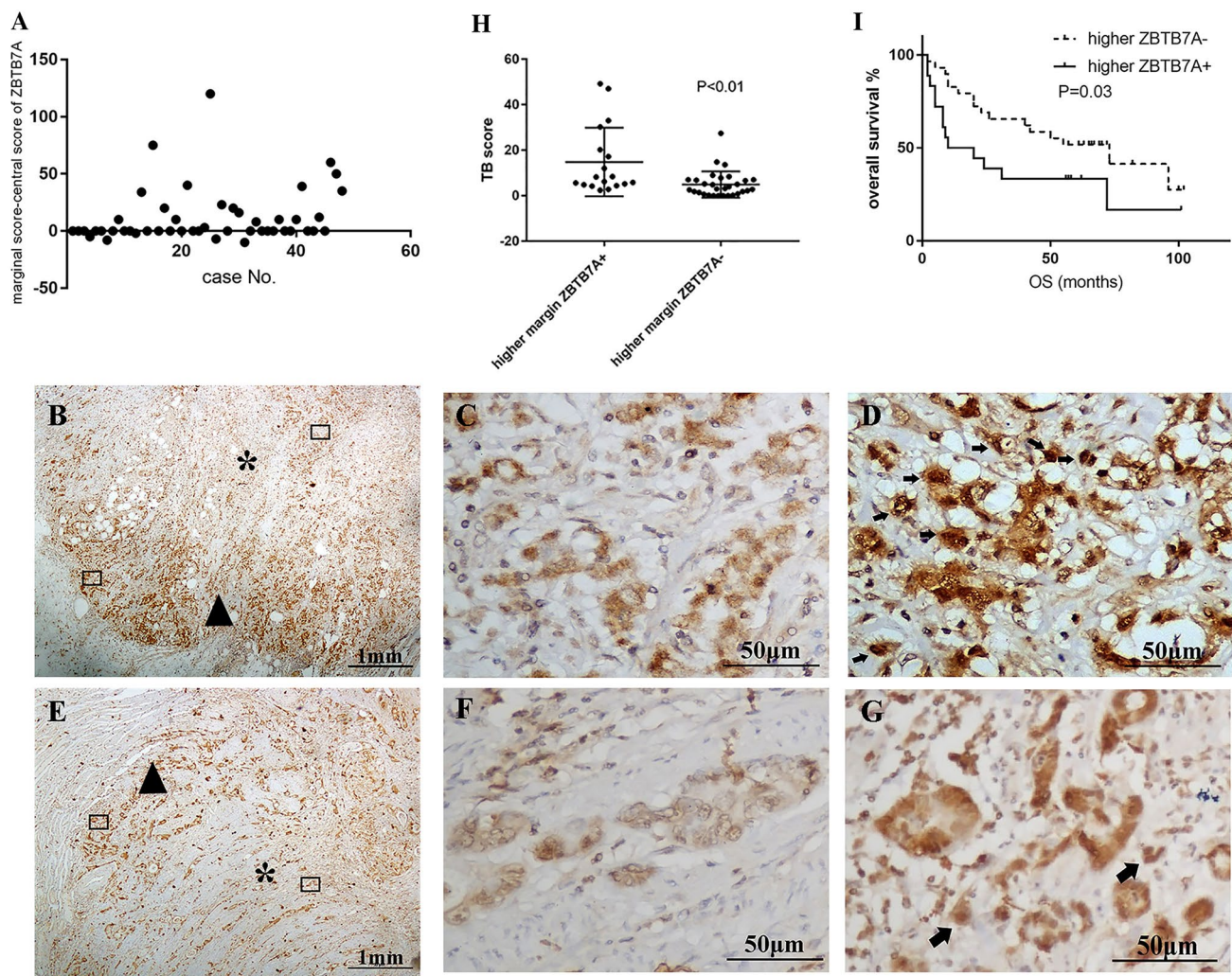


Fig. 3 Higher ZBTB7A expression in the tumor margin and its clinical significance. **a** In intestinal-type GACs, a portion of our cohort have higher ZBTB7A expression in the margin than inside the tumor (marginal score – center score > 0). **b–g** Two representative cases showed stronger immunostaining in the tumor margin than in the tumor center. Image **b, c, d** and **e, f, g** were taken from the two individual cases, respectively. Low-power microscopic images ($\times 20$, **b, e**) and corresponding high-power microscopic images of tumor center

($\times 400$, **c, f**) and tumor margin ($\times 400$, **d, g**) were taken (asterisk: tumor center, arrow head: tumor margin, arrows: identifiable tumor budding, rectangles: magnified area of high-power microscopic images). **h, i** Patients with higher ZBTB7A expression in the tumor margin than the tumor center had more tumor buds in the tumor edge (**h**) and unfavorable prognosis (**i**). Non-paired student's t test was used to analyze the difference between the two groups. Survival was analyzed by the Kaplan–Meier method and compared by log-rank tests

survival. Then we analyzed the predictive role of TB scores in intestinal-type GACs. The results in intestinal-type GACs were similar with whole cohort but to a less extend, as diffused type GACs displaying extensive tumor buds, earlier nodal involvement and frequent recurrence were excluded.

It is still not fully understood how tumor budding forms and how it affects tumor progression. Several genes which are correlated with tumor budding are found to be differentially expressed inside the tumor mass and in the invasive front. In colorectal carcinoma, the expression of miR-200 is largely decreased in the invasive front and is negatively

associated with tumor budding numbers (Martinez-Ciarpaglini et al. 2019). The miR-17/92 cluster members are increased in the invasive front and are positively associated with tumor budding (Jepsen et al. 2018). In the present study, we analyzed the immunostaining pattern of GAGE12I, CTNND1, Kif26B, CREB1 and ZBTB7A in GAC tissues. All five genes were previously found to be oncogenic and is closely related to prognosis by our team. Immunostaining scores of inside the tumor mass as well as in the tumor edge were assigned to each case respectively. Out of the five genes, tumor edge expression of ZBTB7A was positively correlated with TB scores in intestinal types

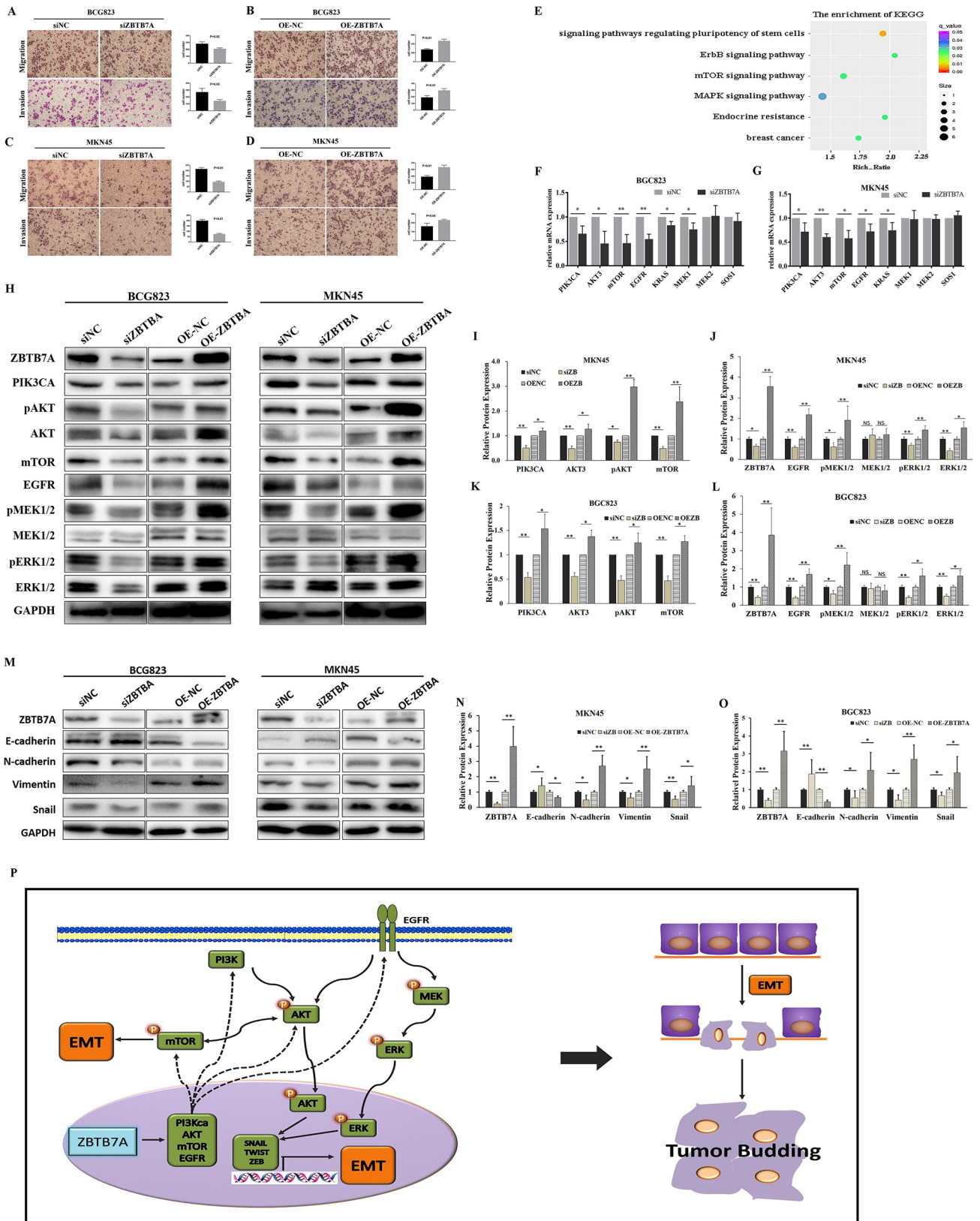


Fig. 4 ZBTB7A enhanced tumor cell mobility and activated PI3K-AKT-mTOR and EGFR-MAPK-ERK pathway. **a–d** Transwell assay showed that ZBTB7A knock-down inhibited migration and invasion of BGC823 cell (**a**) and MKN45 cell (**c**). ZBTB7A overexpression enhanced migration and invasion of tumor cells (**b, d**). **e** KEGG pathway enrichment analysis of RNA-seq data indicated the enrichment of EGFR, mTOR and MAPK pathway. **f, g** RT-qPCR were performed to verify the RNA-seq data of selected mRNAs in tumor cell lines. **h–l** Western blotting confirmed the up-regulation and down-regulation of PI3K-AKT-mTOR and MAPK-ERK pathway-related genes by ZBTB7A overexpression and knockdown respectively. **m–o** Western blotting showed that EMT was initiated upon ZBTB7A manipulation. All the experiments were performed in triplicates. **p** Schematic model of potential mechanisms utilized by ZBTB7A to promote tumor budding formation. * $P < 0.05$, ** $P < 0.01$

GACs. In a portion of our intestinal GAC cohort (18 out of 48 cases, 36%), ZBTB7A was more intensively stained in the tumor edge than that in the tumor body. This portion of patients tends to have more tumor buds in the tumor edge and unfavorable prognosis. Thus, ZBTB7A may play a role in promoting budding formation in GAC.

ZBTB7A is a multifaced gene exerting its function in a context-dependent manner (Caterina Constantinou 2019). It was first reported as an oncogene in T cell lymphoblastic lymphoma/leukemia by Maeda et al. (2005). ZBTB7A works as a suppressive transcription factor to inhibit the expression of the Arf tumor suppressor (p19Arf) gene resulting in decreased p53 activity. Accumulating studies further show that ZBTB7A is overexpressed in several other human cancers (Caterina Constantinou 2019). Previously we have demonstrated for the first time that higher ZBTB7A expression was associated with more advanced tumor stage, positive LNM, diffused type of Lauren's classification and unfavorable prognosis in GAC patients (Shi et al. 2015). In this study, we showed that ZBTB7A expression in the tumor invasive front was correlated with tumor budding formation. We further investigated the underlying mechanism. Upon ZBTB7A knock-down, the mobility of gastric cancer cells was greatly hindered manifested by decreased cell numbers in trans-well assay. ZBTB7A knock-down or over-expression could enrich key regulators or transducers of PI3K-AKT-mTOR pathway and MAPK-ERK pathway, indicating that ZBTB7A may function by modulating the two pathways. The PI3K/AKT and MEK/ERK pathways are both activated in response to various extracellular stimuli and have diverse down-stream targets regulating cell proliferation, migration, and apoptosis. Both the two pathways could transduce the signals from EGFR activation (De Luca et al. 2012). Chan-Chan Lin et al. (2012) reported that ZBTB7A silencing inhibited the PI3K/Akt and Raf/MEK/ERK pathways. Phospho-PEN, a major negative regulator of the PI3K/Akt pathway, and c-Raf, a direct activator of ERK, were increased and decreased respectively by ZBTB7A silencing.

In this study, we showed that ZBTB7A increased the expression of EGFR in both mRNA and protein level. EGFR activation is an up-stream event of both PI3K-AKT-mTOR and MAPK-ERK pathway, so it might be a key step mediating ZBTB7A-induced activation of the two pathways. Moreover, we showed that the expression of AKT3, PIK3ca, mTOR and K-ras, key genes in the PI3K/AKT and MEK/ERK pathways, were up-regulated by ZBTB7A both in mRNA and protein level. We failed to demonstrate any direct binding of ZBTB7A to the promoter region of each gene, so the regulation by ZBTB7A might be indirect.

It is proposed that tumor budding is the visible process of EMT as the buddings display EMT features such as lose of adherence and enhanced cell motility (Koelzer et al. 2016). The relation between tumor budding and the expression of main regulators of EMT has been studied in several human malignancies (De Smedt et al. 2017; Galvan et al. 2015; Karamitopoulou et al. 2010, 2011; Niwa et al. 2014). In this study, we found that the PI3K-AKT-mTOR pathway and MAPK-ERK pathway, two classical EMT regulators, were enriched by ZBTB7A overexpression. In many cancer types, p-ERK1/2 could regulate the expression of ZEB, SNAIL and TWIST, key transcription factors of EMT, leading to decreased expression of E-cadherin and increased expression of N-cadherin, Vimentin and Fibronectin. AKT activation is the center of PI3K-AKT signaling pathway, mediating numerous cellular activities. Recently, it is reported that AKT could promote EMT through suppressing E-cadherin expression via EMT transcription factors. mTOR, which was greatly inhibited by ZBTB7A silencing, can be activated directly by phospho-Akt and can also regulate the EMT by affecting actin cytoskeleton arrangement of the cell through altering the PKC phosphorylation state (Karimi Roshan et al. 2019; Olea-Flores et al. 2019). Interestingly, our data showed that ZBTB7A manipulation greatly altered the expression of EMT markers. This strongly supported our hypothesis that ZBTB7A may enhance tumor cell motility by modulating EMT via PI3K-AKT-mTOR and MAPK-ERK pathway.

In conclusion, we found that tumor budding was a valuable indicator in predicting LNM and prognosis. ZBTB7A, a multi-faceted gene, may be a key regulator of budding formation through enhancing tumor cell EMT via modulating PI3K-AKT-mTOR pathway and MAPK-ERK pathway. Further approaches might be therapeutically and diagnostically promising in cancer research.

Acknowledgements This study was supported by the National Natural Science Foundation of China (Grant no. 81872362 and 81672842) and the Taishan Scholars Program of Shandong Province (Grant no. ts201511096).

Author contributions YS and PG designed the experiments and wrote the paper. YS did the in vitro experiments. YS and JH performed the

data analysis. DBS, HZ, XC and AYY collected the specimens and performed the IHC staining.

Funding This study was supported by the National Natural Science Foundation of China (Grant no. 81872362 and 81672842) and the Taisihan Scholars Program of Shandong Province (Grant no. ts201511096).

Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interests.

Ethics approval This study was approved by the Ethics Committee of Shandong University.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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