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Original Article

High expression of biglycan is associated with poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract: Biglycan (BGN), an extracellular matrix component, has been reported to play a crucial role in the tumor progression of various cancers. However, the relation between the expression of BGN and clinical prognosis has not been studied yet. We therefore carry out the present study to elucidate the role of BGN in predicting outcomes of patients with esophageal squamous cell carcinoma (ESCC). In this study, the expression of BGN in 170 cases of ESCC tissues and matched 46 adjacent non-tumorous tissues was measured by quantitative real-time PCR and immunohistochemistry. Upregulation of BGN occurred in approximately 60% of primary ESCCs compared with their non-tumor counterparts. In addition, high expression of BGN was significantly associated with clinical stage (P = 0.009), tumor invasion (P = 0.006) and lymph node metastasis (P = 0.046). The 5-year disease-specific survival (DSS) in high expression of BGN group is poorer than that in low level expression group (36.8% VS 57.4%, P = 0.006). Stratified analysis according to the pathological stage revealed its discernibility on DSS was only pronounced in patients with advanced clinical stage (P = 0.010). Cox multivariate analysis revealed that pathologic N category (P < 0.001; hazard ratio, 2.482, 95% CI, 1.576-3.909) and BGN expression (P = 0.019; hazard ratio, 1.713, 95% CI, 1.092-2.688) were two independent prognostic factors. The findings of the present study provide evidence that BGN represents a potential novel prognostic biomarker for resected ESCC patients in advanced clinical stage.

Keywords: BGN, ESCC, prognosis

Introduction

Esophageal cancer (EC) has been ranked as the eighth most common malignancy and the sixth leading cause of cancer-related mortality worldwide, with incidence that varies greatly by geographic locations and ethnicity [1, 2]. Histologically, esophageal squamous cell carcinoma (ESCC), the most prevalent pathological type of EC, predominates in east countries, particularly in China, with a proportion of more than 90% of all EC [3, 4]. Despite tremendous progress in diagnosis and therapeutic options, the average overall 5-year survival rate for ESCC is approximately 10-41% [5-7]. To date, extensive molecular biology studies of ESCC have identified a mass of dysregulated molecular events involved in esophageal carcinogenesis, which cover a wide range of genes with diverse functions. However, the reliable biomarkers for high-risk population screening, for clinical diagnosis and prognosis, for evaluation of treatment efficiency are still lacking. Therefore, it is imperative to identify and characterize more effective biomarkers for such purposes.

With the advent of next-generation sequencing technologies in recent years, transcriptome sequencing (RNA-Seq), a new sequencing platform, has been applied to delineate changes at the transcriptomic level in the study of cancer. Recently, our group performed a RNA-Seq to investigate differential gene expression in twelve non-tumor and ESCC clinical samples. About 1,598 up-regulated genes were identified including biglycan (BGN). Extracellular matrix (ECM) is the non-cellular component of tissues, which not only provides mechanical structural support but also takes part in the regulating the behavior of the cells. Every tissue

Table 1. Primer sequences used for qPCR analyses

Gene	Sequence	Accession No.
BGN q-F	5'- CTGGCATCCCCAAAGACCTC -3'	NM_001711.4
BGN q-R	5'- GCTCCCGTTCTCGATCATCC -3'	
GAPDH q-F	5'- ACTTCAACAGCGACACCCACTC -3'	NM_001256799.1
GAPDH q-R	5'- TACCAGGAAATGAGCTTGACAAAG -3'	

Table 2. Association of BGN expression with clinicopathological features in ESCCs

Clinical features	Cases	BGN expression		*P* value
		low level (%)	high level (%)	
Age (years old)				0.913
≤ 59	90	42 (46.7%)	48 (53.3%)	
> 59	80	38 (47.5%)	42 (52.5%)	
Gender				0.762
Male	123	57 (46.3%)	66 (53.7%)	
Female	47	23 (48.9%)	24 (51.1%)	
Location				0.660
Upper	34	18 (52.9%)	16 (47.1%)	
Middle	95	42 (44.2%)	53 (55.8%)	
Lower	41	20 (48.8%)	21 (51.2%)	
Differentiation				0.988
Grade 1	37	17 (45.9%)	20 (54.1%)	
Grade 2	93	44 (47.3%)	49 (52.7%)	
Grade 3	40	19 (47.5%)	21 (52.5%)	
pT category				**0.006**
T1-2	39	26 (66.7%)	13 (33.3%)	
T3-4	131	54 (41.2%)	77 (58.8%)	
pN category				**0.046**
N0	90	49 (54.4%)	41 (45.6%)	
N1	80	31 (38.8%)	49 (61.2%)	
Clinical stage				**0.009**
Early (I-II)	94	53 (56.3%)	41 (43.6%)	
Advanced (III)	76	27 (35.5%)	49 (64.5%)	

Statistical significance ($P < 0.05$) is shown in bold.

has an ECM with unique composition and topology that governs the process of determination, differentiation, proliferation, migration and regeneration of cells [8]. BGN is a member of the small leucine-rich proteoglycan (SLRP) family of proteoglycans found in ECM, which is characterized by a core protein with centrally located leucine-rich repeat motifs flanked by disulfide bond stabilized loops [9]. BGN is distinctly expressed at the cell surface or in the pericellular matrix in various tissues of mainly mesenchymal origin [10], and is functionally involved in matrix assembly, cellular migration and adhesion, and the regulation of growth factor [11, 12]. BGN expression is associated with morphological changes of cellular hypertrophy and well-formed actin stress fibers, characteristic of epithelial-to-mesenchymal transdifferentiation [13, 14]. Recently, overexpression of BGN has been detected in a variety of human epithelial tumors, including colon [15, 16], ovary [17], liver [18], pancreas [19] and odontogenic [20], suggesting an important role of BGN in the pathogenesis, progression and therapy of cancer. Nonetheless, the relationship between BGN expression and clinical prognosis in ESCC need to be further elucidated. Thus, the purpose of present study is to verify the BGN expression in primary ESCC and analyze the correlation with clinical parameters.

Materials and methods

Patients and tissue samples

170 primary ESCC tumor tissues and 46 paired adjacent non-tumorous tissues were collected immediately after surgery resection at Sun Yat-Sen University Cancer Center from March 2002 to October 2008. The inclusion criteria were as follows: (a) histological proof of thoracic ESCC; (b) complete surgical resection (R0); (c) no neoadjuvant or adjuvant treatment; (d) complete follow-up data. Ethical approval for this study was granted by the Medical Ethics Committee of Sun Yat-Sen University Cancer Center. All patients signed informed consent.

Quantitative real-time polymerase chain reaction (qPCR)

The fresh tumorous and non-tumorous samples were taken from regions which macroscopically judged to be neoplastic and normal, respectively. Both of them were immediately stored at dry ice after resection and then frozen at -80°C. Total RNA was extracted from clinical samples

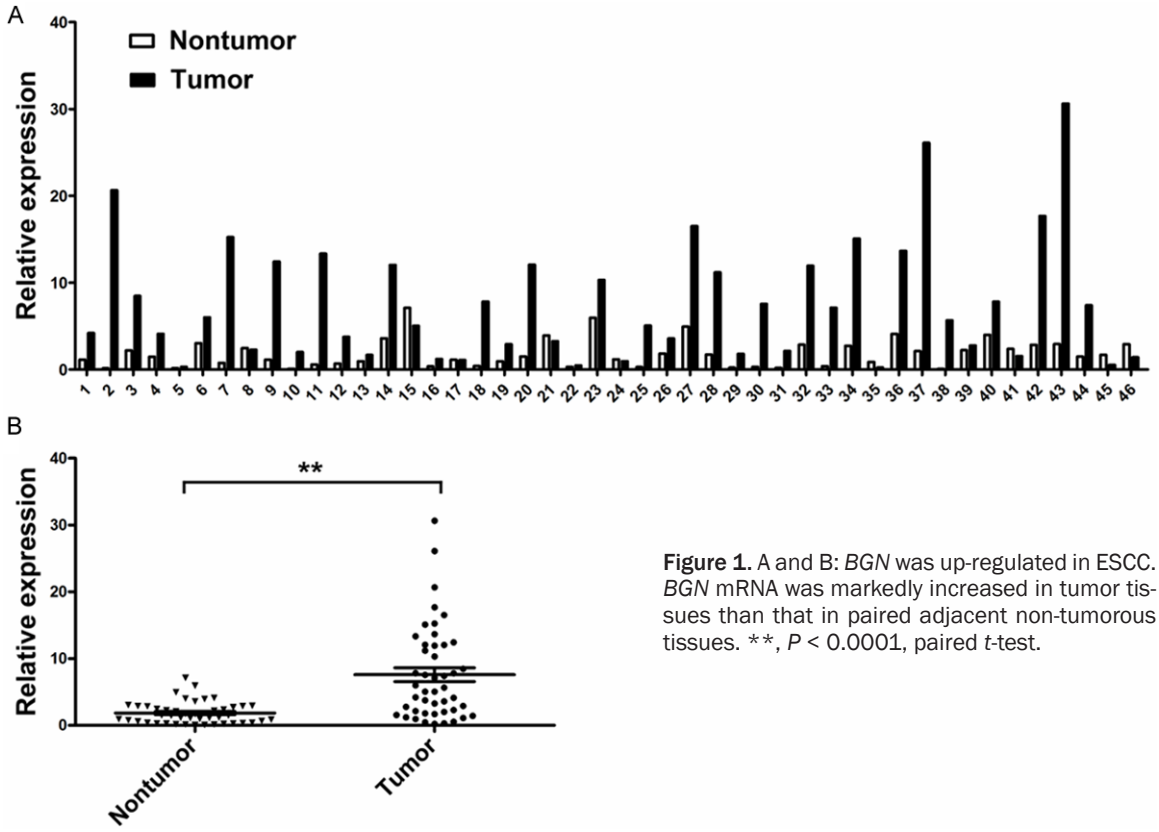

Figure 1. A and B: *BGN* was up-regulated in ESCC. *BGN* mRNA was markedly increased in tumor tissues than that in paired adjacent non-tumorous tissues. **, $P < 0.0001$, paired *t*-test.

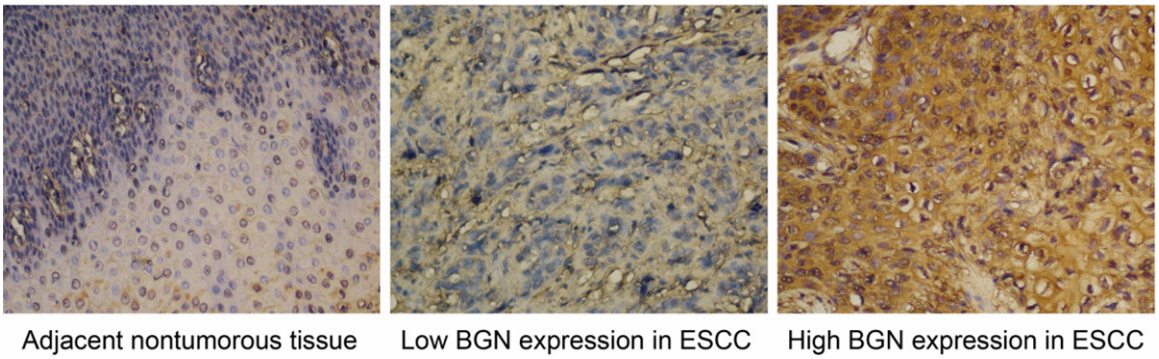

Figure 2. Representative of BGN expression in adjacent non-tumorous tissue and ESCC tumor tissues detected by immunostaining with anti-BGN antibody (brown). The slide was counterstained with hematoxylin (original magnification × 200).

using TRIzol reagent (Invitrogen), and was reverse-transcribed with random primers using an Advantage RT-for-PCR Kit (Clontech Laboratories) according to the manufacturer's instructions. qPCR was performed to detect levels of the corresponding *GAPDH* and *BGN* using a SYBR Green PCR Kit (Applied Biosystems) and an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The *GAPDH* was used as an internal control for *BGN*. The primers of *BGN* and *GAPDH* were listed in **Table 1**. The relative levels of expression were quantified and analyzed by using SDS 2.3 software (Applied Biosystems). The real-time value for each sample was averaged and compared using the Ct method. ΔΔCt(sample) = ΔCt(sample) - ΔCt(calibrator), ΔCt(sample) = Ct(sample) of *BGN* - Ct(sample) of *GAPDH*; ΔCt(calibrator) = Ct(calibrator) of *BGN* - Ct(calibrator) of *GAPDH*; calibrator was

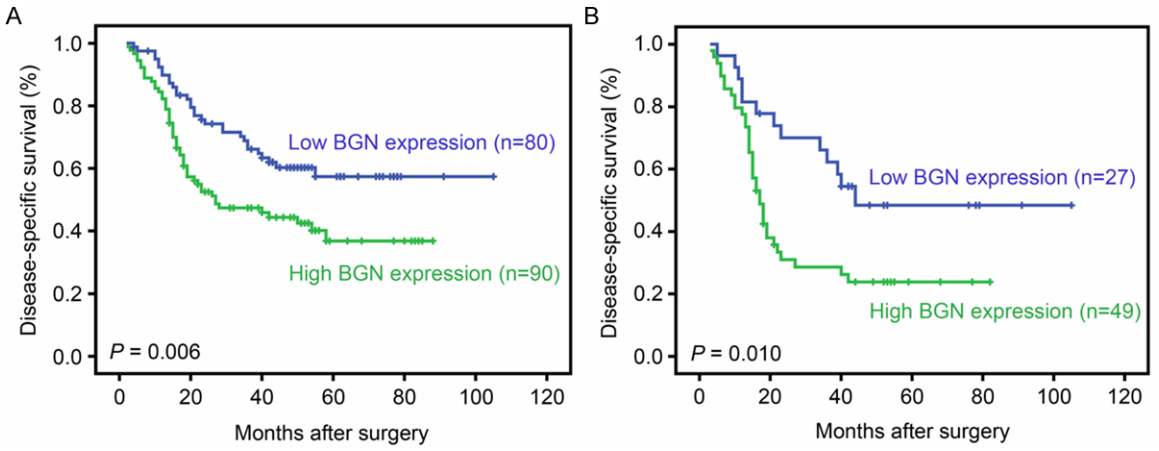

Figure 3. A: The correlation of expression of BGN and prognosis of patients with ESCC. Kaplan-Meier curves with univariate analysis showed that patients with high expression of BGN had a poorer disease specific survival than those with low expression of BGN. $P = 0.006$, log-rank test. B: Stratified survival analysis according to the pathological stage revealed discernibility of BGN expression on DSS was only pronounced in patients with advanced clinical stage (pStage III). $P = 0.010$, log-rank test.

defined as the pooled samples from 46 adjacent non-tumorous tissues.

ESCC tissue microarray (TMA) and immunohistochemical (IHC) staining

A total of 170 formalin-fixed, paraffin-embedded ESCC tumor specimens and 46 corresponding adjacent non-tumorous tissues were selected from Sun Yat-Sen University Cancer Center. The ESCC TMA was constructed as described previously [21]. Briefly, tissue sections with 5 μm thick were cut from the tissue microarray blocks and mounted on microscope slides. The IHC staining was performed using TMA slides that were deparaffinized in xylene, rehydrated through a graded alcohol series and incubated with 3% hydrogen peroxide. For antigen retrieval, TMA slides were boiled by a pressure cooker in 10 mM sodium citrate buffer (pH 6.0) for 15 minutes. The slides were blocked by 10% normal goat serum at room temperature for 30 minutes and then incubated with rabbit polyclonal antibody against BGN (Abgent) at a dilution of 1:200 at 4°C overnight. Immunoreactivity was visualized using an Envision detection system (DAKO), and the nuclei were counterstained with hematoxylin. An immunoreactivity score (IRS) system was applied as described previously [22]. The percentage of BGN-positive cells was scored as 0, < 5%, negative; 1, 5%-25%, sporadic; 2, 25%-50%, focal; 3, > 50%, diffuse. The intensity of BGN-positive staining was scored as 0, negative; 1, weak; 2, moderate; 3, strong. Both the percent of positive cells and cell staining intensity were decided in a double-blinded manner. The total score was determined by the following formula: Staining index = intensity × positive rate. In the present study, staining index ≤ 4 was considered low expression, and staining index > 4 was considered as high expression.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc, Chicago, IL). Paired two-tailed t-test was employed to compare the expression of BGN in primary ESCC tumors and their corresponding adjacent non-tumorous tissues. The correlation between BGN expression and clinicopathologic characteristics was assessed by χ^2 or Fisher's exact tests. Disease-specific survival was calculated from the time of surgery to either the time of death from ESCC or last follow up (31 December 2011). The prognostic value was calculated by the Kaplan-Meier analysis with log-rank test. Univariate and multivariate survival analysis was performed using the Cox proportional hazard model with a forward stepwise procedure (the entry and removal probabilities were 0.05 and 0.10, respectively). A significant difference was considered statistically when P value was < 0.05.

Table 3. Univariate analysis of BGN expression and clinicopathological factors for disease-specific survival in ESCC

Clinical features	Cases	Disease-specific Survival (DSS) (%)			*P* value[a]
		1-year DSS	3-year DSS	5-year DSS	
Age (years old)					0.214
≤ 59	90	83.0	58.0	53.9	
> 59	80	88.8	54.0	37.5	
Gender					0.903
Male	123	84.4	55.4	48.2	
Female	47	89.4	58.1	43.5	
Location					0.655
Upper	34	88.2	60.3	53.7	
Middle	95	88.3	56.5	44.0	
Lower	41	77.7	51.9	38.9	
Differentiation					0.202
Grade 1	37	81.1	63.5	54.1	
Grade 2	93	86.9	59.9	51.7	
Grade 3	40	87.5	40.8	31.1	
pT category					0.272
T1-2	39	89.5	57.9	57.9	
T3-4	131	84.7	55.5	43.2	
pN category					< 0.001
N0	90	91.0	70.8	59.7	
N1	80	80.0	39.7	32.4	
BGN expression					0.006
Low	80	89.8	66.1	57.4	
High	90	82.2	47.4	36.8	

[a]Kaplan-Meier method, log-rank test.

Table 4. Multivariate survival analysis[a] for disease-specific survival in patients with ESCC

Clinical features	*p* value	HR[b]	95% CI[c]
pN category	< 0.001	2.482	1.576-3.909
BGN expression	0.019	1.713	1.092-2.688

[a]Cox's proportional hazards regression analysis (Forward stepwise); [b]HR, hazard ratio; [c]95% CI, 95% confidence interval.

Results

Characteristics of ESCC patients

According to the inclusion criteria, 170 patients with ESCC were recruited in this study. There were 123 male and 47 female patients, with a mean age of 58.1 years (range 30-88 years). Other clinical and pathological parameters were shown in **Table 2**. The follow-up data were obtained from all the patients, with a median survival of 54 months (range 2-105 months). Patients who died from diseases other than ESCC or from unexpected events were excluded from the study. According to the 7th edition AJCC staging system [23] and our demographic data, the clinicopathologic features were dichotomized for statistical analyses as shown in **Table 2**.

The expression of BGN in ESCC and non-tumorous tissues

The mRNA expression of *BGN* was initially tested in 46 pairs of primary ESCC tumors and their corresponding adjacent non-tumorous tissues by qPCR. Up-regulation of *BGN* was detected in 28/46 (60.9%) of ESCC tumors compared with their normal counterparts (defined as a 2-fold higher of *BGN* expression in non-tumor counterparts) (**Figure 1A**). The relative expression level of *BGN* was significantly up-regulated in tumor tissues compared with their non-tumor counterparts ($P < 0.001$, *t*-test; **Figure 1B**). BGN expression in protein level was further studied in 46 primary ESCCs by IHC using a tissue microarray. BGN localized at the cytoplasm of esophageal epithelial cells and ESCC cells. Upregulation of BGN was detected in 26/46 (56.5%) of ESCC tissues compared with their adjacent non-tumor tissues ($P < 0.001$, *t*-test; **Figure 2**).

Clinicopathologic features of BGN in ESCC patients

We next examined the correlation between the expression of BGN and the clinicopathological characteristics of ESCC. High-level expression of BGN was detected in 90/170 (52.9%) of informative ESCC tissues. The correlation between BGN expression status and clinicopathologic features of ESCC was further evaluated, which was summarized in **Table 2**. The results showed that BGN expression was sig-

nificantly associated with pathologic T category (P = 0.006), pathologic N category (P = 0.046) and clinical stage (P = 0.009). No correlation was observed between BGN expression and patient's age (P = 0.913), gender (P = 0.762), tumor location (P = 0.660) and tumor cell histological differentiation (P = 0.988; **Table 2**).

Association between BGN expression and patient survival

Kaplan-Meier analysis showed that a high-level expression of BGN was significantly associated with poorer disease-specific survival (DSS) of resected ESCC patients (P = 0.006). The 5-year DSSs of ESCC patients in high and low level expression groups were 36.8% and 57.4%, respectively (**Figure 3A** and **Table 3**). In a stratified survival analysis according to the pathological stage, no significant difference in DSS was observed in patients with early clinical stage (pStage I and II) (P = 0.704). However, BGN expression could differentiate the prognosis of patients in advanced clinical stage (pStage III) (P = 0.010, **Figure 3B**). Further, by multivariate survival analysis including pathologic N category and BGN expression which had impact on survival of patients, we found that pathologic N category (P < 0.001) and BGN expression (P = 0.019) were two independent prognostic predictors for resected ESCC patients enrolled in this study (**Table 4**).

Discussion

There is ample evidence that the small leucine-rich proteoglycans (SLRPs) are among the key players in the tumor microenvironment and involved in the matrix assembly, cellular migration and adhesion, cell growth, and apoptosis [12, 24]. BGN, as one of the best studied member of the SLPRs, has been reported that its up-regulated expression is associated with cancer progression [15, 17, 18, 20, 25]. Here in this study, we found that approximately 60% of ESCC patients showed elevated BGN expression in their tumorous specimens. *BGN* has been reported to positively regulate cell proliferation, through cdk2- and p27-dependent pathways [26]. It is also proved to enhance the activation of the Wnt/β-catenin pathway through a direct interaction of its core protein with Wnt ligand and its coreceptor, LRP6 [27]. These results lead the hypothesis that *BGN* may induce the proliferation and/or inhibit the apoptosis of ESCC cells. However, other several studies suggest that BGN may serve as a negative growth regulator: anti-proliferative effects on pancreatic tumor cells in vitro by inducing G1 phase cell cycle arrest [19]; anti-apoptotic effects on mesangial cells via inhibiting the activity of caspase-3 [28]. Based on these studies, *BGN* seems to display very contradicting roles dependent on cellular context. Thereby, further studies *in vitro* and *in vivo* are needed to elucidate the precise mechanisms of *BGN* that involved in the progression of ESCC.

Furthermore, in this study, the genetic-clinicopathologic correlation analysis found that a high expression level of BGN was observed more frequently in patients with tumor invasion (P = 0.006), lymph node metastasis (P = 0.046) and advanced clinical stage (P = 0.009). A recent study which indicated that up-regulated *BGN* was associated with tumor metastasis via a microarray profiling of 15 adjacent normal/tumor-matched ESCC specimens confirmed our results [29]. These findings agree with the fact that proteoglycans, as prominent constituents of both the extracellular matrix and the cell surface, are proposed to play roles in cell adhesion, growth factor interactions, and matrix assembly [30, 31]. A hand-full of studies have now been conducted to investigate the molecular mechanism of *BGN* involved in metastasis. Tufvesson et al. has previously reported that *BGN* and decorin could induce morphological and cytoskeletal changes involving signaling by the small GTPases RhoA and Rac1, resulting in lung fibroblast migration [13]. In addition, *BGN* was found to inhibit cell adhesion on type I collagen and fibronectin via its binding to these substrates [28]. In the *BGN* transgenic mice, *BGN* may directly or indirectly activate TGF-β [32]. Of interest, it has been reported that *BGN* is specifically expressed in tumor endothelial cells, and serum *BGN* levels are higher in cancer patients than in healthy volunteers [33]. In light of the present investigation we postulate that BGN secreted from tumor endothelial cells into blood flow might be of diagnostic value in ESCC patients with advanced clinical stage. Nonetheless, further investigation with a larger sample size is needed to confirm these findings. On the other hand, Yamamoto et al. also found that *BGN* could act as an angiogenic factor stimulating TEC migration and tube formation in an autocrine manner through TLR2 and

TLR4 [33]. Thus, this may indicate *BGN* could be a novel target for anti-tumor, as well as anti-angiogenic therapy without injuring normal blood vessels to ESCC patients with advanced clinical stage in future.

Our study also demonstrated that high expression of BGN was one of the most important prognosis factors of poor disease-specific survival in the univariate and multivariate analysis. The 5-year disease-specific survival of patients with high BGN expression was markedly shorter than that with low expression (36.8 vs. 57.4%). Moreover, we analyzed the prognostic value of BGN expression level in selected patient subgroups, and found that in subgroup of patients with advanced clinical stage, higher BGN expression was associated with a poorer DSS. Adjuvant chemotherapy [34, 35] or neoadjuvant chemoradiotherapy [36-40] was proven to improve the clinical outcome of patients with locally advanced ESCC, compared with surgery alone. Therefore, postoperative adjuvant chemotherapy may be recommended for the subset of patients with high expression of BGN to improve their outcome.

In conclusion, the results of present study, for the first time, demonstrated that high expression of BGN in ESCC tumorous specimens indicated aggressive tumor behaviors and predicted a worse clinical outcome. These finding suggested that *BGN* may serve as a potential target for diagnostic and anti-angiogenic therapy to ESCC patients, especially the patients with advanced clinical stage.

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Disclosure of conflict of interest

None.

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