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Expression and significance of biglycan in endometrial cancer

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Abstract
Objective This study aimed to determine the expression level of biglycan in different lesion properties of endometrium and to investigate the possible function and prognostic value of biglycan in endometrial cancer.
Methods Immunohistochemical staining (IHC) and quantitative realtime reverse transcription polymerase chain reaction (qRT-PCR) were used to determine the protein and mRNA levels of biglycan in human normal endometrium, atypical hyperplasia endometrium, and endometrial cancer tissue samples. The expression of biglycan in serum and peritoneal washings was detected by ELISA method. Then we analyzed the correlation of biglycan expression with clinicopathological parameters in endometrial cancer.
Results (1) Biglycan was overexpressed in endometrial cancer, especially in cancerous mesenchyme. Moreover, biglycan expression was significantly correlated with histopathological grade and FIGO stage of endometrial cancer; (2) Biglycan expression level in sera and peritoneal washings was significantly higher in endometrial cancer patients; otherwise, Serum expression correlated with clinicopathological parameters of endometrial cancer; (3) Higher level expression of biglycan in cancerous mesenchyme correlated with poor prognosis of endometrial cancer.
Conclusions Biglycan might play a role in the progression of human endometrial cancer and it might be a useful molecular marker for the prognosis of endometrial cancer. This research is an initial step towards biglycan as a potential prognosis marker in endometrial cancer.

Keywords Biglycan · Endometrial cancer · Cancerous mesenchyme · Prognosis

Y. Liu and W. Li contributed equally.

We also state that we have had full control of all primary data and that we agree to allow the Journal to review our data if requested.

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Introduction

Endometrial cancer (EC) is one of the most common carcinogenic conditions in women. In recent years, the incidence of endometrial cancer is on the rise worldwide. Every year approximately 74,000 women die of endometrial cancer, mainly due to recurrence or metastatic disease [1]. Endometrial carcinogenesis is a complex multistage process that involves constant disruption of homeostatic mechanisms controlling endometrial gland cell proliferation, differentiation and programmed cell death. At present, we are in need of deep understanding of its pathogenesis and biological features so that people can benefit from its early discovery and treatment.

Biglycan (BGN), a member of the family of small leucine-rich proteoglycans (SLRPs), consists of a 38-kDa core

protein and two chondroitin/dermatan sulfate chains [2]. BGN is considered to have an organizing role in the assembly of the extracellular matrix (ECM) and is structurally related to decorin, fibromodulin and lumican. Targeted disruption of the BGN gene results in abnormal collagen fibril morphology [3]. BGN can affect signal transduction pathway during cell growth and differentiation through induction of the cyclin-dependent kinase inhibitor p27Kip1 [4]. Moreover, biglycan is found to be expressed both on the cell surface and in the pericellular space in various tissues of mainly mesenchymal origin. It is released from the ECM by proteolytic digestion or secreted by activated macrophages. Soluble biglycan is capable of interacting with cell surface receptors, thereby causing downstream signaling events [5].

The interaction of cells with extracellular matrix (ECM) has been shown to be vital in the regulation of many fundamental cellular biological behaviors including proliferation, migration, and survival, as well as differentiation and tissue morphogenesis. Loss of cellular homeostasis, a characteristic of diseases such as cancer, is always associated with alterations in the composition and amounts of ECM molecules and is usually related to changes in the ability of cells to sense and integrate ECM-derived signals.

Recently, overexpression of biglycan has been detected in a variety of human epithelial tumors, originating from different organs such as liver [6], ovary [7], odontogenic tissue [8], colon [9] and stomach [10], thus suggesting an important role of biglycan in cancer pathogenesis and progression. However, no report is available on biglycan expression or function in human endometrial cancer. In this study, we investigated the biglycan expression in different endometrial specimens, especially, in sera and peritoneal washings. We explored the potential function of biglycan in the development and progression of endometrial cancer.

Materials and methods

Characteristics of tissue specimens

After approval of the Institution Ethics Review Board, we searched the pathology archives of Qilu Hospital of Shandong University for EC cases from 2005 to 2009. A chart review was conducted, with extraction of clinical information, including patient age at the time of diagnosis, surgical stage, postoperative therapy, disease-free survival time, recurrence time and site, death from the disease, or death from unrelated causes. All the patients underwent a complete surgical staging procedure, including an abdominal hysterectomy with bilateral salpingo-oophorectomy, with or without pelvic and para-aortic lymph node dissection and pelvic washing, depending on the tumor grade and stage.

Fifty-three patients were found to be suitable for evaluation. Patients' age ranged from 29 to 68 (mean age, 53), including 41 (77.36 %) endometrial adenocarcinoma; 4 (7.55 %) adenosquamous carcinoma; 4 (7.55 %) papillary serous carcinoma; 3 (5.66 %) clear cell carcinoma; and 1 (1.89 %) squamous cell carcinoma. All hysterectomy specimens were stained by hematoxylin and eosin and reviewed by an expert gynecologic pathologist for confirmation of the diagnosis, histologic grade, histologic type, and stage on the basis of the 2000 FIGO surgical staging guidelines. According to these guidelines, 30 (56.6 %) were at stage I, 7 (13.21 %) were at stage II, 15 (28.3 %) were at stage III and 1 (1.89 %) was at stage IV. Histologically, 45 (84.92 %) were type I (endometrioid subtype), 8 (15.09 %) were type II (clear cell carcinoma and serous).With regard to histologic grade, 15 (28.3 %) were grade 1 (G1), 21 (39.62 %) were grade 2 (G2) and 17 (32.08 %) were grade 3 (G3). 32 (60.38 %) cases showed myometrial invasion (MI) less than or equal to half the depth of myometrial layer, 21 (39.62 %) were invaded more than half the depth. Lymph node metastasis were positive in 14 (26.42 %), whereas 39 (73.58 %) were negative. In addition, 42 specimens of normal endometrium and 36 cases of atypically hyperplastic endometrium were also included for comparative study. All study subjects have signed an informed consent.

During the follow-up study, 14 patients died of disease, 37 patients survived, and contact was lost with two patients, adding up to a follow-up rate of 96.23 %. Follow-up was mostly executed by telephone or outpatient follow-up service. Follow-up was continued until January 2011 (ranged from 6 to 72 months), with a mean follow-up time of 43.2 months.

RNA isolation and quantitative Real Time-PCR

Total RNA was extracted from tumor tissues and control group using TRIzol reagent (Life Technologies, Inc., Rockville, MD), following the manufacturer's instructions. 5 μg of total RNA was subjected to reverse transcription using the SuperScript^{TM} II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). SYBRGreen reagent (Bio-Rad, Hercules, CA, USA) was used for quantitative real time polymerase chain reaction (qRT-PCR) in a Mastercycler EP realplex Real-Time Quantitative Thermal Block (Eppendorf AG,Hamburg,Germany). For qRT-PCR, each reaction substrate (25 μl) contained 1 μl reverse transcription cDNA product and 100 nM of each primer. The primers for biglycan were as follows: forward primer sequence, 5′-TTTGAGCAGAGAGGCTTCTGG-3′; reverse

primer sequence, 5′-AAAGGACACATGGCGCTGTA G-3′, amplicon size was 140 bp. The specificity of the PCR was confirmed by examining the dissociation reaction plot subsequent to real-time RT-PCR. Real-time PCR data were the normalized expression values with the housekeeping gene GAPDH as the reference gene. Each experiment was conducted in triplicate.

Immunohistochemical staining

Immunohistochemistry staining (IHC) kit was purchased from Santa Cruz Company (California, American). Endometrial tissues were fixed with 10 % formalin, paraffin-embedded, serially sectioned, and stained with hematoxylin and eosin (HE). The expression of biglycan was detected by using immunohistochemical ABC method, following the standard protocol of the reagent kit. Biglycan antibody was purchased from Abcam Company (Cambridge, American) as rat anti-human monoclonal antibody, the concentration of the primary antibody was set at 1:800. Human spleen tissue section was chosen as a positive control, and PBS buffer, instead of primary antibody, was chosen as a negative control. DAB color reagent was used for staining.

All staining tissues were assessed by pathologists blinded to the origination of the samples using a semi-quantitative method. Each specimen was assigned a score according to the intensity and proportion of the staining. Tissue was scored (H-score) based on the total percentage of positive cells and the intensity of the staining (1+, 2+, or 3+), where $H = (\%\ 1+ \times 1) + (\%\ 2+ \times 2) + (\%\ 3+ \times 3)$. A minimum of 100 cells were evaluated to calculate the H-score.

Blood sampling and peritoneal washings

We investigated 57 cases (mean age: 54 years, range: 31–70 years) of endometrial cancer patients from January 2009 to March 2012 from Qilu Hospital of Shandong University, China. The selected patients did not have any other serious medical problems and other malignant tumors, all of them did not undergo chemotherapy, radiotherapy or hormonal therapy before surgery. Patients (including cancer patients and controls) with a history of hysterectomy or bilateral oophorectomy were excluded from the study. In addition, 52 cases of normal endometrium and 38 cases of atypically hyperplastic endometrium were also included for comparative study. Permission for this test was ascertained as the standard written informed consent that was obtained before the time of surgery from each case.

Blood samples were drawn from cancer patients and controls before surgery or any other therapy. Fasting blood samples were obtained and centrifuged at 4 °C in the blood collection tube. Then sera were kept in −80 °C freezer until further processing.

The peritoneal washings were collected routinely during the exploratory laparotomies for suspected gynecological neoplasms or collected prior to hysterectomies for suspected endometrial carcinomas and other benign lesions. 50 ml of sterile saline solution was instilled into the peritoneal cavity immediately after entrying into the peritoneal cavity. Then about 10 ml peritoneal washings was aspirated, centrifuged at 3,000 rpm for 10 min and then sent to the laboratory for further analysis. Only the supernatant from each sample was preserved in −80 °C freezer for further use.

ELISA assay to detect biglycan expression level

ELISA assays for detecting level of biglycan in sera and peritoneal washings were performed and analyzed by commercial, double-antibody, sandwich ELISA kit (Bachem Bioscience, Inc., Prussia, PA) following the manufacturer's procedures. Each sample was examined in triplicates with the average value as the final result. Biglycan concentration was calculated according to the standard concentration and the corresponding optical density value was at 450 nm.

Statistical analyses

SPSS software (Version 10, Chicago, IL) was used for statistical analysis. Variance analysis was used to check the expression differences on IHC H-scoring and RNA expression. Chi-square was used to analyze the association between biglycan expression and clinicopathological data. Survival curves were plotted by using the Kaplan–Meier method and the log-rank test. A probability (p) value <0.05 was considered to be statistically significant.

Results

Biglycan expression levels in different endometrial tissues

To determine biglycan expression levels in different endometrial tissues, we first performed qRT-PCR to determine the transcript levels of biglycan gene in the samples obtained from surgical resection of 20 cases of normal endometrium, 15 cases of atypically hyperplastic endometrium and 24 cases of endometrial cancer. As shown in Fig. 1, biglycan mRNA expression was significantly upregulated in endometrial cancer tissues. To verify whether its functional protein level was also upregulated, IHC staining was conducted and each group samples were also enlarged.

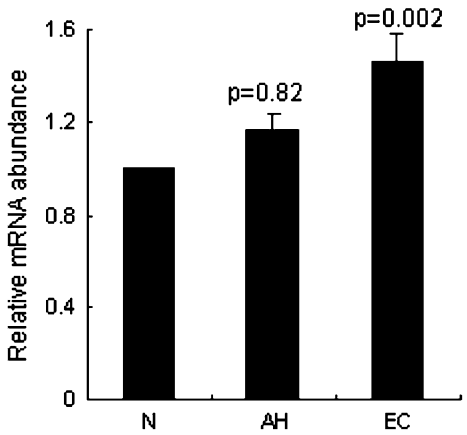

Fig. 1 Biglycan mRNA abundance was analyzed by qRT-PCR in normal endometrium (*N*), atypical hyperplasia endometrium (*AH*) and endometrial cancer (*EC*). (N and AH, $p = 0.082$; N and EC, $p = 0.002$)

The positive biglycan expression is shown by the brown coloring of the tissue. Biglycan was weakly stained implying its low expression in normal endometrium and atypically hyperplastic endometrium, while overexpression was witnessed in endometrial cancer (Fig. 2).We just separatively analyzed relationship of cancerous parenchyma and cancerous mesenchyma with clinicopathological parameters of endometrial cancer patients. Biglycan expression was quantified by H-scoring system as 1.083 ± 0.39 in endometrial carcinoma, while 1.41 ± 0.65 in carcinomatous mesenchyme, there was no significant difference of biglycan expression ($p > 0.05$) between carcinoma and carcinomatous mesenchyme, but there was a significant correlation ($r = 0.406$, $p = 0.003$).

Relationship of biglycan expression levels with clinicopathological parameters of endometrial cancer

We individually analyzed the relationship between the tumor parenchyma and mesenchyma with the clinical parameters involved in endometrial cancer. After statistical analysis, our study indicated that biglycan expression was significantly correlated with histopathological grade, FIGO stage and depth of myometrial invasion in endometrial cancer ($p < 0.05$), but no association was found with age, CA125 level, histological type and lymph node metastasis ($p > 0.05$). Biglycan expression in cancerous parenchyma or mesenchyme of grade 3 was significantly higher than that of grade1-grade2. The similar trend was found in the FIGO stage. The higher stages exhibited higher levels of biglycan expression. Data were showed in Table 1. It showed that biglycan expression was higher in progressive stage, especially in cancerous mesenchyme.

Association of biglycan expression with prognosis of endometrial cancer patients

After surgery, patients were followed-up for overall survival time. The mean follow-up period was 43.2 months (range: 6–72 months). We analyzed its effect on prognosis in carcinoma and cancerous mesenchyme respectively. We just used IHC H-scoring system to analyse its expression level. Then we took a median value 1.8 of H-score in cancerous mesenchyme as critical value, while the median value was 1.2 in carcinoma, and divided the tumour patients into two groups respectively. Survival functions were established between these two groups respectively. It was found that the survival time of patients with higher biglycan expression level in the cancerous mesenchyme was shorter than those with lower expression, whereas patients with lower biglycan expression had better survival rates ($\chi^2 = 5.1$, $p = 0.024$). However, there was no significant difference in prognosis between these two groups when compared its expression in carcinoma (Fig. 3).

Biglycan expression in sera and peritoneal washings of different patients

After finding its overexpression in endometrial cancer tissues, biglycan expression levels in sera and peritoneal washings of endometrial cancer patients were also found to be significantly higher than that of normal control group and atypically hyperplastic group($p < 0.05$); However, there was no significant difference between atypical hyperplasia and normal control group ($p > 0.05$) (Table 2).

Correlation of biglycan expression in sera and peritoneal washings with clinicopathological parameters

Biglycan expression levels in sera of advanced patients were higher than those in early stage patients. Sera biglycan content in patients with lymph node metastasis was obviously higher than those without lymph node metastasis, and there were significant differences in each case ($p < 0.05$). However, biglycan expression levels in peritoneal washings of endometrial cancer patients showed no significant association with FIGO stages, histological grades, depth of myometrial invasion or lymph node metastasis ($p > 0.05$) (Table 3).

Discussion

Current clinical practice in endometrial cancer has no better screening method. However, the curative rate for advanced endometrial cancer remains low and the morbidity rate persists to be high. Despite such facts, several

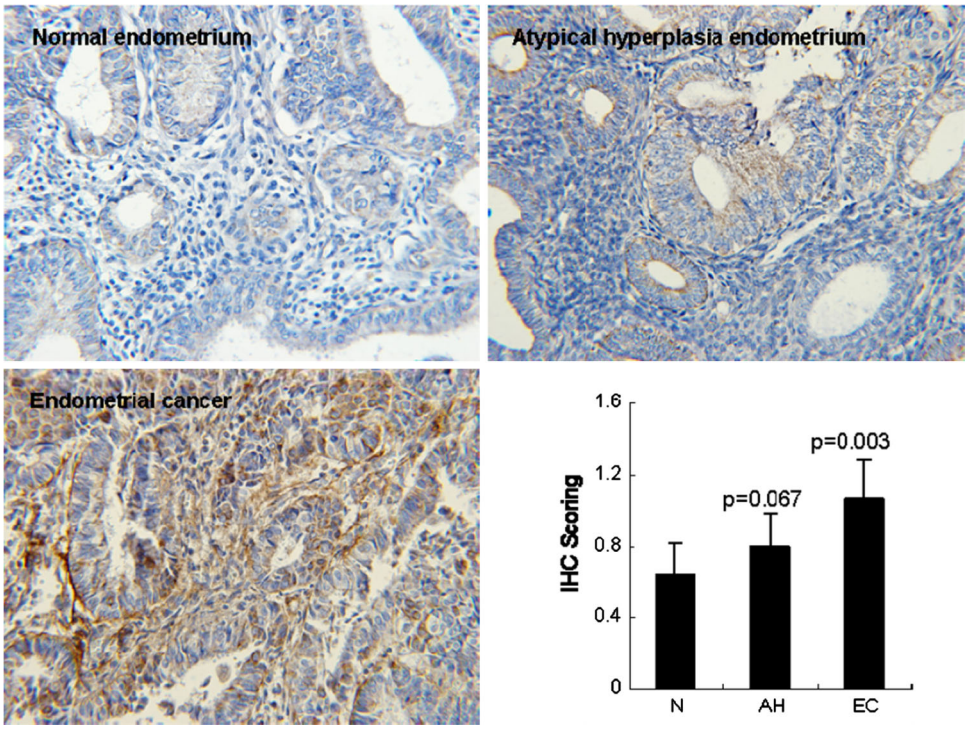

Fig. 2 Biglycan expression was detected on endometrial tissues derived from normal endometrium (*N*), atypical hyperplasia endometrium (*AH*), endometrial cancer (*EC*), (N and AH, $p = 0.067$; N and EC, $p = 0.003$; AH and EC, $p = 0.005$). Original magnification, ×40

Table 1 Relationship between biglycan expression and clinicopathological parameters in endometrial cancer

	N (%)	Cancerous parenchyma		Cancerous mesenchyma	
		IHC scoring	*p* value	IHC scoring	*p* value
Histologic grade					
G1–G2	36 (67.9)	0.93 ± 0.72		1.04 ± 0.59	
G3	17 (32.1)	1.62 ± 0.91	0.002	2.11 ± 0.62	0.000
Histological type					
I type	45 (84.9)	1.15 ± 0.8		1.37 ± 0.58	
II type	8 (15.1)	1.18 ± 1.05	0.672	1.48 ± 0.71	0.361
FIGO stage					
I–II	37 (69.8)	0.85 ± 0.87		1.06 ± 0.76	
III–IV	16 (30.2)	1.36 ± 0.81	0.019	1.68 ± 0.92	0.011
Depth of myometrial invasion					
<1/2	32 (60.4)	0.93 ± 0.73		1.12 ± 0.87	
≥1/2	21 (39.6)	1.49 ± 0.82	0.031	1.59 ± 0.77	0.028
Lymph node metastasis					
No	39 (73.4)	0.91 ± 0.74		1.14 ± 0.83	
Yes	14 (26.6)	1.24 ± 0.89	0.118	1.48 ± 0.82	0.376

clinicopathological parameters such as cancer antigen (CA125) and carbohydrate antigen (CA19-9) have been used as serum markers for endometrial cancer, their sensitivities show variation in different studies and their clinical usefulness remains controversial. Therefore, it is necessary to explore novel markers for estimating the progression of endometrial cancer when designing individualized treatment procedures, which will be helpful to improve the current treatment regimen.

Biglycan is a family member of leucine-rich proteoglycans (small leucine-rich proteoglycan, SLRPs), belonging to the extracellular matrix protein. Biglycan is chiefly expressed in the connective tissue, with high expression on the cell surfaces and extracellular matrix of bones and cartilages. Recently, a number of microarray-based investigations have demonstrated that biglycan is frequently overexpressed in human cancer tissues and is associated with cancer progression [6, 7]. Biglycan was highly expressed in both mouse and human tumour endothelial cells (TECs), and biglycan knockdown inhibited TEC migration. Biglycan might be a novel marker of TECs, and is activated during tumour angiogenesis [11]. It could be a novel target for anti-angiogenic therapy in the future.

Furthermore, the most important finding of the present study is the evidence that the up-regulation of biglycan was significantly associated with poor tumor differentiation and advanced stages of the tumour. These results agree with the fact that biglycan, a transforming growth factor β (TGF-β)-binding protein, is able to increase the probability of an interaction of TGF-β with its specific surface receptors, thereby contributing to the early progression of carcinomatous tumors [12–14].

Our study has found that biglycan is expressed in endometrial cancer cells and carcinomatous stroma; the

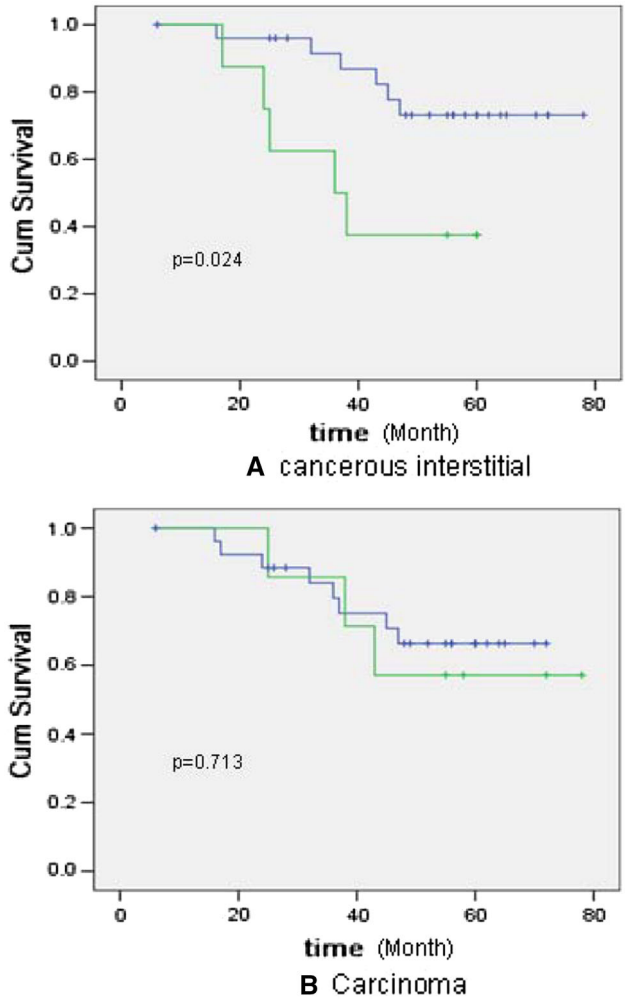

Fig. 3 Survival curves were determined by the Kaplan–Meier product-limit method in carcinoma or cancerous interstitial. It clearly showed that tumours with higher expression of biglycan in mesenchyme had a shorter survival time than those with lower biglycan expression

Table 2 Biglycan expression levels in serum and peritoneal washings of the patients with normal (N), atypical hyperplasia (AH) and endometrial cancer (EC)

Tissue type	*N*	Serum		Peritoneal washings	
		Mean	*p* value	Mean	*p* value
N	52	2.02 ± 0.36		1.92 ± 0.46	
AH	38	2.63 ± 0.47	0.409	2.36 ± 0.51	0.187
EC	57	3.87 ± 1.11	0.002	3.20 ± 1.02	0.0001

expression level of biglycan in stromal cells of cancer in poorly differentiated group is significantly higher than that in the highly and moderately differentiation groups; the cases with higher biglycan expression in the cancer

Table 3 Correlation of biglycan expression levels in serum and peritoneal washings with clinicopathological parameters of endometrial cancer patients

	N (%)	Serum		Peritoneal washings	
		Mean	*p* value	Mean	*p* value
Histologic grade					
G1	30 (52.6)	2.96 ± 0.90		2.96 ± 0.86	
G2	18 (3.60)	4.09 ± 0.79	0.000	3.29 ± 1.02	0.264
G3	9 (1.80)	4.56 ± 1.16	0.005	3.37 ± 0.46	0.085
FIGO stage					
I–II	41 (71.9)	3.33 ± 0.95		3.24 ± 1.12	
III–IV	16 (28.1)	4.19 ± 1.27	0.022	3.10 ± 0.70	0.563
Myometrial infiltration depth					
<1/2	38 (66.7)	3.31 ± 0.94		3.16 ± 0.98	
≥1/2	19 (33.3)	4.09 ± 1.25	0.020	3.08 ± 0.73	0.713
Lymph node metastasis					
No	46 (80.7)	3.35 ± 0.94		3.09 ± 0.94	
Yes	11 (19.3)	4.47 ± 1.21	0.013	3.35 ± 0.60	0.262

interstitium have poor prognosis than those with lower expression; Biglycan expression in cancer tissues and that in the adjacent tissues have a definite correlation. So we can draw an inference that TGF-β1 through stimulation of biglycan synthesis, promotes tumor cell growth and promotes the invasiveness of endometrial cancer [12, 15]. Recent microarray-based studies have documented overexpression of biglycan in different human cancers, which may serve as a potential marker for diagnosis of these cancers [16, 17].

Biglycan expression in sera and peritoneal washings of patients with endometrial cancer were significantly higher than that in normal control group and endometrial atypically hyperplastic groups. Serum expression level of biglycan has correlation with clinicopathological parameters of endometrial cancer patients. Therefore, determination of biglycan content in serum provides us with a certain reference for judging prognosis of endometrial cancer. Further studies are still required for a deeper understanding of the biological function and the specific molecular mechanism of biglycan in endometrial cancer.

Conclusions

Biglycan might play a role in the progression of human endometrial cancer and it might be a useful molecular serum marker for the prognosis of endometrial cancer. Further studies are needed to clarify the mechanisms by which biglycan is involved in the progression of

endometrial cancer and its exact roles in endometrial carcinogenesis by functional analysis. To the best of our knowledge, endometrial cancer is a hormone sensitive tumor and it requires further in-depth exploration in order to understand the exact relationship of biglycan expression level with steroid hormones. Furthermore, its role in the pathogenesis of advanced endometrial cancer could be another scope of research and probably provide some guidelines for the prognosis of endometrial cancer.

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Conflict of interest We declare that we have no conflict of interest with other people or organisations. All authors have contributed significantly to this work, and that all authors are in agreement with the content of the manuscript.

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