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Original Article

Increased NUCKS expression is a risk factor for poor prognosis and recurrence in endometrial cancer

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Abstract: Nuclear ubiquitous casein and cyclin-dependent kinases substrate (NUCKS) was reported to function as a potential biomarker in various tumors. Thus, we aimed to explore the expression of NUCKS in endometrial cancer (EC) and its clinical significance using quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC). qRT-PCR results showed that NUCKS mRNA expression gradually elevated from normal endometrium to atypical endometrial hyperplasia, and to EC ($P < 0.05$ between each group). NUCKS overexpression was strongly associated with FIGO stage ($P = 0.002$), histologic grade ($P = 0.029$), lympho-vascular space involvement ($P = 0.014$), lymph node metastasis ($P = 0.019$), and recurrence ($P < 0.001$). Cox multivariate analysis revealed that NUCKS overexpression was an independent factor for overall survival and recurrence-free survival ($P < 0.001$ for both). Multivariate logistic regression suggested that recurrence was independently correlated with NUCKS overexpresion ($P = 0.039$), FIGO stage ($P = 0.002$), and lymph node metastasis ($P = 0.002$). In summary, NUCKS overexpression may function as a potential biomarker for prognosis especially for recurrence in ECs.

Keywords: Endometrial cancer, nuclear ubiquitous casein and cyclin-dependent kinases substrate (NUCKS), recurrence, prognosis

Introduction

Endometrial carcinoma (EC) is the most common malignancy of the female genital tract [1], with an increasing incidence. Most women are diagnosed at an early stage because of irregular vaginal bleeding, with 5-year survival rates of 80-82% [2-4]. However, women with advanced stage disease or high-risk histopathologies have a poor prognosis [4, 5], with 5-year survival rates of 57% for regional disease and 19% for distant spread [2]. Although usually diagnosed at an early stage with uterine-confined disease and an overall favorable prognosis, 13-20% of women will still develop recurrent disease [6, 7]. Prognostic factors such as advanced stage, deep myometrial invasion, adnexal metastasis and lymph node metastasis [8, 9] are not sufficient to predict prognosis accurately for EC. Thus, there is a necessity to investigate reliable biomarkers for identifying patients who are susceptible to relapse.

Nuclear ubiquitous casein and cyclin-dependent kinases substrate (NUCKS), similar to the HMG (high-mobility group) protein family and being located on human chromosome 1q32.1, is a highly phosphorylated protein [10]. NUCKS is a substrate for the second messenger kinases, cyclic AMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase II, and calcium/phospholipid-dependent protein kinase in vitro [11]. It is also a substrate for DNA-activated protein kinase in vitro, which is involved in DNA repair [12]. In addition, NUCKS played an important role in cell growth, cell proliferation and the regulation of cell cycle especially rapidly growing cells [13-15]. Although the ubiquity and abundance of NUCKS was observed in various types of cancers [16-20], however, little is known about its expression and clinical significance in ECs. In current paper, we aimed to investigate the expression of NUCKS and its clinical significance in ECs.

Materials and methods

Patients and tissue specimens

A total of 175 EC patients who underwent hysterectomy, bilateral salpingo-oophorectomy, pelvic and/or para-aortic lymphadenectomy and

Table 1. Expression of NUCKS in different endometrial tissues

	No. of cases	NUCKS expression status		*P*
		High	%	
Normal endometrium	20	1	5	0.003[a]
Atypical endometrial hyperplasia	20	3	15	0.598[b]
Endometrial cancer	175	67	38.3	0.040[c]

[a]endometrial cancer versus normal endometrium, $P = 0.003$; [b]atypical endometrial hyperplasia versus normal endometrium, $P = 0.598$; [c]endometrial cancer versus atypical endometrial hyperplasia, $P = 0.040$.

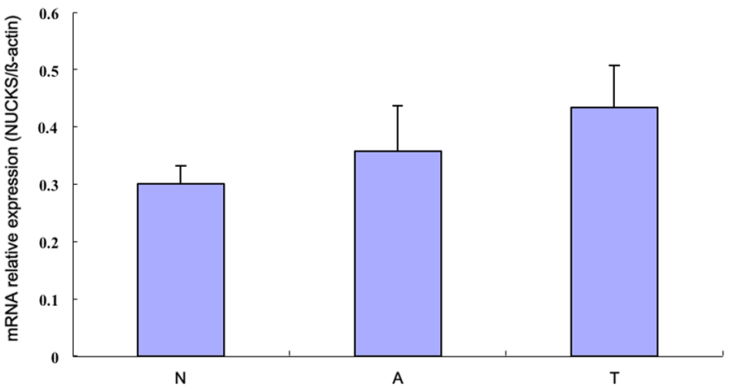

Figure 1. Quantitative real-time PCR assay of NUCKS mRNA expression. Histogram of pooled data from normal endometrium tissues (N), atypical endometrial hyperplasia tissues (A), and endometrial cancer tissues (T). The expression of NUCKS was elevated in endometrial tissues, and highest in endometrial cancers (A vs. N: $P = 0.004$; T vs. A: $P = 0.003$; T vs. N: $P < 0.001$).

peritoneal washing for cytology at the Department of Gynecology of the Third Affiliated Hospital of Harbin Medical University, China, from January 2008 to December 2008 were enrolled in this study. The demographic and clinical characteristics of ECs are displayed in **Table 1**. Tumor stages were evaluated according to the International Federation of Gynecology and Obstetrics (FIGO) 2009 staging system. Specimens of normal endometrium and atypical endometrial hyperplasia were obtained from patients who received hysterectomy for benign uterine disease. Frozen fresh tissue sections obtained from 20 patients with EC, 20 with atypical endometrial hyperplasia, and 20 with normal endometrium were used in the quantitative real-time PCR (qRT-PCR) analysis. Each patient had no history of adjuvant or neoadjuvant therapy before surgery.

The informed consent was provided by each patient, and the study was approved by the Ethics Committee of Harbin Medical University.

qRT-PCR

Total RNA were isolated from 20 EC tissues, 20 atypical endometrial hyperplasia tissues and 20 normal endometrium tissues using RNA simple Total RNA Kit (DP419; Tiangen, Beijing, China) according to the manufacturer's protocol. The primers to NUCKS were designed as follows: forward, 5'-TCTGATGATGCAGATGAAGATTA-3'; reverse, 5'-CTGCTGAGTGAGAATCATCC-3'. β-actin was applied as the internal reference; its primers were as follows: forward, 5'-CTTAGTTGCGTTACACCCTTTCTTG-3'; reverse, 5'-CTGTCACCTTCACCGTTCCAGTTT-3'. We quantified the RNA concentration using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and performed complementary DNA synthesis with 2× Power Taq PCR MasterMix kit (PR1702; BioTeke, Beijing China). We then performed qRT-PCR with the Exicycler 96 real-time RT-PCR system (Bioneer, Daejeon Korea) and SYBR Green mastermix (SY1020; Solarbio, Beijing China). Experiments were performed in triplicate in the same reaction.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded samples were sectioned at 4°C and stained with hematoxylin-eosin for tumor confirmation. Sections adjacent to the hematoxylin-eosin-stained sections were used for immunohistochemical staining. Using the Two-Step IHC Detection Reagent Kit (Zhong Shan Golden Bridge Biological Technology Inc., Beijing, China) following standard procedures, the sections were dewaxed in xylene and rehydrated through graded alcohol concentrations. The slides were incubated in 3% H_2O_2 for 10 min and then immersed in buffered ethylenediaminetetraacetic acid (pH 8.0) for 2 min in a pressure cooker. After washing with phosphate-buffered saline (PBS), the sections were incubated with

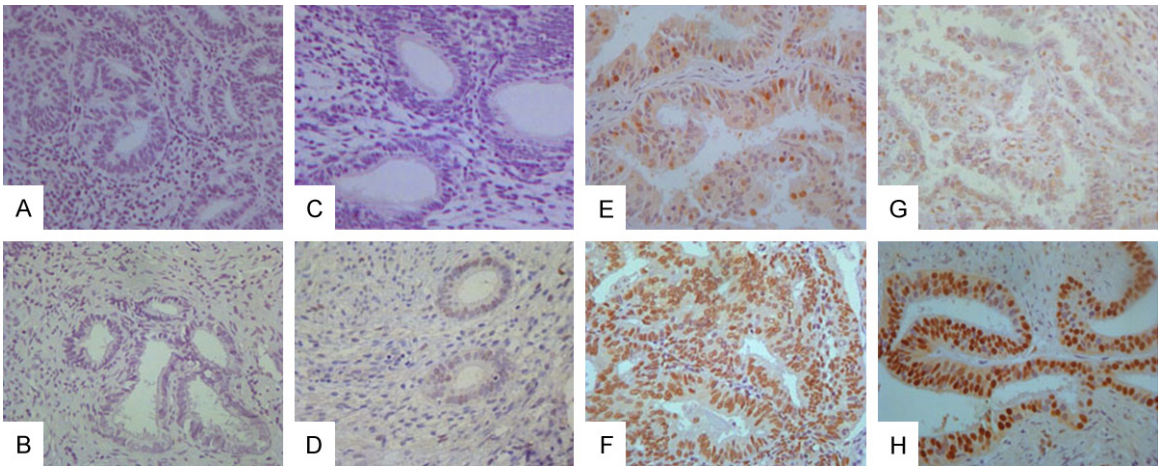

Figure 2. Representative immunostaining of NUCKS in normal endometrium tissues (A), negative control (B), atypical endometrial hyperplasia tissues (C: low expression; D: high expression), endometrial cancer tissues (E, G: low expression; F, H: high expression). Original magnification ×400.

NUCKS antibody (Abcam, diluted at 1:800) overnight at 4°C and a secondary antibody for 20 min at room temperature, followed by incubation with dispensed diaminobenzidine solution.

Immunoreactivity was assessed in 10 high power fields and scored by counting the percentage of positive cells as follows [16]: 0, fewer than 10% of positive nuclei of epithelial cells were detected; 1, 11 to 30% were detected at low staining intensity; 2, more than 30% but less than 60% were detected with moderate staining intensity; 3, more than 60% of positive nuclei were detected with high staining intensity. For statistical analysis, the score of NUCKS expression was defined as low expression if the overall score was 0-1, and high expression if the sum was 2-3.

The scoring procedure was performed independently by two pathologists who blinded with the clinicopathological data. In cases with disagreement, the slides were re-reviewed by the original two pathologists until a final consensus was reached.

Follow up

All patients were followed until death or February 2015. The median follow-up time was 65 months (rang, 4-84 months). The overall survival (OS) was defined as the interval from the end of surgery to death or the date of recent follow-up; recurrence-free survival (RFS) was defined as the period from the end of surgery to the presence of local recurrence or distant metastasis.

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software (SPSS, Chicago, IL, USA). The chi-square test was performed to assess the association between NUCKS overexpression and clinicopathologic parameters. Survival curves were plotted by the Kaplan-Meier method and assessed using log-rank test. To evaluate the increment statistical power of the individual covariates as indicator of unfavorable prognosis, we performed Cox regression analyses. Univariate and multivariate logistic regression were used to estimate the effect of NUCKS overexpression on recurrence. A two-sided $P < 0.05$ was considered statistically significant.

Results

NUCKS protein and mRNA expression in endometrial tissues

qRT-PCR analysis showed that the level of NUCKS mRNA expression gradually increased from normal endometrium to atypical endometrial hyperplasia, and to EC. The statistical differences between each group were observed (**Figure 1**).

To investigate the expression of NUCKS protein in endometrial tissues, immunohistochemistry was initially performed in 175 paraffin-embed-

Table 2. Stratification analysis of the correlation of high NUCKS expression with clinicopathological features

Characteristics	No. of cases	NUCKS expression status		*P*
		Low (n = 108)	High (n = 67)	
Age (years)				0.650
< 55	85	51	34	
≥ 55	90	57	33	
FIGO Stage				0.002
I	95	67	28	
II	45	28	17	
III-IV	35	13	22	
Histologic grade				0.029
G1	68	50	18	
G2	65	37	28	
G3	42	21	21	
Histological type				0.956
EC	157	97	60	
non-EC	18	11	7	
Depth of MI				0.120
< 50%	94	63	31	
≥ 50%	81	45	36	
LVSI				0.014
No	128	86	42	
Yes	47	22	25	
Lymph node metastasis				0.019
No	141	93	48	
Yes	34	15	19	
Recurrence				< 0.001
No	137	94	43	
Yes	38	14	24	
BMI (kg/m^2)				0.430
< 25	98	63	35	
≥ 25	77	45	32	
ER				0.917
Negative	74	46	28	
Positive	101	62	39	
PR				0.621
Negative	72	46	26	
Positive	103	62	41	

International Federation of Gynecology and Obstetrics: FIGO; G1: Well; G2: Moderate; G3: Poor; Endometrioid cancer: EC; Myometrial invasion: MI; Lympho-vascular space involvement: LVSI; Body mass index: BMI; Estrogen receptor: ER; Progesterone receptor: PR.

ded, archival EC samples, 20 atypical endometrial hyperplasia samples, and 20 normal endometrium samples. Positive NUCKS protein expression was mainly observed in the nuclei of tumor cell and normal epithelial cells (**Figure 2**). Weak or moderate NUCKS expression was also observed in cytoplasm of tumor cell. A total of 67 (38.3%) cases showed high expression of NUCKS protein. The ratio of NUCKS overexpression was higher in EC tissues compared with atypical endometrial hyperplasia (P = 0.040) and normal endometrium (P = 0.003) tissues (**Table 1**). However, there was no significant difference between atypical endometrial hyperplasia and normal endometrium tissues (P = 0.598).

NUCKS protein overexpression correlates with clinicopathologic features

The correlation between the protein expression of NUCKS and clinicopathologic variables of EC were listed in **Table 2**. NUCKS expression was significantly correlated with FIGO stage (P = 0.002), histologic grade (P = 0.029), lympho-vascular space involvement (LVSI) (P = 0.014), lymph node metastasis (P = 0.019), and recurrence (P < 0.001). While, no significant relationships of NUCKS expression with other clinicopathologic factors were also observed (P > 0.05).

NUCKS protein overexpression indicates poor survival in ECs

Univariate Kaplan-Meier analysis demonstrated that NUCKS overexpression predicted an unfavorable OS (**Table 3**, P < 0.001; **Figure 3A**) and RFS (**Table 4**, P < 0.001; **Figure 3B**). In addition, FIGO stage (P < 0.001 for both), histologic grade (P = 0.002 and P = 0.003, respectively), lympho-vascular space involvement (LVSI) (P = 0.001 and P < 0.001, respectively), and lymph node metastasis (P < 0.001 for both) correlates with poor prognosis (**Tables 3** and **4**).

Multivariate analysis suggested that NUCKS overexpression (P = 0.003 and P = 0.001, respectively) together with FIGO stage (P < 0.001 for both) and LVSI (P = 0.015 and P = 0.023, respectively) were independently prognostic factors for both OS and RFS (**Tables 3** and **4**) in ECs.

Table 3. Univariate and multivariate analyses with regard to OS

Variables	Univariate analysis	Multivariate Cox regression		
	P	HR	95% CI	P
Age (years)	0.771			
< 55				
≥ 55				
FIGO Stage	< 0.001			< 0.001
I		1.000 (reference)		
II		3.126	1.318-7.414	0.010
III-IV		6.479	2.921-14.368	< 0.001
Histologic grade	0.002			
G1				
G2				
G3				
Histological type	0.080			
EC				
non-EC				
Depth of MI	0.067			
< 50%				
≥ 50%				
LVSI	0.001			0.015
No		1.000 (reference)		
Yes		2.187	1.162-4.117	
Lymph node metastasis	< 0.001			
No				
Yes				
BMI (kg/m^2)	0.161			
< 25				
≥ 25				
ER	0.819			
Negative				
Positive				
PR	0.440			
Negative				
Positive				
NUCKS expression	< 0.001			0.003
Low		1.000 (reference)		
High		2.926	1.458-5.873	

Overall survival: OS; hazard ratio: HR; confidence interval: CI; International Federation of Gynecology and Obstetrics: FIGO; G1: Well; G2: Moderate; G3: Poor; Endometrioid cancer: EC; Myometrial invasion: MI; Lympho-vascular space involvement: LVSI; Body mass index: BMI; Estrogen receptor: ER; Progesterone receptor: PR.

NUCKS protein overexpression independently predicts recurrence in ECs

We first performed univariate analysis of clinicopathogical factors for recurrence. The recurrence was positively associated with FIGO stage ($P < 0.001$), LVSI ($P = 0.005$), and lymph node metastasis ($P < 0.001$).

A multivariate logistic regression analysis suggested that NUCKS overexpresion ($P = 0.039$), FIGO stage ($P = 0.002$), and lymph node metastasis ($P = 0.002$) independently correlated with recurrence (**Table 5**).

Discussion

In this study, we analyzed the status of NUCKS mRNA expression and we found that NUCKS mRNA expression gradually increased from normal endometrium to atypical endometrial hyperplasia, and to EC. This is consistent with the results that in colon cancer and breast cancer [17, 21]. Also, IHC results showed that the ratio of NUCKS overexpression was higher in EC tissues compared with atypical endometrial hyperplasia and normal endometrium. Furthermore, NUCKS overexpression in breast tissues was observed in both atypical ductal hyperplasia and ductal carcinoma in situ [16]. All the above demonstrate that NUCKS which is associated with high levels of transcription [22] plays a pivotal role in neoplastic progression.

Although occurring in almost all types of human cells [10, 23], NUCKS plays a significant role in tumor growth and metastasis [22, 24]. In our

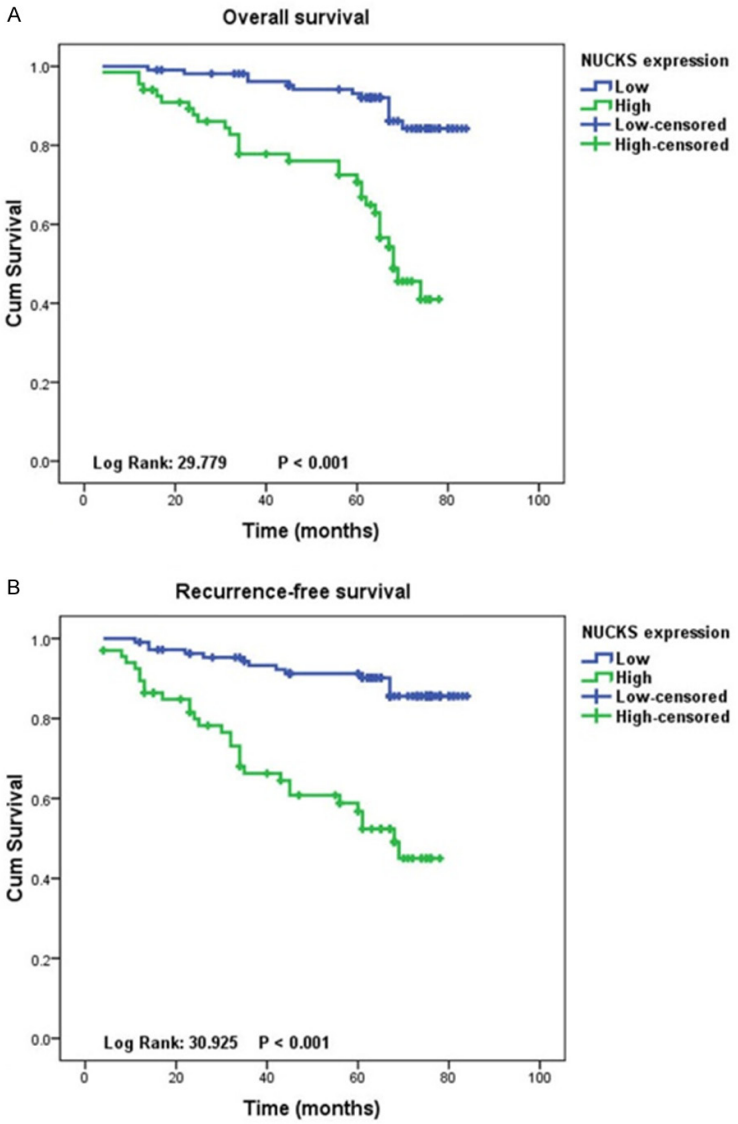

Figure 3. Kaplan-Meier analysis of overall survival (A) and recurrence-free survival (B) in relation to NUCKS expression in 175 endometrial cancer patients.

reports, we found that NUCKS overexpression was positively associated with FIGO stage, histologic grade, lympho-vascular space involvement, lymph node metastasis, and recurrence. Our previous study also showed that NUCKS overexpression was associated with tumour progression and recurrence in cervical squamous cell carcinoma [18]. Kikuchi et al. [17] reported that NUCKS involved in the distant metastasis of colorectal cancer. Moreover, in gastric cancer, increased NUCKS expression was significantly associated with TNM stage, and depth of invasion [19]. Thus, the flexibility of NUCKS indicates that it is multifunctional and may reflect its distribution in an invasive phenotype [23, 25, 26]. Our results have shown that high NUCKS expression can be correlated with prognosis of EC especially tumor recurrence. In colorectal cancer and gastric cancer, it was reported that NUCKS was related to prognosis [17, 19]. Previous study also indicated that NUCKS was independently associated with recurrence in cervical squamous cell carcinoma [18]. The current data demonstrated that increa sed NUCKS expression plays a critical role in disease progression and poor survival of EC and meanwhile suggested that NUCKS could be a valuable biomarker for the indication of EC prognosis especially recurrence.

Elevated expression and amplification of copy number of NUCKS has been observed in human lung and mammary tumor cell lines [27, 28], and also in mouse lung cell strains [29]. NUCKS involves in cell growth, proliferation, and cell cycle through being phosphorylated by CK-2 and Cdk1, 2, 4, 6, respectively [13-15]. The presentation of NUCKS was observed higher than Ki67 in malignant skin tumors [20]. In contrast, there was no association between NUCKS and Ki67 expression neither in tissue sections nor in primary cell cultures [16]. This may suggest that NUCKS involves in proliferation of tumor cell independent of Ki67. By binding to single stranded and double stranded DNA in vitro [27], NUCKS functions as a substrate for DNA-activated protein kinase which is necessary for DNA repair [12]. In addition, it might participate in the induction of apoptosis following photodynamic therapy in MCF-7 cells [30]. Recently, NUCKS was identified to be as a novel Tat coactivator which is required for Tat-mediated HIV-1 transcription

Table 4. Univariate and multivariate analyses with regard to RFS

Variables	Univariate analysis	Multivariate Cox regression		
	P	HR	95% CI	P
Age (years)	0.617			
< 55				
≥ 55				
FIGO Stage	< 0.001	1.000 (reference)		< 0.001
I		2.557	1.094-6.071	
II		8.375	3.738-18.763	0.030
III-IV				< 0.001
Histologic grade	0.003			
G1				
G2				
G3				
Histological type	0.127			
EC				
non-EC				
Depth of MI	0.069			
< 50%				
≥ 50%				
LVSI	< 0.001			0.023
No		1.000 (reference)		
Yes		2.089	1.107-3.943	
Lymph node metastasis	< 0.001			
No				
Yes				
BMI (kg/m^2)	0.194			
< 25				
≥ 25				
ER	0.660			
Negative				
Positive				
PR	0.580			
Negative				
Positive				
NUCKS expression	< 0.001			0.001
Low		1.000 (reference)		
High		3.385	1.689-6.785	

Recurrence-free survival: RFS; hazard ratio: HR; confidence interval: CI; International Federation of Gynecology and Obstetrics: FIGO; G1: Well; G2: Moderate; G3: Poor; Endometrioid cancer: EC; Myometrial invasion: MI; Lympho-vascular space involvement: LVSI; Body mass index: BMI; Estrogen receptor: ER; Progesterone receptor: PR.

and replication and be implicated in HIV-1 reactivation in latently HIV-1 infected cells [31].

Interestingly, it was reported that NUCKS mRNA level was significantly related to obesity markers in all obese patients with breast cancer [21]. With regard to that obesity is a risk of EC, our study showed that high NUCKS protein level correlated with high level of body mass index (BMI), though there was no differences between them. Conversely, there was an inverse association between NUCKS protein levels and BMI in humans [32]. So what is the exact relationship between them? To solve this problem, further studies and more samples will be required to confirm the relationship of NUCKS with obesity in EC.

In conclusion, we indicate that NUCKS is overexpressed in a large proportion of EC and elevated NUCKS expression can be associated with tumor progression and unfavorable prognosis especially recurrence. These results suggest that NUCKS may be a potential therapeutic target for the treatment of EC.

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Disclosure of conflict of interest

None.

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Table 5. Multivariate analysis of the relationship of recurrence with NUCKS overexpression in 175 patients with endometrial cancer

Variables	B	S.E.	P	OR	95% CI
FIGO Stage			0.002		
I					
II	0.869	0.551	0.115	2.385	0.810-7.026
III-IV	1.975	0.557	< 0.001	7.025	2.417-21.475
Lymph node metastasis					
No					
Yes	1.537	0.497	0.002	4.651	1.758-12.309
NUCKS expression					
Low					1.046-6.035
High	0.921	0.447	0.039	2.513	

International Federation of Gynecology and Obstetrics: FIGO; the parameter estimator of association coefficient and its standard error: B and S.E.; confidence interval: CI; odds ratio: OR.

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