Expression of FBXW7 in endometrial cancer negatively correlates with mTORC1 activity and tumor progression

W. Liping[1*], W. Yuping[1*], W. Xuefei[1], Z. Huihui[1], C. Zhenguo[2], Z. Mei[1]

[1]Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, Guangzhou
[2]Department of Cell Biology, School of Basic Medical Sciences, Southern Medical University, Guangzhou (China)

Summary

Purpose of Investigation: The authors aimed to investigate the relationship between FBXW7 expression and mTORC1 activity in endometrial cancer (EC) patients and determined their relation to tumor differentiation, invasion, nodal metastasis, and the underlying mechanism. *Materials and Methods:* The expression of FBXW7 and phospho-S6 (S235/S236) was examined by immunohistochemistry in 1.5 mm tumor cores from 97 EC patients and five adjacent normal endometrial tissue samples using tissue microarray technology. *Results:* The positive expression rate of phospho-S6 was significantly higher in EC than in control tissues. Age was highly correlated with the expression of phospho-S6. However, FBXW7 was expressed at higher levels in normal tissues than in tumor samples, and its expression correlated with low tumor histological grade. Importantly, the expression of P-S6 (S235/236) in EC had a significant negative correlation with FBXW7. *Conclusion:* FBXW7 negatively correlates with mTORC1 activity and tumor progression in EC. FBXW7 is a potential prognostic marker and a target for the treatment of EC.

Key words: Endometrial cancer; mTOR; FBXW7; Tissue microarray; Immunohistochemistry.

Introduction

Endometrial cancer (EC) is one of the most common gynecological malignancies in developed countries and the fourth most common cancer among women in Western countries[1]. In China, the estimated rates of new cases and deaths were 63.4% and 21.8%, respectively, in 2015 [2, 3]. The incidence of EC is currently increasing and there are two different pathological types: namely type I or estrogen-dependent with endometrioid morphology, and type II, which is non-estrogen-dependent, with serous papillary or clear cell morphology [4]. Type I accounts for approximately 70–80% and type II accounts for 10–20% of all EC cases [5]. The pathogenesis of EC remains unclear. Standard treatment consists in primary hysterectomy and bilateral salpingo-oophorectomy; lymph node surgical strategies are contingent on histological factors (including subtype, tumor grade, and involvement of lymphovascular space), disease stage (including myometrial invasion), patient characteristics (such as age and comorbidities), and national and international guidelines [6]. The extent of local invasion and tumor grade are important factors in determining disease outcome. To predict disease progression and improve therapeutic interventions, it is important to explore the molecular mechanisms underlying targeted therapy to determine the optimal approach to the treatment of EC.

Several signal pathways are involved in endometrial carcinogenesis, and improving our understanding of the underlying oncogenic mechanisms may lead to the discovery of novel therapeutic targets and improve the quality of life of patients with EC. The mammalian target of rapamycin (mTOR) is a conserved Ser/Thr protein kinase. mTORC1 regulates many key cellular processes, including cell growth and metabolism, primarily by regulating cap-dependent protein translation initiation [7]. Therefore, the mTOR pathway is considered a promising target for EC therapy [8]. Preclinical studies examined the effect of mTOR pathway inhibitors in a panel of EC cell lines. An activated status of p70 S6K1 and S6 could accelerate the translation of mRNAs bearing a 5′-terminal oligopyrimidine tract, which encodes the translational apparatus including ribosomal proteins, the elongation factors eEF1A and eEF2, and the poly A-binding protein [9, 10]. By contrast, the inactivation of S6K1 decreases the synthesis of ribosomal proteins and other translation factors [9, 11, 12]. mTOR inhibition can induce autophagy. The induction of autophagy simultaneously increases AMPK phosphorylation levels in parallel with a reduction in the phosphorylation of the S6 kinase p70 subtype (p70S6K) [13, 14]. Therefore, p70S6K (T389) and S6 (S235/236) may predict the activity of the mTORC1 signaling pathway [10, 15].

FBXW7 (also known as FBW7, hCDC4, Sel-10, and hAGO) is thought to be a component of a conserved SCF

*These authors contributed equally to this work.

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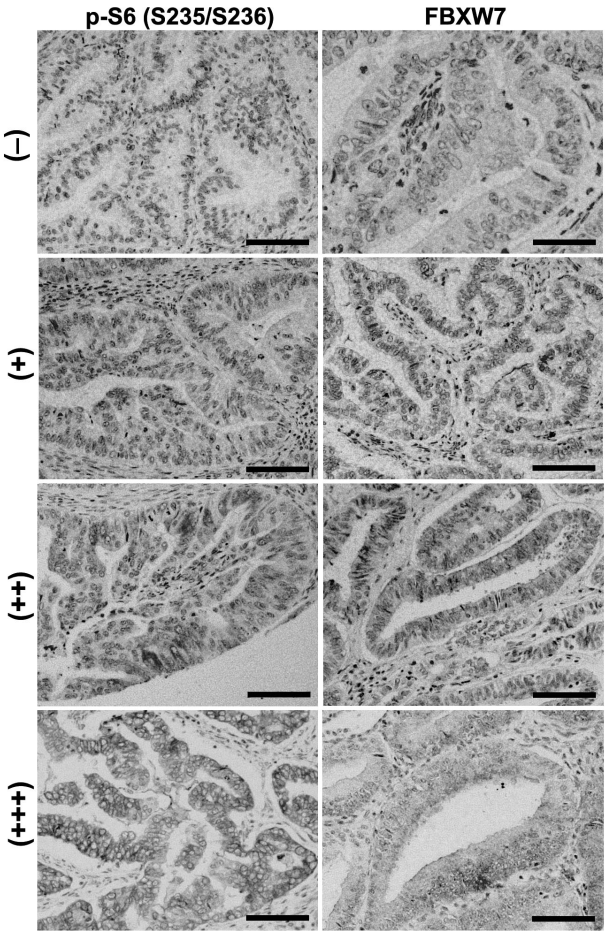

Figure 1. — Representative immunohistochemical images of P-S6 (S235/236) and FBXW7 in endometrioid adenocarcinoma. (-) negative, (+) weakly positive, (++) moderately positive, (+++) strongly positive. Scale bar = 100 μm.

(complex of SKP1, CUL1, and F-box protein)-type ubiquitin ligase [16]. FBXW7 plays an important role in tumorigenesis. It functions by targeting the degradation of several proto-oncogenes involved in cellular growth and division pathways [16]. FBXW7 is thought to act as a tumor suppressor. It can also accelerate the degradation of mTOR through the ubiquitin-proteasome pathway. The attenuation of mTORC1 induced by FBXW7 leads to the activation of autophagy [17]. mTOR and p-mTOR (phosphorylated mTOR) protein levels, along with the downstream mTOR target S6-kinase (PS6K), are increased when FBXW7 is depleted [18].

Phosphatidylinositol 3-kinase (PI3K) pathway aberrations occur in > 80% of endometrioid ECs [1]. The PI3K/mTOR pathway is considered as a promising target for the treatment of EC [19]. However, the mechanism underlying the relationship between FBXW7 and mTORC1 activity and EC development remains unclear.

Table 1. — Clinicopathologic characteristics of 97 cases of endometrioid adenocarcinoma.

Item		Percentage (%)
Age	≤ 56 years	50/97 (51.5)
	> 56 years	47/97 (48.5)
Tumor grade	I	29/97 (29.9)
	II	47/97 (50.5)
	III	17/97 (15.5)
	IV	0/97 (0)
	Data not applicable	4/97 (4.1)
Primary tumor	T1	90/97 (92.8)
	T2	4/97 (4.1)
	T3	3/97 (3.1)
	T4	0/97 (0)
Lymph nodes metastasis		0/97 (0)
Distant metastasis		0/97 (0)

To investigate the role of the FBXW7/mTOR axis in tumor differentiation, invasion, nodal metastasis, and the clinicopathologic features of EC, the expression of the mTOR-related proteins P-S6 (S235/236), and FBXW7 was assessed by immunohistochemistry in an EC tissue microarray. The involvement of FBXW7 and the mTOR signal pathway in the development of EC was also analyzed.

Materials and Methods

The study used a tissue microarray containing 97 EC and five adjacent normal tissue samples. All cases were reviewed and classified according to the criteria of the National Comprehensive Cancer Network classification. Pathologic staging was reviewed based on the Tumor Node Metastasis (TNM) staging system of the American Joint Committee on Cancer [20]. Patients were grouped based on age, TNM stage, tumor invasion, presence of lymph node metastasis, presence of distant metastasis, and histologic grade. The mean age of the patients was 56-years-old. The patients had no history of chemotherapy, radiotherapy, or immunotherapy prior to surgery. According to the tumor grade, 29 patients were classified as Stage I (29.9%), 47 (50.5%) patients as Stage II, 17 (15.5%) patients as Stage III, and four (4.1%) were not applicable (Table 1). The TNM staging system revealed 90 (92.8%) cases of T_1, four (4.1%) cases of T_2, and three (3.1%) cases of T_4. There were no patients with lymph node metastasis or distant metastasis (Table 1). One 1.5 mm tumor core per case and one 1.5 mm representative normal tissue core were sampled for tissue microarray. Briefly, 4-μm-thick multiple sections were cut from the tumor tissue using a microtome for immunohistochemical staining.

Immunohistochemical staining was performed on slides prepared using standard procedures from paraffin embedded tissues. After deparaffinization and rehydration, slides were heated in a microwave oven at 100°C for 15 minutes with citrate buffer at pH 6.0 and treated with 3% H_2O_2 for 20 minutes to inhibit the activity of endogenous hydrogen peroxidase. Slides were blocked with 5% goat serum for one hour and incubated with primary antibodies [P-S6 (S235/S236, 1:50]) and FBXW7 (1:50) at 4°C overnight. The secondary antibody was added for 60 minutes at 37°C with diaminobenzidine stain. After immunostaining, slides

Table 2. — *Scale of p-S6 and FBXW7 immunoexpression.*

Scale	p-S6			FBXW7		
	Tumor	Normal	*p* value	Tumor	Normal	*p* value
Negative (-)	31/97	4/5	0.034	28/97	1/5	0.017
Low (+)	45/97	1/5		35/97	2/5	
Moderate (++)	16/97	0/5		24/97	2/5	
High (+++)	5/97	0/5		10/97	0/5	

Wilcoxon signed-rank test was used to test the difference expression between normal and tumor group.

Table 3. — Distribution of p-S6 and FBXW7 and positive immunoexpression in relation to clinicopathological characteristics.

	p-S6		FBXW7	
	Positive (%)	*p* value	Positive (%)	*p* value
Age		0.009		0.479
≤ 56 years	28/50(56.0)		23/50(46.0)	
> 56 years	38/47(80.9)		25/47(53.2)	
Tumor grade		0.189		0.004
I	20/29(69.0)		19/29(65.5)	
II	36/47(76.6)		28/47(59.6)	
III	9/17(52.9)		3/17(17.6)	
Primary tumor		0.266		0.505
T1	64/90(71.1)		46/90(51.1)	
T2	2/4(50.0)		1/4(25.0)	
T3	1/3(33.3)		1/3(33.3)	

The distribution is presented as percentage (%), Chi square test was used.

were counterstained with hematoxylin and examined under a microscope. The immunohistochemical expression of P-S6 and FBXW7 was assessed in tissue sections using the following scoring system based on the staining intensity: 0 (achromatic color), 1 (pallide-flavens), 2 (deep yellow), and 3 (brown). The percentage of positive cells was scored as 0: 0-5% of stained cells, 1: 5-25% of stained cells, 2: 26-50% of stained cells, 3: 51-75% of stained cells, and 4: > 75% of stained cells. The final staining scores for P-S6/FBXW7 consisted in the sum of the staining intensity and percentage of positive cells and ranged from 0 to 8. Tumors with a final staining score of 0 were considered negative (-), 1-2 was weakly positive (+), 3-5 was moderately positive (++), and 6-8 was strongly positive (+++)[21].

Data were analyzed using SPSS 17.0 statistical software. The Wilcoxon signed-rank test was used to determine the differences in expression between the normal and tumor groups. The Chi square test was used to test two groups of cases for one variable. Pearson's $\chi 2$ test was used to test the correlations between P-S6 and FBXW7. $p < 0.05$ was considered statistically significant.

Results

The expression of P-S6 (S235/236) and FBXW7 was assessed by immunohistochemical in five normal endometrial tissue samples and 97 patients with EC. P-S6 and FBXW7 showed comparable rates of expression in EC samples (68% and 71.1%, respectively); however, the staining intensity and area of FBXW7 were lower than that of P-S6 (Figure 1).

P-S6 protein expression was higher in tumor samples than in normal endometrial tissues, with positive expression rates of 68% (66 of 97 cases) and 20% (1 of 5 cases), respectively ($p = 0.034$). By contrast, the expression of FBXW7 was lower in tumor samples (71.1%) than in normal endometrial tissue 4 (80%) ($p = 0.017$) (Table 2).

The distribution of positive immunoexpression in EC showed that P-S6 was not significantly related to histologic tumor grade ($p = 0.189$) or depth of invasion ($p = 0.266$), whereas it was correlated with age ($p = 0.009$). By contrast, the distribution of FBXW7 positive immunoexpression in EC was not significantly related with age ($p = 0.479$) or the depth of the primary tumor ($p = 0.505$), whereas it was correlated with tumor grade ($p = 0.004$) (Table 3).

Analysis of the expression of P-S6 and FBXW7 in EC showed a significant negative correlation between with P-S6 and FBXW7 ($p = 0.014$, R= -0.267) (Table 4).

Discussion

Previous studies identified many risk factors associated with the development of EC. These risk factors include exposure to endogenous and exogenous estrogens associated with obesity, diabetes, early age at menarche, nulliparity, late-onset menopause, older age (≥ 55 years), and use of tamoxifen [6, 22-27]. Activation of the PI3K/AKT/mTOR pathway was reported to promote cancer initiation by increasing chromosomal instability [28, 29]. The radical hysterectomy surgical technique is used for the treatment of EC when affecting the cervix [30]. Adjuvant radiotherapy plus chemotherapy is associated with a higher five-year disease-specific survival rate and lower recurrence rate com-

Table 4. — *Expression status and correlation of p-S6 and FBXW7.*

	FBXW7			
p-S6	Positive	Negative	R □□	*p* □□
Positive	20	29	-0.267	0.014
Negative	25	11		

Correlation between p-S6 and FBXW7 was analyzed by Pearson's χ^2 tests.

pared with radiotherapy alone and chemotherapy alone in high-risk endometrial cancer patients [31]. It is reported that inhibitors of PI3K/Akt/mTOR signaling can be used for the treatment of EC. A dual inhibitor of PI3K and mTOR, BEZ235, has shown efficacy in the treatment of EC [32]. However, the effect of P-S6 (S235/236) and FBXW7 on the prognosis of EC remains unclear. In the present study, immunohistochemical analysis was used to examine the expression of P-S6 (S235/236) and FBXW7 in human EC and its relationship with clinicopathologic factors.

P-S6 (S235/236) is a downstream signaling target of mTORC1 [33]. Direct targets of mTORC1 include ribosomal protein S6 kinase 1 (S6K1), which regulates protein translation [34]. S6K1 and S6K2 are upregulated in many malignancies including breast cancer [35]. However, P-S6 (S235/236) is also downstream of S6K1. In the present study, the authors showed that the expression of P-S6 (S235/236) was significantly higher in EC tissues than in normal endometrial tissue ($p = 0.034$). FBXW7 mediates mTOR degradation, and the loss of FBXW7 increases the levels of total and activated mTOR [18, 36]. The present authors showed that the expression of FBXW7 was lower in EC than in normal endometrial tissues. Furthermore, the expression of P-S6 and FBXW7 showed a significant negative correlation in EC ($p = 0.014$, R= -0.267). These findings suggest that the activity of the PI3K/AKT/mTORC1 pathway in EC is increased.

High expression levels of p-mTOR are associated with tumor progression and poor survival in EC [37]. In the present study, the authors assessed the relationship between the expression of P-S6 (S235/236) and FBXW7 and clinicopathologic parameters. Increased expression of P-S6 (S235/236) was correlated with age ($p = 0.009$) but not with tumor histological grade. By contrast, altered expression of FBXW7 was significantly correlated with low tumor histological grade. These findings indicated that FBXW7 functions as a tumor suppressor during EC carcinogenesis.

Laboratory research and clinical trials demonstrated that mTOR inhibitors have anti-tumor effects and clinical activity in EC [38]. However, the relationship between the expression of P-S6 (S235/236), FBXW7, and overall survival in EC remains to be explored. The risk of recurrence is evaluated on the basis of pathological findings, such as stage, lymph node metastasis, myometrial invasion, and tumor grade [39]. A high endometrial carcinoma recurrence score is significantly associated with the presence of deep myometrial infiltration, vascular invasion, and a higher vascular invasion score [40, 41]. However, the molecular mechanisms underlying EC remain unclear. Aberrant expression of the genes analyzed in the present study was associated with carcinogenesis in EC, underscoring the need to design relevant experimental models to demonstrate that the function of FBXW7 in human EC cells is necessary.

Conclusion

The expression of P-S6 (S235/236) was higher in EC than in adjacent normal endometrial tissues. The present findings demonstrate that elevated expression of the P-S6 (S235/236) protein has a strong relationship with the development of EC. Positive expression of P-S6 (S235/236) was positively correlated with age. By contrast, the expression of FBXW7 in EC showed the opposite expression pattern, supporting its inhibitory effect on the mTOR signaling pathway. These findings indicate that FBXW7 is a potential tumor suppressor in EC and could be developed as a prognostic marker and a target for the treatment of EC.

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Corresponding Author:
MEI ZHONG, M.D.
Department of Obstetrics and Gynecology
Nanfang Hospital
North Guangzhou Avenue, 1838
Guangzhou 510515 (China)
e-mail: czg1984@smu.edu.cn