

Original Article

Immunohistochemical expression of glypican 3 in endometrial carcinoma and correlation with prognostic parameters

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Abstract: Background: Carcinogenesis is associated with several critical regulatory molecules which are involved in different signaling pathways such as the WNT signaling pathways. Among which the β -catenin dependent pathway has been associated with human endometrial cancer. Genetic and biochemical studies have demonstrated that glypicans can regulate several signaling pathways including those triggered by Wnts. Glypican 3 is one of six mammalian members of the glypican family of proteoglycans. Overexpression of glypican 3 has been reported in some types of cancers but only few data are available about its expression in endometrial carcinoma and its role in endometrial carcinogenesis. The aim of this study was to examine the immunohistochemical expression of glypican 3 in endometrioid endometrial carcinoma (EEC) and serous endometrial carcinoma (SEC), and to correlate its expression with prognostic factors of endometrial carcinoma. Materials and methods: Immunohistochemical expression of glypican 3 was studied in fifty two EEC and nineteen SEC cases. Results: Glypican 3 expression showed a significant difference between EEC and SEC ($P = 0.027$) and it was significantly correlated with tumor grade, stage and myometrial invasion ($P = 0.001$). Conclusion: Glypican 3 expression can be used as an adjunct in the differentiation between EEC and SEC. Glypican 3 is associated with poor prognostic parameters in both EEC and SEC, and it can be a promising molecule for targeted immunotherapy in positive cases.

Keywords: Glypican 3, endometrial carcinoma, immunohistochemistry

Introduction

Endometrial adenocarcinoma is the most common gynecologic malignancy [1]. The most common form of endometrial carcinoma (EC) is the endometrioid endometrial carcinoma (EEC) or type I which accounts for more than 80% of all EC. Uterine EEC is usually accompanied by endometrial hyperplasia and usually have favorable prognosis [2]. In contrast, type II endometrial cancers are more aggressive, non estrogen related, high grade, and lack association with hyperplasia [3]. The most common histological subtype in the second group is the serous endometrial carcinoma (SEC); it constitutes about 10% of all endometrial cancers and has a higher fatality than the endometrioid counterpart [4]. It usually develops in an atrophic endometrium and do not respond to conventional chemotherapy, radiotherapy and hormone therapy [3]. Factors associated with poor

prognosis in endometrial carcinomas are patient's age, tumor grade, stage, histological subtype and depth of myometrial invasion [5].

Carcinogenesis of EC is associated with several critical regulatory molecules which are involved in different signaling pathways. The WNT signaling pathway is one of the most evolutionary-conserved signal transduction pathways. The WNT signaling pathways include β -catenin dependent WNT signaling pathway (*i.e.* canonical WNT/ β -catenin) and β -catenin independent WNT signaling pathway (*i.e.* Non-canonical, such as WNT/JNK pathway, WNT/calcium pathway). Among these WNT signaling pathways the β -catenin dependent pathway has been associated with human endometrial cancer [6].

Glypican 3 is a cell surface heparan sulfate proteoglycan that binds to the cell membrane via glycosyl-phosphatidyl-inositol anchors [7]. Gly-

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glypican 3 was first identified in patients with Simpson-Golabi-Behmel syndrome, a rare x linked disorder characterized by prenatal and postnatal overgrowth caused by mutation in glypican 3 gene [8]. Several studies elucidated the role of Glypican 3 in regulation of cell proliferation and apoptosis during normal development [9]. Overexpression of Glypican 3 has been reported in some types of cancers such as hepatocellular carcinoma, squamous cell carcinoma of the lung and testicular germ cell tumors [10]. In hepatocellular carcinoma several studies aimed to target Glypican 3 by immunotherapeutic approaches and to inhibit its growth by blocking Glypican 3 function [11]. Genetic and biochemical studies have demonstrated that glypicans can regulate several signaling pathways including those triggered by Wnts [12, 13], bone morphogenetic proteins (BMPs) [14, 15] and fibroblast growth factors [16].

Since the Wnt/ β -catenin dependent pathway has been associated with human endometrial cancer [6], and Glypican 3 can regulate several signaling pathways including those triggered by Wnts So it is of interest to study the immunohistochemical expression of Glypican 3 in endometrial carcinoma especially that the data available on this subject are very limited. This can help in better understanding of endometrial carcinogenesis and possible therapeutic targeting.

The aim of this study was to examine the immunohistochemical expression of Glypican 3 in endometrioid endometrial carcinoma (EEC) and serous endometrial carcinoma (SEC), and to correlate its expression with prognostic factors of endometrial carcinoma.

Materials and methods

Tissue and patient data

The current study was conducted on 52 EEC and 19 SEC. All endometrial carcinoma cases received in the Pathology Lab of Ain Shams Specialized Hospital during the period from January 2008 to June 2015 were retrieved. After exclusion of cases with insufficient information or inadequate material a total number of 52 EEC and 19 SEC were included in our study. All patients underwent total abdominal hysterectomy and bilateral salpinge-oophorectomy. The hematoxylin and eosin slides were

reviewed by both authors to confirm the diagnoses and to select the representative sections. The histological classification was done according to the World Health Organization classification [17]. Staging was defined according to the International Federation of Obstetricians and Gynecologists (FIGO) staging system [18].

All patients who participated in this study signed a written informed consent before surgery. The study was approved by the Research Ethical Committee at Faculty of Medicine, Ain Shams University.

Immunohistochemical procedure

Four micrometer sections of formalin-fixed and paraffin-embedded samples from all cases were prepared. They included the tumor and the adjacent normal endometrial tissue in some specimens. Immunohistochemical staining was performed using primary antibody glypican 3 mouse monoclonal antibody (1:200, clone IG 12, Cell Marque, Burlington, VT, USA). Avidin-Biotin immunoperoxidase complex technique was used according to Hsu et al. [19] by applying the super sensitive detection kit (Biogenex, CA, USA). The prepared tissue sections were fixed on poly-L-lysine coated slides overnight at 37°C. They were deparaffinized and rehydrated through graded alcohol series. Then the sections were heated in a microwave oven in 10 mM citrate buffer (pH 6.0) for 20 min. After the blocking of endogenous peroxidase and incubation in Protein Block Serum-Free Solution (Dako Cytomation) for 20 min, the sections were incubated overnight at 4°C with primary antibodies. Biotinylated anti-mouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were then added. Finally, 3, 3'-diaminobenzidine as the substrate or chromogen was used to form an insoluble brown product. Finally, the sections were counterstained with hematoxylin and mounted. Sections from hepatocellular carcinoma were used as positive control for Glypican 3. Negative control sections were incubated with normal mouse serum instead of the primary antibody.

Interpretation of immunohistochemical staining

Immunohistochemical analysis of Glypican 3 was performed by both authors separately. Any discrepancies were resolved by consensus using a multihead microscope. Both distribu-

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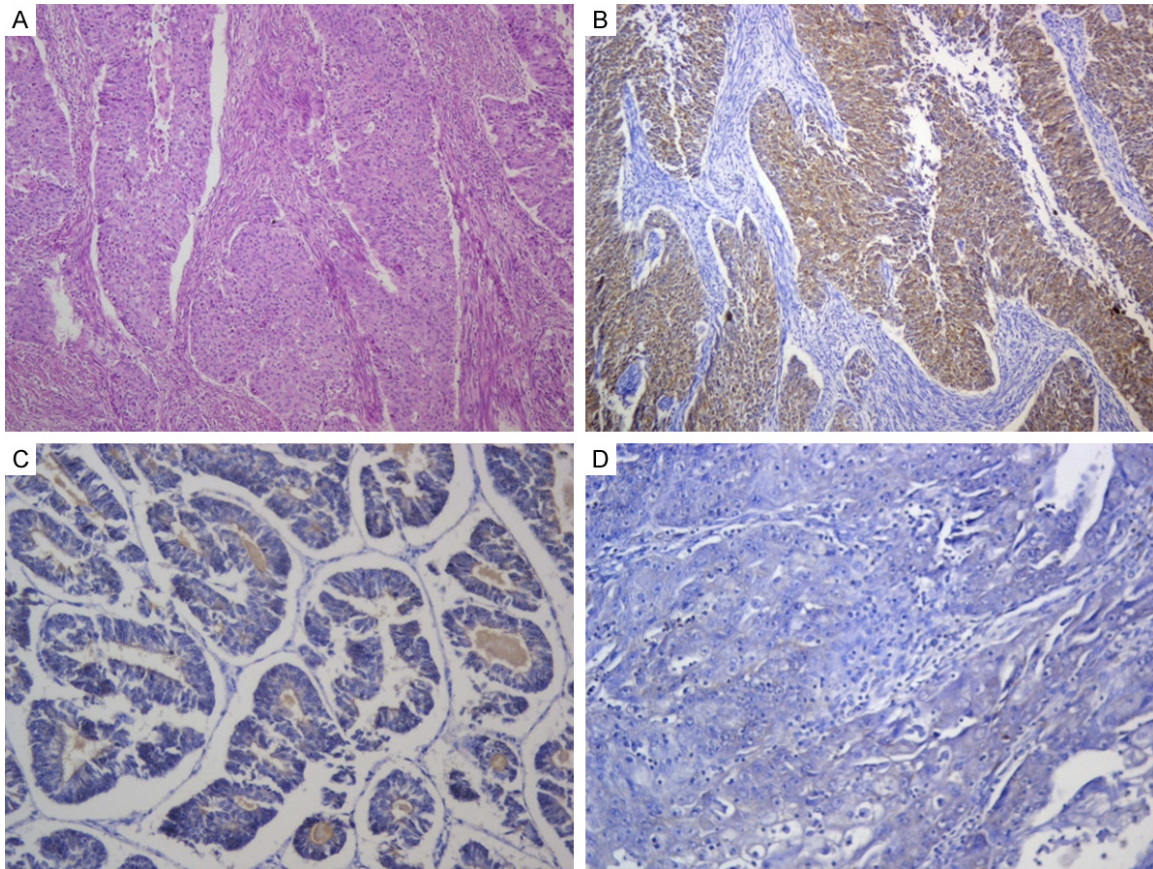


Figure 1. Endometrioid endometrial carcinoma. A. High grade EEC showing deep myometrial invasion (H&E \times 100). B. High grade EEC with myometrial invasion positive for Glypican 3 (Glypican 3 \times 100). C. Low grade EEC negative for Glypican 3 expression (Glypican 3 \times 200). D. Grade 3 EEC negative for Glypican 3 (Glypican 3 \times 200).

tion and intensity of the stain were evaluated. The distribution was defined as the percent of the stained cells and was scored as: positive > 10% positive cells, 1: 10-50% positive cells, 2: > 50% stained cells. The staining intensity was scored as 1: Faint staining (light yellow), 2: Moderate staining (brown), 3: Strong staining (dark brown). Combined scores were obtained by adding the intensity and distribution scores and were considered as 0 in the negative cases.

Statistical analysis

Continuous variables are expressed as mean and SD or as median (interquartile range) in cases of skewed distributions. Categorical variables are expressed as frequencies and percentage. Differences between independent groups were tested using the Student t test for Continuous variables. Categorical variables were compared using the fisher exact test. Spearman's correlation was used to assess the

correlation between variables. A significance level of $P < 0.05$ was used in all tests. All statistical procedures were carried out using SPSS version 20 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Clinicopathological results

The age of patients with endometrioid endometrial carcinoma ranged from 53 to 57 years (mean = 55.02, SD 1.24), while that of patients with serous endometrial carcinomas ranged from 59 to 68 years (mean = 62.74, SD 3.30). Tumor size in EEC ranged from 4 cm to 7 cm (mean = 5.02, SD 0.82) and in SEC from 6 to 11 cm in longest dimension (mean = 9.13, SD 1.44). The 52 EEC included grade 1 (32 cases), grade 2 (13 cases) and grade 3 (7 cases). The 19 SEC included moderately differentiated tumors i.e. grade 2 (8 cases) and poorly differentiated tumors i.e. grade 3 (11 cases). The

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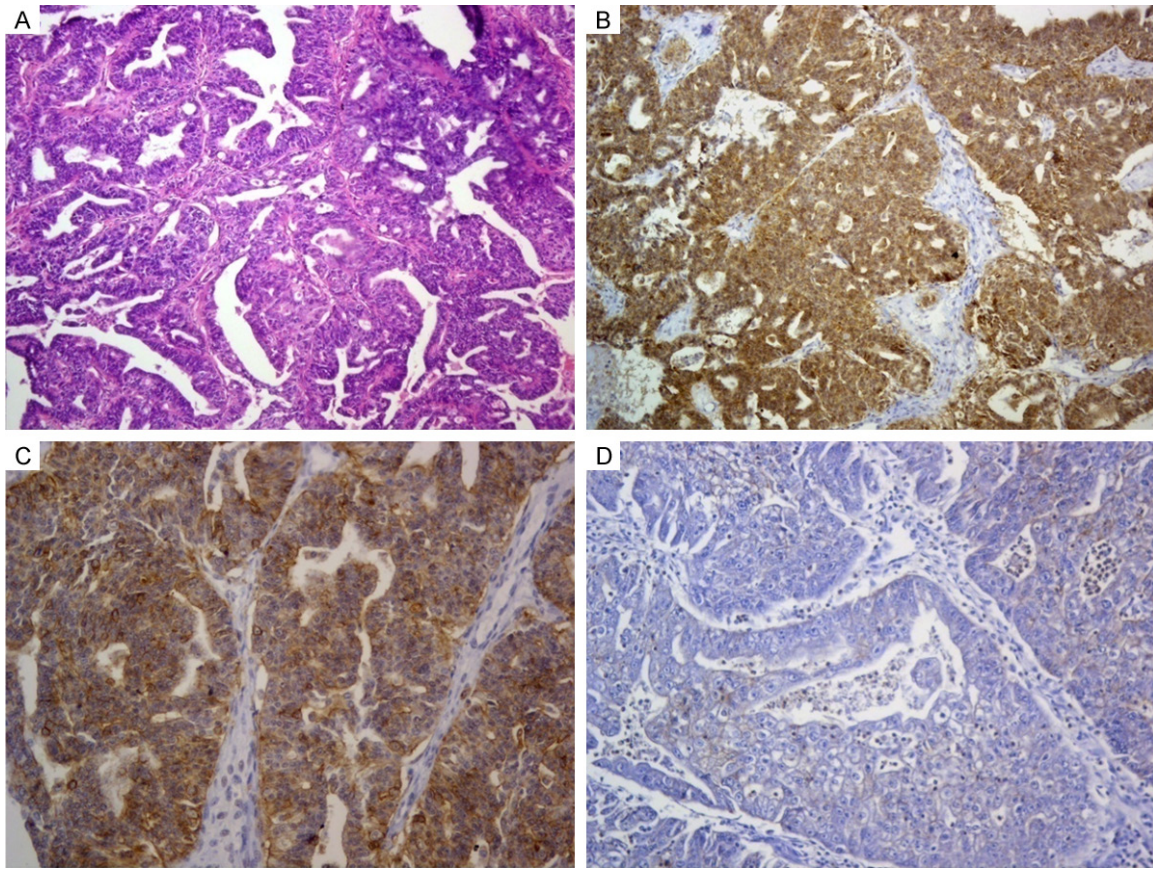


Figure 2. Serous endometrial carcinoma. A. Complex papillary architecture in SEC (H&E $\times 100$). B. Moderately differentiated SEC positive for Glypican 3 expression (Glypican 3 $\times 100$). C. Glypican 3 positive SEC showing foci of cell membrane staining in addition to predominant cytoplasmic staining (Glypican 3 $\times 200$). D. SEC negative for Glypican 3 expression (Glypican 3 $\times 200$).

moderately differentiated cases showed a well-defined papillary architecture with fibrovascular connective tissue cores with solid areas of tumor cells $< 50\%$ of the tumor, while the poorly differentiated carcinomas showed a predominance of solid areas with poorly defined papillae. The nuclei in all SEC were highly atypical with frequent mitotic figures. Myometrial invasion was seen in 5/7 grade 3 EEC, 2/13 grade 2 EEC and in 16/19 SEC (including the 11 poorly differentiated cases and 5/8 moderately differentiated cases). FIGO staging in EEC was stage 1 (47 cases), stage 2 (3 cases), stage 3 (2 cases) while in SEC it was stage 2 (12 cases), stage 3 (6 cases) and stage 4 (1 case).

Immunohistochemical results

Three out of fifty two EEC (5.7%) were positive for Glypican 3. The staining distribution was 2+ in the three cases (i.e. in more than 50% of the tumor cells). The intensity was moderate (2+) in

two cases and faint (1+) in one case. The combined scores were 4, 4, and 3. Myometrial invasion was seen in all positive cases. All positive cases were WHO grade 3 (**Figure 1**). The FIGO staging of the positive cases were (T2a N1 Mx), (T3a N1 Mx), and (T3b N1 Mx).

Five out of the nineteen SEC (26.3%) were positive for Glypican 3. The staining distribution was 2+ in four cases and 1+ in one case. The staining intensity was moderate (2+) in the five cases. The combined scores were 4, 4, 4, 4, and 3. All positive SEC cases showed myometrial invasion and all were WHO grade 3 (**Figure 2**). The staging in the positive cases were (T3a N1 Mx) in four cases and (T4 N1 Mx) in one case.

The stain was localized in the cytoplasm with cell membrane staining in some tumor areas (**Figure 2C**). Normal tissue adjacent to tumors was present in 13 cases and was totally negative for Glypican 3.

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Table 1. WHO grading and the FIGO staging for the examined cases

	WHO grading			FIGO staging			
	Grade 1	Grade 2	Grade 3	Stage 1	Stage 2	Stage 3	Stage 4
EEC (n = 52)	32	13	7	47	3	2	-
SEC (n = 19)	-	8	11	-	12	6	1

Table 2. Clinicopathological and immunohistochemical results for the positive Glypican cases

Positive cases	Age	Tumor size*	Grade	Myometrial invasion	Stage (TNM)	Glypican 3 distribution	Glypican 3 intensity	Glypican 3 score
EEC 3/52 (5.7%)	1 54	4	3	+	T2a N1 Mx	2+	1+	3
	2 53	5.5	3	+	T3a N1 Mx	2+	2+	4
	3 53	6.5	3	+	T3b N1 Mx	2+	2+	4
SEC 5/19 (26.3%)	1 62	7.5	3	+	T3a N1 Mx	2+	2+	4
	2 65	11	3	+	T3a N1 Mx	1+	2+	3
	3 59	8	3	+	T3a N1 Mx	2+	2+	4
	4 67	10.5	3	+	T4 N1 Mx	2+	2+	4
	5 60	10	3	+	T3a N1 Mx	2+	2+	4

*Tumor longest dimension.

Table 3. Comparison between EEC and SEC as regard clinicopathological data

		Group				P	Sig
		EEC (N = 52)		SEC (N = 19)			
		Mean	± SD	Mean	± SD		
Age		55.02	1.24	62.74	3.30	0.001*	HS
Size (cm)		5.02	.82	9.13	1.44	0.001*	HS
Stage	1 (n %)	46	88.5%	0	0.0%	0.001**	HS
	2 (n %)	3	5.8%	12	63.2%		
	3 (n %)	3	5.8%	6	31.6%		
	4 (n %)	0	0.0%	1	5.3%		
Grade	1 (n %)	32	61.5%	0	0.0%	0.001**	HS
	2 (n %)	13	25.0%	8	42.1%		
	3 (n %)	7	13.5%	11	57.9%		
Myometrial invasion	Negative (n %)	45	86.5%	3	15.8%	0.001**	HS
	Positive (n %)	7	13.5%	16	84.2%		

*Student t test. N = number of cases. **Fisher exact test.

Table 1 summarizes the WHO grading and the FIGO staging for the examined cases.

Table 2 summarizes the clinicopathological and immunohistochemical results for the positive Glypican 3 cases.

Statistical results

A statistically significant correlation was found between EEC and SEC regarding age, tumor size, stage, grade and myometrial invasion being higher in SEC than EEC (P = 0.001 for each parameter) (**Table 3**). A statistically significant

difference was found between Glypican 3 expression in EEC and SEC (P = 0.027) (**Table 4**). There was a high statistically significant correlation between tumor grade, stage, myometrial invasion and Glypican 3 positivity (P < 0.001 in each parameter), all positive cases in EEC and SEC were of high grade, stage and all of which showed myometrial invasion (**Table 5**).

Discussion

Carcinogenesis is associated with several critical regulatory molecules that are involved in

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Table 4. Comparison between EEC and SEC as regard Glypican 3 expression

		Group				P	Sig
		EEC		SEC			
		N	%	N	%		
Expression of gp3	Negative	49	94.2%	14	73.7%	0.027*	S
	Positive	3	5.7%	5	26.3%		

*Fisher exact test. S = significant.

Table 5. Relation between myometrial invasion, tumor stage, and grade with Glypican 3 expression in all examined endometrial carcinoma cases (EEC and SEC)

		Glypican 3 expression				P	Sig
		Positive		Negative			
		N	%	N	%		
Myometrial invasion	Positive	8	34.8%	15	65.2%	.001*	HS
	Negative	0	0.0%	48	100.0%		
Stage	1	0	0.0%	46	100.0%	.001*	HS
	2	1	6.7%	14	93.3%		
	3	6	66.7%	3	33.3%		
	4	1	100.0%	0	0.0%		
Grade	1	0	0.0%	32	100.0%	.001*	HS
	2	0	0.0%	21	100.0%		
	3	8	44.4%	10	55.6%		

*Fisher exact test.

different signaling pathways [20]. Glypican 3 is one of six mammalian members of the Glypican family of proteoglycans [21]. It regulates cell growth and apoptosis by interacting with various morphogenic or growth factors such as Wnt, fibroblast growth factor 2, and bone morphogenic protein [22]. It is normally expressed in trophoblastic and fetal tissues but has a limited expression in adult tissue [23]. Overexpression of Glypican 3 has been reported in several malignant tumors such as hepatocellular carcinoma, malignant melanoma, squamous cell carcinoma of the lung and testicular germ cell tumors [10]. However very few data are available about the expression of Glypican 3 in endometrial carcinoma.

The Wnt signaling pathways represent a group of pathways that comprise of proteins involved in the transduction of signals via cell surface receptors. First identified in 1982 by Nusse in mouse models of mammary cancer, they can be divided into two major groups: canonical and non-canonical pathways, with the differentiating factor being the involvement of β -catenin in the former [24]. Both are activated by the

binding of a Wnt-protein ligand to a Frizzled family receptor (Fz), which in turn transfers the signal to the intracellular protein, Dishevelled (Dsh) [25]. Canonical Wnt activity has been shown to play a role in cancer progression in many tumor types including hepatocellular carcinoma [26]. It has also been associated with human endometrial carcinoma [20].

Because Glypican 3 interacts with Wnts it has been proposed that it stimulates signaling by increasing the amount of Wnt at the cell membrane and thus facilitating the interaction between this growth factor and its signaling receptor Frizzled [27]. Therapies targeting the Wnt pathway may play an essential role in the future of anticancer therapeutics either alone or in conjunction with traditional therapies [28].

Since only few data are available about Glypican 3 in endometrial carcinoma, so in our study we examined the immunohistochemical expression of Glypican 3 in EEC and

SEC, we compared its expression in both types and we correlated it with prognostic parameters of endometrial carcinoma. Our results revealed Glypican 3 expression in 5.7% of EEC and in 26.3% of SEC. This was close to the findings of Baumhoer, et al. in EEC (6%) but higher in SEC cases (13% in contrast to 26.3% in our study) [29]. To the best of our knowledge our study is the first one to investigate the correlation between Glypican 3 expression and prognostic factors in endometrial carcinoma. We found Glypican 3 to be significantly associated with poor prognostic parameters as high grade, myometrial invasion and advanced stage (P = 0.001). In the study of Bing et al. [30] Glypican 3 reactivity in malignant mixed mullerian tumors was detected in both sarcomatous and poorly differentiated epithelial component, and in accordance to our study and the study of Baumhoer, et al. [29] the normal tissue adjacent to tumors was totally negative for Glypican 3 [30].

Despite the few number of positive cases in EEC and SEC there was a statistically significant difference between Glypican 3 expression

in both types being more expressed in SEC so it can be used as an adjunct with other markers in their differentiation in problematic cases. The small percent of positive Glypican 3 in EEC suggests that Glypican 3-Wnt interaction has a limited role in its carcinogenesis. The larger percent of Glypican 3 positivity in SEC (in which Wnt pathway is not a major player in its carcinogenesis [31]) suggests involvement of Glypican 3 in serous endometrial carcinogenesis through a different regulatory pathway other than Wnt. Further molecular studies are needed to clarify the role of Glypican 3 in endometrial carcinogenesis.

We concluded that Glypican 3 expression is significantly higher in SEC than EEC and it can be used as an adjunct in their differentiation. Glypican 3 is associated with poor prognostic parameters in both EEC and SEC, and it can be a promising molecule for targeted immunotherapy in positive cases. Further studies are needed to clarify the possible Glypican 3 role in endometrial carcinogenesis.

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Disclosure of conflict of interest

None.

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