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Immunohistochemical study of SLCO4C1 and FGF21 in endometrial lesions

Maram El-Hussieny[1], Huda I. Marey[1], Nisreen Abd El-Tawab Abd El-Gaber[1], Mariana F Gayyed[1], Rehab Kamal Mohammed[1]

[1] *Faculty of Medicine, Minia University, Egypt.*

Abstract

Endometrial carcinoma (EC) is one of the major malignant tumors of the female reproductive system. In recent years, the incidence and mortality rate of EC have increased. SLCO4C1 acts as a tumor suppressor gene for primary head and neck squamous cell carcinoma and is related to the proliferation and differentiation of prostate malignant cells. In EC, it regulates the proliferation, apoptosis, migration, and other characteristics. The fibroblast growth factor member 21 (FGF21) is a protein encoded by the FGF21 gene and proved to be a metabolic regulator with potential anti-diabetic effect. In addition, FGF21 affects cell proliferation and migration via the PI3K/AKT signaling pathway. Associated expression of these two markers is not examined in endometrial hyperplasia and EC yet. The aim of this study was to investigate the expression and prognostic roles of SLCO4C1 and FGF21 in endometrial hyperplasia (EH) and EC. The immunohistochemical expression of both SLCO4C1 and FGF21 was examined in 40 cases of EH and 100 cases of EC tissues. The association between expression patterns of these markers and clinico-pathologic parameters and disease-free survival were evaluated. SLCO4C1 was highly expressed in the membrane and cytoplasm of tumor cells in (70%) of AEH cases and (48%) of EC cases. Increased expression of SLCO4C1 in EC was associated with lower tumor grade ($P = 0.03$). FGF21 was highly expressed in the cytoplasm of (75%) of AEH cases and (56%) of EC cases. Increased expression of FGF21 in EC was inversely associated with advanced tumor stage ($P = 0.007$). Higher disease-free survival rates were associated with low expression of SLCO4C1 and high expression of FGF21 in EC patients. These findings suggest that expression of SLCO4C1 and FGF21 could be considered as prognostic markers in EC patients.

Keywords: SLCO4C1, FGF21, immunohistochemistry, endometrial hyperplasia, endometrial carcinoma, prognosis.

Full length article **Corresponding Author*, e-mail: Huda I. Marey. Hoda.ibrahiemm@mu.edu.eg

1. Introduction

EC is the sixth most common cancer between females worldwide, the fourth most common malignancy in developed countries. It is the most common tumor of the female genital tract [1,2] and the second most common gynecologic malignancy in developing countries after cervical cancer [3]. The incidence rate in Egypt (3.5/100,000) is the lowest compared to other countries in the Middle East. In addition, low stage at diagnosis of EC is noticed compared to other female cancers in Egypt [4,5]. SLCO4C1 has been found to act as a tumor suppressor gene for primary head and neck squamous cell carcinoma, related to the proliferation and differentiation of prostate cancer cells and a potential molecular marker for the classification of associated pulmonary hypertension secondary to pulmonary fibrosis. SLCO4C1 is a human kidney-specific organic anion transporting polypeptide located in the basolateral membrane of the proximal tubule epithelium [6,7]. Researchers have found that SLCO4C1 controls the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, which in turn regulates the proliferation, apoptosis, migration, and other characteristics of endometrial cancer cells [8]. The fibroblast growth factor member 21 (FGF21) is a protein that in mammals is encoded by the FGF21 gene [9]. FGF21 is expressed in several tissues such as liver [10], adipocytes [11], pancreas (12), and brain where it passes the blood-brain barrier [13]. Aberrant deregulation of FGFR signaling is related to tumorigenesis by the upregulation of Mitogen-Activated Protein Kinase (MAPK) signaling or phosphatidylinositol 3-kinase (PI3K) axis [14,15]. Upregulation of FGFR signaling induces not only tumor progression, but also resistance to anticancer therapy in thyroid and other various cancer types [16,17]. Our aim was to assess the immunohistochemical expression of both markers for the first time in different endometrial lesions. Also, we aimed at assessing their association with prognosis and survival.

2. Materials and Methods

2.1 Patients and tissue samples

This study enrolled 20 paraffin blocks of EH without atypia, 20 paraffin blocks of AEH and 100 paraffin blocks of endometrioid EC which were taken from the Pathology Department Laboratory, Minia University in the period between January 2015 to January 2020. The specimen type was endometrial curettage for EH cases and total abdominal hysterectomy for endometrioid EC cases. The patient mean age ± SD, median age and age range for each group included in this study was 49.9±2.5 years, 50 years and 46-55 in EH without atypia, 56.2±3.8, 56 years and 50-63 for atypical EH and 58.48 ±8.91 years, 57 years and 38-82 for endometrioid EC. Ethics approval was obtained from the Institutional Review Board (IRB) of Faculty of Medicine at Minia University (MUFMIRB 405-04-2021). Grading and staging of endometrioid EC cases was determined based on the FIGO 2023 criteria [18]. Tumor size was classified as above 2 cm and below 2 cm as the greatest diameter [19]. The patient's clinico-pathological characteristics are showed in **table (1**).

2.2 Immunohistochemical procedure

Four μm sections were prepared on positive charged slides. Immunohistochemical staining for SLCO4C1 and FGF21 primary antibodies was performed by utilizing the avidin biotin-peroxidase complex method with diaminobenizidine (DAB) chromagen detection system using universal immunostaining kit (Lab Vision laboratories). Sections were heated at 60 ^{o}C for 10 minutes, dewaxed in two changes of xylene and rehydrated in descending graded alcohol. Sections were then immersed in a 3% solution of hydrogen peroxide and incubated for 15 minutes at room temperature then slides were rinsed gently with BPS buffer solution and were placed in fresh buffer bath for five minutes. Sections were treated in microwave by immersion of the slides in citrate buffer solution (pH 6) for 2 times (10 minutes each), and then slides were allowed to cool, and reach room temperature then washed with PBS buffer for 5 minutes. Slides were then incubated overnight at 4 C with the primary antibodies as follows: SLCO4C1 (polyclonal rabbit antibody, 100 μ, concentrated, cat. no. FNab07977, Fine Test Laboratories, China), and FGF21 (polyclonal rabbit antibody, 100 μ, concentrated, cat. no. A3908, AB clonal laboratories, China) primary antibodies were used at a dilution (1:100 in PBS, for each). Secondary biotinylated antibody was added for each slide for 30 minutes at room temperature. After that, slides were rinsed in buffer solution for 5 minutes; streptavidin reagent was then applied to cover each section for 30 minutes at room temperature. The slides were rinsed gently and placed in PBS for 5 minutes. Diaminobenzidine tetrachloride (DAB) substrate and chromogen prepared in a ratio of 1:50 and mixed well. DAB substrate-chromagen solution was applied on sections. Lastly, sections were counterstained in Mayer haematoxylin, rinsed gently in distilled water, dehydrated in ascending grades of alcohols (70%, 95% and 100% alcohol), then cleared in xylene and, mounted using an aqueous-based mounting medium, Disterene plasticizer xylene (DPX) and covered slips. Sections of normal human kidney were used as a positive control for SLCO4C1, while for FGF21; human thyroid cancer tissue was used as a positive control. The negative control was used to verify specificity of the primary antibody.

2.3 Interpretation and scoring of immunohistochemical staining

Slides were examined by two pathologists blinded to patient characteristics and outcome. SLCO4C1 expression was membranous and/or cytoplasmic whereas FGF21 expression was cytoplasmic. The cases were examined by light microscopy (Olympus, Tokyo, Japan) and the IHC score were assessed by the widely accepted H score system calculated using the following equation: H score=ΣPi (I), where I represents the staining intensity score (i.e. 0=no staining,1=weak staining, 2=moderate staining, and 3=strong staining) and Pi represents the percentage of stained cells (from 0 to 100%). The final H score ranged from 0 to 300 [(20)] and expression levels were classified as negative/low expression and high expression using a cut-off value equals the median of the total scores defining a final immunostaining score of > 114 as high SLCO4C1 protein expression and of > 129.5 as high FGF21 protein expression.

2.4 Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS software version 25). Raw data were compiled and used to determine the means ± standard deviations (SDs), median and range of various features. The Chi- square and Fisher's exact tests were used to compare categorical features. P value of < 0.05 was considered significant. Kaplan -Meier, log-rank and Cox Regression tests were used to analyze survival.

3. Results

3.1 Immunohistochemical expression of SLCO4C1 in different histopathological groups

SLCO4C1 immunostaining was cytoplasmic and/or membranous. In EH without atypia, high SLCO4C1 expression was detected in 6/20 (30%) of cases whereas low expression was seen in 14/20 (70%) of cases. In atypical EH, high SLCO4C1 expression was seen in 14/20 (70%) of cases while 6/20 (30%) of cases showed low expression. In endometrioid EC, high SLCO4C1 expression was seen in 48/100 (48%) of endometrioid EC cases, whereas 52/100 (52%) of cases showed low expression. A statistically significant association was detected between SLCO4C1 expression in EH cases and EC cases (p value= 0.040*). These data were demonstrated in table (2) and figure (1; a, b, c, d and e).

- ### *3.2 Association between SLCO4C1 expression and clinico-pathological data in endometrioid EC cases*

Regarding the tumor grade, a statistically significant inverse correlation was detected between SLCO4C1 expression and the tumor grade (P <0.03*) in the studied cases where 64.3 % of grade I tumors, 48% of grade II tumors and 27.3% of grade III tumors exhibited high SLCO4C1 expression. No significant association was found between SLCO4C1 expression and patient's age, tumor stage, myometrial invasion, tumor size, tumor necrosis and LVI (p= 0.236, p=0.2, p= 0.6, p= 0.8, p= 0.6, p= 0.4 respectively). These data were demonstrated in table (3).

3.3 Immunohistochemical expression of FGF21 in different histopathological groups

In the present study, FGF21 immunostaining was cytoplasmic. In EH without atypia, high FGF21 expression

was detected in 5/20 (25%) of cases whereas low expression was seen in 15/20 (75%) of cases. In AEH, high FGF21 expression was seen in 15/20 (75%) of cases while 5/20 (25%) of cases showed low expression. In endometrioid EC, 44/100 cases (44%) exhibited low FGF21 expression, whereas 56/100 cases (56%) revealed high expression. In the present study, there was a statistically significant association between FGF21 expression in EH cases and EC cases (p= 0.005*). These data were demonstrated in table (4) and figure (1; f, g, h, i and j).

3.4 Immunohistochemical expression of FGF21 endometrioid EC cases and its association with clinicopathological data

Regarding expression of FGF21 in EC, the present study, 44/100 cases (44%) exhibited low cytoplasmic FGF21 expression, whereas 56/100 (56%) revealed high expression. The correlation between FGF21 immunoexpression and various clinicopathological variables is summarized in **table (5).** FGF21 expression was significantly inversely associated with tumor stage (p =0.007) where 40/62 cases (64.5%) of stage I, 14/26 cases (53.8%) of stage II and 8/8 cases (100%) of stage III showed high FGF21 expression**.** A statistically significant association was detected between FGF21 expression and tumor necrosis (p=0.0001) where 40/56 (71.4%) of cases with positive tumor necrosis showed high FGF21 expression. There was statistically significant inverse association between high FGF21 expression and LVI (p=0.002) where 50/78 (64.1%) of cases with negative LVI showed high FGF21 expression. No significant association was found between FGF21 expression and patient's age, tumor grade, size and myometrial invasion (p=0.206, p= 0.6, p= 0.5 and p= 0.2 respectively).

3.5 Survival analysis

The median follow-up of patients was 48.5 months (range 6-60 months) with a mean and standard deviation of 42.29 $\pm$ 14.11 months. Correlation between SLCO4C1, FGF21 and disease-free survival was determined using the Kaplan – Meier method and log-rank test. With regard to patient outcome, it was found that low expression of SLCO4C1 and high expression of FGF21 was associated with a higher disease-free survival rate ($p < 0.000$ for SLCO4C1 and $p < 0.005$ for FGF21) (Figure 2). The relation between expression of SLCO4C1 and FGF21 in EC patient's prognosis was evaluated using univariate and multivariate Cox regression analysis (table 6 and table 7). The multivariate regression analysis for SLCO4C1 revealed that lower tumor grades is 5.25 times more likely to have a higher expression than high grades with an adjusted odd 5.25 (1.54-17.96). The multivariate regression analysis for FGF21 revealed that presence of necrosis, negative LVI and low tumor size <2cm is 1.12, 23.13, 7,58 and 6.33 times more likely to have a higher expression than negative necrosis, positive LVL and higher size with an adjusted odds 23.13 (5.34-100.23) for necrosis, 7.58 (1.48-38.86) for LVI and 6.33 (1.11-35.95) for size.

4. Discussion

Endometrial carcinoma (EC) is one of the common malignancies of the female genital system. In our study, we explored the immunohistochemical expression of SLCO4C1 and FGF21 in endometrial lesions for the first time to the best of our knowledge. Notably, SLCO4C1 exhibited varying expression across our studied cases. High expression was detected in 6/20 (30%) in EH without atypia, 14/20 (70%) in atypical EH cases and 48/100 (48%) in EC cases with statistically significant differences observed (p-value = 0.040). This aligns with findings by Hu et al., 2020, indicating strong SLCO4C1 expression in EC tissues compared to non-malignant endometrial tissue [8], the only study that evaluated immunohistochemical expression of SLCO4C1 in endometrial lesions up to our knowledge. This may be due to the tumor-suppressor action of SLCO4C1, so its expression is related to tumorigenesis. Analyzing the relation between SLCO4C1 immunoexpression and clinico-pathological variables in EC patients, a significant association was found with tumor grade (p-value = 0.03) where 64.3% of grade I tumors and 27.3% of grade III tumors showed high expression. This contrasts with the findings of previous studies which reported a correlation between elevated SLCO4C1 expression and higher-grade tumors in ovarian cancer, suggesting a different molecular mechanism across different gynecological malignancies [21]. The previous study conducted by Hu et al., observed that strong SLCO4C1 immunostaining was observed in EC tissues compared to weak or no staining in non-malignant endometrium (p value=0.001) [8]. He also proved that low SLCO4C1 expression was linked to suppressed migration and invasion capacities in endometrial cell lines, aligning with its role in regulating epithelial-mesenchymal transition (EMT) and metastasis in EC cells. This finding matches our results where 50% of stage III and stage IV cases showed high SLCO4C1 expression whereas 30.8% of stage II cases showed high SLCO4C1 expression, although these findings didn't reach the statistical significance due to low number of cases with advanced stages. No significant association could be detected between SLCO4C1 expression and other studied clinicopathological variables in EC cases. Expanding our insights into FGF21 expression, we investigated the immunohistochemical expression of FGF21 in EH and EC cases and found that high FGF21 expression was in 5/20 (25%) of EH without atypia cases, 15/20 (75%) of atypical EH cases and in 56/100 (56%) of EC cases. The difference between these lesions was statistically significant (p-value = 0.005*)*. Regarding expression of FGF21 in EC, its expression was significantly inversely associated with tumor stage (p-*value* =0.007) where 40/62 cases (64.5%) of stage I showed high FGF21 expression. A statistically significant association was detected between FGF21 expression and tumor necrosis (p-*value* =0.0001) where 40/56 (71.4%) of cases with positive tumor necrosis showed high FGF21 expression. There was statistically significant inverse association between high FGF21 expression and LVI (p=0.002) where 50/78 (64.1%) of cases with negative LVI showed high FGF21 expression. These results coincide with what was proved by Dai et al., [22] who found that FGF21 can inhibit proliferation and promotes autophagy in prostate cancer. Our study aligns with the broader context of its role in cancer prognosis.

Table 1: Clinico-pathological characteristics of endometrioid EC patients enrolled in this study (No.=100)

Clinicopathological variables	N (%)
Age (years) Mean ± SD; 58.48 ±8.91 <58.5±8.91 years ≥58.5±8.91 years	 52 (52%) 48 (48%)
Size <2cm ≥2cm	 16 (16%) 84 (84%)
Grade I II III	 28 (28%) 50 (50%) 22 (22%)
FIGO Stage Stage I Stage II Stage III Stage IV	 62 (62%) 28 (28%) 6 (6%) 4 (4%)
Myometrial invasion <50% ≥50%	 44 (44%) 56 (56%)
Necrosis Absent Present	 44 (44%) 56 (56%)
LVI Negative Positive	 78 (78%) 22 (22%)

-FIGO: *International Federation of Gynecology and Obstetrics*
-LVI: *lympho-vascular invasion*

Table 2: SLCO4C1 immunostaining in different histopathological groups

	SLCO4C1 Expression		
Histopathological groups	**Low (%)**	**High (%)**	***p value***
EH without atypia (20 cases)	14 (70%)	6 (30%)	0.040*
Atypical EH (20 cases)	6 (30%)	14 (70%)	
Endometrioid EC (100 cases)	52 (52%)	48 (48%)	

Test of significance: Chi-square test
Significant p-value < 0.05

Table 3: Association between SLCO4C1 expression and clinicopathological data of the endometrioid EC cases (n=100)

Clinicopathological data	SLCO4C1		*p* value
	Low expression (n=52)	High expression (n=48)	
Age (years) Mean ± SD 58.48 ±8.91 < 58.48 ±8.91 ≥ 58.48 ±8.91	 30 (57.7%) 22 (45.8%)	 22 (42.3%) 26 (54.2%)	0.236
Tumor grade Grade I Grade II Grade III	 10 (35.7%) 26 (52%) 16 (72.7%)	 18 (64.3%) 24 (48%) 6 (27.3%)	0.03*
FIGO stage Stage I Stage II Stage III Stage IV	 28 (45.2%) 18 (69.2%) 4 (50%) 2 (50%)	 34 (54.8%) 8 (30.8%) 4 (50%) 2 (50%)	0.2
Myometrial invasion < 50% ≥ 50%	 24 (54.5%) 28 (50%)	 20 (45.5%) 28 (50%)	0.6
Tumor Necrosis Negative Positive	 24 (54.5%) 28 (50%)	 20 (45.5%) 28 (50%)	0.6
LVI Negative Positive	 42 (53.8%) 10 (45.5%)	 36 (46.2%) 12 (54.5%)	0.4
Tumor size <2 cm ≥2 cm	 8 (50%) 44 (52.4%)	 8 (50%) 40 (47.6%)	0.8

Test of significance: Chi-square test
Significant p-value < 0.05
-FIGO: International Federation of Gynecology and Obstetrics
-LVI: lympho-vascular invasion

Table 4: FGF21 expression in different histopathological groups

	FGF21 Expression		
Histopathological groups	***Low (%)***	***High (%)***	***p value***
EH without atypia (n=20)	15 (75%)	5 (25%)	0.005*
Atypical EH (n=20)	5 (25%)	15 (75%)	
Endometrioid EC (n=100)	44 (44%)	56 (56%)	

Test of significance: Chi-square test
Significant p-value < 0.05

Table 5: Association between FGF21 expression and clinicopathological data of endometrioid EC cases (n=100)

Clinicopathological data	FGF21		*p* value
	Low expression (n=44)	High expression (n=56)	
Age (years) Mean ± SD; 58.48 ±8.91 < 58.48 ±8.91 ≥ 58.48 ±8.91	 26 (50%) 18 (37.5%)	 26 (50%) 30 (62.5%)	0.208
Tumor grade Grade I Grade II Grade III	 14 (50%) 22 (44%) 8 (36.4%)	 14 (50%) 28 (56%) 14 (63.6%)	0.6
FIGO stage Stage I Stage II Stage III Stage IV	 22 (35.5%) 12 (46.2%) 8 (100%) 2 (50%)	 40 (64.5%) 14 (53.8%) 0 (0%) 2 (50%)	 0 0.007*
Myometrial invasion < 50% ≥ 50%	 22 (50%) 22 (39.3%)	 22 (50%) 34 (60.7%)	0.2
Tumor Necrosis Negative Positive	 18 (63.6%) 16 (28.6%)	 16 (36.4%) 40 (71.4%)	0.0001*
LVI Negative Positive	 28 (35.9%) 16 (72.7%)	 50 (64.1%) 6 (27.3%)	0.002*
Tumor size <2 cm ≥2 cm	 6 (37.5%) 38 (45.2%)	 10 (62.5%) 46 (54.8%)	0.5

Test of significance: Chi-square test
*Significant p-value < 0.05**
-FIGO: *International Federation of Gynecology and Obstetrics*
-LVI: *lympho-vascular invasion*

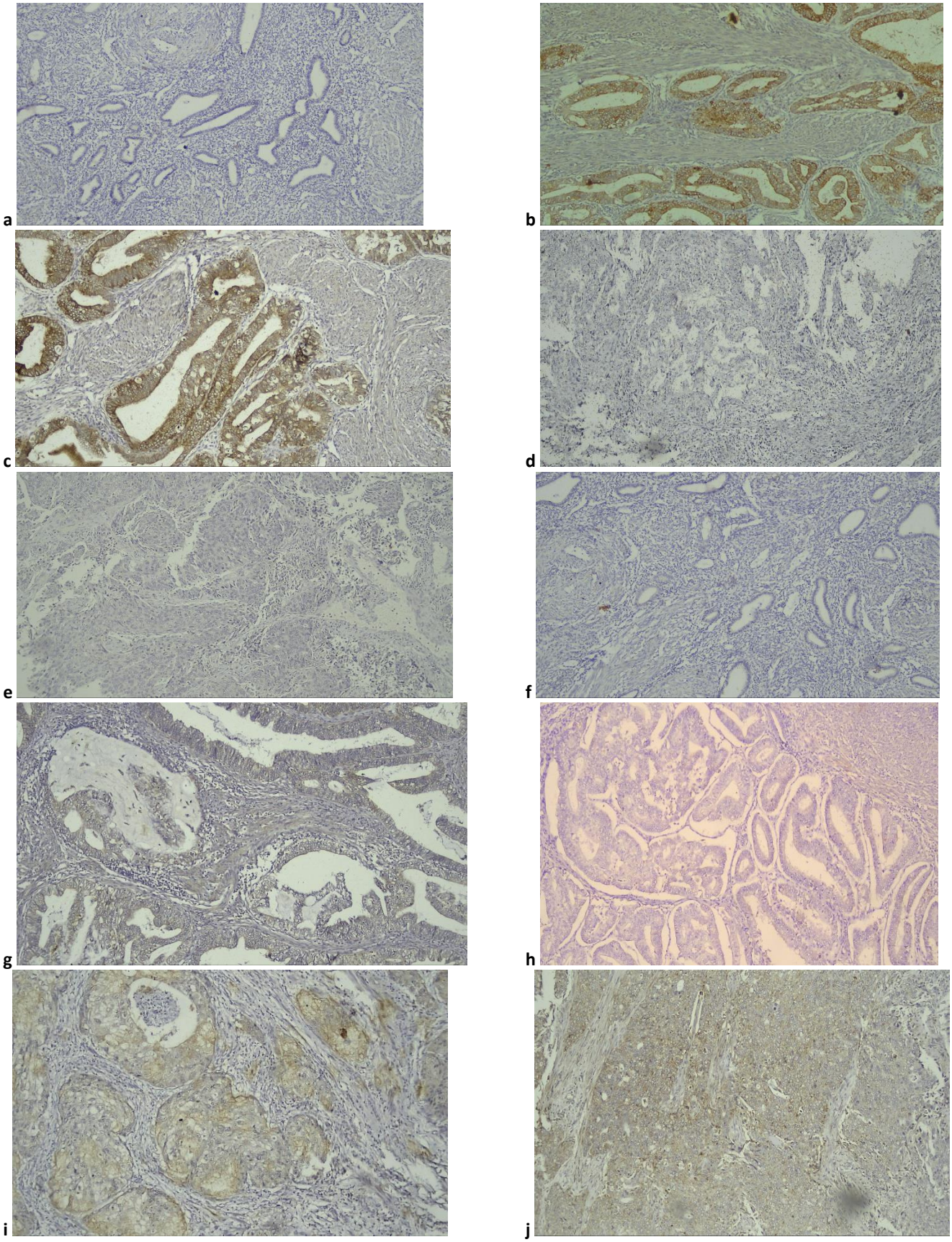

Figure 1: Immunohistochemical expression of SLCO4C1 and FGF21 in different endometrial tissues; (a) Low expression of SLCO4C1 in EH without atypia (b) High expression of SLCO4C1 in AEH (c) High SLCO4C1 expression in grade I (d) Low expression of SLCO4C1 in grade II (e) Low expression of SLCO4C1 in grade III (f) Low expression of FGF21 in EH without atypia (g) High expression of FGF21 in AEH (h) Low expression of FGF21 in grade I (i) High expression of FGF21 in grade II (j) High expression of FGF21 in grade III (Streptavidin-biotin-immunoperoxidase 100x).

Table 6: Univariate and Multivariate logistic regression about factors affecting SLCO4C1 expression

		univariate				multivariate			
		Sig.	Exp(B)	95% C.I. for EXP(B)		Sig.	Exp(B)	95% C.I. for EXP(B)	
				Lower	Upper			Lower	Upper
	Age	.368	1.05	1.00	1.10	.357	1.07	1.01	1.13
	Grade Category (low)	.03*	3.11	1.10	8.79	.01*	5.25	1.54	17.96
	Stage (advanced)	.43	1.71	.45	6.49	.30	2.36	.46	12.06
	Myometrial Invasion (>50%))	.65	1.20	.54	2.65	.50	1.38	.54	3.51
	Necrosis (positive)	.65	1.20	.54	2.65	.47	1.42	.55	3.67
	LVI (positive)	.49	1.40	.54	3.62	.64	1.35	.39	4.69
	Size (<2cm)	.86	1.10	.38	3.21	.65	1.33	.39	4.51
	Constant	.002	1.02	1.01	1.04	.002	.003		

Table 7: Univariate and Multivariate logistic regression about factors affecting FGF21 expression

		univariate				multivariate			
		Sig.	Exp(B)	95% C.I. for EXP(B)		Sig.	Exp(B)	95% C.I. for EXP(B)	
				Lower	Upper			Lower	Upper
	Age	.235	1.08	1.03	1.14	.256	1.12	1.03	1.22
	Grade Category (high)	.42	1.50	.57	3.98	.58	1.61	.30	8.76
	Stage (early)	1.00	2660782585.35	.00	.	1.00	2806532224.29	.00	.
	Myometrial Invasion (>50%)	.28	1.55	.70	3.43	.06	3.32	.95	11.67
	Necrosis (positive)	.00	4.37	1.88	10.18	.00*	23.13	5.34	100.23
	LVI (negative)	.00	4.76	1.67	13.56	.02*	7.58	1.48	38.86
	Size (<2cm)	.57	1.38	.46	4.13	.04*	6.33	1.11	35.95
	Constant	.002	1.02	1.01	1.04	1.00	.00		

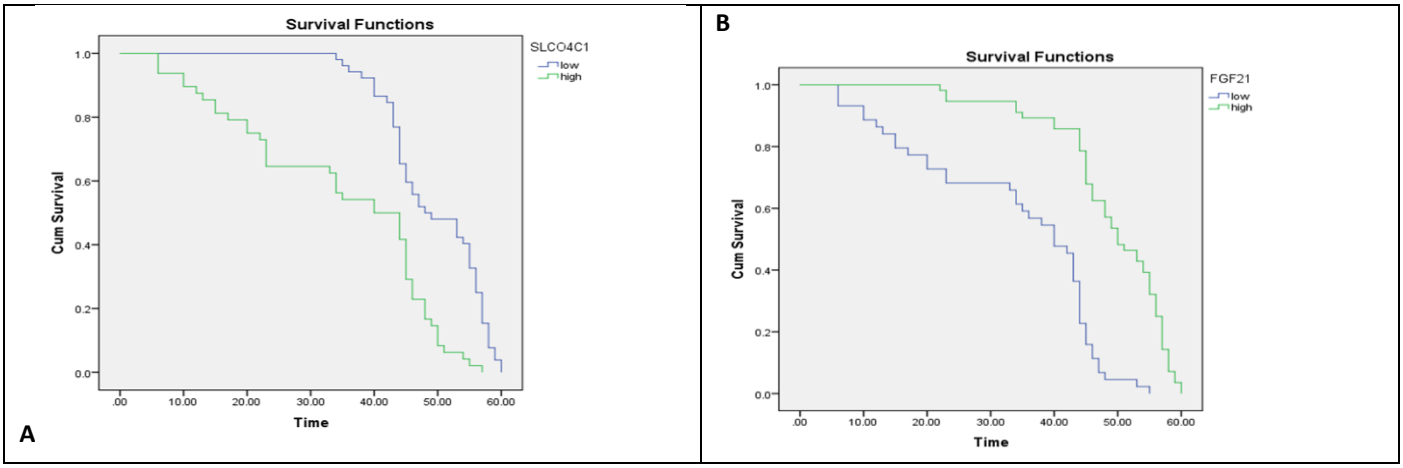

Figure 2: Kaplan-Meier curves for disease-free survival stratified according to SLCO4C1 and FGF21 expression. Longer disease-free survival is associated with low levels of SLCO4C1 expression (A) and high levels of FGF21 expressions (B).

A meta-analysis by Chen et al., 2022, encompassing various cancer types, indicated that high FGF21 expression is generally associated with improved survival [23]. This aligns with our findings of an inverse association between FGF21 expression and adverse clinico-pathological features in EC, supporting its potential role as a favorable prognostic marker. Regarding the relation between the expression of markers with disease-free survival in EC patients, we found that higher disease-free survival rates were associated with low expression of SLCO4C1 and high expression of FGF21. In conclusion, our findings suggest that expression patterns of SLCO4C1 and FGF21 may be used as prognostic tool in EC patients. We anticipate further investigations combining immunohistochemistry with molecular studies with larger sample size and different representative cases in each grade and stage, a deeper insight into the pathophysiology and progression of atypical endometrial hyperplasia (AEH) into invasive EC may emerge, shedding light on the pivotal roles of SLCO4C1 and FGF21 in this complex process and targeting it could be a promising therapeutic strategy for preventing tumor metastasis in patients with EC.

Conflict and interest

None

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