Identification and characterization of endometrial carcinoma with tumor markers HE4 and CA125 in serum and endometrial tissue samples

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Abstract

Objective: Diagnosis of endometrial cancer (EC) is made by biopsy sampling with pathological analysis, but it is extremely important to make an accurate diagnosis in order to plan the specific treatment. We hypothesized that human epididymis protein 4 (HE4) in endometrial tissue and in serum could be beneficial for a more precise diagnosis.

Material and Methods: This prospective study compared patients with EC against non- EC, matched through several variables. The inclusion criteria were: females older than 18 years who accepted to participate; who had never undergone surgery for other oncological pathologies (ovarian, colon, cervical carcinoma or uterine sarcoma); none of them had received preoperative chemo- or radio-therapy; and no participant had any severe renal or liver pathology. All had pre-surgery blood sampling and then underwent hysterectomy. Histopathological assessment of endometrial samples was made by a pathologist who compared normal histopathological staining with HE4-antibody staining.

Results: In total there were 34 cases and 35 controls recruited. There was poor correlation between tissue HE4 in patients with and without carcinoma. However, serum HE4 was significant for the diagnosis of endometrial carcinoma (median EC: 123.1 U, median NE: 64.67 U, p=0.002), although the carbohydrate antigen 125 level was not significant (p=0.208).

Conclusion: The findings concerning the utility of HE4 contrast with earlier reports. However, the conclusions for serum measurements are positive and suggest that the tumor marker HE4 seems to be able to diagnose EC. (J Turk Ger Gynecol Assoc 2021; 22: 161-7)

Keywords: CA125, diagnosis, endometrial cancer, HE4, tissue

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Introduction

Human epididymis protein 4 (HE4), also known as acid protein (WFDC2), was first identified in the epithelial cells of the epididymal duct and plays a role in natural immunity and in sperm maturity (1-3). In 2001, the FDA approved HE4 as a serum tumor marker of ovarian cancer. Uterus, fallopian tubes and ovaries derive from the urogenital crest and, in turn, the first two arise from the paramesonephric tissue. Therefore, they have similar embryological properties suggesting that they could be related.

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Currently, only serum carbohydrate antigen 125 (CA125) is being used as a biomarker in endometrial cancer (EC), although serum HE4 has shown good results (4). Some positive results have already been shown in meta-analysis assessment of serum HE4 in relation to EC (5), though the sensitivity was not sufficient to make a firm recommendation for its routine use. To date, there are few publications investigating the utility of HE4 in tissue samples (6). The published studies show an association between HE4 concentration and worse prognosis or adverse clinicopathological variables (7-9).

The primary objective of this study was to identify and characterize HE4 in endometrial tissue samples obtained from patients diagnosed with EC. Moreover, comparison was made between samples of endometrial tissue from non-EC patients with tissue samples from EC patients. Similar comparison was made between serum levels of HE4 from EC and non-EC cancer groups and between tissue and serum levels. Finally, comparison was made between HE4 staining or serum HE4 and several prognostic variables.

Material and Methods

This prospective study was a case and control, nested in a hospital-based cohort. Initial comparison was made between EC patients (cases) and healthy patients (controls). Each sampling case was matched with one control selected from patients undergoing hysterectomy for non-oncological reasons.

The study was conducted at a tertiary hospital in Spain, during the period July 2017 to April 2018. All the targeted patients (in both arms) fulfilled the following criteria: diagnosed with EC; older than 18 years; and wanted to participate in the research study and signed consent forms voluntarily. Exclusion criteria were the following: patients who underwent surgery for other oncological pathologies, whether for ovarian, colon, cervical carcinoma or uterine sarcoma; and none of them had received pre-operative chemo- or radio-therapy. In addition, no participant had severe renal or liver pathology. The inclusion and exclusion criteria for the control patients were exactly the same, except none had a diagnosis of cancer.

The study was performed in compliance with the medical Declaration of Helsinki. Participation was voluntary and would not affect the standard of medical care the patients received in any way. All participants would be fully anonymized. This study was approved by the Leon Clinical Research Ethics Committee (approval number: 17104).

The recruitment of patients took place when the diagnosis of EC was made following a previous endometrial biopsy. Furthermore, controls were females undergoing elective hysterectomy for non-oncological reasons. Every patient who fulfilled the criteria, and from whom we were able to request the preoperative test was selected. There was ongoing recruitment from July 2017 to April 2018.

After accurate diagnosis, patients were contacted and the study was explained. If the patient understood, accepted and signed the informed consent, then they were included in the study and blood samples were obtained pre-operatively for analysis of CA125 and HE4. As usual, after surgery, the tissue samples were analyzed in the histopathology laboratory. Furthermore, histological assessment of HE4, Ki67 and p53 in endometrial tissue were carried out.

Variables used for matching patients were: parity, hypertension, obesity and diabetes. Data were collected from the medical record and by interview. Data items included: date of surgery; pre-operative images, tests and results; pre-operative staging according to FIGO guidelines; type of surgical procedure; and whether lymphadenectomy was performed. Pathological outcomes included: histological type; cell differentiation; size of tumor; myometrial invasion; vascular or lymphatic invasion; perineural or stromal invasion; invasion of other tissues; and final FIGO staging with node metastasis.

The main variable was the tissue HE4 (H-score determination). To analyze the H-score, tissue samples were routinely processed and paraffin embedded. Sections of 3 μm thick were produced and stained with hemotoxylin-eosin (H&E), and antibodies against HE4, Ki67 and some samples with p53. The calibration of the technique was designed according to the optimal result when the target tissue was human epididymis. The definitive dilution was 1:20.000, as it was necessary to modify it from the trading house, which was used at the beginning, to set it with the epididymis. Immunohistochemical staining of endometrial tissue sample was performed using recombinant rabbit monoclonal Anti-HE4 antibody [EPR16658] of Abcam® on a Ventana Benchmark IHC processor. Representative areas were chosen from H&E stained sections.

Immunohistochemistry results were semi-quantitatively assessed to assign an H-score to tumor samples. Cytoplasmic staining was graded for intensity (0-weak, 1-moderate and 2-strong) and the percentage of positive cells was scored as 0 (0-33%), 1 (34-66%) and 2 (67-100%).

A single scale, with scores 0-4, was obtained by multiplying the intensity and the percentage staining score, and a total score was calculated by grouping score 0 in total score 0, 1-2 in total score 1, and 3-4 in total score 2.

Serum HE4 was determined using HE4 enzyme immunometric assay using a monoclonal antibody. Measuring range was 15-900 ppmol/L.

Serum CA125 was identified by electrochemiluminescence immunoassay using two monoclonal antibodies. Measuring range was 0.6-5000 U/mL.

Immunohistochemistry results were assessed by a semi quantitative approach used to assign an H-score (Figure 1) to tumor samples. Cytoplasmic staining was graded for intensity (0-negative, 1-weak and 2-strong) and the percentage of positive cells were scored as 0 (0%), 1 (1-50%) and 2 (51-100%). Tissue Ki67 determination was carried out semi-quantitatively, as recommended in the "International Ki67 in Breast Cancer Working Group". The measure of Ki67 was conducted through the counting of stained cores in the studied area (200 cores) without taking into account the intensity of the immuno-staining and excluding the counting of cores of other cells. The cell proliferation index was established as the average of the values obtained in three different areas (including areas of more and less proliferation). Ki67 was analyzed as a continuous variable setting the cut-off point at 25% (10).

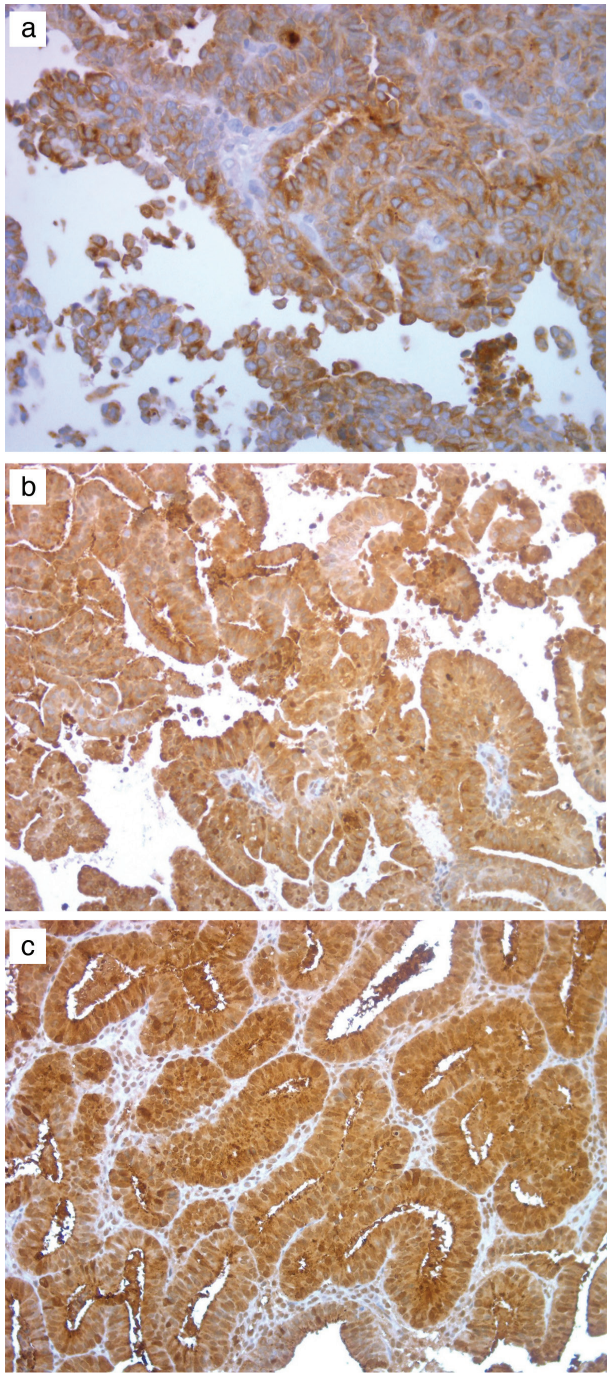

Figure 1. Representative immunohistochemical staining for HE4 in tissue microarrays of endometrial carcinoma: (a) H-score 0, endometrial endometrioid type adenocarcinoma FIGO G1; (b) H-score 1, endometrioid carcinoma FIGO G1; (c) H-score 2, FIGO G1 area of a G3 endometrioid carcinoma.

HE4: Human epididymis protein 4

Statistical analysis

Continuous variables were reported as mean ± standard deviation or median (25^{th}-75^{th} percentiles), according to the normality of their distribution, which was assessed by the Kolmogorov-Smirnov test. Categorical variables are reported as count (percentage). Comparisons of categorical variables between case-control groups were assessed using Fisher's exact test. For comparing continuous variables between groups, the Student's t-test was used if the samples were normally distributed or their variances were homogeneous; otherwise, the Mann-Whitney U test was used. Correlations between continuous variables were assessed using the Pearson (r) or the Spearman (ρ) rank correlation test. Possible biomarkers and H-score were compared using receiver operating characteristics curves and the corresponding area under the curve, whose differences were assessed using the DeLong test. Statistical analysis was performed using SPSS software, version 20 (IBM Inc., Armonk, NY, USA) and two-sided p-values $p<0.05$ were considered significant.

Results

There were 34 cases collected and 35 controls. Unfortunately, serum HE4 was not measured in all because recruitment was completed after preoperative analysis. So, in total pre-operative serum HE4 levels were available in 45 (65.2%) patients, 33 (94.3%) controls and 12 (35.3%) cases. Possible confounders were menopausal status (which was significantly higher in the cases group), the age at treatment (because patients with EC were older), and other variables (10) related to EC which, by definition would also be more frequent in the cases group.

Demographic features of both cases and controls are shown in Table 1, and the cohort of patients with EC treated, including the matching variables. Our sample consisted of 38% of all EC cases presenting during the study period. There were no differences between the participants with EC and the other EC cases who were not eligible for the study, suggesting that the risk of sample bias was low.

The expression of HE4 in endometrial tissue from patients with cancer was significantly weaker than in those without cancer (p=0.035). However, the difference between median serum HE4 levels in non-EC (64.67 U) compared with ECs (123.1 U) was statistically significant (p=0.002), although the CA125 level was not significant (p=0.208) as shown in Table 2. The comparison between modified H-score and different variables measured in pathological terms only shows statistical difference with a few variables related to the staining that are part of the staging itself. There was no difference among G1-G2-G3 cellular differentiation, with Ki67 or when comparing p53 (Table 2).

HE4 showed a considerably higher sensitivity compared with CA125 for detecting EC, 38.5% vs 7.7% and similar specificity of 84.8% compared with 90.9% for CA125 (Figure 2). However, this calculation was based on the reference ranges used in

Table 1. Demographic features of the study population

	Total cases during 2017/2018 (n=92)	Cases (n=35)	Controls (n=34)	p-value between C-C	p-value between total-cases
Age at treatment (years)	67.2±12.7	66.6±13.3	57.4±13.9	**0.006**	0.448
Parity	1.8±1.3	1.9±1.2	1.7±1.2	0.592	0.181
Menopause	85 (92.4%)	32 (91.4%)	20 (58.8%)	**0.002**	0.738
Hypertension	37 (40.2%)	12 (34.3%)	7 (20.6%)	0.282	0.241
Obesity	15 (16.3%)	8 (22.9%)	5 (14.7%)	0.540	0.153
Diabetes	18 (19.6%)	6 (17.1%)	2 (5.9%)	0.259	0.724
Other related to endometrial cancer	29 (31.5%)	9 (25.7%)	2 (5.9%)	**0.045**	0.250

Table 2. The association between HE expression in endometrial tissue and pathological parameters

		Cases	Controls		p-value
H-score					
0		5 (7.2%)	0		0.035
1		8 (11.6%)	5 (14.7%)		
2		22 (31.9%)	29 (42%)		
Serum HE4		123.1 (63.7-156.2)	62.05 (54.5-74.6)		0.002
Serum CA125		21.04±11.27	17.08±8.678		0.208
		Modified H-score			
		0	**1**	**2**	
Nuclear grade	G1	2 (5.7%)	2 (5.7%)	9 (25.7%)	0.729
	G2	1 (2.9%)	4 (11.4%)	9 (25.7%)	
	G3	2 (5.7%)	2 (5.7%)	4 (11.4%)	
Ki67	<25%	2 (6.1%)	2 (6.1%)	11 (33.3%)	0.586
	>25%	2 (6.1%)	5 (15.2%)	11 (33.3%)	
Staining macroscopic intensity	1	5 (7.2%)	0	0	<0.001
	2	0	8 (11.6%)	10 (14.5%)	
	3	0	5 (7.2%)	41 (59.4%)	
Cellular staining area	Cytoplasm	3 (4.3%)	2 (2.9%)	3 (4.3%)	0.005
	Cytoplasm + Nucleus	2 (2.9%)	11 (15.9%)	48 (69.6%)	
Staining tissue area	Apical	2 (2.9%)	1 (1.4%)	4 (5.8%)	0.124
	Diffuse	3 (4.3%)	12 (17.4%)	47 (68.1%)	
Staining	Homogeneous	2 (2.9%)	2 (2.9%)	51 (73.9%)	<0.001
	Heterogeneous	3 (4.3%)	11 (15.3%)	0	
p53	Normal	2 (5.7%)	6 (17.1%)	17 (48.6%)	0.267
	Aberrant	3 (8.6%)	2 (5.7%)	5 (14.3%)	

HE4: Human epididymis protein 4, CA125: Carbohydrate antigen 125

our laboratory which are 0-35 UI/mL for CA125 and for HE4, 0-70 pmol/L in postmenopausal women and 0-140 pmol/L in premenopausal ones.

The relationship between serum HE4 levels and the clinicopathological features of the EC patients are shown in Table 3. Higher serum HE4 levels were not significantly associated with any of the variables collected. However, serum CA125 was associated with being menopausal.

Discussion

EC is the most frequent malignant tumor of the female reproductive system in developed countries, although it is not the female reproductive cancer with the highest mortality rate (11). The mortality rate of EC has been reported to be approximately 20% in 5 years (12,13). In the USA, the estimated incidence of EC is 26.5 for every 100.000 per year, and accounts for 3.6% of all diagnosed cancer (12-15). Prognostic factors include histologic differentiation, deep myometrial invasion, non-endometrioid histologic subtype, lymphovascular invasion, lymph node status, cervical involvement, and the presence and extent of extra-uterine disease (11).

Localized EC treatment consists of hysterectomy and double adnexectomy. Moreover, the identification of those patients that should undergo a pelvic and/or para-aortic lymphadenectomy depends on several parameters (16-19).

Therefore, an endometrial biopsy and an abdomino-pelvic imaging technique (MRI as first election) are necessary for diagnosis and local extension prediction. Despite this, a significant proportion of EC cases require an extension of the planned surgery. For this reason, it was thought useful to investigate the role of HE4 as an efficient diagnostic test (20,21). However, the H-score did not have any significant result compared to any important variable studied. The only parameters with significance were pathological descriptions of the staining itself. This contrasts with the literature and some of the results are even negative, with the controls score more that the cases (14,17).

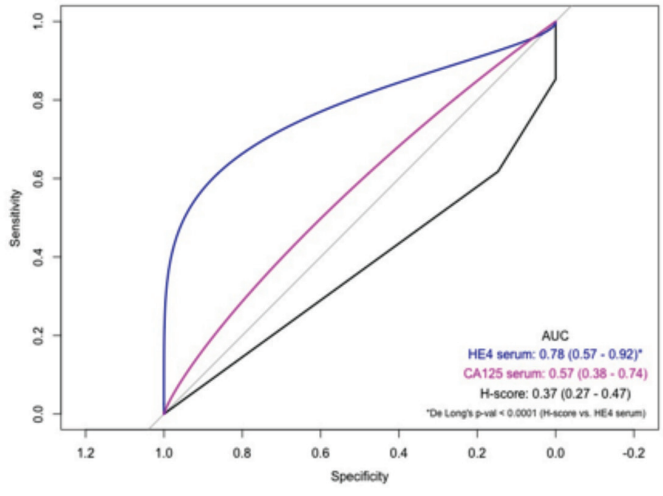

Figure 2. ROC curve: Modified H-score, serum HE4 and serum CA125

ROC: Receiver operating characteristics, HE4: Human epididymis protein 4, CA125: Carbohydrate antigen 125

Nevertheless, serum HE4 gave a positive result and we suggest that HE4 may represent a possible future biomarker for EC. HE4 is currently being studied by many research groups (18-25) and the outcomes are encouraging. There is still a requirement for additional studies of the role of serum HE4 in EC so that this can become a robust and useable biomarker. Moreover, we believe that HE4 may replace CA125 as a prospective and prognostic marker for EC because HE4 appears to have much greater specificity for EC while exhibiting a similar sensitivity to CA125.

The articles analyzed were homogeneous in terms of the number of patients, the H-score method and their general results. Furthermore, the prognosis with tissue HE4 was studied in the studies of Li et al. (8) and Deng et al. (9), but Bignotti et al. (7) only mentions serum HE4. The follow-up of patients in these three studies, as well as reported survival results are very unequal (survival of 14%, 18% and 33% respectively).

The sample of EC cases included in the study appear to be representative of our population of EC cases. However, it should be highlighted that the classical H-score, as described in the previous articles, was modified, so it was similar for basic criteria used but with fewer grades. This is because we were not able to distinguish more than 3 grades (modified H-score 0, 1 and 2), so, we designed a "modified H-score" which is the one described and used in our study.

Regarding bias, patients were interviewed by more than one researcher which will result in inconsistency in data acquisition. In addition, selection of cases was not randomized, as the incidence of EC was not high enough. As it is a case control design, there are several typical biases present including in selection and information, as well as not providing appropriate data for determining the incidence or prevalence of EC.

Study limitation

The most significant limitation of the study was that the sample size was too small. This was because our results were so discouraging for tissue HE4 in preliminary analysis, that recruitment was stopped before the expected recruitment number was obtained. Despite this, the results for measuring HE4 in serum as a biomarker for EC were encouraging.

Conclusion

The sensitivity and specificity of serum HE4 was not sufficient to recommend its adoption as a robust biomarker for EC.

Table 3. The relationship between serum HE4 and CA125 levels and the clinicopathological features

Variable	n	Serum HE4 median (Q1-Q3)	p-value	Serum CA125 ($\overline{X}$ ± SD)	p-value
Age (years)	45	67.3 (54.9-101.4) Rho= 0.247	0.102	19.1±9.9 r=-0.107	0.519
Diabetes					
Yes	6	71.4 (54.7-142.9)	0.616	19.0±13.4	0.991
No	39	64.9 (54.7-101.3)		19.1±95	
Hypertension					
Yes	14	70.6 (57.9-126.9)	0.384	19.3±11.9	0.924
No	31	64.4 (54.5-86.9)		19.0±8.9	
Menopause					
Yes	30	70.6 (55.9-111.8)	0.354	16.4±9.4	**0.029**
No	15	61.7 (54.7-82)		23.4±9.4	
Obesity					
Yes	8	66.6 (55.9-156.7)	0.449	20.0±7.8	0.783
No	37	67.3 (54.6-94.1)		18.9±10.4	
Parity					
Yes	40	69.2 (54.8-101.45)	0.448	18.6±9.5	0.373
No	5	58.8 (47.3-103.5)		23.3±13.3	
Other related to EC					
Yes	6	65.3 (57.2-121.6)	0.726	17.8±11.4	0.731
No	39	67.3 (54.7-101.3)		19.3±9.8	
Pelvic Lymphadenectomy					
Yes	5	101.3 (62.3-137.5)	0.181	15.6±0.5	0.402
No	40	64.7 (54.6-85.7)		19.6±10.3	
Paraaortic lymphadenectomy					
Yes	4	109 (67.6-147.3)	0.151	16.9±6.6	0.653
No	41	64.9 (54.6-84.5)		19.3±10.2	
Myometrial invasion >50%					
Yes	6	112.2 (55.1-156.0)	0.361	19.3±6.1	0.875
No	5	142.5 (93.1-210.3)		20.4±16.2	
Vascular, lymphatic or perineural invasion					
Yes	2	84.7 (51.4-)	0.182	12.5±4.1	0.182
No	6	132.8 (93.0-182.6)		18.4±10.6	
Adnexal affection					
Yes	1	-	0.333	-	0.660
No	11	138.2 (101.3-156.8)		20.1±11.0	
Lymph node status					
Affected	2	106.7 (56.3-)	1	20.2±7.5	0.958
Not affected	9	123.1 (84.8-156.2)		19.7±12.2	
Final FIGO stage					
I-II	6	132.8 (93.0-182.6)	0.286	18.4±10.6	0.490
III-IV	2	84.7 (51.4-)		12.5±4.1	
Second surgery					
Yes	1	-	0.397	-	0.891
No	44	66.1 (54.9-94.2)		19.1±10.0	

HE4: Human epididymis protein 4, CA125: Carbohydrate antigen 125, SD: Standard deviation, EC: Endometrial cancer

However, in our opinion it is essential to calculate a correct cut-off for EC and not to use the cut-off appropriate for ovarian cancer. This would allow correct comparison in positive and negative cases. In any case, it would be necessary to obtain data from larger studies in order to test the validity of our hypothesis.

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