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ORIGINAL ARTICLE

Tissue HE4 Expression Discriminates the Ovarian Serous Carcinoma but Not the Uterine Serous Carcinoma Patients. A New Adjunct to the Origin of the Tumor Site

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Abstract

Both uterine serous carcinoma (USC) and ovarian serous carcinoma (OSC) are presented at advanced stage at the first admittion and dissseminated disease makes the anatomical site of the tumor origin imposible. CA125 and p53 are reliable markers that are useful for differentiating both uterine serous and ovarian serous carcinoma from their most common subtypes (endometrioid type carcinoma of ovary and uterus) but so far there is no histopathologic marker that differentiates USC from OSC. On the other hand, Trastuzumab (Herceptin) increases progression-free survival among USC patients, but not OSC patients and makes the histopathologically assigning the origin of the tumor important. So, the aim of this study was to evaluate the immunohistopathological discriminative value of the human epididymis secretory protein 4 (HE4) between OSC and USC patients. Patients with a diagnosis of OSC and UTC were enrolled. HE4 expression was evaluated by immunohistochemistry. The results were compared between groups. Of the tumor tissues studied, HE4 immunostaining was seen in the majority of ovarian serous carcinoma cases (89.65%), while endomatrial serous carcinoma cases were devoid of HE4 immunostaining. HE4 immunostaining was seen in 39.1% uterin serous carcinoma cases and this difference was statistically significant ($p = 0.001$). Our study demonstrated for the first time the potential of HE4 expression to predict the anatomical site of tumor origin. HE4 is a novel tumor marker that differentiates USC from OSC.

Keywords HE4 · Serous · Carcinoma · Ovary · Endometrium · HER2

Background

Uterine serous carcinoma (USC), classified as type II cancer has aggressive behavior and poor prognosis, accounting for more than 40% of endometrial cancer deaths [1]. One reason for the poor prognosis of USC is its association with extrauterine disease. The incidence of extrauterine disease has been reported at 37% in type II endometrial cancer cases [2]. Peritoneal dissemination is also common in Type II high grade ovarian serous cancer (OSC) as well. Besides extrauterine or peritoneal dissemination of these two tumors, synchronous primary endometrial and ovarian cancers are found in 10% of women with ovarian cancer and 5% of women with endometrial cancer [3].

Typically, the tumor predominates in one or the other organ. When involves both the ovary and uterus equally, it may be difficult or impossible to determine the primary site of the tumor. Both tumors have the same histopathological similarity, ie: Papillary structures or tufts with markedly cytological atypia and hobnail type cells. Immunohistochemical profile of tumors are commonly used to trace the primary anatomical origin of the tumor in pathology practice but P53 and CA125, immunohistochemical biomarker commonly used for serous carcinoma diagnosis [4], are positive in both tumors and cannot help differentiate one from the other. Although

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Wilms tumor 1 (WT1) expression between OSC and USC has been discussed formerly, later-on studies observed higher expression rate in USC as in OSC (100% of OSC compared to 64–75% of USC) [5].

Despite the similar morphology regardless of whether they originate from the ovary or the uterus and same immunohistochemical markers, treatment with HER2 based therapies makes difference between these two tumors that also share the same name: Trastuzumab plus carboplatin and paclitaxel show incremental benefit for HER2 positive USC patients but not in OSC patients [6]. So it is necessary to distinguish the anatomical site of the serous carcinoma which is difficult most of the time due to the extrauterine disease at the time of diagnosis and there is a need for a histopathological marker that could be used to differentiate these two tumors.

Human epididymis 4 (HE4) protein belongs to whey acidic 4-disulfide center protein family [7]. The protein shows characteristics of a secretory protein, with an acidic and cysteine-rich polypeptide [8, 9]. Formerly found in epididymis, it is now shown that HE4 plays an important role in cultured ovarian cell adhesion and motility [10]. There is an interact between HE4 and steroid hormones: Treatment of ovarian cancer cells with steroid hormones promoted nuclear translocation of HE4 and cells became less responsive to hormonal therapy, which was restored by blocking HE4 from entering the nucleus [11]. Its presence at the cellular level was also demonstrated in malignant ovarian tumors and in a wide range of benign and borderline ovarian lesions [12, 13]. It is also found in endometrial carcinoma patients but to our knowledge, its presence in the USC tissue was examined in only one study and the expression rate was found 38.1% [14].

Our research team formerly demonstrated that HE4 was overexpressed exclusively in lung adenocarcinoma and ovarian serous carcinoma [15, 16]. Subsequently, we studied its role in the development of intestinal metaplasia and its potential for gastric tumor progression [17]. In this study, we analyzed the immunohistochemical expression of HE4 protein in uterine (endometrial) serous carcinoma tissues (Group I) and ovarian serous carcinoma tissues (Group II). In order to predict its potential for predicting the anatomical origin of the tumor, we compared our results to the expression of HE4 between Group I and Group II.

Materials and Method

A total of 53 consecutive patients who were diagnosed with high grade serous carcinoma and who underwent surgical excision at Health Science University, Antalya Hospital were retrospectively enrolled in the study. After obtaining approval from institutional Ethics Committee (2016–187, 30/06/2016), one representative tumor block containing sufficient tumor tissue from 24 USC and from 29 OSC were chosen retrospectively. Exclusion criteria were tumors with <10 tumor cells and tumors from metastatic focuses. Patient information, histopathological parameters, and previous WT1, CA125, p53 immun results of each patient were obtained from the relevant pathology reports and from the hospital data basis. Tissue sections of normal human epididymis processed in a comparable manner provided as positive control. Negative controls were obtained by omitting the primary.

Immunohistochemical Procedure

Formalin-fixed, paraffin-embedded sections were de-waxed with xylene an rehydrated through gradient ethanol into a phosphate buffered solution (PBS). Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol for ten minutes at room temperature. At the same time 2 ml Tris-EDTA Buffer (abcam, ab93684) was added to 198 ml of distilled water, and swirled. Prepared retrieval solution was added to the microwaveable vessel. When the time elapsed, slides were washed in PBS three times and placed into the microwaveable vessel. The vessel was placed inside the domestic microwave, set to full power for ten minutes, at a second highest power for five minutes, and at medium power for five minutes. The procedure was monitored for evaporation and watched for boiling over during the procedure and did not allow the slides to dry out. When the retrieval solution evaporated during the boil, hot retrieval solution was added. When 20 min elapsed, the vessel was removed. When it cooled, the slides were washed in PBS three times before application of the rabbit polyclonal antibody to HE4 (anti-HE4 antibody [EPR16658] [ab200828], 1:2000 dilution). After two hours incubation with the primary antibody, the slides were washed in PBS and biotinylated goat anti-rabbit IgG secondary antibody was applied and incubated for ten minutes at room temperature. Slides were washed three times in PBS and streptavidin peroxidase was applied for ten minutes at room temperature. At the same time 20 μl DAB chromogen was added to 1 ml of DAB substrate and swirled. When the time elapsed, the slides were washed in PBS three times and prepared chromogen was applied to the tissues for ten minutes at room temperature. Slides were then washed in PBS three times and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. The tissue sections of the human epididymis were processed in a comparable manner and provided a positive control (Fig. 1a, inset). Negative control was obtained by omitting the primary antibody (Fig. 1b, inset). Protein expression was then defined as negative, and positive. Cytoplasmic staining was graded for intensity (0-negative, 1-weak, 2-moderate and 3-strong) and percentage of positive cells (0, 1 (1–24%), 2 (25–49%), and 3 (50–100%). The grades were multiplied to determine an H-score. The H-scores for tumors with multiple cores were averaged. Protein

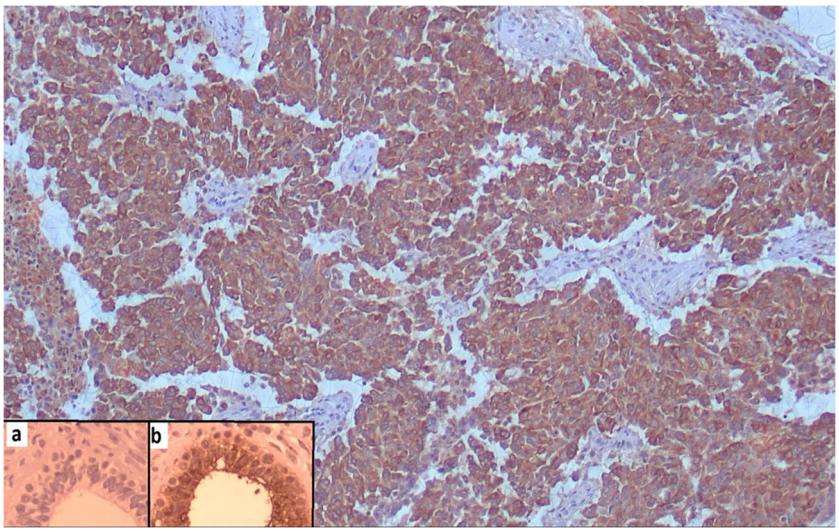

Fig. 1 Strong (3+) intracytoplasmic staining of anti-HE4. The immunostaining seen here was obtained from an ovarian serous carcinoma tissue and from human epididymis (inset, B). The negative control was obtained by omitting the anti-HE4 (inset, A) (tumor: ×10, inset: ×20)

expression was then defined as negative (H-score = 0), weak (H-score = 1–3), or strong (H-score ≥ 4).

Statistical Analysis

All data obtained in the study were evaluated with SPSS version 11.5. Contionuous variables were described as mean ± standard deviation and categorical variables were decribed as frequency (percentage). The normality check of continuous variables were performed with the Shapiro-Wilk test. Two-group comparisons were performed with the Student's t test or the Mann-Whitney U test depending on normality of distribution. For the comparison of categorical variables, the Chi-squared tests were used. *P* values <0.05 were considered to be significant in all tests.

Results

The study retrospectively recruited 53 serous carcinoma cases. One USC case was failed to get immunostained in spite of several attempts and excluded from the study. Alltogether 29 ovarian serous carcinomas and 23 uterine serous carcinomas were analyzed. Among 23 USC cases (Group I) successfully stained with HE4, there were two weak, and seven strong immunostaining, whereas fourteen (60.9%) were negative with HE4. Overall, HE4 tissue expression among Group I has been 9 out of 23 cases and the sensitivity of the test has been 39.1% for Group I.

Among 29 OSC cases (Group II) successfully stained with HE4, there were five weak, and twenty-one strong immunostaining (Fig. 1), whereas three cases (10.3%) were negative with HE4. Overall, HE4 tissue expression among ovarian serous carcinoma has been 26 out of 29 cases and the sensitivity of the test has been 89.65% for Group II) (Tables 1 and 2. The frequency of HE4 immunostaining was significantly higher in Group II as compared to Group I ($p = 0.001$).

We also assessed whether coordinate immunoexpression of p53, WT1, and HE4 could distinguish USC and OSC. WT1 plus HE4 positivity was observed in 17 out of 20 OSC cases (85%), while p53 plus HE4 positivity was seen in 8 out of 15 OSC cases (53.3%) (Table 3). Any two positive test results (WT1, p53, or HE4) had 95.65% sensitivity for OSC cases (22 out of 23 cases). For USC cases, WT1 results were inadequate to coordinate, and the result of coordinate immunoexpression of p53 plus HE4 remained inconclusive due to the universal positivity of p53 in USC cases.

Discussion

Endometrial cancer (EC) has been the most common female genital tract malignancy in the USA. The incidence is of 61,380 cases and 10,920 deaths in 2017 [18]. It is classified into type I and type II diseases inwhich etiology, tumor characteristics, and prognosis are different. Type II EC develops from the atrophic endometrium and is seen in the elderly. It has a worse prognosis than type I EC. Type II EC includes serous (USC), clear cell, and grade 3 endometrioid carcinoma types. Serous type is the most biologically aggressive variant of type II EC [19]. It has a predilection for deep myometrial invasion, and intra-abdominal as well as a distant spread at the time of diagnosis. Histopathologically, CA125, p53,p16, and HMGA2 are immune markers that are specific to USC, but WT1 is also expressed in USC [20]. Among the immune markers applied during the initial examination, 19/20 (95%), and 9/15 (60%) OSC cases were positive with WT-1 and p53 antibody respectively, while 3/3 (100%), and 9/10 (90%) USC cases were positive with WT-1 and p53 antibody respectively (Table 2).

Table 1 HE4 expression between groups. Strong (3+) immunostaining was three times frequent in tumors originated from the ovary

HE4 expression				
H-score				
	0 (negative) *(%)*	1–3 (weak)	≥ 4 (strong)	*p*
Group I (USC)	14 *(60.9)*	2	7	$p = 0.001$
Group II (OSC)	3 *(10.3)*	5	21	

Ovarian cancer (OC) has been the second most common female genital tract malignancy in the USA but it is the most common cause of cancer-related mortality among gynecological malignancies. The incidence is of 22,440 cases and 14,080 deaths in 2017 [18]. Like EC, OC is also classified into type I and type II, with a different etiology, tumor characteristics, and prognosis. Type II OCs are aggressive and present in an advanced stage. Type II tumors include high-grade serous, high-grade endometrioid, carcinosarcoma, and undifferentiated carcinoma. Of them, ovarian high-grade serous carcinoma (OSC) is more likely to disseminate to the abdomen and has a lower survival compared to other histological types [21]. Histopathologically, CA125, p53, p16, WT1, and HMGA2 are immune markers that are specific to OSC.

The key role of HE4 in ovarian cancer cell adhesion and motility has long been known [10, 22–26]. Tissue expression of HE4 in ovarian tumors was shown immunohistologically in previous studies [13, 27]. Our group demonstrated that it served as a surrogate marker for p53 in high-grade OSC [15]. We also observed that it was superior to p53 and CA125 for OSC diagnosis. HE4 overexpression in EC cell lines had been observed in vitro [28]. For endometrial carcinoma patients, the elevation of HE4 in the serum was also observed [29]. Moreover, its serum level differentiated ECs from benign endometrial tumors [30], predicted the depth of myometrial invasion [14, 31], overall survival [32], and recurrence of EC cases [33]. Immunohistochemical expression of HE4 in overall EC cases has been 84.62% [14]. However, the studies above and many more studies measured patients who had endometrioid type EC. There is only one study that separated "serum" level of HE4 according to the histological type [29]. This study revealed that increased HE4 level that predicted recurrence was found to limit to patients with endometrioid cell type, not the serous type. Concordant with the above result, we observed lower level of HE4 in our USC patients. Li X et al. sub-classified EC cases in the same manner and sought HE4 immunohistochemically: Even though HE4 expression in the overall EC cases was high (84.6%), it was 71.43% in the USC cases and this rate dropped to 38.10% when an intense (3+) immunostaining was sought [14]. The 3+ immunostaining in their study had been lower compared to the present study.

Several biomarkers have been proposed to improve the differentiation of USC and OSC. One of the oldest studies compared to p53 and WT1. WT1 immunoreactivity was seen in 10 of 16 USC and in 24 of 28 OSC [34]. When correlated with p53, there was no statistical significance for serous carcinomas. One year later a gene expression analysis was published. It couldn't identify a gene set distinguishing serous cancers (across organ site) from endometrioid and clear cell subtypes [35]. In a more recent study, Estrogen receptor, progesterone receptor and WT1 genes were examined in USC and OSC cases and triple positive markers were identified in 33.6% of OSC but in none of USC [36]. Although specificity and positive predictive value (PV) were 100%, its sensitivity and negative PV were merely 33.6% and 19% respectively. The present study, on the other hand, had a higher sensitivity rate in the differential diagnosis between OSC and USC, ie: positivity for HE4 as a single marker favored an extrauterine origin whereas negativity supported an endometrial origin. Furthermore, the combined use of a triple marker (WT1, p53, and HE4), instead of HE4 as a single marker, made a difference of only 6% in the sensitivity for OSC (89.65% vs 95.65%).

During the past decade, cancer type-specific treatment gained great interest. Amplification or overexpression of HER2 was identified in up to 40% of USC and 10% of OSC [37–39]. In vivo studies also supported these in vitro studies. In a randomized phase II trial, adding Trastuzumab (Herceptin) to the same chemotherapy regimen increased median progression-free survival from 8.0 months to 12.6 months in USC patients [40]. The promising result observed on USC patients taking Traztuzumab is not seen on OSC patients and results are disappointing [40, 41]: A phase II trial indicated a response rate of 7.3% in a cohort of OSC patients [42]. It is clear that treatment response depends on the anatomical site of the tumor origin and histological resembling of these "serous" carcinomas confounds treatment decision. Moreover, when serous cancer is the case, it is not uncommon to face with a

Table 2 WT1, CA125, and p53 expression between groups. Note that there were no diffuse WT1 and CA125 expressions in tumors originated from the uterus

ovary $n = 25$						
NA		negative	false	focal +ve	diffuse +ve	
WT1	5	1	0	**2**	**17**	19/20
CA125	0	0	1	**3**	**21**	24/24
p53	9	6	1	**0**	**9**	9/15
uterus $n = 11$						
	NA	negative	false	focal +ve	diffuse +ve	
WT1	6	2	0	**3**	**0**	3/3
CA125	8	0	0	**3**	**0**	3/3
p53	0	1	1	**0**	**9**	9/10

disseminated intraabdominal tumor which can be derived from ovary, uterus, fallopian tube, omentum, or peritoneum [43]. Besides, approximately 5–10% of women with ovarian and uterine cancer harbor simultaneous ovarian or uterine cancer [3]. Histopathologically and tracing the origin of the tumor immunohistologically are a challenge for the pathologist and there is a necessitate for an adjunct marker. In this study, we demonstrated that there was a statistically significant correlation with the tumor site and HE4 immunostain ($p < 0.005$) and HE4 could be a useful marker for differentiating OSC from USC.

Our study had several limitations. Initially, a limited number of included USC cases may decrease the power of this analysis. Secondly, we couldn't determine the specificity of HE4, as there was no individuals without disease (control group). Thirdly, this was a pathology-based study and the clinical characteristics were not incorporated into the study. Fourthly, due to the retrospective design of our study, we didn't measure the serum level of the HE4 protein in patients. Finally, HE4 expression in serous tumors derived from the fallopian tube, omentum, and peritoneum were not assessed. Additional studies particularly to evaluate HE4 expression in USC is necessary to get more data to improve the quality of our research.

Table 3 Coordinate immunophenotipic pattern of ovarian serous carcinoma cases

	n	no *(%)*	yes *(%)*
WT1+/HE4+	20	3 *(15)*	17 *(85)*
p53+/HE4+	15	7 *(46.7)*	8 *(53.3)*
any 2+	23	1 *(4.35)*	22 *(95.65)*

In conclusion, these data are a preliminary result of HE4 in USC patients and suggest that seeking HE4 as a single, cost-effective marker in serous tumor tissue may provide additional value for the recognition of a tumor origin with additional cost-saving.

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Author's Contributions Conceived and designed the study: BC; Immunohistochemical assistance: SK; Analyzing data: BC, TB, ADY; Writing paper: BC.

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Compliance with Ethical Standards

Financial Disclosures None reported.

Conflict of Interest The authors report no conflicts of interest relevant to this manuscript.

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