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RESEARCH ARTICLE

HE4: a new potential early biomarker for the recurrence of ovarian cancer

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Abstract Human epididymis protein 4 (HE4) has recently been described as a new marker for the early diagnosis of ovarian cancer (OC). The objective of this study was to evaluate (a) the expression of HE4 vs. OC mucin CA125 in 32 patients with OC compared to 163 patients with other malignant or benign pathologies (b) HE4 as indicator of the recurrence of the disease in eight patients followed-up for 20 months after OC diagnosis. Serum HE4 and CA 125 levels were determined by ELISA and IRMA, respectively. At diagnosis, the patients with OC demonstrated high levels of both biomarkers with 96.9% sensitivity for HE4 and 85.7% for CA125. In the other pathologies there was 3.7% positivity for HE4 and 21.0% for CA125. The follow-up study showed an increase of HE4 5–8 months before CA125 increment in five of the eight patients, this early expression being strictly associated to a relapse of the disease. In conclusion, this study showed that HE4, compared to CA125, potentially is a better marker for the diagnosis of OC and could be an important early indicator of the recurrence of the disease.

Keywords Ovarian cancer · Biomarkers · HE4 · CA125

Introduction

Ovarian carcinoma (OC) is the leading cause of death from gynecologic cancer for women in industrialized countries [1]. At present, the global incidence is approximately 165,000 cases per year [2, 3]. If the carcinoma is diagnosed at an early stage, it has an excellent prognosis since it can be treated before spreading to surrounding tissue. However, due to its biological characteristics and the lack of screening tools, OC is diagnosed in most cases (≥80%) when it is already in advanced stages (FIGO III-IV). Unfortunately, in these patients, the median survival ranges from 18 to 24 months with an 80% probability of recurrence of the disease within 5 years [4, 5].

In the last decades, several tumor markers have been detected in the blood of patients with OC but their sensitivities and specificities for predicting this form of cancer were no better than those of mucin CA125 alone [6, 7]. CA125 was originally identified following the development of the OC125 antibody and was found to be elevated in about 80% of patients with cancer of ovary and in 30% of patients with any primary cancer with extensive intra-abdominal disease [8]. Therefore, even if CA125 generally has a high sensitivity, its clinical use in confirming OC is limited because it is also frequently increased in women with benign gynecological diseases as well as benign diseases associated with inflammatory cells of the pleura, pericardium, and peritoneum [9–11]. For these reasons, numerous studies have been conducted in order to identify a marker with high specificity and sensitivity for OC diagnosis.

Recently, attention has been focused on a new biomarker called *Human epididymis-specific protein* 4 (HE4). HE4 was initially identified in the epithelium of the distal epididymis and originally predicted to be a protease inhibitor involved in sperm maturation [12–14]. It is also called WFDC2 because it contains two whey acid protein domains and a "four disulphide bond core" made up from eight cysteine residues [15].

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It has also been reported that HE4 is commonly over expressed in ovarian neoplastic tissue and it is elevated in the serum of patients with cancer of the ovary. Recent studies have demonstrated high sensitivity of HE4 compared with CA125 especially in the early stages of the disease [16–20].

Since, as already mentioned, CA125 has limitations as a marker for OC, because it is also expressed in both other malignant and benign diseases, the levels of both HE4 and CA125 have been measured in a group of patients with diagnosis of OC compared with a group of patients affected by different oncological or non oncological pathologies. In addition, in order to understand the relevance of HE4 in the clinical response to treatment or remission from OC, the expression of this biomarker and that of CA125 have been retrospectively evaluated in a group of patients followed for a time of between 16 and 20 months after the diagnosis of the disease.

Patients and methods

Patients

All subjects included in the study were all patients admitted to our laboratory as a result of a suspicion of neoplastic disease. Serum samples were collected from a total of 267 subjects subdivided as follows:

- Thirty-two ovarian cancer (age range 33–86 years, median 66 years)
- Seven colorectal cancer (age range 46–76 years, median 64 years)
- Sixteen breast cancer (age range 41–79 years, median 59 years)
- Twenty-six cervical cancer (age range 39–83 years, median 61 years)
- Eighty-six benign ovarian tumors (age range 16–82 years, median 45 years)
- Twenty-eight other benign pathologies (pneumonia, fibroma, vaginitis, appendicitis, and renal colic; age range 21–74 years, median 53 years
- Seventy-two normal blood donors (age range 29–63 years, median 48 years).

All patients and subjects gave informed consent to the work.

Follow-up study

Eligibility for inclusion in the study required (a) availability of sera at diagnosis and at intervals during the subsequent 16–20 months after surgery, and (b) clinical data to correlate the date of serum collection to the clinical status of the patients. Thus, 46 serum samples obtained from eight patients with advanced disease diagnosed between 2005 and 2007 at the Sapienza University of Rome were retrospectively tested for the evaluation of the levels of HE4 and CA125. The clinical characteristics of the patients are summarized in Table 1.

Sample preparation

All sera were acquired following a standard collection protocol. Briefly, samples were collected in a Red Top Vacutainer, clotted 60–90 min and centrifuged for 10 min at 1,300×*g*. The serum fractions were aliquoted and stored at −80°C until analysis.

Biomarker assays

HE4 determination

HE4 levels were determined using the HE4 EIA assay (Fujirebio Diagnostics). The HE4 EIA is a solid phase, non competitive immunoassay based upon the direct "sandwich" technique using two monoclonal antibodies, 2H5 and 3D8, directed against two epitopes in the C-WFDC domain of HE4. Controls or patient serum samples and standards were incubated with biotinylated anti-HE4 monoclonal antibody 2H5 aliquots in streptavidin-coated microstrips. HE4 present in standards or serum samples was adsorbed to the streptavidin-coated microstrips by the biotinylated anti-HE4 monoclonal antibody during the incubation period. The strips were then washed and incubated with HRP labeled anti-HE4 monoclonal antibody 3D8. After washing, buffered substrate/chromogen reagent was added to each well and the enzyme reaction was allowed to proceed. During the enzyme reaction, a blue color developed if the antigen was present. The intensity of the color was directly proportional to the amount of HE4 present in the samples. According to the manufacturer's indications, normal values of HE4 were considered to be less than 150 pmol/L.

Table 1 Characteristics of patients affected by ovarian cancer and included in the follow-up study

Patient	Hoistology	FIGO stage	Age	Dead from disease
1.	Serous	III C	52	
2	Serous	III C	56	x
3.	Serous	II A	73	x
4.	Endometrioid	III C	68	x
5.	Serous	III C	69	
6.	Serous	II A	49	
7.	Endometrioid	II A	78	
8.	Serous	III C	65	

CA125 determination

CA125 levels were evaluated by a one-step "sandwich" radioimmunoassay (Radim, Netherlands). Polystyrene beads coated with M11 capture antibody reacting with molecules containing OC 125 reactive determinants were incubated with control or patients' serum samples, standards and tracer (^{125}I-labeled mouse monoclonal OC 125 antibody) aliquots. The bound radioactivity observed was proportional to the concentration of the OC 125 reactive determinant (antigen). Normal levels of CA125 were considered to be less than 35 U/ml.

Biomarker distribution in groups

The values above the reference limit of both markers determined in all groups of patients were arbitrarily distributed in quartiles as follows. HE4 quartile I, values from 150 to 249 pmol/L; quartile II, values from 250 to 349 pmol/L; quartile III, values from 350 to 449 pmol/L; quartile IV, values from 450 to >850 pmol/L. CA125 quartile I, values from 37 to 136 U/ml; quartile II, values from 127 to 236 U/ml; quartile III, (values from 237 to 336 U/ml); quartile IV, values from 337 to >500 U/ml.

Comparison of the biomarker diagnostic assay accuracy

To assess the specificity and sensitivity of the methods for the determination, of the levels of both markers the determined values of HE4 and CA125 were compared in two groups of patients (n=125) with homologous organ pathologies, in this case, cancer of the ovary vs. benign diseases of the ovary.

Statistical analysis

The statistical significance of difference between the number of patients affected by OC and that of patients affected by other malign and benign pathologies showing values of the two biomarkers above the limits of positivity were assessed using chi-square test for categoric variables. In terms of the diagnostic accuracy of the assays, the performance was assessed by estimation of receiver-operating characteristic (ROC) curve for OC cases versus ovarian benign cases. The area under the ROC curve (AUC) was calculated by MedCall V 4.30 Software.

Results

HE4 is expressed predominantly in patients with ovarian carcinoma

HE4 levels above the cut-off value were detected in 96.9% (31/32) of the patients with OC. In the other oncologic and benign pathologies, high serum HE4 levels were detected only in 3.7% (5/163) of the patients distributed as follows: three patients with cancer of the breast (two of whom also with high CA125 levels), two patients with cancer of the uterus (one of whom also with high CA125 level). The two percentages were statistically significant different (p<0.0001). None of the 72 healthy controls was positive for HE4.

The values of HE4 in the 31 patients with OC were distributed in the quartiles as follows: 11 in quartile I, four in quartile II, three in quartile III, and 13 in quartile IV By contrast, the 5 patients suffering from other oncological diseases and benign pathologies showed levels of HE4 belonging only to quartile I (Table 2).

CA125 is expressed in ovary cancer and other benign and malignant diseases

CA125 values above the cut-off were detected in the 87.5% (28/32) of patients affected by OC whereas pathologic CA125 levels were detected in 21% of patients affected by other pathologies and distributed as follows: three out of seven with colorectal cancer, eight out of 16 with breast cancer, four out of 26 with cervical cancer, ten out of 86 with ovarian benign tumors, and nine out of 28 with other benign diseases. The two percentages were statistically significant (p<0.0001). None of the 72 healthy controls was positive for CA125.

The patient CA125 distribution in quartiles is shown in Table 3. Patients with OC were distributed as follows: ten in quartile I, three in quartile II, three in quartile III, and 12 in quartile IV. Differently from what was found with HE4, CA125 values in patients affected by pathologies other than ovarian cancer (OC) were distributed in all four quartiles.

HE4 and CA125 diagnostic potential

HE4 statistical specificity and sensitivity analysis studied in the two selected groups of patients showed that the area under the ROC curve was 0.992 (95% CI 0.954–0.999).

Table 2 Subdivision into quartiles of the positive HE4 values observed in the patients included in the study

	I quartile (150-249)	II quartile (250-349)	III quartile (350-449)	IV quartile (450->850)
Ovarian cancer	11	4	3	13
Colon cancer	–	–	–	–
Breast cancer	3	–	–	–
Cervix cancer	2	–	–	–
Ovarian benign disease	–	–	–	–
Other benign disease	–	–	–	–

Table 3 Subdivision into quartiles of the positive CA125 values observed in the patients included in the study

	I quartile (37-136)	II quartile (137-236)	III quartile (237-336)	IV quartile (337->500)
Ovarian cancer	10	3	3	12
Colon cancer	2	–	1	–
Breast cancer	7	1	–	–
Cervix cancer	2	2	–	–
Ovarian benign disease	10	–	–	–
Other benign disease	7	–	1	1

The CA125 statistical analysis in the same two group of patients showed that the area under the ROC curve was 0.938 (95% CI 0.878–0.974; Fig. 1).

The expression of HE4 is an earlier indicator of recurrence of disease than CA125

All eight patients included in the follow-up study had a diagnosis of disease (FIGO II and III) and showed high levels of HE4 and CA125, except one patient that showed a weak positive value of CA125. After surgery, all patients showed a normalization of the values of HE4 and CA125 and underwent chemotherapy. After being followed at regular intervals for a period of up 20 months, five of the patients (including the one who at diagnosis had high values of HE4 but not of CA125) showed a rise in the levels of the tumor markers during the observation period. In these patients, the increase of HE4 level about the cut-off always precedes the rise of CA125 by 5–8 months. This increase coincided with the resumption and worsening of the disease confirmed by instrumental diagnostic analysis and by the death of three of the five patients (Fig. 2). The remaining three patients, that at present do not show remission from the disease, did however maintain, throughout the observation period, levels of HE4 and CA125 within the range of normality (Fig. 3).

Discussion

OC is the second most common form of gynecological cancer and first cause of death from gynecological malignancy in the western hemisphere. Moreover, OC is the fourth cause of death from cancer among women in industrialized countries [1–4]. Given its biological characteristics and the lack of effective screening tools in more than 80% of the cases, the diagnosis is made in the advanced stages of disease (FIGO III-IV) [5]. The median survival period ranges from 18 to 24 months with an 80% probability of the recurrence of the disease within 5 years [21]. This type of cancer affects women in peri- and post-menopausal with a peak between 55 and 65 years [22].

Many biomarkers are currently used at the time of diagnosis to better define the stage of the cancer. Amongst the numerous biomarkers studied which have revealed only a limited utility in clinical practice, the most recognized has, until now, been CA125. This marker's diagnostic importance is however somewhat reduced since it has been shown to be frequently expressed in other malignant and benign diseases [23].

Recently, HE4, a new OC biomarker, has been found to be highly expressed in the early stages of the disease. Moreover, by virtue of its selective presence in OC, it could help discriminate between benign and malignant diseases, thus reducing the diagnostic ability of CA125 [24] .

The present study has confirmed these early findings of HE4 since it has demonstrated that the marker is often expressed at diagnosis in nearly all patients affected by OC and that only a small percentage of non-OC patients had levels above the cut-off of the marker. By contrast, CA125,

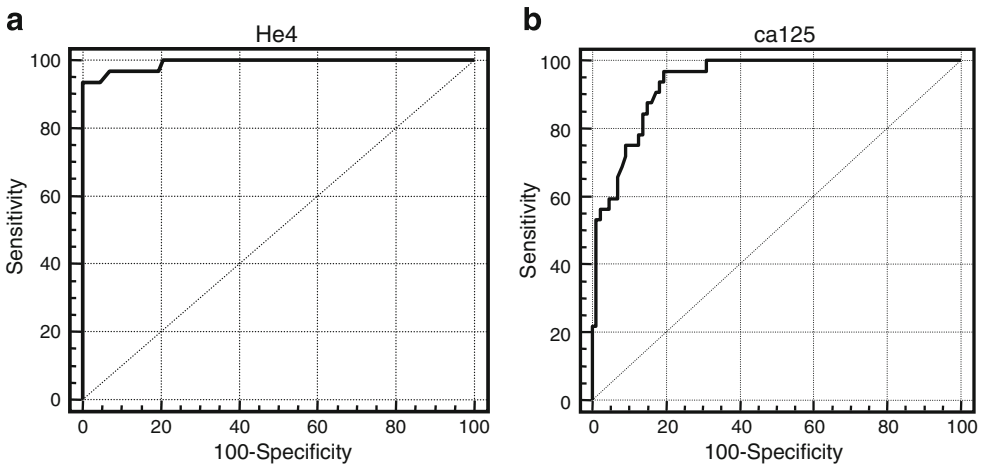

Fig. 1 (*Left*) HE4 statistical analysis showed that the area under the ROC curve was 0.992 (95% CI 0.954–0.999). (*Right*) CA125 statistical analysis showed that the area under the ROC curve was 0.938 (95% CI 0.878–0.974)

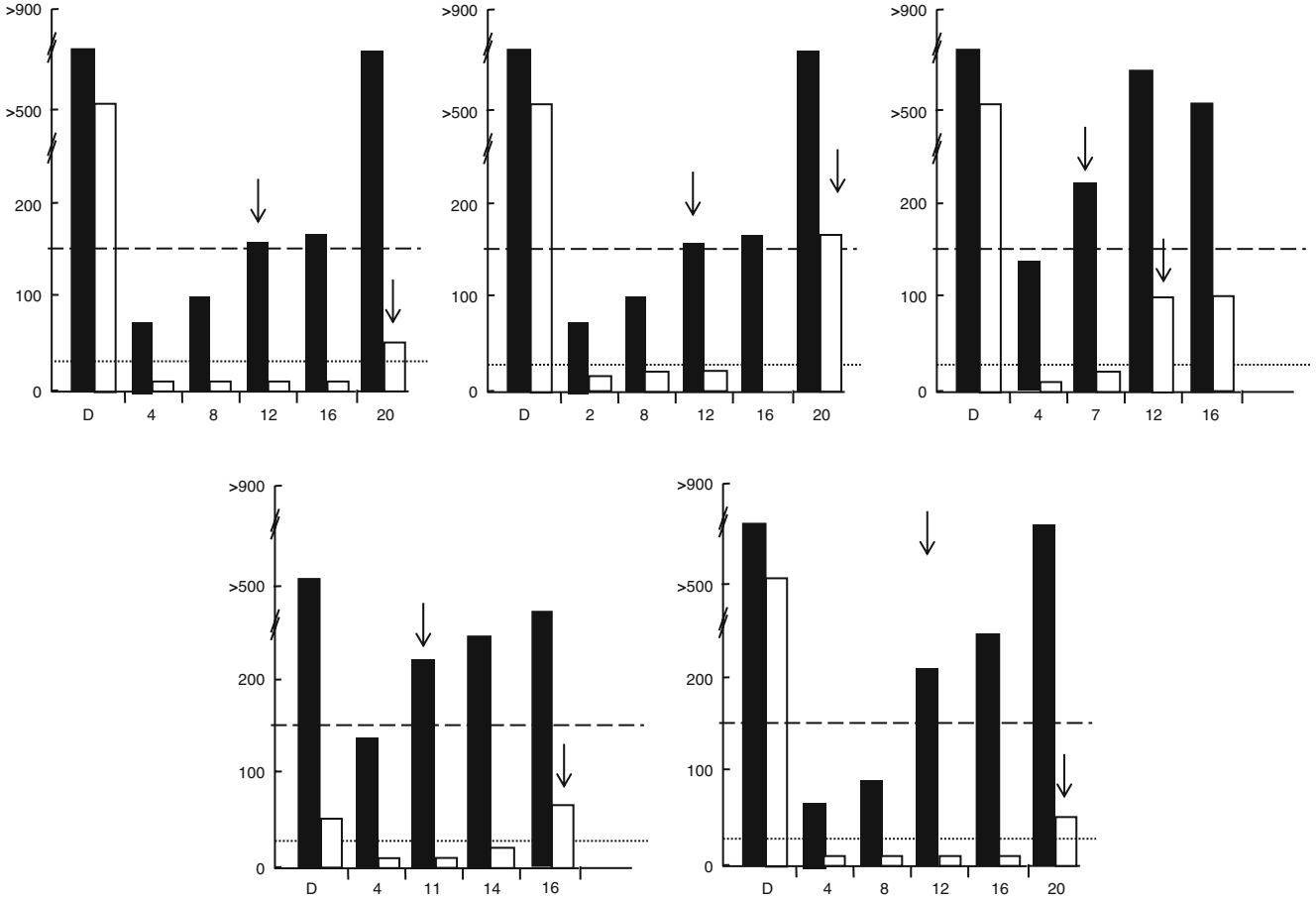

Fig. 2 Follow-up study of the five patients with ovarian cancer disease and with relapse of disease. The *black bars* represent the values of HE4 and the *white bars* those of CA125. The *dashed line* indicates the cut-off of HE4 while the *dotted line* indicates the cut-off for CA125. *D* is for diagnosis and numbers indicate the months after the surgical intervention. The *arrows* indicate the time in which the level of the marker was found to be above the limit of positivity

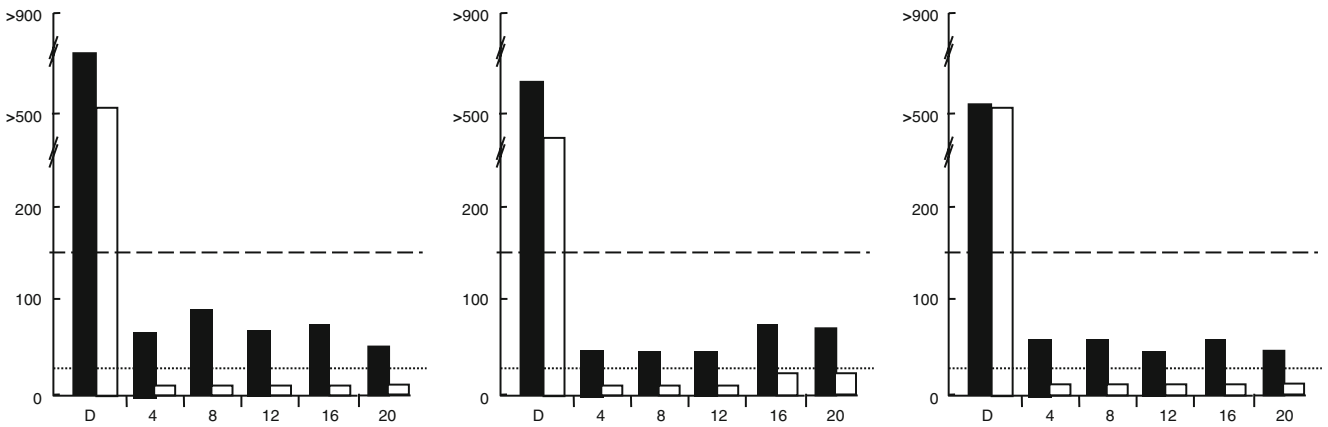

Fig. 3 Follow-up study of the three patients with ovarian cancer disease without relapse of disease. The *black bars* represent the values of HE4 and the *white bars* those of CA125. The dashed line indicates the cut-off of HE4 while the *dotted line* indicates the limit of positivity for CA125. *D* is for diagnosis and numbers indicate the months after the surgical intervention. The *arrows* indicate the time in which the level of the marker was found to be above the limit of positivity

although found in a large percentage of newly diagnosed OC patients, was also expressed, in some cases at high levels (quartiles III and IV), in a significant number (over 20%) of non-OC patients.

In addition to the search of specific markers for the determination of the early stages of any cancer form, it is just as important to find markers capable of following the remission from disease as response to therapy and in the event of the unfortunate recurrence of the disease. The suggestion that HE4 is a good indicator for the remission from the disease was recently reported by a follow-up study, in which it was shown that the values of HE4 correlated with the clinical response to treatment or remission from the disease, as documented by CT imaging [25].

In this study, we had the opportunity to retrospectively assess in eight patients the expression of CA125 and HE4. Three patients are currently free of disease as demonstrated by the normal values of both CA125 and HE4 measured throughout the study. Much more interesting, from a clinical point of view, were the results obtained in the five patients who had a relapse of the disease. The study of the expression of HE4 has shown that in case of recurrence of the disease, the expression of HE4 preceded by up to 5–8 months the rise in expression of CA125. This means that HE4 is not only a good indicator for the remission from illness but, being able to anticipate its expression, compared to that of CA125, is an ideal OC marker for therapeutic strategies against relapse.

Although a high percentage of positivity for HE4 among patients suffering from OC was found and a low positivity percentage in other pathologies, it was however of interest to see that three patients affected by breast cancer were also positive for HE4, the values belonging to the range defined by the first quartile.

This finding suggests the existence, in some cases, of common pathologic mechanisms which may concur in the development of these two types of cancer. It would therefore be of importance to investigate, by molecular approaches, these or eventually similar patients, in order to exclude the existence of a mutation in the BRCA1 and BRCA2 genes, since it is widely reported in the literature that the mutation of these genes is associated both with the cancer of the breast and that of the ovary [26, 27]. Thus, in the presence of an alteration of the genetic background, the early expression of HE4 would probably be relatively less important but it could act as an alarm signal.

In conclusion, this study has shown that HE4 may be able to provide a valuable contribution both for the diagnosis of OC and for controlling its recurrence. Thus, the search for biomarkers which can predict as early as possible the insurgence of ovarian or other gynecological cancers and can foresee, in spite of treatment, the recurrence of these diseases, has done a further fundamental step for their control or, even better, for their definitive cure.

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Conflict of interest statement None.

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