RESEARCH COMMUNICATION

HE4 and Mesothelin: Novel Biomarkers of Ovarian Carcinoma in Patients with Pelvic Masses

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Abstract

Objectives: To evaluate the utility of novel serum tumor markers, HE4 and mesothelin either alone or in combination with CA125 in diagnosis and early detection of ovarian carcinoma in patients with pelvic masses. Subjects and methods: Sera were obtained preoperatively from 65 women underwent surgery for a pelvic mass and 25 age- and menopausal status-matched healthy women. All samples were analyzed for levels of CA125, HE4, and mesothelin by serum based immunoassays and patients results were compared to final pathology findings. Results: Of 65 patients with pelvic masses; 41 had histologically diagnosed ovarian cancer, and 24 had benign ovarian diseases. The studied tumor markers were significantly increased in malignant compared to benign cases and healthy subjects, and in benign cases compared to healthy subjects ($p<0.001$). Based upon Receiver operator characteristic (ROC) curves analysis, HE4 had the highest sensitivity as a single marker in detecting ovarian malignancy (82.9%) and early stage malignancy (76.9%), followed by CA125, then mesothelin. The combination of HE4 and CA125 gave the highest sensitivity in detecting ovarian carcinoma and early stage disease (90.2%, 84.6% respectively). Addition of mesothelin to this combination did not show any improvement in the sensitivity. Conclusions: As a single marker, HE4 had the highest sensitivity for detecting ovarian carcinoma specially early stage disease. Combined CA125 and HE4 was a more accurate predictor of ovarian malignancy than either alone.

Key Words: Tumor markers - CA 125 - HE4 - mesothelin - ovarian cancer - pelvic mass

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Introduction

Ovarian cancer is one of the important leading causes of cancer deaths among women (Jemal et al., 2008). Early stage ovarian cancer has an excellent prognosis if treated, but 70% of patients are diagnosed in advanced stage which is associated with a poor survival rate of only 10–30% (Schink, 1999). Given the limitations of treatment for advanced ovarian cancer and the success of treatment for early stage disease, a screening test is intuitively appealing. However, the low prevalence of ovarian cancer limits the achievable sensitivity and specificity of any single screening test (Havrilesky et al., 2008).

The majority of cancer-associated antigens used as serum tumor markers in common solid malignancies are neither fully sensitive nor specific, and CA125 in ovarian cancer is no exception. Any primary cancer resulting in extensive intra-abdominal disease can raise CA125 levels, as well as other metastatic solid tumors without peritoneal involvement, e.g. breast cancer (Eagle and Ledermann, 1997). At the time in which many benign conditions including endometriosis, some ovarian masses, hepatic cirrhosis and peritonitis may cause increased its serum level, more than half of patients with early stage ovarian cancer do not exhibit elevated CA125 levels (Zurawski et al., 1988; Nagele et al., 1995). In addition, there is a group of women with epithelial ovarian cancers, mostly those with mucinous tumors, in whom CA125 levels are never increased (Palmer et al., 2006). There is a pressing need for novel markers which are sensitive and specific and can improve diagnosis when used in combination with CA125 or can replace it.

Real-time PCR on an independent set of benign and malignant tissues was performed to characterize amplified genes. Two genes, WDFC2 (HE4) and MSLN (mesothelin family), were confirmed as overexpressed in ovarian cancers but not in normal tissues. Importantly, the former gene was not amplified in any of 19 tissues from women with benign ovarian masses who had elevated serum levels of CA125 (Kojima et at., 1995).

The WFDC2 gene was initially identified in epithelial cells of human epididymis and referred to as an epididymis-specific, fertility-related protein, HE4. HE4 is an 11 kDa protein belongs to a "four-disulfide core" family. It is made up of two whey acidic protein domains and a 4 disulfide core (Bast et al., 1983). Some members of the four-disulfide core family of proteins are protease inhibitors. However, no protease inhibitory activity has been identified for HE4, whose function remains unknown (Hellstrom et al., 2003).

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Mesothelin is a cell surface protein present on normal mesothelial cells lining the body cavities. It is highly expressed in several cancers, including mesotheliomas, ovarian and pancreatic cancers, and some squamous cell carcinomas (Chang et al., 1992; Chang and Pastan, 1996). Human mesothelin is made as a 69 kDa polypeptide with a hydrophobic sequence at the carboxyl end that is removed and replaced by phosphatidylinositol. This glycosyl-phosphatidylinositol linkage anchors mesothelin to the cell membrane (Chang and Pastan, 1996; Hassan et al., 2004). Mesothelin is shed like many other cell membrane proteins (Censullo and Davitz, 1994). Scholler et al. (1999), and Robinson et al (2003) have described a 42 to 44 kDa protein, called soluble mesothelin-related (SMR) protein. Soluble mesothelin-related peptides are members of the megakaryocyte potentiating factor (MPF) family and have been detected in both the serum and urine of patients with ovarian cancer (Scholler et al., 1999). A recent study presented evidence that mesothelin binds CA125 and may, therefore, play a role in the dissemination ovarian cancer in the peritoneal cavity (Rump et al., 2003).

Subjects and Methods

The study included 65 newly diagnosed women presented with pelviabdominal swelling attending Obstetric and Gynecology Department, Zagazig University Hospitals, and 25 age- and menopausal status–matched healthy women. Patients were diagnosed clinically as well as radiologically. All patients underwent surgical removal of the ovarian mass or cyst, histological diagnosis along with surgical staging for malignant cases. The study was approved by the institution Ethics Review Board, and written informed consent was obtained from all participants.

Blood samples from patients were collected before surgical intervention. Within 4 hours of collection, sera were separated and frozen at -80˚C for determination of CA125, HE4, and mesothelin.Serum CA125 was determined on Cobas e 411 analyzer (Roch, Tokyo, Japan). It is an electrochemilumenesence immunoassay based on sandwich principle using two monoclonal antibodies, a biotinylated monoclonal CA125-specific antibody, and a monoclonal CA125-specific antibody labeled with a ruthenium complex. The within run precision is 1.4-3.3%, total precision is 2.5-4.2%, and measuring range is 0.6-5000 U/ml.

Serum HE4 was determined using HE4 enzyme immunometric assay (Fujirebio Diagnostics, Inc.). The HE4 assay is a solid-phase immunoassay based upon the direct sandwich technique utilizing biotinylated anti-HE4 monoclonal antibody (MAb), streptavidin coated microstrips, and HRP labeled anti-HE4 MAb. Detection was accomplished by addition of substrate/chromogen reagent (hydrogen peroxide and tetra-methylbenzidine). Measuring range is 15-900 pM.

Methoselin was determined using Mesomark™, an ELISA assay produced by Fujirebio Diagnostic Inc. based upon sandwich principle. Two separate monoclonal antibodies were used; one for capturing and the other for detection of mesothelin. Detection is accomplished by addition of a standard chromogenic substrate that binds to the HRP- labeled monoclonal antibody. The within run precision is 1.1-5.3%, total precision is 4.0-5.3%, and measuring range is 0.3-32 nM.

Statistical analysis

Data were statistically described in terms of median, range; mean ± standard deviation (± SD), frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison between normally distributed quantitative two variables was done using Student t test. Non normally distributed quantitative variables were compared by Mann-Whitney test for comparing two groups and Kruskal-Wallis test for comparing more than two groups. For comparing categorical data, Chi square (c2) test was performed. Receiver operator characteristic (ROC) analysis and 95% confidence interval (CI) were used to determine the optimum cutoff value for the studied diagnostic markers. Diagnostic performance was represented using the terms sensitivity, specificity, likelihood ratio of positive (LR+), likelihood ratio of negative (LR-), overall accuracy. Correlations between tumor stages and grades in one side and the studied tumor markers were done using Spearman rank correlation. A probability value (p value) less than 0.05 was considered statistically significant. All statistical calculations were done using SPSS computer program (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

Results

Twenty four patients were diagnosed histologically as benign ovarian disease and 41 as ovarian carcinoma. The mean age for patients with benign tumors was significantly lower than that among patients with malignant tumors (p=0.02). Although the number of postmenopausal women was increased in patients with ovarian malignancy, it did not reach statistical significance (p=0.18). The most common benign neoplasm was the serous type which accounted for 66.7% of women with benign disease. Of malignancies, there were 63.4% serous, 14.6% mucinous, 12.2% endometroid, and 9.8% mixed tumors. In patients with ovarian carcinoma, 13 (31.7%) were surgically staged as early stage disease (stage I & II), and 28 (68.3%) as late stage disease (stage III & V) (Table 1).

The median serum levels of CA125, HE4 and mesothelin were significantly elevated in both benign and malignant cases compared to healthy subjects and in malignant compared to benign cases (p<0.001) (Table 2). The area under receiver operating characteristic curve (ROC-AUC) was determined for individual serum marker levels for differentiating benign from malignant cases. Analysis of ROC-AUC revealed that HE4 had the highest AUC (0.95& 95%CI 0.90-0.995) followed by CA125 (0.90& 95%CI 0.82-0.97), then mesothelin (0.89& 95%CI 0.81-0.96) (Figure 1a).

A standard cutoff value of 35 U/ml for CA125 was already established. CA125 was elevated in 30 out of 41 ovarian cancer patients and in 5 out of 24 benign ovarian diseases. The best cutoff points were determined for HE4

Table 1. Patients Demographic and Ovarian Tumour Characteristics

Variables		Benign (n=24)	Malignant (n=41)
Age[a] (years)		49.3±11.2 (31-69)	55.3±9.2* (33-72)
Menopausal[b]	Pre-	9 (37.5%)	9 (22.0%)
	Post-	15 (62.5%)	32 (78.0%)
Type	Mucinous	6 (25.0%)	6 (14.6%)
	Endometroid	2 (8.3%)	5 (12.2%)
	Serous	16 (66.7%)	26 (63.4%)
	Mixed	-	4 (9.8%)
Grade	Well differentiated	-	8 (19.5%)
	Moderately diff	-	10 (24.4%)
	Poorly differentiated	-	15 (36.6%)
	Undifferentiated	-	8 (19.5%)
Stage	I	-	2 (4.9%)
	II	-	11 (26.8%)
	III	-	20 (48.8%)
	IV	-	8 (19.5%)

Data are [a]mean ±SD,[b]n (%); *Significant (p=0.02)

Table 2. Comparison of CA125, HE4, and Mesothelin

Markers	Healthy (n=25)	Benign (n=24)	Malignant (n=41)	P-value
CA125 (U/ml)	10.0 (3.0-40.0)	23.5* (10.0-70.0)	130.0* (22.0-700.0)	<0.001[a]
HE4 (pM)	30.0 (12.0-70.0)	47.5* (35.0-120.0)	230.0* (47.0-742.0)	<0.001[a]
Mesothelin(nM)	0.43 (0.13-1.2)	0.87* (0.14-2.2)	3.4* (0.6-33.0)	<0.001[a]

Data are median (range); [a]Kruskal-Wallis test; *Significant

Table 3. Validity Tests of the Studied Markers for Diagnosis of Malignant Ovarian Disease

Markers	Accuracy	LR-	LR+	Specificity	Sensitivity
CA125	75.4%	0.34	3.52	79.2%	73.2%
HE4	84.6%	0.19	6.63	87.5%	82.9%
Mesothelin	75.4%	0.35	4.2	83.3%	70.7%
CA125&HE4	86.2%	0.12	4.33	79.2%	90.2%
CA125&Meso	76.9%	0.31	3.63	79.2%	75.6%
Ca125&HE4&meso	86.2%	0.12	4.34	79.2%	90.2%

Table 4. Comparison of CA125, HE4, and Mesothelin

Markers	Benign (n=24)	Early Malignant (n=13)	P-value
CA125(U/ml)	23.5 (10.0-70.0)	90.0* (27.0-230.0)	<0.001a
HE4(pM)	47.5 (35.0-120)	96.0* (56.0-456.0)	<0.001a
Mesothelin(nM)	0.87 (0.14-2.2)	1.5*(0.75-8.0)	0.004a

Data are median (range); [a]Mann-Whitney test; *Significant

Table 5. Validity Tests of the Studied Markers for Diagnosis of Early Stage Malignant Ovarian Disease

Markers	Accuracy	LR-	LR+	Specificity	Sensitivity
CA125	73.0%	0.49	2.96	79.2%	61.5%
HE4	83.8%	0.26	6.15	87.5%	76.9%
Mesothelin	73.0%	0.55	3.22	83.3%	53.8%
CA125&HE4	81.1%	0.19	4.07	79.2%	84.6%
CA125&Meso	75.7%	0.39	3.33	79.2%	69.2%
CA125& HE4&Mesothelin	81.1%	0.19	4.07	79.2%	84.6%

and mesothelin utilizing (ROC) curve analysis. HE4 at cutoff of 72 pM was able to detect 34 of malignant cases and misclassified 3 of benign cases as positive. Mesothelin levels were elevated (>1.4 nM) in 29 patients with malignant disease and in 4 of benign disease.

Using the data from individual biomarkers analysis, various combinations of the markers was evaluated. The combination of CA125 with HE4 gave the best differentiation between benign and malignant cases with sensitivity 90.2%, while CA125 and mesothelin combination showed sensitivity of 75.6%. Interestingly addition of mesothelin to the first combination did not show any improvement in the sensitivity (Table 3).

When the three studied tumor markers were compared between patients with benign and early stage malignant ovarian patients, they were significantly elevated in patients with early stage malignancies (Table 4). The ROC-AUC (95%CI) values were 0.90 (0.80-1.0) for HE4, 0.87 (0.76-0.99) for CA125, and 0.81 (0.67-0.95) for mesothelin (Figure 1b). For individual markers, HE4 was the best single marker in detecting early stage ovarian malignancy detecting 10 out of 13 early malignant cases followed by CA125 detecting 8 cases, then mesothelin detecting 7 cases. The dual marker combination, CA125 and HE4 remained the most sensitive predictor of early stage ovarian malignancies (Table 5).

When the relationship between histology and the three

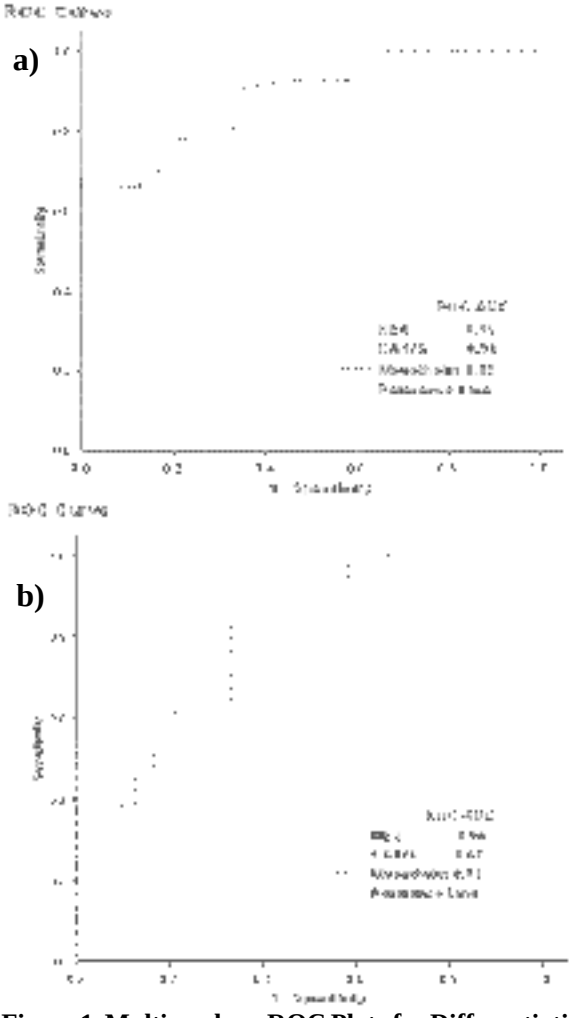

Figure 1. Multimarkers ROC Plate for Differentiation. a) benign from malignant ; b) benign from early malignant

Table 6. Correlation of the Studied Tumor Markers with Stages and Grades of Ovarian Malignant

Marker	Stage		Grade	
	r	p	r	p
CA125	0.39*	0.012	0.51**	0.001
HE4	0.51**	0.001	0.57**	<0.001
Mesothelin	0.36*	0.021	0.47**	0.002

*,**Significant at the 0.05 and 0.01 levels (2-tailed)

tumor markers were examined, none of the mucinous tumors, 3 of the 5 endometroid tumors (60%), 25 of the 26 serous tumors (96.2%), and 2 of the 4 mixed tumors (50%) had elevated CA125. With regard HE4, elevated levels were found in 4 of mucinous tumors (66.7%), 2 of endometroid tumors (40%), 25 of serous tumors (96.2%), and 3 of mixed tumors (75%). Mesothelin levels were elevated in 1 of endometroid tumors (20%), 25 of serous tumors (96.2%), 3 of mixed tumors (75%) but none of mucinous tumors.

Serum levels of CA125, HE4 and mesothelin showed significant positive correlations with both the stage and grade of cancer in ovarian carcinoma patients (Table 6).

Discussion

CA125 is the most widely used serum biomarker among patients with ovarian cancer. Its utility in determining response to treatment or as a marker for the detection of recurrent disease is well established (Hising et al., 1991). CA125 levels are elevated in 80% of patients with epithelial ovarian cancer but in only half of patients with early stage disease (Bast et al., 1983; Zurawski et al., 1988). Unfortunately, the sensitivity and specificity of CA125 alone for the detection of early stage disease are too low to be of clinical value (Einhorn, 1992; DePriest et al., 1993). The results of the present study revealed that CA125 level at 35 U /ml had a sensitivity of 73.2% and a specificity of 79.2%, which is comparable with that achieved by others (Havrilesky et al., 2008; Moore et al., 2008) at the same cutoff for predicting the presence of ovarian malignancy. The sensitivity and specificity of any single or multiple serum biomarker assays would need to be significantly higher than that achieved with serum CA125 alone in order to be useful as a triage test before surgery.

The levels of several novel tumor markers have been investigated in patients with ovarian cancer. However, as a stand-alone test, the sensitivity and specificity values of these serum biomarkers are of limited value in identifying patients with ovarian malignancies (Moore et al., 2008). Mesothelin and HE4 levels were found to be elevated in women with ovarian cancer, adding to the group of possible biomarkers for this disease (Scholler et al., 1999; Hellstrom et al., 2003). We examined whether these two biomarkers can be used in combination with CA125 to improve its sensitivity as a tool for detecting ovarian carcinoma specially early stage disease.

Of the studied tumor markers in the current study, HE4 showed an increase in the AUC-ROC when compared to CA125 and mesothelin. HE4 (at 72 pM cutoff) had the highest sensitivity and specificity as a single marker with 82.9% sensitivity and 87.5% specificity, findings consistent with those reported by Hellstrom et al. (2003) and Moore et al.(2008). Our study revealed that the combination of the two serum biomarkers CA125 and HE4 increased the sensitivity (90.2%) when compared to either marker alone.

Prior studies have investigated combinations of biomarkers and statistical models for predicting ovarian cancer. Moore et al (2008) examined a panel of biomarkers and found the dual marker combination of HE4 and CA125 produced the highest sensitivity of the various tumor marker combinations and increased the sensitivity of CA125 alone. An algorithm utilizing HE4 and CA 125 successfully classified patients into high and low risk groups (Moore et al., 2009). Serum HE4 has previously been reported to have an advantage over CA125 (Hellstrom et al., 2003) and to complement the expression of CA125 (Scholler et al., 2006) in the detection of epithelial ovarian cancer. CA125 suffers from a lack of specificity secondary to its tendency to be elevated in many common benign gynecologic and non gynecologic conditions. Because HE4 is not falsely elevated in many of these conditions (Hellstrom et al., 2003), it may complement CA125. As well, for the 20% of epithelial ovarian cancer that express little, if any, CA125, a single marker is not sufficient. Notably, HE4 levels are elevated in greater than 50% of tumors that do not express CA125 (Moore et al., 2008). Therefore, the addition of HE4 to CA125 enables the detection of malignancies in patients with tumors that do not express CA125 and will be missed by algorithms that employ CA125 alone.

The current study reported that serum mesothelin at 1.4 nM cutoff had a lower sensitivity than CA125 in detecting ovarian malignancy (70.7%). Combined use of CA125 and mesothelin does not improve the sensitivity so much (75.6%). This combination is lower to that achieved by combining CA125 and HE4. Interestingly, adding mesothelin to CA125 and HE4 combination did not increase the sensitivity at all.

Mesothelin levels are elevated in sera from 60% to 77% of women with ovarian cancer (Cole and Nam., 1989; Scholler et al., 1999; McIntosh et al., 2004). Several studies investigated the use of CA125 and mesothelin as single and combination markers in patients with ovarian cancer. As a single marker, CA125 had a higher sensitivity than did mesothelin alone (McIntosh et al., 2004; Moore et al., 2008). Although mesothelin cannot serve as a stand-alone marker for detection of ovarian cancer, it might be used in combination with CA125 to achieve an appropriate sensitivity. As mesothelin is elevated in a fraction of patients with normal serum CA125, the most useful application of this marker may be in combination with CA125 to detect ovarian cancer (McIntosh et al., 2004). Combined CA125 and mesothelin improve the sensitivity compared with that of CA125 alone in detecting ovarian cancer patients (McIntosh et al., 2004; Moore et al., 2008). Badgwell et al.(2007) document that urinary levels of mesothelin provide a more sensitive marker than serum levels of mesothelin for distinguishing patients with early and late stage ovarian cancer from healthy controls.

When the relationship between histology and the three tumor markers was examined, they were all elevated in most of serous tumors. Serous tumors are the most frequently occurring histological type among the invasive ovarian cancers (Badgwell et al., 2007) and the observation that a large fraction of serous cancers can be detected using the studied markers is encouraging. CA125 and mesothelin were not elevated in any of mucinous tumors while HE4 was elevated in 4 of the 6 of this histotype, making an advantage of using HE4 in combination with either CA125 or mesothelin not to miss this type of tumor. However, the number of tumors included in the present study is too small to allow any conclusions to be made from these results. CA125 levels are previously reported to be never elevated in mucinous ovarian tumors (Palmer et al., 2006). Badgwell et al. (2007) reported that urinary mesothelin was elevated in none of mucinous ovarian carcinoma, 11% of endometroid, 77.6% of serous, and 71% of mixed tumors.

Current limitations of biomarkers for ovarian cancer screening relate to the relatively poor sensitivity and specificity for detection of early stage disease. Detection of disease in early stage is likely to have a greater impact as it clearly has a survival advantage when compared to late stage disease (Havrilesky et al., 2008). For patients with early stage disease, the detection sensitivity was lower for all individual tumor markers and tumor marker combinations in this study. HE4 alone had the highest sensitivity in early stage disease followed by CA125, then mesothelin (76.9%, 61.5%, 53.8% respectively). Interestingly, the dual marker combination of HE4 and CA125 remained the most sensitive predictors of early stage ovarian malignancy with a sensitivity of 84.6%. This observation suggests that HE4 may be useful in a multiple marker screening panel designed for the early detection of ovarian cancer. However, the implication of this finding are limited by small number of early stage disease in our study (n=13). HE4 was reported to have the highest sensitivity of a panel of nine biomarkers for the detection of ovarian cancer, especially stage I disease, in patients with a pelvic mass (Moore et al., 2008). Similarly, other studies reported a combination of HE4 and CA125 or HE4 alone has been shown to have greater sensitivity in patients with early stage disease compared with CA125 (Hellstrom et al.,2003; Moore et al., 2008).

HE4 and mesothelin are significantly correlated with tumor stage and grade in ovarian cancer. These biomarkers may have utility to monitor disease status in patients with recurrent ovarian cancer. Havrilesky et al. (2008) reported that a subset of biomarker panel ,HE4, matrix metalloproteinase-7,and glycodelin predicted disease recurrence prior to elevation of CA125 in 56% and residual disease in 50% compared to 0% for CA 125 of early ovarian cancer patients. Hassan et al. (2006) reported that serum mesothelin levels are elevated in patients with ovarian cancer and decreased after surgical therapy. Therefore, they considered it useful as a tumor marker for diagnosis and to follow response to therapy. However, these results were considered preliminary and should be subjected to further evaluation. The efficacy of longitudinal serum monitoring using these two biomarkers should ideally be evaluated prospectively to evaluate response to treatment and to determine the role for such tests in the setting of possible disease recurrence.

In summary, the analysis of multiple biomarkers in this study demonstrates that HE4 was the best single marker in detection of malignant ovarian disease and early stage malignancy. The addition of HE4 to CA125 improves the sensitivity over that of CA125 alone. However, the triple combination of mesothelin, CA125, and HE4 did not increase the sensitivity over the dual combination. Lower specificity may be acceptable in higher risk populations, such as women with a strong family history of ovarian cancer, or within a population of women presenting with a pelvic mass.

Screening strategy using elevated levels of CA 125 and HE4 in addition to transvaginal sonography could improve the diagnostic sensitivity of ovarian carcinoma in patients with a pelvic mass. Future studies including larger number of patients have to be done to evaluate HE4 and mesothelin as prognostic markers for ovarian carcinoma.

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