Using Human Epididymis Protein (HE4) as an Alternative Biomarker for Ovarian Cancer Detection in Women

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A. INTRODUCTION

Ovarian cancer has one of the highest mortality rates for cancers among women due to late-stage diagnosis. According to the National Cancer Institute, the incidence of ovarian cancer is 10.3 per 100,000 women per year, and the average mortality rate is 6.3 per 100,000 women annually, which leaves limited time for intervention. Early identification of the cancer is challenging due to the absence of early symptoms, and detection typically occurs at more advanced stages, leading to a higher chance of death. Reflected in a study summarized by DMC Women's Health: "Nearly 60% of epithelial ovarian cancers are diagnosed at a late stage, at which time five-year survival is only 29%. In contrast, for the 15% of ovarian cancers diagnosed at a localized stage, five-year survival is 92%" (Doherty et al., 2022). Early detection is key to improving patients' prognosis, and research into assays with higher sensitivity and specificity is fundamental in achieving this goal.

Cancer Antigen 125 (CA-125) is the most commonly used biomarker for ovarian cancer detection. However, CA-125 suffers current limitations, such as low sensitivity and false positives. It detects the recurrence of the disease [ovarian cancer], only 4.8 months before clinical symptoms come to fruition, meaning that the biomarker doesn't provide a big window of "turn-around" to assist doctors. A recent article by Gland Surgery on OC biomarkers presented that "...none of the biomarkers in clinical use for early detection of OC, including carcinoembryonic antigen (CEA), CA125, carbohydrate antigen 19-9 (CA19-9)...are effective due to a lack of sensitivity or specificity," (Giampaolino et al. 2020). Ovarian cancer biomarkers have not been able to detect neither the mutated gene nor cancer cells, making late diagnosis a common trend. NCBI revealed that with the CA-125 biomarker, "...50 percent of patients with

stage I tumors remain undetected," (Radu et al. 2021) Additionally, genetic factors can predispose specific individuals with a higher likelihood of diagnosis. Studies conducted by the American Cancer Society show that "Mutations in BRCA1 and BRCA2 are … responsible for most inherited ovarian cancers. Mutations in BRCA1 and BRCA2 are about 10 times more common in those who are Ashkenazi Jewish than those in the general U.S. population" (American Cancer Society, 2021).

HE4 has shown greater effectiveness than existing biomarkers, especially CA-125, in several aspects of OC detection. This section presents the findings from several studies which compare the performance of several OC biomarkers. Biomarker levels were evaluated for benign, borderline, and malignant ovarian tumor conditions. Sensitivity, specificity, and predictive values were also analyzed. The results show that HE4 outperforms CA-125 in distinguishing between conditions, demonstrating the overall superior diagnostic performance of HE4 compared to CA-125. One instance of HE4's superiority stems from its stability and reduced susceptibility to interference from various gynecological conditions. According to Yanaranop et al. in a research study evaluating HE4's effectiveness as a biomarker, he states " [31] reported a specificity of 86% for HE4, and the AUC was higher than CA125 alone, with values of 0.893 and 0.865, respectively [32]. These data...showed that HE4 for diagnosing ovarian epithelial cancer appeared more reliable than CA125." HE4 poses, according to the research study, as resulting in higher levels of accuracy in terms of specificity of 0.893 compared to the lower value of specificity of 0.865 for CA-125. CA-125's limitations especially affect premenopausal women, where benign conditions can cause elevated CA-125 levels.

Additionally, HE4 proves to be more valuable for early detection of ovarian cancer. Earlier diagnosis can reduce mortality by 10–30%, and when ovarian cancer is confined to the ovaries (stage I), it can be cured in up to 90% of patients (Elias et al. 2019). In tumor marker sensitivities among patients with Stage I ovarian cancer, HE4 consistently demonstrated higher sensitivities than CA-125 (Anton et al. 2012). This makes HE4 particularly valuable in the early stages of ovarian cancer, where early intervention is crucial for better patient outcomes. Although HE4 is well-established as a more specific and sensitive biomarker to ovarian cancer than CA125, there are a number of considerations to be made. The National Cancer Institute indicates that ovarian cancer recurs in 7 in 10 patients (Elia 2022). If HE4 could be used for

ongoing surveillance, many patients could experience a much higher quality of life and more timely intervention.

B. MATERIALS AND METHOD

This method employs specific antibodies and enzyme-antibody conjugates that selectively bind to HE4, allowing for quantification of the protein based on enzyme activity linked to the detection antibody.

Procedure:

1. Dilute both antibodies (monoclonal antibody 3b1 and human serum sample from ovarian cancer patients) in coating buffer at 0.5, 1, 2, and 5 µg/ml and add 100 µl of each concentration to 24 wells of the 96-well microtiter plate.
2. Incubate the plate containing the capture antibody overnight at 4°C and continue the experiment the next day.
3. Remove the unbound capture antibody solution from the microtiter plates by aspirating or dumping the plate., and add 200 µl of blocking buffer to each well of the 96-well microtiter plate. Incubate the plate for one hour at room temperature.
4. Remove the blocking buffer from the plate by aspirating or dumping the plate. Determine the desired working range of the analyte. This will give you the high and low concentrations to incubate with each capture antibody dilution. The zero analyte wells will give you the non-specific binding (NSB).
5. Add 100 µl of the analyte to each well in the microtiter plate and incubate for 2.5 hours at room temperature.
6. Wash the plates 3 times with a wash buffer.
7. Dilute the detection antibody serially at 1:200, 1:1000, 1:5000 and 1:25000 in diluent.
8. Add 100 µl of detection antibody to each well plate and incubate for 1.5 hours at room temperature.
9. Wash the plates 3 times with a wash buffer. Dilute streptavidin-HRP (if detection antibodies are biotinylated) or appropriate secondary antibody (if capture and detection antibodies are from different species) according to manufacturer instructions in antibody diluent and add 100 µl to each well in the microtiter plate and incubate for 1 hour at room temperature.

10. For HRP readout add TMB as a substrate to allow color development and incubate for 10-20 minutes at room temperature.
11. Add acid stop reagent to stop the enzyme reaction. Read at 450 nm for TMB/HRP.

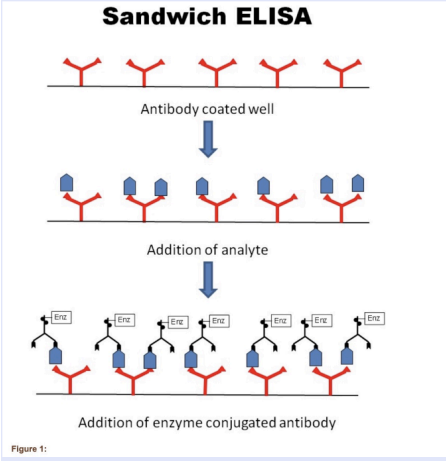

Figure 1: Sandwich ELISA

This figure illustrates the principle of ELISA, a widely used method for detecting specific proteins, such as the HE4 biomarker, in serum samples.

Reagents:

1. Two antibodies that recognize different epitopes on the analyte
2. The optimal antibody pair for the sandwich assay was determined empirically in the experiment above
3. Greiner immunoassay plate
4. Buffers- Coating buffer: PBS, Blocking buffer: 1% BSA, TBS, 0.1% Tween-20, Antibody diluent buffer: 1% BSA, PBS or TBS, or 0.1% Tween-20, Wash buffer: PBS or TBS 0.1% Tween-20
5. TMB and HRP are used for enzyme/substrate readout
6. Acid stop buffer

Detection Antibody	Capture Antibody											
	2 µg/ml			1 µg/ml			0.5 µg/ml					
1:1000	H	L	0	H	L	0	H	L	0			
	H	L	0	H	L	0	H	L	0			
1:5000	H	L	0	H	L	0	H	L	0			
	H	L	0	H	L	0	H	L	0			
1:25000	H	L	0	H	L	0	H	L	0			
	H	L	0	H	L	0	H	L	0			

Table 1: Detection Ability of ELISA

Coating Buffers:

- 50 mM sodium bicarbonate, pH 9.6 & 0.2 M sodium bicarbonate, pH 9.4
- PBS - 50 mM Phosphate, pH 8.0, 0.15 M NaCl
- Carbonate-bicarbonate
- Phosphate Buffer: 1.7 mM NaH_2PO_4, 98 mM $Na_2HPO_4 \cdot 7H_2O$, 0.1% NaN_3, pH 8.5
- TBS - 50 mM TRIS, pH 8.0, 0.15 M NaCl

Blocking Buffers:

- 1% BSA or 10% host serum in TBS, or TBS with 0.05% Tween-20
- Phosphate Buffer: 73 mM Sucrose, 1.7 mM NaH_2PO_4, 98 mM $Na_2HPO_4 \cdot 7H_2O$, 0.1% NaN_3, pH 8.5
- 1% HSA in PBS
- Casein Buffer: Pierce Blocker cat# 37528 & Protein Free Block: Pierce cat# 37573
- Heterophilic Blocking Reagent (HBR): Scantibodies Laboratory, Inc., cat# 3KC533

Wash Buffers:

- PBST, 0.05% Tween-20 & TBST, 0.05% Tween-20

Antibody Diluents Buffers:

- 1% BSA or 10% host serum in TBS, or TBS with 0.05% Tween-20 & 1% BSA or 10% host serum in PBS, or PBS with 0.05% Tween-20
- 50 mM HEPES, 0.1 M NaCl, 1% BSA, pH 7.4
- Blocking buffer

C. RESULTS

HE4 has shown greater effectiveness than existing biomarkers in several aspects of OC detection, as shown by the findings of several studies. Biomarker levels were evaluated for benign, borderline, and malignant ovarian tumor conditions. Sensitivity, specificity, and predictive values were also analyzed. Results indicate that HE4 outperforms CA-125 in distinguishing between conditions, early detection, sensitivity, and specificity, demonstrating the overall superior diagnostic performance of HE4 compared to CA-125.

Biomarker Levels Across Conditions

Table 1: Biomarker Levels across conditions

Biomarker	Benign (Median, Range)	Borderline (Median, Range)	Malignancy (Median, Range)	Key Observations
HE4 (pM/L)	49.01 (8.19–137.3)	63.97 (41.45–236.3)	245.9 (30.97–3270)	Clear distinction between benign, borderline, and malignancy with minimal overlap.
CA-125 (U/mL)	21.27 (2.84–994.9)	64.69 (3.81–2053)	368 (6.77–12494)	Significant overlap in ranges, especially between benign and borderline groups.

A study published in *Current Problems in Cancer* by Zhang et al. examined the median levels of CA-125 and HE4 across benign, borderline, and malignant tumor conditions (Zhang et al., 2019). The study found that HE4 displayed the following median levels for each condition: 49.01 pM/L for benign, 63.97 pM/L for borderline, and 245.9 pM/L for malignancy, with minimal overlap in ranges. In contrast, CA-125 showed a greater overlap between the ranges for different categories, especially between benign (median 21.27 U/mL) and borderline (median 64.69 U/mL) cases, which compromises its precision in differentiating between these groups (Zhang et al., 2019). This clear distinction in HE4's levels suggests it may be a more reliable biomarker for ovarian cancer diagnosis than CA-125.

Sensitivity and Specificity of Biomarkers

Table 2: Sensitivity and Specificity of CA-125 and HE4

Biomarker	Sensitivity	Specificity
CA-125	73.2%	71.5%
HE4	95.4%	81.3%

The sensitivity and specificity of both biomarkers were assessed in a study by Englisz et al. (2024) as shown in Table 2. HE4 demonstrated a significantly higher sensitivity of 95.4% compared to CA-125's 73.2%, alongside a higher specificity of 81.3%, versus 71.5% (Englisz et al., 2024). Therefore, HE4 can correctly identify malignancy while minimizing false positives, a crucial advantage when considering cases such as endometriosis, where CA-125 often yields misleading false-positive results. This data shows HE4's superior diagnostic performance.

Stage I Sensitivity Analysis (Early Detection)

Table 3: HE4 and CA-125 sensitivities in patients with Stage I OC

Biomarker	ROC-EUC	Sensitivity at 90% Specificity	Sensitivity at 95% specificity	Sensitivity at 98% specificity
CA-125	70%	23.1%	15.1%	7.7%
HE4	76.5%	46.2%	45.9%	30.8%

According to this study by Moore et al., HE4 shows higher sensitivity across specificity values for Stage I OC, indicating that it is a more reliable marker for early detection (Moore et al., 2008). Additionally, the data shows that HE4 maintains its performance at higher specificities compared to CA-125. For example, at 90% specificity, HE4 has a sensitivity of 45.9%, while CA-125 drops to 15.1%. This suggests that HE4 is a more effective biomarker for identifying Stage I ovarian cancer, particularly when a high level of specificity is required.

Comparative Analysis of Predictive Values

A study by Hamed et al. (2013) presented the positive predictive value (PPV) and negative predictive value (NPV) for CA-125 and HE4 (see Table 4). HE4 exhibited a significantly higher PPV of 93.1%, compared to 80.7% for CA-125, indicating that HE4 is more reliable in confirming malignancy. Both biomarkers had similar NPVs, suggesting that they are equally effective in ruling out ovarian cancer (Hamed et al., 2013). This further solidifies HE4's utility in diagnosing ovarian cancer, offering higher confidence in detecting true positives without an increase in false positives.

Table 4: PPV and NPV of CA-125 and HE4

Biomarker	PPV (%)	NPV (%)
CA-125	80.7	87.2
HE4	93.1	92.7

D. CONCLUSION

OC's high mortality rate, numerous risk factors, and lack of reliable biomarkers make its detection a significantly critical issue. Biomarkers with elevated sensitivity and specificity can enable earlier diagnosis, improved risk stratification and an increased survival rate. Currently, CA-125 is utilized as the gold standard for OC detection biomarkers. However, its limited specificity, late detection, and susceptibility to false positive detection reveal the biomarker to be

ineffective. CA-125's limitations highlight the need for alternative markers. HE4 demonstrates higher precision when differentiating between tumor types, increased specificity, higher sensitivity for early OC stages, and an overall greater diagnostic accuracy in comparison to existing biomarkers. Future clinical research should focus on further validating HE4 as an effective biomarker for OC and aim to assess its performance across longer periods of time and diverse patient populations. Additionally, recent research has shown the combination of both CA-125 and HE4 to be more effective than other single markers or dual-marker combinations (Barr et al.). While further exploration of this combination is required, it shows promise in further enhancing OC diagnostic accuracy and speed. By prioritizing the integration of HE4 into clinical diagnostic protocols, the medical community can not only save countless lives, but also make significant strides in diagnosing and combating OC.

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