Development and evaluation of candidate dual biomarker for detecting circulating tumor cells of ovarian cancer using ovarian cancer cell line; HE4 and EpCAM

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Running title: Trial application of CTC biomarker for OC cells.

ABSTRACT

Objective: Detecting ovarian cancer in early stages is challenging; therefore, various research tools have been developed to overcome this challenge. Previous studies have used circulating tumor cells (CTCs) to detect ovarian cancer, and CTC markers have been evaluated for this application. Reported markers of ovarian cancer CTCs are mainly focused on the only CTCs. However, coverage of reported marker (EpCAM, Cytokeratin etc.) is not enough to detect whole CTCs, because properties of CTCs are changed by biological pathways. Therefore, newly ovarian cancer CTCs markers are needed.

Study Design: For development of newly ovarian CTCs markers, the applications of EpCAM, cytokeratin, cancer antigen 125 (CA-125) and human epididymis 4 protein (HE4) (ovarian cancer markers) were evaluated using ovarian cancer cell lines (OVCAR3, SKOV3, SNU-251, and SNU-8). Furthermore, we examined the feasibility of EpCAM and HE4 as dual markers using a spike-in test with the ovarian cancer cell lines OVCAR3 and SNU-251.

Results: EpCAM usually known as CTC epithelial gold standard marker but it had differences of expression level between ovarian cancer cell lines. The reason why we choose EpCAM and HE4 are dual marker for complementary to each other. (HE4 expression levels were as follows: OVCAR3, 84.7%; SKOV3, 97.3%; SNU-251, 99.2%; and SNU-8, 93.2%. EpCAM expression levels were as follows: OVCAR3, 97.7%; SKOV3, 49.4%; SNU-251, 28.5%; and SNU-8, 28.1 %.) The staining coverage rate of EpCAM was 61.8% in OVCAR3 and 68.3% in SNU-251, while that of HE4 was 97.7% in OVCAR3 and 90.1% in SNU-251.

Conclusion: These results demonstrated that the combination of EpCAM and HE4 can be used as dual markers for detecting ovarian cancer CTCs.

Key words: HE-4, EpCAM, Ovarian cancer, Circulating tumor cell, Spiking test

1. Introduction

Ovarian cancer is one of the gynecological cancers and its early diagnosis has been a challenge, with approximately 70% of patients diagnosed at stage 3 or later [1]. Many biomarkers have been examined to overcome this hurdle; however, none of them have been applicable except for cancer antigen 125 (CA-125; mucin16) [2]. Although CA-125 is the most popular biomarker for ovarian cancer, 20% of total patients with ovarian cancer and 50% of patients with early stage ovarian cancer show a normal range of CA-125 serum levels [3]. Furthermore, CA-125 serum levels can be increased in nonmalignant conditions, resulting in low sensitivity [4]. Many researches have been performed to overcome the limitation of CA-125 in diagnosis of ovarian cancer, and human epididymis 4 protein (HE4) has been proposed as a biomarker for ovarian cancer. HE4 is highly expressed in ovarian carcinomas, and it was detected in 50% of patients with normal range of CA-125, and more sensitive than other ovarian cancer markers including CA-125 [5]. HE4 was approved by the Food and Drug Administration (FDA) to monitor ovarian cancer patients. However, exact biomarker of ovarian cancer has not been found yet.

To diagnose and monitor cancer, many studies have used circulating tumor cells (CTCs). For these studies, efficient isolation of CTCs is essential. We developed a CTC enrichment platform comprising a size-based filtration system using a high-density microporous (HDM) chip and CTCs negative selection [6]. This platform can capture CTCs with various markers, thereby increasing the CTC detection rate. Furthermore, capturing live

CTCs enables serial liquid biopsy and genetic analysis [7, 8]. However, unfortunately, it is difficult to isolate CTCs in ovarian cancer because epithelial cell adhesion molecule (EpCAM), that is the universal CTC marker, is not homogeneously expressed in ovarian cancer CTCs.

In this study, to find the specific CTCs marker in ovarian cancer, EpCAM, cytokeratin, CA-125, and HE4 were evaluated as specific biomarkers in ovarian cancer cells using four different kinds of ovarian cancer cell lines. The feasibility of specific markers based on CTCs was evaluated through a CTC enrichment platform using a spike-in test with ovarian cancer cell lines.

2. Materials & Methods

Cell culture

SNU-251 (originated from endometroid adenocarcinoma), SNU-8 (serous cystadenocarcinoma), OVCAR3 (papillary serous adenocarcinoma), and SKOV3 (clear cell adenocarcinoma) cell lines were cultured using a RPMI1640 medium (supplemented with 10% fetal bovine serum, 10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml Fungizone TM) at 37°C and a 5% CO2 incubator.

Spike-in test

Whole blood samples from healthy donors were spiked with the appropriate number of cultured cancer cells. To count the number of cancer cells, serially diluted cancer cells and a hemocytometer were used. A 10-μl aliquot of each dilution was placed on a glass slide, and

the number of cells was counted. To achieve the appropriate concentration, 1 ml of whole blood was spiked with ovarian cancer cells (100 cells). Whole blood spiked with cancer cells was layered on Ficoll-Hypaque gradients with a density of 1.077 g/ml (GE Healthcare, Piscataway, NJ) and centrifuged for 30 min at 400 × g at room temperature, and the PBMC layer was collected in a new tube. Staining coverage was definition as the number of cells stained for each biomarker divided by the number of total cells.

Negative enrichment by filtration

The collected PBMC layer was mixed with PBS and then passed through the HDM chip. The enriched cells were recovered from the chip and layered on a glass slide. Cells on the glass slide were air dried for 30 min and fixed with 4% paraformaldehyde in PBS.

Immunostaining

Cells on a glass slide were permeabilized in 0.2% Triton X-100 (Sigma-Aldrich, MO) for 10 min and quenched using 3% H_2O_2 for 30 min. After washing thrice with PBS, cells were blocked with 1% BSA in PBS for 30 min at room temperature.

Cells were incubated with primary antibodies (anti-EpCAM, Cell Signaling Technology, Danvers, MA; anti-Cytokeratin, DAKO, Carpinteria, CA; anti-CA125, Santa Cruz Biotechnology, Inc; anti-HE4, Abcam, Boston, MA). After washing with PBS, the Tyramide Signal Amplification Kit was used (Invitrogen), according to the manufacturer's instructions. Next, the cells were incubated with anti-CD45 antibody (Cell Signaling Technology, Danvers, MA), followed by incubation with Alexa Fluor 594-conjugated secondary antibody (Invitrogen). Finally, the cells were counterstained with DAPI (Immunoscience, Washington, USA) to visualize nuclei.

3. Results

Determining biomarker candidates for ovarian cancer CTCs

The typical epithelial CTC markers (EpCAM and cytokeratin) and ovarian cancer markers (CA-125 and HE4) were analyzed using four different ovarian cancer cell lines (OVCAR3, SKOV3, SNU-251, and SNU-8) to evaluate the feasibility of diagnostic biomarker candidates (Table 1 and Figure 1).

Cytokeratin, the typical CTC marker, was not detected in SNU-8, while EpCAM was detected in all cell lines, even with variable staining intensity rates. EpCAM expression coverage was as follows: 97.7% in OVCAR3, 49.4% in SKOV3, 28.5% in SNU-251, and 28.1% in SNU-8. In the case of SNU-8, cytokeratin was not detected. CA-125, the most traditional ovarian cancer maker, revealed the lowest staining coverage rate in cell lines, except in OVCAR3; the expression coverage of CA-125 was 98.3% in OVCAR3, but that was below 20% in other cell lines. HE4 displayed a high staining coverage rate in all cell lines. HE4 expression coverage was as follows: 84.7% in OVCAR3, 97.3% in SKOV3, 99.2% in SNU-251, and 93.2% in SNU-8. These results demonstrated that EpCAM, the gold standard CTC marker, and HE4, the new ovarian cancer marker, can be used together for detecting ovarian cancer CTCs.

Dual screening of EpCAM and HE4 for detecting ovarian cancer CTCs

The spike-in test was performed with ovarian cancer cell lines to evaluate the feasibility of EpCAM and HE4 as ovarian cancer CTC markers (Figure 2 and Figure 3). Two

ovarian cancer cell lines, OVCAR3 and SNU-251, were spiked with normal blood from healthy volunteers and enriched using Cytogen's CTC enrichment platform. The enriched cells were immunofluorescent stained for EpCAM and HE4 and analyzed for staining coverage rate. The staining coverage of EpCAM was 61.8% in OVCAR3 and 68.3% in SNU-251, while that of HE4 was 97.3% in OVCAR3 and 90.1% in SNU-251.

4. Comment

Various diagnostic approaches have been applied to achieve early diagnosis of ovarian cancer; however, no accurate diagnostic method has been established [9]. Although CA-125 is the most popular biomarker for diagnosing ovarian cancer, there are many studies regarding its low sensitivity and specificity [10]. A study demonstrated the combined use of CA-125 and HE4 with an increased sensitivity for early diagnosis of ovarian cancer. In addition, Lokich *et al.* [11] reported that HE4 had high expression levels in patients with ovarian cancer and correlation with platinum resistance. As mentioned above, CA-125 and HE-4 are widely used as the diagnostic biomarkers of ovarian cancer, but limitations of CA-125 and HE-4 have been reported [10].

Although many studies have attempted to use CTCs for diagnosing various cancers, studies using CTCs for diagnosing ovarian cancer have not been sufficient. CTCs undergo an epithelial-to-mesenchymal transition, and during this process, they change their cell type-specific markers [7, 8, 12-20]. There are many studies regarding CTCs not expressing EpCAM and cytokeratin in various cancer types. The study to identify novel markers for CTCs in patients with epithelial ovarian cancer showed that two thirds were identified by

overexpression of the cyclophilin C gene (PPIC), and just a few by EpCAM overexpression. Interestingly, the presence of CTCs was correlated with the elevated blood CA-125 and HE4 levels, but they did not investigate the possibility of CA-125 and HE4 as the novel markers for CTCs in ovarian cancer [10].

Therefore, in this study, we attempted to identify the accurate ovarian cancer CTC marker by cross-validating CTC epithelial markers and ovarian cancer-specific markers. For this purpose, we selected EpCAM and cytokeratin as CTC epithelial markers and CA-125 and HE4 as ovarian cancer makers.

The detecting coverage rates of HE4 were over 80% in all four cell lines and those of CA-125 were high only in OVCAR3. The staining coverage rates of EpCAM were over 90% in OVCAR3, but below 50% in other cell lines. However, cytokeratin was not detected in SNU-8, implying that EpCAM is a better marker than cytokeratin for detecting ovarian cancer CTCs. Although HE4 revealed an overall high staining coverage rate in all cell lines, EpCAM displayed a much higher coverage rate in OVCAR3 than HE4. These results suggest that combining two markers can increase the detection coverage rate of heterogeneous ovarian cancer CTCs.

Cytogen's CTC enrichment platform comprises a size-based filtration system and CTC negative selection [7, 8]. Therefore, CTCs enriched using this platform can be captured, regardless of specific markers. We performed the spike-in test with ovarian cancer cell lines by enriching the cells using Cytogen's CTC platform and performing immunofluorescent staining for EpCAM and HE4 as dual biomarkers. The results revealed that the majority of spiked cells were detected by the combined use of EpCAM and HE4.

In conclusion, EpCAM and HE4 could be used as dual biomarkers for detecting CTCs for early diagnosis and monitoring of ovarian cancer. However, our research is a just preliminary stage at present, because we identify the ovarian cancer cell markers using only ovarian cancer cell lines and suggest their possibility as ovarian cancer CTC markers through the spike-in test using CTC enrichment platform. Further studies with real blood samples from patients with ovarian cancer will be required to confirm the feasibility of EpCAM and HE4 as dual biomarkers for early diagnosis of ovarian cancer in a clinical setting.

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Table 1. Staining coverage rate of candidate cancer cell markers using ovarian cancer cell lines.

Staining coverage rate (%)*	EpCAM	Cytokeratin	CA-125	HE4
OVCAR3	97.7	97.4	98.3	84.7
SKOV3	49.4	77.8	6.3	97.3
SNU-251	28.5	38.2	18.3	99.2
SNU-8	28.1	ND**	6.5	93.2

*Staining coverage rate (%): (stained cell by each antibody)/(Total cells) × 100

**ND, Not Detected

Figure legends

Figure 1. Immunofluorescence image of ovarian cancer cell lines that were stained by typical CTC epithelial markers (EpCAM and cytokeratin) and clinical ovarian cancer markers (CA-125 and HE4).

Figure 2. Immunostaining results of spiked OVCAR3, SNU-251, and white blood cell (WBC). WBC was only stained using the CD45 hematopoietic marker. Immunofluorescence signals of EpCAM and HE4 were detected in OVCAR3 and SNU-251.

Figure 3. Immunostaining coverage rate of EpCAM and HE4 in spiked OVCAR3 and SNU-251.

Figure. 1.

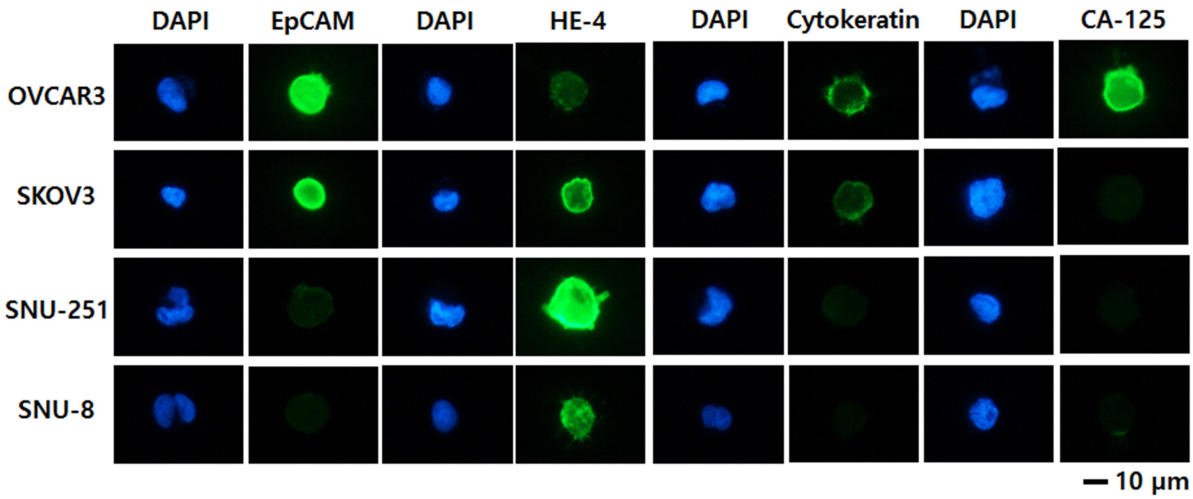

DAPI
EpCAM
DAPI
HE-4
DAPI
Cytokeratin
DAPI
CA-125
OVCAR3
SKOV3
SNU-251
SNU-8
10 μm

Figure. 2.

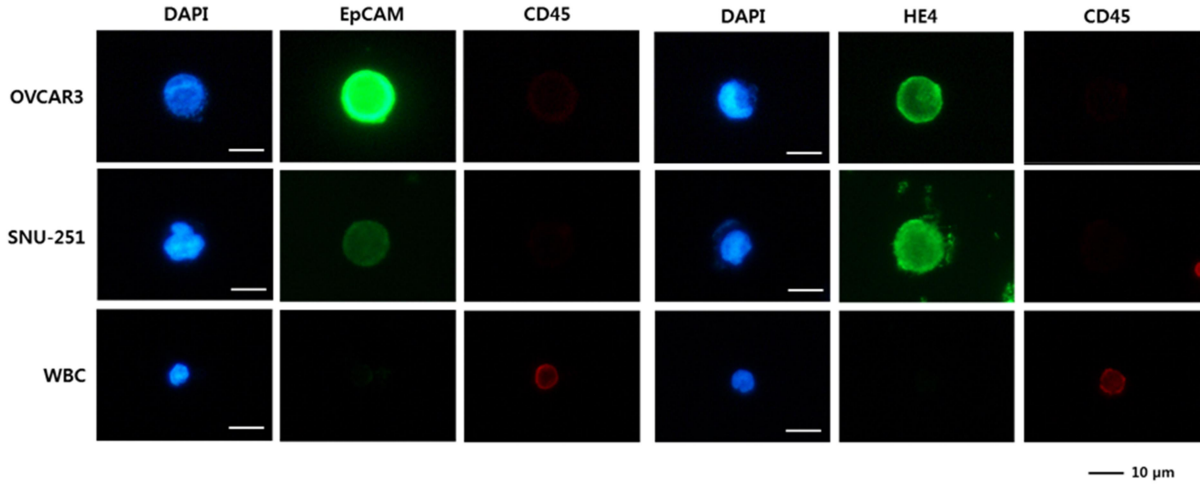
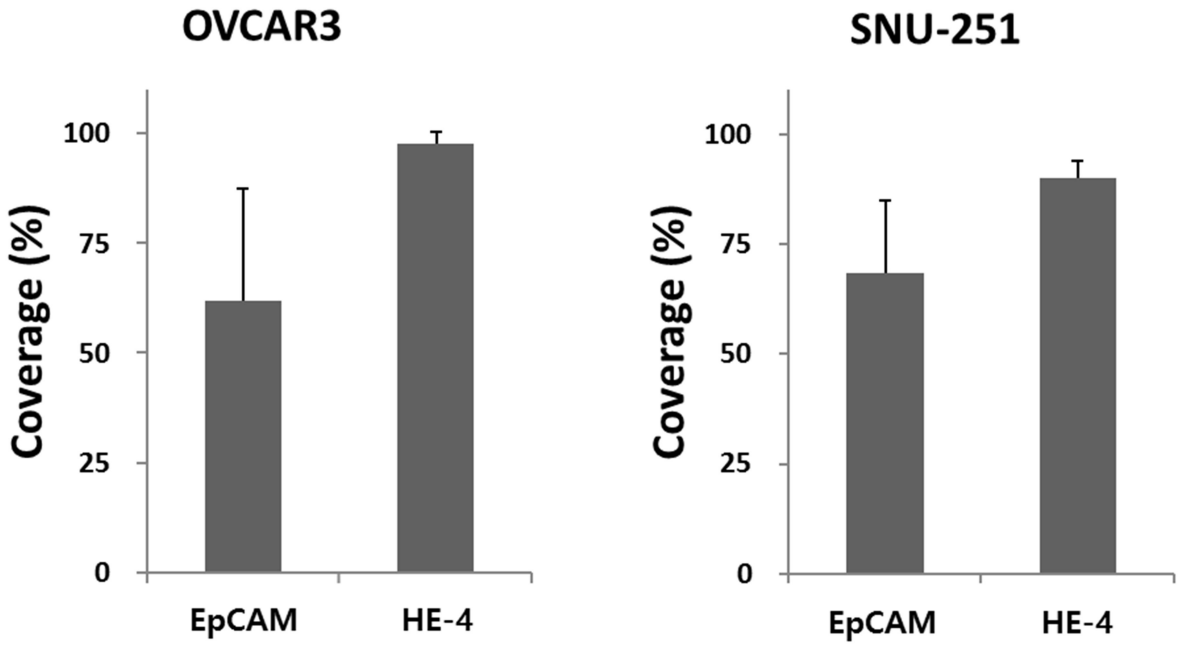


Figure. 3.


OVCAR3
Coverage (%)
100
75
50
25
0
EpCAM
HE-4
SNU-251
Coverage (%)
100
75
50
25
0
EpCAM
HE-4