Original Article

Detection of Circulating Tumor Cells in Peripheral Blood by Use of The High-Gradient Magnetic Separation Technique in Ovarian Tumor Patients

Chamnan Tanprasertkul[1,2 *], Charintip Somprasit[1], Komsun Suwannarurk[1], Nipattha Vinayanuvattikhun[1], Prapat Suriyaphol[3], Sebastian Chakrit Bhakdi[4]

Abstract

Objective: To evaluate the performance of the high-gradient magnetic separation (HGMS) technique in detecting circulating tumor cells (CTCs) for the preoperative diagnosis of ovarian cancer.

Methods: Women who had ovarian tumors and were admitted to Thammasat University Hospital during January 2018-December 2019 were enrolled into the study. Ten milliliters of fresh peripheral blood for HGMS were collected within 24 hrs prior to surgery. After healthy cell depletion by HGMS, the remaining cells including CTCs were spun onto gelatinized standard laboratory slides and stained with a panel of specific antibodies against CD45, CD31, CD34, CD73, CAM5.2, C-11, VIM and PKM2. The findings were classified into five classes, as based on cell types and their quantities: Classes I-III were categorized as a negative test and Classes IV-V were categorized as a positive test. The CTCs findings were compared to the final histopathological report.

Results: There were 67 participants in the study, with a mean age of 44.8 years. The detection rate of the test was 72.92%. Overall sensitivity and specificity were 45.45% and 94.12%, respectively. The accuracy of this method was 85.48%, with a negative predictive value of 88.89% and a positive predictive value of 62.50%.

Conclusion: The HGMS technique has a promising capacity for detecting ovarian cancer CTCs in patients with ovarian tumors. This technique should be optimized further and utilized, instead of a tumor markers, as a preoperative method for detecting ovarian cancer in the near future.

Keywords: circulating tumor cells, ovarian cancer, peripheral blood, high-gradient magnetic separation

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[1] Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Thailand
[2] Advanced clinical innovative research in obstetrics and gynecology group, Thammasat University, Thailand, 12120
[3] Division of Bioinformatics and Data Management for Research, Research Group and Research Network Division, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand ,10400
[4] Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand; X-ZELL, 133 Cecil Street, #06-02 Keck Seng Tower, Singapore 069535, Singapore
***Corresponding author:** Tanprasertkul C., Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand 12120; Tel. 02-9269343; E-mail: chamnandoctor@gmail.com

Introduction

Ovarian cancer is a common gynecologic cancer, not only in Thailand, but globally. It has a high mortality rate because there are no obvious symptoms[1] and consequently, the delayed diagnosis often leads to a substantially higher mortality rate. In the preoperative assessment of ovarian tumors, gynecologists may fail to accurately diagnose ovarian cancer due to the limited precision of existing diagnostic tools.

The current screening method uses serum tumor markers like CA 125 and HE4 in peripheral blood, along with trans-vaginal ultrasonography. The results from the use of these investigational tools still have limitations in terms of accuracy. Moreover, this method has a high false-positive rate and lacks value in health economics.[2]

With the development of biotechnology, previous studies have found that circulating tumor cells (CTCs) in cancer patients can be detected from an early to an advanced stage of the disease. This detection of cancer cells in the bloodstream may be used to support the diagnosis and assist the decisions of gynecologists in planning management, especially for surgical procedures. In addition, CTCs could be used to monitor the stage of the disease or inform the clinician of its prognosis.[3,4,5] However, there are still no conclusive methods for detecting CTCs.

The measurement for the presence of cancer cells in the bloodstream is highly complex and difficult, especially because the number of such cells is so very small. Currently, the method of searching for cancer cells in the bloodstream is to capture and pull cancer cells directly from the bloodstream. Most research relies on the use of immunological methods, for example, an antibody to the epithelial cell adhesion molecule (EpCAM).

The challenge associated with the aforementioned method lies in the significant heterogeneity of cancer cells possibly present in the bloodstream, particularly in terms of the variety of surface markers. As a result, the direct trapping approach for identifying circulating tumor cells (CTCs) frequently fails to capture cells that exhibit divergent properties. Moreover, studies for detecting CTCs found that even with the same patient's blood samples, different methods produced different results.[6,7,8]

This study aims to evaluate the high-gradient magnetic separation (HGMS) technique, which is a newly developed method for searching for cancer cells in the bloodstream, or CTCs, in the preoperative prediction of ovarian cancer.

Methods

The study is based on a diagnostic design with prospective data collection. It was conducted at the Department of Obstetrics and Gynecology of Thammasat University Hospital, Thailand, during January 2018 - December 2019 following approval from the ethical institute committee.

We enrolled women with ovarian tumors who had to undergo ovarian surgery, using either laparoscopy or laparotomy. They were 18 - 65 years old, completely understood the process of this study and had given their written informed consent to the study. Any with suspected or confirmed other organ cancers of other types were excluded from the study. The sample size was calculated by the formula $n = \frac{Z^2 p\,(1 - p)}{d^2}$. The proportion (p) of ovarian cancer in our institution was 0.20. We used an error (d) = 0.10 and alpha (α) = 0.05. The participant number was then set at 62. We compensated for a data loss of 15%, and the final total needed sample size was set at 72.

An additional 10 mL of blood was drawn from each patient on the day of hospitalization prior to surgery. This blood sample was collected into a container pre-filled with either EDTA or heparin by qualified nursing staff or specialized laboratory personnel. All specimens were accurately labeled in accordance with the trial protocol, including pertinent information about the participating patients. Clinical data were meticulously recorded in case report forms. On the same day as collection, the blood samples were stored at a temperature of 4°C and promptly transported to the Laboratory of Bioinformatics and Research Data Management Unit within the Research Center at the Faculty of Medicine, Siriraj Hospital, Mahidol University. Cellular analyses were conducted on the same day, and investigators responsible for these analyses were blinded to both the operative outcomes and the pathological findings.

Laboratory Technique

All samples were processed as follows. The blood samples of the participants were subjected to hemolysis by using of a lysing buffer solution. The blood was then washed with a phosphate buffer saline (PBS) and mixed with paramagnetic nano-beads for 10-15 min. These nano-beads bound to white blood cells without binding to cancer cells. The cell suspension was then placed in a column so that the normal blood cells that were magnetically labeled became fixed in the column. The cancer cells in the blood passed out of the column and were stored for further HGMS separation. Cells were spun onto gelatinized standard laboratory slides and stained with a panel of specific antibodies against CD45, CD31, CD34, CD73, CAM5.2, C-11, VIM and PKM2.

The slides were examined under a fluorescence microscope. The findings were classified into five classes, as based on cell types and their quantities: negative for malignancy (class I), atypical cells found but negative for malignancy (class II), suspicious for malignant cells (class III), strongly suggestive for malignant cells (class IV) and conclusive for malignant cells (class V). The CTCs Cytopathological Criteria were classified as the followings:

I	• No CD45- cells
II	• CD45- cells without positive markers (CEC/CTC vs plasma cells) • Less than five giant polyploidic cells: CD31+CD34=CD73-VIM=CK-PKM2-CD45- (Megakaryocyte lineage) • Cells with aneuploidy: CD31+CD34-CD73-VIM-CK-PKM2-CD45-
III	• Less than five single cells: CD31=CD34+CD73-VIM+CK-PKM2-CD45- (angiogenic tip cell: tumor-derived vs inflammatory) • Five or more giant polyploidic cells: CD31+CD34=CD73-VIM=CK-PKM2-CD45- (Megakaryocyte lineage) • More than one large cell: CD31-CD34=CD73-VIM+CK-PKM2-CD45- (mesenchymal CTC vs hematopoietic stem cell) • Both conditions with or without aneuploidy
IV	• Binucleated cells: CD45- • Less than five single cells: CD31-CD34-CD73-VIM=CK+PKM2=CD45-* • One clump: CD31=CD34+CD73=VIM=CK-PKM2=CD45-** • One or more large cells with aneuploidy: CD45- • More than one cells with pronounced aneuploidy: CD31=CD34+CD73-VIM+CK=PKM2=CD45- • More than five single cells: CD31=CD34+CD73-VIM+CK-PKM2=CD45- • One or more single cell: CD31=CD34+CD73+VIM=CK=PKM2=CD45-
V	• One clump: CD31=CD34=CD73=VIM=CK+PKM2=CD45-** • More than one clump: CD31=CD34+CD73=VIM+CK=PKM2=CD45- • More than five CD31-CD34-CD73-VIM=CK+PKM2=CD45- cells* • One or more CD45- cell in atypical mitosis*** • One or more aneuploidic cell: CD31=CD34+CD73+VIM+CK=CD45- • One or more cell with or without aneuploidy: CD31=CD34+CD73+VIM=CK=CD45-

The CTCs findings were compared to the final histopathological reports. All the interpreters were blinded to the concluding results. Classes I-III were categorized as a negative test and Classes IV-V were categorized as a positive test.

Results

Sixty-seven participants, with a mean age of 44.8 years, were included. 62 cases had the complete data for analysis. From the pathological test results, 82.26% of the cases were not found to have ovarian cancer. Most of these were diagnosed with endometrioma and benign cystic teratoma. Of the remaining patients, 11 out of 62, or 17.74%, were confirmed to have ovarian cancer. The majority of these were characterized by the presence of epithelial cells, including types such as serous cystadenocarcinoma, clear cells, and endometrioid cells.

Table 1 presents a classification of circulating tumor cells (CTCs) for the purpose of comparing non-ovarian cancer patients with ovarian cancer patients. In non-cancer patients, most of the detection of CTCs, as much as 94.11%, was found in classes I to III, a true negative. Specifically, there were CTCs detections of 76.47%, 5.88% and 11.76% of patients in classes I, II, and III, respectively. Only 3 out of 62, or 4.83%, were found with CTCs in class IV, which was a false positive. None of the benign ovarian tumor patients were found in class V. In the patients with ovarian cancer, CTCs were detected at 45.45% (true positive) with 9.09% and 36.36% in classes IV and V, respectively. The false negatives in CTC classes I, II & III were 18.18%, 9.09% & 27.27%, respectively, totaling 54.54 %. The ROC curve of detection of CTCs was shown in Figure 1, in which the detection rate of the test was 72.92 %. Overall sensitivity and specificity were 45.45% and 94.12%, respectively. The accuracy of this method was 85.48%, with a negative predictive value of 88.89% and a positive predictive value of 62.50%.

Table 1 Comparison of CTCs occurrences between non-ovarian cancer and ovarian cancer patients

CTCs Class	Non-ovarian cancer		Ovarian cancer	
	No.	%	No.	%
I	39	76.47	2	18.18
II	3	5.88	1	9.09
III	6	11.76	3	27.27
IV	3	5.88	1	9.09
V	0	0.00	4	36.36
Total	51	100.00	11	100.00

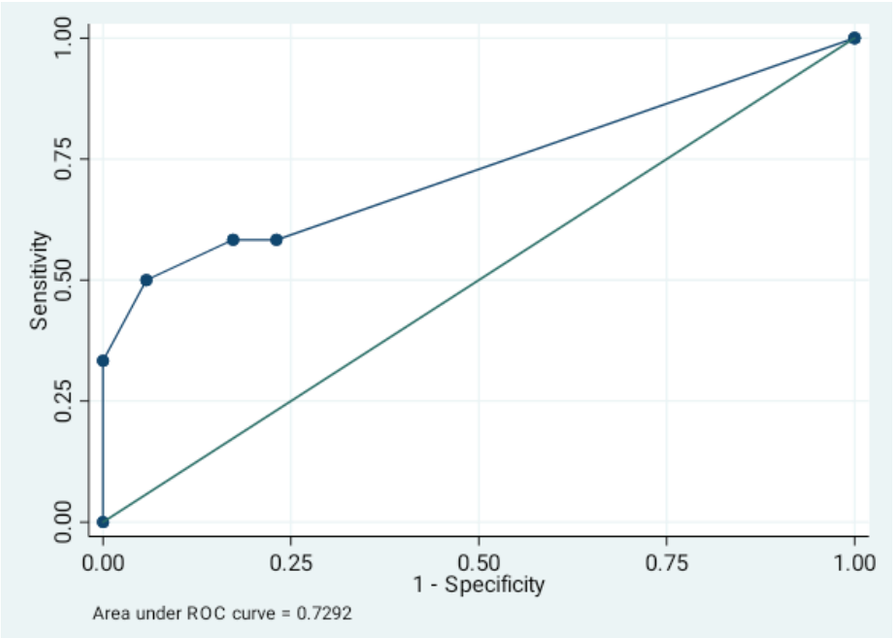

Figure 1 Graphical plot showing diagnostic performances of high-gradient magnetic separation (HGMS) technique as the area under the curve of detection of circulating tumor cells (CTCs)

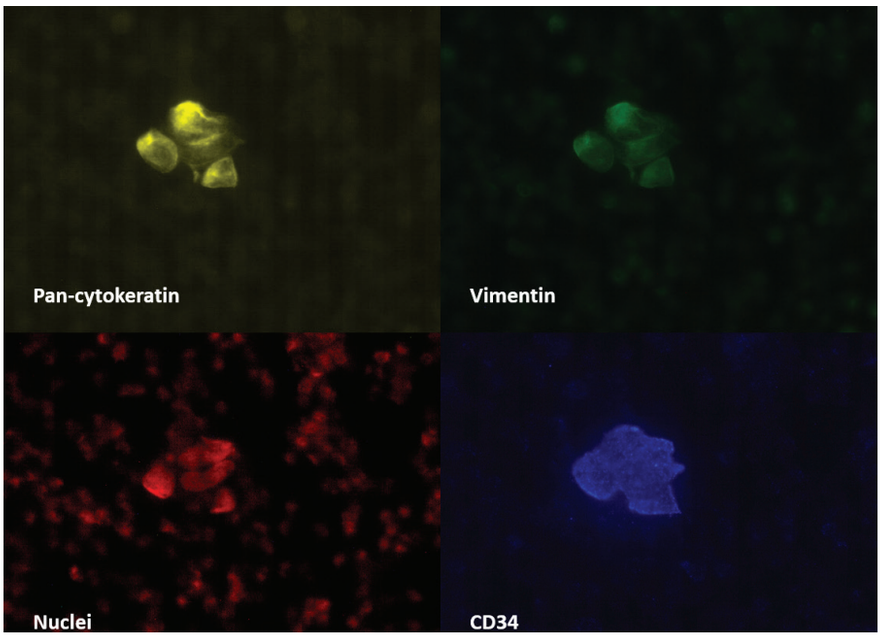

Figure 2 Image of a pan-cytokeratin positive ovarian cancer cell clump, or giant polyploidic cell, in peripheral blood detected and examined under a fluorescence microscope. Note the cytosolic localization of cytokeratin and vimentin as opposed to the pan-membrane staining of CD34, homogenously covering the complete surface of the clump. The nuclear view shows smaller white blood cell nuclei surrounding the four largest, strikingly anisokaryotic cancer cell nuclei. Fluorophores: pan-cytokeratin PE, vimentin AlexaFluor488, CD34 Brilliant Violet 421, nuclei Draq5.

Discussion

Our study results demonstrate the ability of the HGMS technique to detect CTCs in the blood of ovarian tumor patients. The percentage of patients in the ovarian cancer group with true-positive diagnoses was 45.45%, which means that almost half of the preoperative ovarian cancer cases would be well prepared with a specialist team and gynecologic oncologist. Also, this test had an accuracy of 72.92% with a confidence interval between 55.32% to 90.52% (Figure 1), which was sufficient to differentiate benign tumors from malignancies. This outcome is consistent with previous reports[9, 10], which found CTCs in ovarian cancer patients, yet those reports differed from our current study, because prior research was conducted in confirmed ovarian cancer cases, while our study consisted only of suspected or newly identified cases. In our study, we utilized a mixed population comprising both benign and malignant ovarian tumors, which resulted in a low prevalence rate of ovarian cancers. Additionally, the study encompassed a diverse range of cell types and cancer stages. As a consequence, the performance of detection was not particularly high. With statistical calculation, the sensitivity and specificity of this test were found to be 45.45% and 94.12%, respectively. The negative predictive and positive predictive values were 88.89% and 62.50%, respectively. These predictive values could serve as a forecast of non-cancer cases with high predictive performance. HGMS is the cell isolation technique for the separation of large numbers of cells, as based on specific cell-surface markers. The present study adopted high-gradient magnetic separation for the removal of white blood cells, or in other words, it applied a negative rare-cell isolation approach. Use of this technique was followed by highly multiplexed immunostaining on standard laboratory slides. The combination of these two techniques allowed us to isolate tumor-associated cells in a manner independent of the expression of a single antigen and to analyze epithelial, mesenchymal and endothelial antigens on the single-cell level simultaneously (Figure 2). Applying classical cytopathological criteria of malignancy to cells characterized in this way seems to show promise as an adjunct tool in the diagnostics of ovarian cancer.[11, 12]

There were several limitations in this study. The finding of CTCs did not show an association with ovarian cell types, stages of malignancy or serum tumor markers such as CA 125 or HE4, because of the small sample sizes and the variety of ovarian cancer cell types. Moreover, the likelihood of a positive diagnosis of cancer was rather low, possibly because our objective was based on a strategy targeting newly diagnosed ovarian cancer cases.

Conclusion

The HGMS technique was utilized for the pre-operative detection of CTCs in newly diagnosed ovarian cancer patients. The accuracy of the technique was satisfactory, and this method has a promising capacity. Optimizing the HGMS technique might allow it to be utilized, instead of tumor markers, as a pre-operative method for detecting ovarian cancer.

Conflict of interest

The authors declare that there is no conflict of interest

Acknowledgement

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