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Tumor-Induced GIMAP8 Expression in Peripheral Blood Mononuclear Cells as a Novel Marker for Epithelial Ovarian Cancer Detection

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Abstract

Cancer cells change the properties of surrounding cells *via* secretion signaling. The effects of tumor communication were found to cause molecular alterations in peripheral blood mononuclear cells (PBMCs). These alterations could be applied in a blood-based test for cancer detection, especially with epithelial ovarian cancer (EOC). This cancer has less effective screening tools and nonspecific symptoms leading to high mortality rates in patients. Therefore, a novel biomarker for screening is required. A simulated model of cancer cell signaling was performed by the co-culture of normal PBMCs with ovarian cancer cell lines. Transcriptome analysis was then performed using RNA sequencing (RNA-seq). In addition, we retrieved expression microarray (GSE31682) data from GenBank and combined this expression data with the two groups of RNA-seq data using Connection Up- and Down-Regulation Expression Analysis of Microarrays extension (CU-DREAMX). The most upregulated gene, GTPase IMAP family member eight (GIMAP8), was selected for validation by quantitative reverse transcription polymerase chain reaction in PBMCs from 16 ovarian cancer patients compared with 15 healthy controls. The GIMAP8 expression was significantly increased in ovarian cancer patients (p-value < 0.0001). Interestingly, there was high expression in all three cases of clear cell and four cases of serous adenocarcinoma. We determined that PBMCs changed their gene expression as a result of ovarian cancer cell signaling. Furthermore, the expression level changes in GIMAP8 could be applied for cancer screening, diagnosis, and treatment monitoring purposes.

Keywords: Epithelial ovarian cancer; Peripheral blood mononuclear cells; RNA sequencing; Expression array; GIMAP8 expression

Introduction

Communication between cancer cells and the normal surrounding cells plays a role in providing the proper tumor microenvironment for cancer survival and progression [1-5]. The molecular properties of the normal surrounding cells are modified through the secretion of factors such as cytokines or intermediate metabolites from cancer cells [1]. Many studies have reported that stromal cell alterations were referring in the molecular levels [3,6]. Our previous study revealed that epigenetic regulation causes differential gene expression in plasma cells as a result of signaling from breast cancer cells [7]. Therefore, cancer cells may secrete substances to regulate the expression of white blood cells (WBCs) that are also found in the bloodstream. The expression changes in WBCs could be applied in a blood-based test for cancer detection.

Ovarian cancer is a silent killer of women [8]. It is the fifth leading cause of cancer deaths among American women and the seventh leading cause of new cancer patients among Thai women [9]. The nonspecific symptoms of ovarian cancer can result in late diagnosis [8]. Moreover, this leads to a more severe stage of cancer that responds poorly to treatment, in USA the five-year survival rate from 2006 to 2012 was 46.2%. Ovarian cancer is a heterogeneous disease and epithelial ovarian cancer (EOC) is the most common diagnosis, with approximately 90% of cases [10]. The histological subtype is primarily used to classify epithelial ovarian cancer as serous, clear cell, mucinous, and endometrioid adenocarcinoma [11]. To date, only carbohydrate antigen 125 (CA125, sensitivity 79.6% and specificity 82.5%) and human epididymis protein 4 (HE4, sensitivity 81.7% and specificity 85.1%) are used to assess ovarian cancer in women with a pelvic mass [12,13], however, these biomarkers are still limited in their sensitivity and specificity. A more effective screening tool is needed for ovarian cancer.

To discover novel biomarkers, changes in molecular biology activities were widely identified by microarrays that analyzed the differential expression of genes between disease and normal conditions [14]. Analyses of white blood cells (WBCs) in cancer patients were conducted to interpret patients abnormalities in many cancers such as lung [15,16], colon [17,18], liver [19], head and neck [20], and ovary [21]. This data is known as the GEO dataset in GenBank and can be integrated along with other experiments using the Connection Up- and Down-Regulation Expression Analysis of Microarrays extension (CU-DREAMX) program [22]. In addition, RNA sequencing (RNA-seq) was utilized for transcript identification and quantification of gene expression with more biological information [23]. This data was combined with disseminated experiments to explore a novel approach for cancer screening.

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In this study, we hypothesized that the ovarian tumors may be synthesizing some hormone-like or soluble substances that cause molecular changes in WBCs, particularly peripheral blood mononuclear cell (PBMCs). Alteration of these cells must be prevalent enough to be detectable from circulating PBMCs and be useful in distinguishing ovarian cancer patients from their healthy counterparts. Differential gene expression in PBMCs that are influenced by cancer would be found in ovarian cancer patients and could serve as tumor biomarkers. In addition, protein modifications in immune cells could be future targets of immunotherapy [24,25].

Materials and Methods

Blood collection and PBMC preparation

For the co-culture model, 24 ml of EDTA blood samples were collected from healthy females without a record of familial ovarian cancer and isolated for PBMCs by lymphocyte separation medium according to the manufacturer's instructions (Axis-Shield PoC AS, Oslo, Norway). Briefly, blood samples were loaded into a 15 ml tube with separation medium and centrifuged to separate the mononuclear cell layer. The cells were then washed twice with phosphate-buffered saline (PBS) before co-culturing in the next step. For the validation step, blood from ovarian cancer patients was collected from department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Thailand. PBMCs were isolated from blood samples (16 cases of epithelial ovarian cancer and 15 female controls) with the method described above. All detailed sample data is presented in Table S1.

Cell lines and co-culture

Ovarian cancer cell lines were purchased from the JCRB cell bank in Japan, including OVISE (JCRB1043) and OVKATE (JCRB1044). Both cancer cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Merck KGaA, Darmstadt, Germany) with 10% fetal bovine serum (FBS) (Thermo ScientificTM, Waltham, MA, USA) and 1% antibiotic-antimycotic 100X (Thermo ScientificTM, Waltham, MA, USA) at 37°C, 5% CO_2, and standard humidity conditions. Both cancer cell lines were harvested with 0.05% Trypsin, washed, and resuspended in PBS. The co-culture model consisted of healthy PBMCs co-cultured with the cancer cell lines to allow signaling between cells. Ovarian cancer cell lines were seeded with 2 ml DMEM media (1×10^6 cells per well) in 6 well-culture plates (Corning Incorporated, Corning, NY, USA) for 2 experiment groups including OVISE and OVKATE. The plates were incubated overnight in cell culture conditions. The control group had no cancer cell lines in the culture system. Next, two million healthy PBCMs were collected from three different donors. For each experiment and sample, these PBCMs in 1.5 ml DMEM media were loaded onto a 0.4 µm transmembrane (Corning Incorporated, Corning, NY, USA) in triplicate and cultured with cancer cells for four hours. Co-cultured PBMCs were then collected to extract RNA.

RNA extraction

PBMC cell pellets were collected by centrifugation at 700 g, 4°C for 10 min and washed twice with PBS. RNA was extracted using 1 ml TRIzol LS reagent (Thermo ScientificTM, Waltham, MA, USA) according to the manufacturer's instructions. RNA was then separated by chloroform, precipitated by 100% isopropanol with glycogen, and washed with 75% ethanol. Total RNA in 30 µl of Diethyl pyrocarbonate (DEPC) water was confirmed to be of satisfactory quantity by a Qubit® Fluorometer (Thermo ScientificTM, Waltham, MA, USA) and RNA integrity quality was verified by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The samples with a minimum RNA concentration of 1 µg and a RNA Integrity Number above seven were used for next generation sequencing.

RNA sequencing and a transcript level expression analysis

A RNA sample library was prepared using NEBNext® Ultra™ RNA Library Prep Kit for RNA sequencing according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Messenger RNA isolation and fragmentation was performed for the samples. The fragmented RNA was then synthesized to cDNA that had both ends modified with an adaptor. Subsequently, the adaptor-ligated DNA was selected and purified using an AxyPrep Mag PCR Clean-up (Corning Incorporated, Corning, NY, USA). Each sample was amplified by PCR for 11 cycles using P5 and P7 primers. Purified PCR products were validated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and quantified by a Qubit 2.0 Fluorometer (Thermo Scientific™, Waltham, MA, USA). Lastly, the libraries were loaded onto an Illumina HiSeq instrument according to the manufacturer's instructions (Illumina, Inc., San Diego, CA, USA). Sequencing was carried out using a 2×150 bp paired-end (PE) configuration.

RNA-seq raw data was cleaned of the interference information (adapters). A quality control tool, FastQC, was used to filter high quality data (Q score > 20). The data was then processed by the HISAT, StringTie, and Ballgown analysis pipeline [26]. HISAT version 2.1.0 was used to align reads to the reference human genome (GRCh38.p10). Read alignments were assembled into transcripts by StringTie version 1.3.3b. DESeq2 version 1.16.1 was used instead of Ballgown to analyze differential expression. The differential expression of each gene between experiment and control groups was calculated. Finally, we had two groups of primary candidate genes from RNA-seq, including group one genes (OVISE treatment) and group two genes (OVKATE treatment). Each group was classified into two subgroups as upregulated or downregulated genes, followed by a calculation of fold change and *p*-value.

Retrieving data from GenBank

The gene expression profiling was selected and downloaded from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/gds) by keyword, including ovarian cancer and white blood cells. GSE31682 analyzed the differences in gene expression in PBMCs from 48 ovarian cancer cases and 20 healthy controls. This data (genes from expression microarray GSE31682) was called group three and included in the analysis with the primary candidate gene groups.

Connection Up- and Down-Regulation Expression Analysis of Microarrays Extension (CU-DREAMX) Program

We combined and analyzed RNA-seq expression microarray data from each group using the CU-DREAMX program. Significant genes from the CU-DREAMX analysis, with a *p*-value of less than 0.05 and an odds ratio more than one, were collected. The function of these genes was classified in terms of biological process by an online database available from the protein analysis through evolutionary relationships (PANTHER) classification system (http://pantherdb.org/). Finally, we selected candidate genes from those with the intersection between the three data groups.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

To validate gene expression differences in patients, we chose the highest expressing gene, GTPase IMAP family member eight (*GIMAP8*), as a target gene to evaluate expression in PBMCs from ovarian cancer

patients and control females. First, RNA was extracted from PBMCs and diluted to a final concentration of 500 ng. Then, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™, Waltham, MA, USA) following the manufacturer's instructions. The target gene, *GIMAP8*, was amplified from cancer patients and healthy controls using PowerUp™ SYBR® Green Master Mix (Thermo Scientific™, Waltham, MA, USA) containing the forward primer 5'-CAGAGAAAAAGAAACCCTGAAC-3' and the reverse primer 5'-CTCCCCAGGATAGAGTTCC-3'. *GIMAP8* expression was quantified by an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Thermo Scientific™, Waltham, MA, USA) with 35 cycles for denaturation 95°C 15 seconds and annealing/Extension 58°C 60 seconds. To determine the fold change in gene expression, the target gene was normalized to an endogenous reference gene, GAPDH (forward primer 5'-TGGAAGGACTCATGACCACAG-3' and reverse primer 5'-TTCAGCTCAGGGATGACCTT-3'). All samples were amplified in duplicate and analyzed by the $2^{-\Delta\Delta Ct}$ method [27].

Statistical analysis, ROC curve, and diagnostic test

SPSS software for Windows version 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses in this study. We used chi-squared test to determine the distribution of up- and down-regulated genes. The student's t-test was performed to compare mean expression levels between experiment and control data from cell lines as well as between samples from cancer and healthy patients. All *p*-values are two-sided and *p*-values of less than 0.05 were considered to be statistically significant. We verified the ability to use *GIMAP8* expression levels for ovarian cancer differentiation, using a receiver-operating characteristic (ROC) curve analysis and diagnostic test.

Results

RNA-sequencing analysis demonstrated that signaling from ovarian cancer cells causes gene expression changes in PBMCs

To discover novel biomarkers in PBMCs, this study followed the

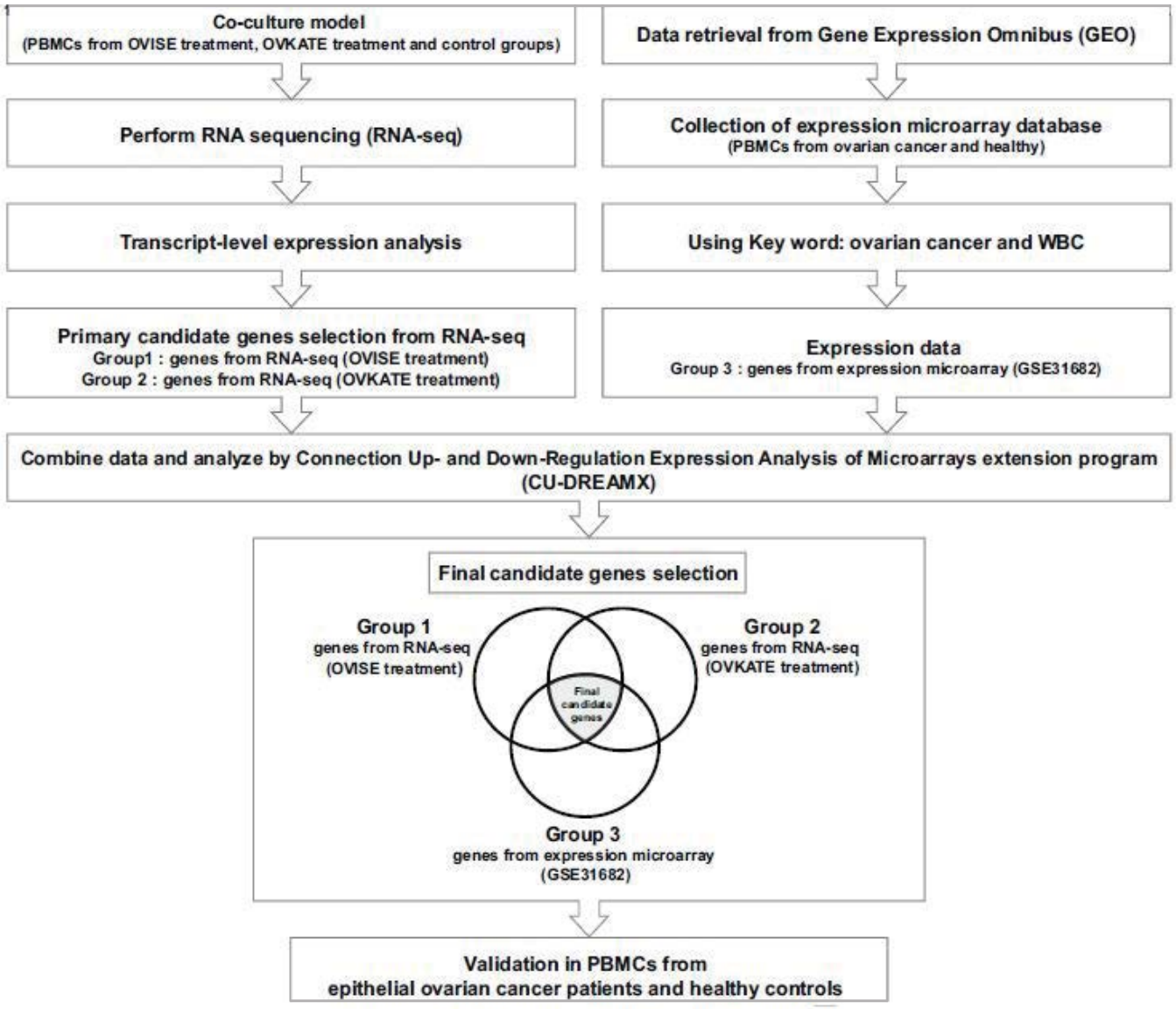

Figure 1: Summary of the experimental design for epithelial ovarian cancer detection in peripheral blood mononuclear cells (PBMCs).

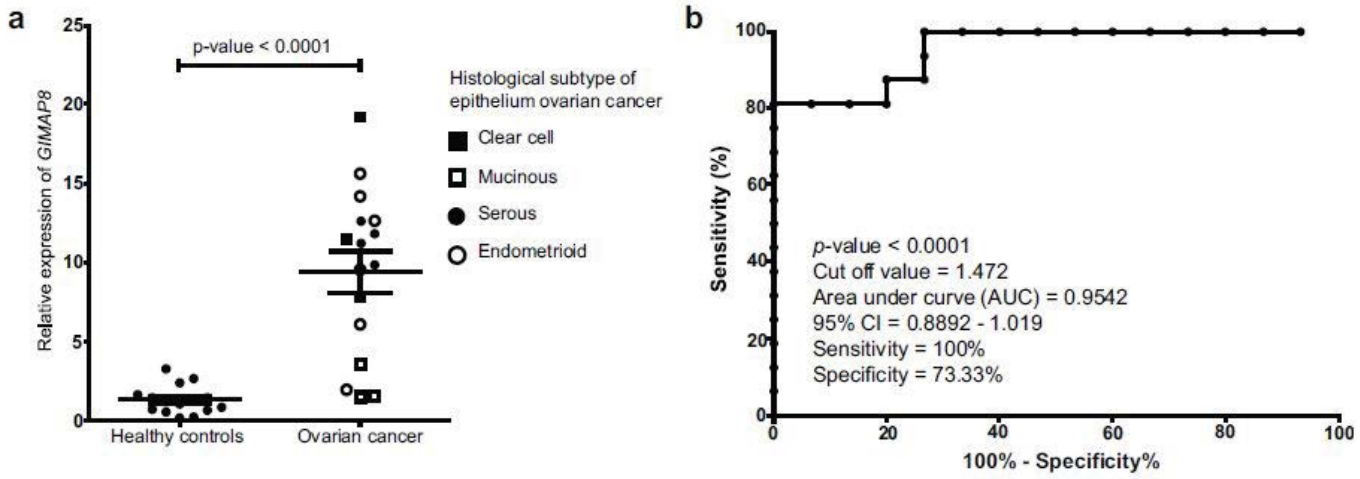

Figure 2: *GIMAP8* expression in PBMCs. (a) Comparison of epithelial ovarian cancer patients and healthy controls. (b) The receiver-operating characteristic (ROC) curve of *GIMAP8* expression for differentiation.

steps of analysis that are summarized in Figure 1. The RNA sequences from our co-culture model were high quality, as shown in Table S2. After a differential expression analysis, the primary candidate genes from group one (OVISE experiment) included 234 up-regulated genes and 235 down-regulated genes with a p-value < 0.05. The number of significant up- and down-regulated genes from group two (OVKATE experiment) were 203 and 171 genes, respectively (Table S3).

Bioinformatics analysis of PBMCs expression from RNA sequencing and expression profiling

The significantly up- and down-regulated genes from RNA-sequencing of group one were integrated together with the expression profiling data from group three. A combined analysis of the data from both groups resulted in 56 significantly associated up-regulated genes with an odds ratio of 1.457 and a p-value of 0.021. A similar analysis combining groups two and three resulted in 42 significantly associated up-regulated genes with an odds ratio of 1.638 and a p-value of 0.010. The down-regulated genes from all data groups were not significantly associated (Table S4). The significantly up-regulated genes that intersected from the expression array and both groups of RNA-sequencing, including the OVISE and OVKATE experiments, were categorized by function in biological process terms. Gene ontology indicated that cancer induced PBMCs demonstrated expression changes in genes associated in cellular and metabolic process (Figures S1 and S2). Furthermore, combination of the three data groups showed that cyclin dependent kinase inhibitor 1B (*CDKN1B*), GTPase IMAP family member 8 (*GIMAP8*), and Stannin (*SNN*) were the final candidate genes. These genes are highly expressed in PBMCs co-cultured with ovarian cancer cell lines as well as in PBMCs from ovarian cancer patients.

GIMAP8 expression in PBMCs from ovarian cancer patients and healthy controls

Since it had the highest expression in PBMCs, *GIMAP8* was selected to validate gene expression in ovarian cancer patients and healthy controls. PBMCs from 16 ovarian cancer cases had a mean *GIMAP8* expression of 9.410 ± 1.321, while PBMCs from 15 healthy controls had a mean *GIMAP8* expression of 1.310 ± 0.230. *GIMAP8* expression was high in ovarian cancer patients when compared with healthy controls (p-value < 0.0001) as shown in Figure 2. This confirmed the bioinformatics finding. We then evaluated the feasibility of using the differences in expression of *GIMAP8* in PBMCs from ovarian cancer patients and healthy controls as a diagnostic clinical test. The relative expression of *GIMAP8* in PBMCs could differentiate ovarian cancer patients from healthy controls with a sensitivity of 100%, a specificity of 73.33% (Figure 2), a positive predictive value of 80%, and a negative predictive value of 100% by using a cut off at 1.472. We also analyzed data by histological subtypes of EOC, there are three cases of clear cell, three cases of mucinous, four cases of serous, and six cases of endometrioid adenocarcinoma. The results revealed that all patients with serous and clear cell adenocarcinoma were detected by this marker with 100% sensitivity and specificity using a cut off at 5.518.

Discussion

Our study demonstrated that ovarian cancer cells synthesize and secrete signaling molecules that can influence PBMCs' gene expression. These secreted substances can be various compounds depending on the type of cancer, tumor aggressiveness, or other factors [4,28]. Nevertheless, ovarian cancer cells may release common signals that effect gene regulation in PBMCs. We focused on altered PBMCs as a representative target for cancer screening detection. This approach is conducive to an easy and non-invasive technique. We designed a co-culture model to simulate directed communication between ovarian cancer cells and PBMCs. The goal of our experiment was to identify gene expression changes in PBMCs as a result of exposure to cancer cells. We performed RNA sequencing and analyses for PBMCs from the co-culture model. Compared to a microarray, this technique provides more coverage of transcript expression analysis and provides a lot of data for further interpretation. The effect of cancer cells on PBMCs in a patient may be different from that of our co-culture experiment. Therefore, we supported our cell culture evidence with expression profiling of ovarian cancer patients' PBMCs. Modifications in PBMCs from patients could be a result of cancer, but other factors such as infection or inflammation may also play a role.

To determine if gene expression changes in cancer induced PBMCs could be used as a novel biomarker, we combined data from RNA-sequencing with expression microarray data. Both the PBMC data sources explained some molecular biology transformations within

PBMCs due to the influence of ovarian cancer cells and both displayed gene expression changes. Indeed, up-regulated genes were significant in ovarian cancer induced PBMCs. These genes function mainly in cellular and metabolic processes. Importantly, the combined results identified three genes, including *CDKN1B*, *GIMAP8*, and *SNN*.

We demonstrated that the evaluation of gene expression changes of *GIMAP8* in PBMCs may serve as a potential biomarker for ovarian cancer detection. The relative expression of *GIMAP8* was significantly increased in PBMCs derived from patients with various subtypes of epithelial ovarian cancer, especially serous and clear cell adenocarcinoma, when compared to healthy controls. The quantitative evaluation of *GIMAP8* in PBMCs was clear because these subtypes of cancer were used in our co-culture signaling model. The different subtypes of ovarian cancer demonstrated ambiguous patterns of gene expression associated with inflammation [29]. Thus, some molecular factors from ovarian cancer could be involved with alteration of PBMCs or WBCs. These include TNF, CXCL12, and IL6, which play important roles in angiogenesis and immune suppression for the supporting ovarian cancer microenvironment [30,31]. Additionally, our previous research reported that the decreased methylation of long interspersed elements (LINE-1) in ovarian cancer tissues was important in multistep carcinogenesis [32]. Accordingly, molecular modifications in cancer-induced PBMCs may be due to epigenetic regulation.

GIMAP8, also known as immune-associated nucleotide-binding protein 9, encodes a protein in the GTP-binding superfamily and immuno-associated nucleotide (IAN) subfamily. The predicted sequence of this gene has three GTP-binding domains and a mass of 77 kDa. The *GIMAP* gene family plays a role in immune reactions and controlling cell survival and death [33]. There is no previously reported association of *GIMAP8* with ovarian cancer, but analyses of non-small cell lung cancer revealed that *GIMAP8* had decreased expression in the tumor, while high levels of *GIMAP8* were showed in non-tumor surrounding tissue when compared with control lung tissues [33]. This finding was similar to expression changes in plasma cells in the lymph nodes of breast cancer metastasis [7] that suggested immune cells were responding to cancer cell interactions.

A clinical diagnosis of ovarian cancer commonly involves evaluating CA125 blood serum levels. Increased CA125 levels are found in only 50% of patients with early stage disease, while 80% of patients with late stage are detected [13,34]. Additionally, elevated CA125 levels may be caused by benign conditions such as endometriosis, renal dysfunction, and hepatic disease [12,13]. HE4 is one of the serum biomarkers with more specificity than CA125 [12]. Expression of HE4 protein is specific to endometrioid and serous epithelial ovarian cancer [34]. CA125 and HE4 were examined in women with a pelvic mass and 79.6% and 81.7% of sensitivity and 82.5% and 85.1% of specificity was reported, respectively [13]. Recently, we developed an approach to identify an alternative target for ovarian cancer detection and increase the advantage for screening asymptomatic women. The result showed an increased level of *GIMAP8* expression in PBMCs with a sensitivity of 100% and specificity of 73.33% in differentiating epithelial ovarian cancer patients and healthy controls. We also examined *GIMAP8* expression in each subtype of epithelial ovarian cancer. Mucinous adenocarcinoma did not demonstrate significant differences in *GIMAP8* expression compared to healthy controls, but prognosis of this subtype is good. On the other hand, *GIMAP8* expression showed 100% sensitivity and specificity in the serous and clear cell adenocarcinoma subtypes of ovarian cancer. Therefore, access to this test will be very important for those patients.

Conclusion

In conclusion, substances secreted from ovarian cancer cells have the ability to alter gene expression regulation in PBMCs. Most notably, the differential up-regulation of GIMAP8 in PBMCs could be utilized in further studies of ovarian cancer screening, therapy and prognosis.

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Ethics Approval and Consent to Participate

All of the protocols were reviewed and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Thailand (IRB no. 313/60).

Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available in the [Gene Expression Omnibus (GEO)] repository [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110745].

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