의학박사 학위 논문

상피성 난소암에서 면역세포와 암줄기세포의 상관관계

Correlation of cancer stem cells with immune cells in epithelial ovarian cancer

울 산 대 학 교 대 학 원

의 학 과

이 건 우

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지 도 교 수　김 용 만

이 논문을 의학박사 학위 논문으로 제출함

2023년 8월

울 산 대 학 교　대 학 원

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감사의 글

오랫동안 본 논문을 준비하면서 많은 분의 도움이 있었습니다. 모든 분께 심심한 감사의 말씀을 드리며, 본 논문의 작은 열매가 성숙하기까지 도움을 주신 분들을 생각하며, 감사의 글을 시작하고자 합니다.

많은 시간과 노력을 쏟으며 제게 지속적인 지도와 격려를 해주신 김용만 교수님, 이신화 교수님께 무한한 감사를 표합니다. 스승님의 다양한 지식과 경험, 아낌없는 지도는 저의 학문적 성장에 많은 도움이 되었으며, 헌신적인 지도와 지원 없이는 이 연구를 완성할 수 없었을 것입니다. 또한, 인자한 웃음으로 따뜻하고 말씀으로 저를 격려해주신 김영탁 교수님과 김대연 교수님께도 깊은 감사의 마음을 표현합니다. 교수님께서 말씀해주신 소중한 조언은 이 논문을 크게 향상하게 시키는 데 도움이 되었습니다. 마지막으로 이 연구에 필요한 자료를 제공하고, 도움을 주신 강성완 연구원에게도 진심으로 감사드립니다.

이 모든 분께 감사의 말씀을 전하며, 이 논문이 모두의 노력과 지원을 바탕으로 완성되었다는 사실에 큰 감사와 자부심을 느낍니다.

Abstract

Correlation of cancer stem cells with immune cells in epithelial ovarian cancer

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Objective: The aim of this study was to identify mechanisms for overcoming t he resistance to immunotherapeutic strategies in the treatment of ovarian cancer The author sought to elucidate the correlation between cancer stem cells, which are considered the primary cause of resistance, and the tumor microenvironment, characterized by inflammation, invasion exclusion, and immune deserts. In epithel ial ovarian cancer, the author's goal was to understand this correlation by confir ming the significance of cancer stem cell markers (CD133, ALDH, and CD44) a nd immune cell markers (CD3, CD4, CD8, Foxp3, and CD56), and PD-L1.

Materials & Methods: The author utilized paraffin blocks from patients aged 1 9 and above who underwent surgical treatment for epithelial ovarian cancer at S eoul Asan Hospital, affiliated with Ulsan University College of Medicine. Tissue microarray (TMA) slides were prepared from these blocks. The Opal multiplex i mmunohistochemistry assay, was used to stain the prepared slides, and the stain ed samples were quantitatively analyzed using the Vectra 3.0 Automated Quantit ative Pathology Imaging System. Immunofluorescence staining with Opal™, was performed and the correlation between each marker in the immunofluorescently s tained tissues was analyzed. Patient information was used to examine the correl ation between disease-free survival (DFS) and overall survival (OS).

Results: The author compared the correlation between cancer stem cells and im mune cells separately. Pearson correlation coefficients raging 0.2 to 0.5 were obs erved in CD3/CD44, CD4/CD8, and Oct4/ALDH12. A significant correlation was f

ound between CD 44 (p=0.43) and tumor-infiltrating lymphocytes (CD3, and CD 8). Regression analysis was conducted for each group, revealing a highly signific ant positive correlation (p<0.001) between CD8 and CD4 counts. Statistical signif icant differences were observed for CD3, CD4, tumor-infiltrating lymphocytes (C D3+CD4+CD8), CD44, Oct4, ALDH12, CD133, Foxp3. DFS was significantly asso ciated with CD44/CD3 expression (p=0.024), while OS showed significance in Oc t4 (p<0.001), CD133 (p=0.044), CD44/CD3 (p=0.014), and CD8/Foxp3 (p=0.034).

Conclusion: This study evaluated the correlation between immune cells and can cer stem cells in ovarian cancer, providing a research methodology that reduces subjective interpretation by pathologists during conventional immunohistochemical staining. While previous studies have explored the correlation between cancer st em cells and prognosis in ovarian cancer, this study is unique in its investigatio n of the relationship between immune cells and cancer stem cells. It identifies m arkers with positive or negative correlations.

Key words: ovarian cancer, tumor stem cell, immune cell, cell surface marker

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Introduction

Ovarian cancer ranks as the third most common cancer affecting the female reproductive system and the ninth most prevalent cancer globally[1]. Epithelial ovarian cancer, which constitutes the majority of ovarian cancer cases, is associated with the highest mortality rates. Early stages of the disease, typically lack specific symptoms, resulting in approximately 75% of cases being diagnosed at advanced stages, notably stages 3 and 4, discouraging 5-year survival rate of 20-25%. Standard treatment primarily involves surgical intervention and platinum-based chemotherapy. Nevertheless, more than 70% of patients experience relapse[2]. The growing elderly population and Westernization have contributed to increased incidence, rates, highlighting the inadequacy of conventional cytotoxic chemotherapy and the pressing need for effective treatment modalities and strategies to enhance their efficacy.

Recently immune checkpoint inhibitors have garnered substantial interest and are being increasingly applied. However, similar to traditional treatments, the development of resistance poses a significant challenge. Currently, no well-defined biomarkers or mechanisms are associated with this resistance. To overcome resistance to immunotherapy, it is imperative to elucidate the mechanism by which cancer stem cells contribute to treatment resistance and explore the tumor microenvironment, characterized by inflammation, immune exclusion, and immune deserts.

Tumor-infiltrating lymphocytes (TILs) exhibit vacations depending on the cancer type or tumor compartment. Inflammatory cancers are marked by abundant CD8+ T cells, which can be modulated through immunotherapies targeting immune tolerance. Conversely, immune desert tumors lack T cells, which heightens the risk of immunotherapy failure T cell priming[3].

1. Cell Markers

CD3

CD3 is an integral component of the T cell receptor (TCR) complex and plays a pivotal role in signal transduction and activation of T cells. T cells are vital for the immune system, as they eliminates infected or aberrant cells, recognize antigens, and initiate immune responses. Upon antigen recognition, CD3 binds to the TCR, facilitating signal transduction and T cell activation, Comprising four protein subunits, the CD3 complex forms complexes with TCRs. The CD3 complex amplifies TCR signal transmission T cell survival, differentiation, proliferation, and cytotoxicity.

CD4

CD4 is a crucial receptor found on T cells within the immune system. Its primary function is to recognize antigens and facilitate the activation of T cells. CD4+ T cells also serve as surface markers for T helper cells. A decline in the quantity of CD4+ T cells can result in immune deficiency, potentially fostering the emergence and advancement of diverse cancers. Additionally, CD4+ T cell count serves as an indicator to assess the efficacy of cancer treatments. Reduced CD4+ T cells counts can diminish the effectiveness of cancer treatment, underscoring their significance as vital markers for evaluating both cancer treatment outcomes and prognosis.

CD8

CD8 is a T cell receptor present in the immune system, serving as a surface marker for cytotoxic T cells responsible for eliminating tumor cells or infected cells. CD8+ T cells, upon antigen recognition, directly destroy tumor cells or aid in destruction. These CD8+ T cells play essential roles in cancer prevention and treatment. A reduction in the number of CD8+ T cells can result in immunodeficiency, thereby increasing the susceptibility to cancer. Conversely, an increase in the number of CD8+ T cells following cancer treatment can indicate the effectiveness of the anti-cancer effects. Tumor cells often employ immune evasion mech

anisms, but CD8+ T cells are capable of recognizing and eliminating these tumo r cells. While both CD8 and CD4 act as T cell receptors and are critical compon ents of the immune system, CD4 serves as a mediator of the immune response, distinguishing it from CD8.

CD56

CD56, also referred to as the Neural Cell Adhesion Molecule (NCAM), is expres sed in natural killer (NK) cells, CD8+ cells, and dendritic cells. It possesses the ability to recognize and induce the apoptosis of tumor cells without requiring pri or stimulation.

Foxp3

Foxp3 is a transcription factor that plays a crucial role in the proliferation of re gulatory T cells, which are responsible for suppressing autoimmune responses a nd maintaining immune system homeostasis. This transcription factor is vital for the development and functional of regulatory T cells (Tregs). It has been identifi ed as an inducing factor that converts naive T cells into a CD4+CD25+ regulato ry T cell phenotype, suggesting its involvement in the regulation of immune tole rance. In individuals with ovarian cancer, Foxp3 expression is observed in cance r cells, primarily through regulatory T cells, and it promotes the growth of canc er cells.

CD44

CD44 is crucial transmembrane glycoprotein acting as the primary receptor for h yaluronate. Its functions encompass cell-to-cell interactions, cell adhesion, and m igration. CD44 has been implicated in various processes, including lymphocyte ac tivation, recirculation, homing, hematopoiesis, anti-cancer chemotherapy, and the development of drug resistance in tumor progression[4,5,14]. In ovarian cancer, the interaction between CD44 and hyaluronate, triggers Nanog-Stat-3 activation[5]. St udies utilizing paclitaxel and CD44 short interfering RNA (siRNA) to target CD4 4 have demonstrated induced cell apoptosis, reduced tumor size, and minimal si de effects[6]. Extensive research on CD44 has been conducted in breast, prostate, pancreatic, and head and neck squamous cell carcinomas. Notably, in ovarian ca

ncer, the fusion of dendritic cells with ovarian cancer-initiating cells. It has been decrease in CD44+ cancer stem clusters, CD44 has been identified as a potential marker in combination with other ovarian cancer stem cells[6,7].

CD133

CD133 is a well known marker present in hematopoietic stem cells and progenitor cells, and its expression has been identified in various types of stem cells. It has been reported to play a role in inhibiting differentiation[8,9]. The study of CD133 expression in ovarian cancer was initially conducted by Ferradina et al., who observed higher levels of the antigens CD133-1 and CD133-2 in malignant tumors compared to normal ovarian tissues and benign tumors[10-13]. CD133 is commonly utilized as a marker to distinguish tumor cells from other cells. In ovarian cancer, CD133 positive (CD133+) stem cells exhibit a greater capacity for clone formation and proliferation than CD133 negative (CD133-) stem cells, Furthermore, CD133+ cells display higher expression levels of Sox2, Nanog, and Oct3/4 compared to CD133+ cells[12]. Historically, CD133 was the first stem cell surface marker to be extensively investigated, and its involvement in ovarian cancer has provided evidence regarding the progression of this disease[15,16].

ALDH(Aldehyde dehydrogenase)

Aldehyde dehydrogenase (ALDH) amplifies the features of cancer stem cells in epithelial ovarian cancer and imparts resistance to anticancer drugs. Moreover, ALDH is pivotal in immune-based anticancer therapies. ALDH positive(ALDH+) tumor cells prominently participate in immune evasion mechanisms and are implicated in suppressing anti-cancer immune responses, thereby contributing to an unfavorable prognosis. However, ALDH alone is inadequate as a marker for cancer stem cells. Notably, studies combining CD44 with other markers have yielded valuable findings in this regard.

Oct4

Oct4 is a transcription factor that governs the expression of target genes and plays crucial role in the self-renewal and differentiation of embryonic stem cells. It is also expressed during various cellular processes, including cell differentiatio

n, migration, growth, survival, and division. In the context of epithelial ovarian c ancers, Oct4 is frequently upregulated or overexpressed, potentially contributing t o the recurrence and chemoresistance observed in this cancer type. This pattern aligns with other cancer stem cells. Oct4 is associated with tumors exhibiting u ndifferentiated phenotypes and sharing similar characteristics.

PD-L1

PD-L1 is a protein present in immune cells, certain cancer cells, and normal cell s. However, it does not serve as a marker for cancer stem cells or immune cell s. When expressed on the surface of tumor cells, PD-L1 inhibits T cell activatio n, leading to evasion of the immune system. This expression of PD-L1 by tum or cells can suppress immune response against the tumor and impair immune memory, consequently promoting resistance to treatment. As a result, PD-L1 e xpression has been associated with resistance to immune checkpoint blockade th erapy.

The objective of this study, was to establish the correlation between various ca ncer stem cell markers (CD133, ALDH, CD44, CD56, and Oct4) and immune cell markers relevant to cancer cells (CD3, CD4, CD8, Foxp3, and CD56), along and PD-L1 in ovarian cancer. Additionally, we sought to investigate the significance of these markers in relation to patient prognosis.

Materials and Methods

1. Patients and specimen collection

The study cohort consisted of patients diagnosed with epithelial ovarian cancer who underwent primary cytoreductive surgery at Asan Medical Center, affiliated with Ulsan University College of Medicine, between 1998 and 2013. A total of 186 patients with complete data were included in this study. The median age of the patients was 51 years, with a range of 25 to 78 years. More than 80% of the patients were classified as FIGO stages III-IV, and 82.5% received combined therapy with paclitaxel and carboplatin following primary cytoreductive surgery. Neoadjuvant chemotherapy was administered to 7.5% of the patients, and optimal debulking achieved in 66.8% oif the cases. The median progression-free survival (PFS) was 17.0 months, and the median overall survival (OS) was 58.0 months.

2. Tissue microarray

Tissue microarrays were conducted as a prerequisite for performing multiplex immunofluorescence, To ensure enhanced precision and reliability of the analysis, regions of the primary tumor tissue comprising over 70% cancerous tissue were meticulously selected based on microscopic evaluation performed by a gynecologic pathologist. Cores measuring 0.6mm in diameter were extracted from these regions and subsequently subjected to formalin-fixation and paraffin-embedding (FFPE). FFPE technique is widely employed in pathology as it allows for long-term preservation of tissue without compromising its integrity. This standardized method facilitates the application of various staining techniques and ensures accurate evaluation of pathological features by preserving intercellular structures. By employing tissue microarrays in conjunction with FFPE, hundreds of distinct tissue samples can be analyzed on a single slide, leading to cost and time efficiency while enabling repeated experiments using the same tissue. Hence, this methodology was employed in the current study.

3. Multiplexed immunofluorescene

Multiplex immunofluorescence is employed to simultaneously visualize multiple a ntigens. Each antigen binds to a distinct fluorochrome and emits a unique fluore scent color. To ensure accurate detection, the emission spectra of the fluorochro mes must not overlap. Although OPAL™ allows for up to eight concurrent mea surements, typically only seven antigens are detected in practice. This is becaus e the 8th fluorochrome-(4′,6-diamidino-2-phenylindole (DAPI)) is used to labele d nuclear DNA. DAPI serves as a potent nuclear stain enabling the identification and quantification of cells, while the specific antigens labeled by other fluorochro mes can be correlated with each individual cell. Although additional fluorochrome s could be used, their emission spectra must be distinguishable. Currently, techn ological limitations make adjusting the emission spectrum of fluorochromes challe nging. Consequently, in practice, immunofluorescence detection typically involves only 7 additional antigens.

FFPE sections were subjected to multiplex immunofluorescence staining. Tissue microarray blocks were cut to standardized thickness of 4μm using rotary microt ome. This thickness allowing for optical observation and visualization of tissue s tructure and cells under an optical microsope. It is important to maintain this thi ckness to minimize the risk of compromising the structural characteristics of the tissue. Furthermore, this thickness is globally standardized, ensuring comparabilit y and reproducibility of research finding.

The sections were placed on positively charged slides, immunofluorescent stainin g was performed using a Leica Bond Rx™ Automated Stainer (Leica Biosystems, Wetzlar, Germany). The slides were dewaxed with Leica Bond Dewax solution (AR9222, Leica Biosystems, Wetzlar, Germany), followed by a 30-minute antigen retrieval process using Bond Epitope Retrieval 2 (AR9640, Leica Biosystems). St aining was conducted sequentially starting with blocking using an antibody dilue nt/block (ARD1001EA, Akoya Biosciences, Marlborough, MA, USA). This was f ollowed by a 30 minute incubation with the primary antibody and a 10-minute i

ncubation with the OPAL™ polymer HRP Ms+Rb (ARH1001EA, Akoya Biosciences, Marlborough, MA, USA). Visualization of the antigen was achieved using Tyramide Signal Amplification (TSA, Akoya Biosciences, Marlborough, MA, USA) for 10 minutes, Subsequently, Bond Epitope Retrieval 1 (AR9961, Leica Biosystems, Wetzlar, Germany) was applied for 20 minutes before proceeding to the next step in the staining sequence.

The primary antibodies used for each protein and their corresponding TSA are as follows (Figure 1): anti-CK (AE1/AE3, NBP2-29429, Novus Biologicals, Centennial, CO, USA) with CK at 780, anti-CD4 (EPR6855, Ab133616, Abcam, Cambridge, UK) with CD4 at 480, anti-CD8 (4B11, MCA1817, Bio-Rad, Hercules, CA, USA) with CD8 at 520, anti-CD56 (EP2567Y, Ab75813, Abcam, Cambridge, UK) with CD56 at 570, anti-Foxp3 (236A/E7, Ab20034, Abcam, Cambridge, UK) with Foxp3 at 620, anti-PD-L1 (E1L3N, 13684S, Cell Signaling Technology, Denver, MA, USA) with PD-L1 at 690, anti-CD3 (2GV6, 790-4341, Ventana Medical Systems, Oro Valley, AZ, USA) with CD3 at 480, anti-CD133 (Ab19898, Abcam, Cambridge, UK) with CD133 at 570, anti-CD44 (Ab157107, Abcam, Cambridge, UK) with CD44 at 620, anti-Oct4 (Ab18976, Abcam, Cambridge, UK) with Oct4 at 690, and anti-ALDH12 (H-8, sc-166362, Santa Cruz, Dallas, TX, USA) with ALDH12 at 520.

The process from the blocking stage to the antigen retrieval step was repeated for all antibody stains. After the final round of antigen retrieval, nuclei were stained with DAPI (62248, Thermo Scientific, Waltham, MA, USA) to provide contrast staining. To protect the fluorescent samples, minimize loss of fluorescent signals, and reduce background noise, the slides were covered with ProLong Gold antifade reagent (P36935; Invitrogen, Waltham, MA, USA).

4. Multispectral image processing and data analysis

The slides stained with multiple markers were scanned at 20x magnification using the Vectra Polaris Automated Quantitative Pathology Imaging System (Akoya Biosciences, Marlborough, MA, USA). Regions of interest (ROI) were selected

within the tissue microarray (TMA) using Phenochart software (Akoya Biosciences, Marlborough, MA, USA), and analyzed was performed using image Analysis software (InForm 3.0; Akoya Biosciences). Spectral library compounding was not employed for the multispectal images. Each individual cell was segmented based on DAPI staining (nuclear spectral elements), and phenotypes were assigned based on the intensity and expression compartments of each marker in the panel. The same algorithm was applied in batch mode. The count of CD3, CD4, CD8, CD56, Foxp3, PD-L1, CD133, CD44, Oct4, ALDH, and CK positive cells per square millimeter was determined in each core of the tissue microarray slides.

5. Statistical Analysis

GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) was utilized for both graphical and statistical analyses, Additionally SPSS 26.0 (IBM Corporation, Armonk, NY, USA) was employed for statistical evaluations. The correlation between marker expression intensity and patient survival was assessed through regression analysis and Kaplan-Meier survival analysis ($p<0.05$).

Results

The experimental group comprised 186 samples, derived from tumor cells of pati ents diagnosed with epithelial ovarian cancer. Only tissues specifically associated with epithelial ovarian cancer were included in the analysis. The distribution of cancer stem cell markers in the samples was as follows: CD44 (38.3%), Oct4 (3 1.5%), ALDH12 (21.1%), CD133 (8.4%), and PD-L1 (0.7%). Among the immune cell markers examined, the percentages were as follows: CD3 (40.0%), CD56 (19. 8%), CD4 (19.3%), CD8 (13.1%), and Foxp3 (7.8%).

Analysis of Cancer Stem Cell Markers and Immune Stem Cell Markers

The author investigated the correlation between cancer stem cells and immune s tem cells. Although no strong associations were observed with Pearson correlati on coefficient values of 0.5 or higher, wer did identify moderately correlated rela tionships with values raging from 0.2 to 0.5 in CD3/CD44, CD4/CD8, and Oct4/A LDH12. Notably, tumor-infiltrating lymphocytes (CD3, and CD8) exhibited signifi cant correlation with CD44 (p=0.43) (see Table 1). This correlation is visually re presented in Figure 2.

A regression analysis was performed for each group, and the significant results are depicted in Figure 3. There was a highly significant positive correlation bet ween CD8 and CD4 (p<0.001). Similarly, significant correlations were observed b etween CD3 and CD44, tumor-infiltraing lymphocytes (TIL) including (CD3, CD 4, and CD8), CD44, Oct4 and ALDH12. The expressions of CD133 and ALDH12 were statistically significant (p=0.02), as were Foxp3 and CD4 (p=0.0), and CD3 and Oct4 (p=0.02). Although not reaching statistically significance, CD44 and CD 133 (p=0.07) demonstrated some suggestive association.

Progression Free Survival and Overall Survival

Irrespective of platinum sensitivity, the participants had a median overall surviva

l 58.5 months. The median overall survival for the platinum-sensitive group was 74 months, and while for the platinum-resistant group, it was 23.5 months. The progression-free survival was 14 months, regardless of platinum sensitivity, with the platinum-sensitive group demonstrating 23 months of progression-free surviv al and the platinum-resistant group showing 2.5 months.

An analysis was conducted to assess the progression-free survival of the target group and the associated markers. The expression levels of each marker were c ategorized into high and low expression groups based on the median value. The classification into high and low groups was determined by comparing the overall values to the median value.

Regarding the analysis of overall survival, a highly significant difference was ob served between the two groups for Oct4 (p<0.001), and there was a significant difference for CD133 (p=0.044). When the markers were combined and analyzed, CD44/CD3 exhibited a significant difference (p=0.014), as did CD8/Foxp3 (p=0.03 4). However, the remaining markers did not show significant differences betwee n the two groups (Figure 4).

In terms of the analysis of progression-free survival, Oct4 (p=0.53) and CD133 (p=0.70) did not exhibit significant values, but while only CD44/CD3 showed a s ignificant value (p=0.024) (Figure 5).
When stratified according to platinum sensitivity, the platinum-sensitive group s howed demonstrated a significantly higher expression (p=0.034) of the CD8/Foxp 3 group (Figure 6).

Table 1. Correlation table between tumor stem cell markers and immune cell markers

		CD3(%)	CD4(%)	CD8(%)	CD133(%)	CD44(%)	ALDH12(%)	CD56(%)	Oct4(%)	Foxp3(%)	PDL1(%)
CD3(%)	Pearson correlation	1	.025	.033	.100	.235**	.098	-.009	-.149*	.014	.044
	Significance probability (two-sided)		.701	.616	.126	.000	.133	.891	.022	.825	.498
CD4(%)	Pearson correlation	.025	1	.467**	-.011	.107	.036	-.047	.011	.143*	.078
	Significance probability (two-sided)	.701		.000	.863	.098	.585	.473	.865	.027	.226
CD8(%)	Pearson correlation	.033	.467**	1	.022	.020	.019	-.040	.051	-.010	.058
	Significance probability (two-sided)	.616	.000		.731	.757	.773	.533	.438	.876	.375
CD133(%)	Pearson correlation	.100	-.011	.022	1	.117	.147*	.025	.016	-.041	-.008
	Significance probability (two-sided)	.126	.863	.731		.070	.023	.702	.806	.533	.901
CD44(%)	Pearson correlation	.235**	.107	.020	.117	1	.007	-.007	-.036	-.062	.121
	Significance probability (two-sided)	.000	.098	.757	.070		.919	.920	.576	.338	.063
ALDH12(%)	Pearson correlation	.098	.036	.019	.147*	.007	1	-.018	.444**	.023	-.004
	Significance probability (two-sided)	.133	.585	.773	.023	.919		.784	.000	.720	.952
CD56(%)	Pearson correlation	-.009	-.047	-.040	.025	-.007	-.018	1	-.006	-.022	.017
	Significance probability (two-sided)	.891	.473	.533	.702	.920	.784		.932	.736	.792
Oct4(%)	Pearson correlation	-.149*	.011	.051	.016	-.036	.444**	-.006	1	.040	-.056
	Significance probability (two-sided)	.022	.865	.438	.806	.576	.000	.932		.535	.390
Foxp3(%)	Pearson correlation	.014	.143*	-.010	-.041	-.062	.023	-.022	.040	1	.015
	Significance probability (two-sided)	.825	.027	.876	.533	.338	.720	.736	.535		.812
PDL1(%)	Pearson correlation	.044	.078	.058	-.008	.121	-.004	.017	-.056	.015	1
	Significance probability (two-sided)	.498	.226	.375	.901	.063	.952	.792	.390	.812	

**.Correlations were significant at the 0.01 level (two-tailed).

*. Correlations were significant at the 0.05 level (two-tailed).

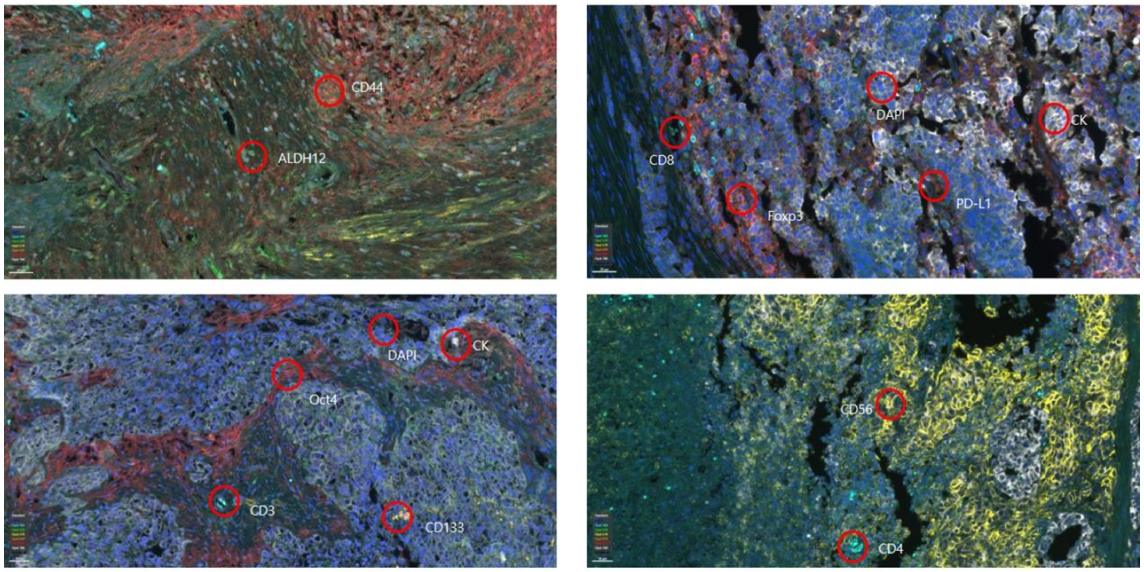

Figure 1. In the above picture, the left and right sides correspond to each group. 780 for anti-CK and CK, 480 for anti-CD4 and CD4, 520 for anti-C D8 and CD8, 570 for anti-CD56 and CD56, 620 for anti-Foxp3 and Foxp3, anti-PD-L1 and 690 for PD-L1, 480 for anti-CD3 and CD3, 570 for anti-C D133 and CD133, 620 for anti-CD44 and CD44, 690 for anti-Oct4 and Oct 4, and 690 for anti-ALDH12 and ALDH12 It is 520.

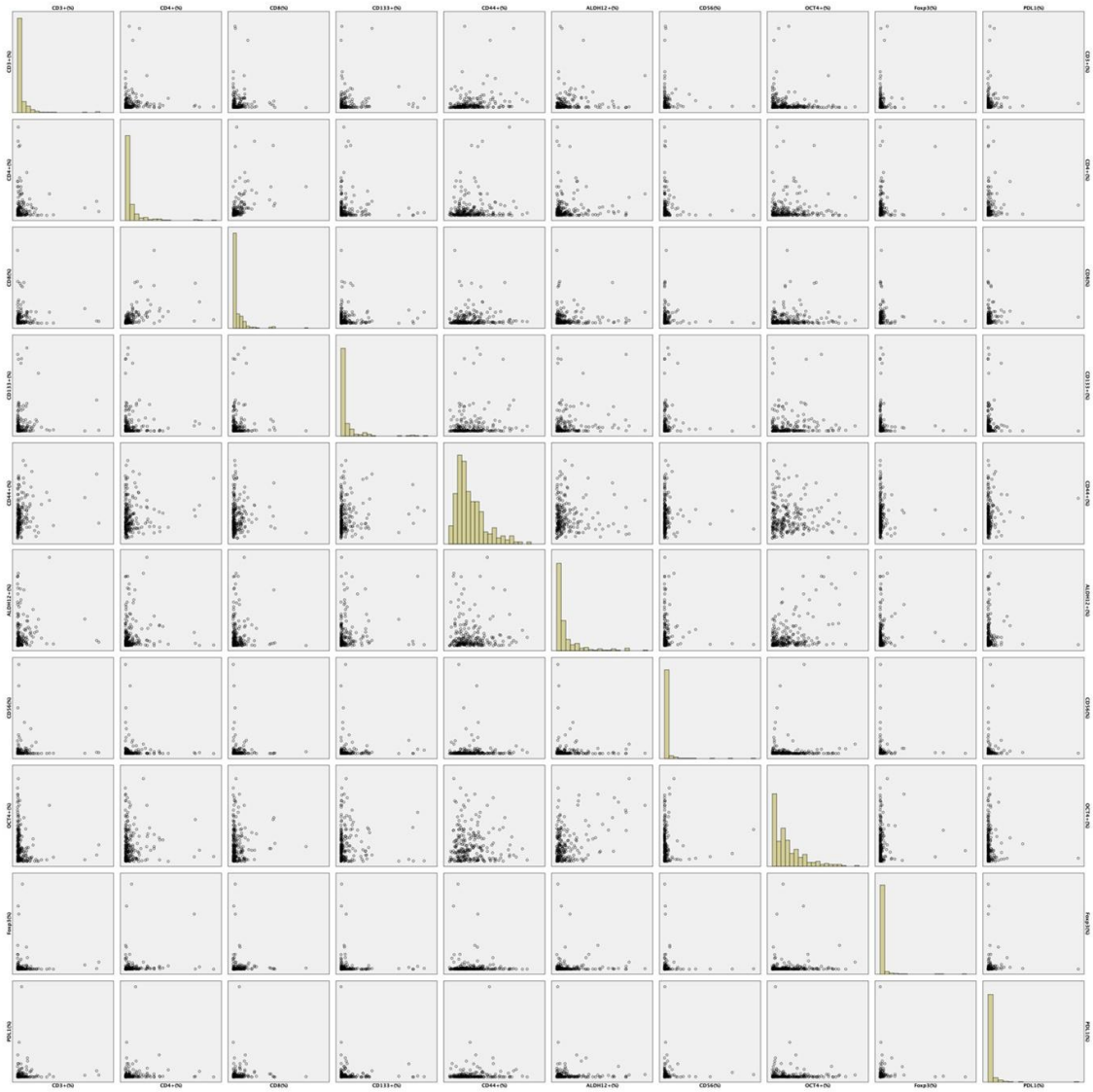

Figure 2. Scatter plot of tumor stem cell markers and immune cell markers

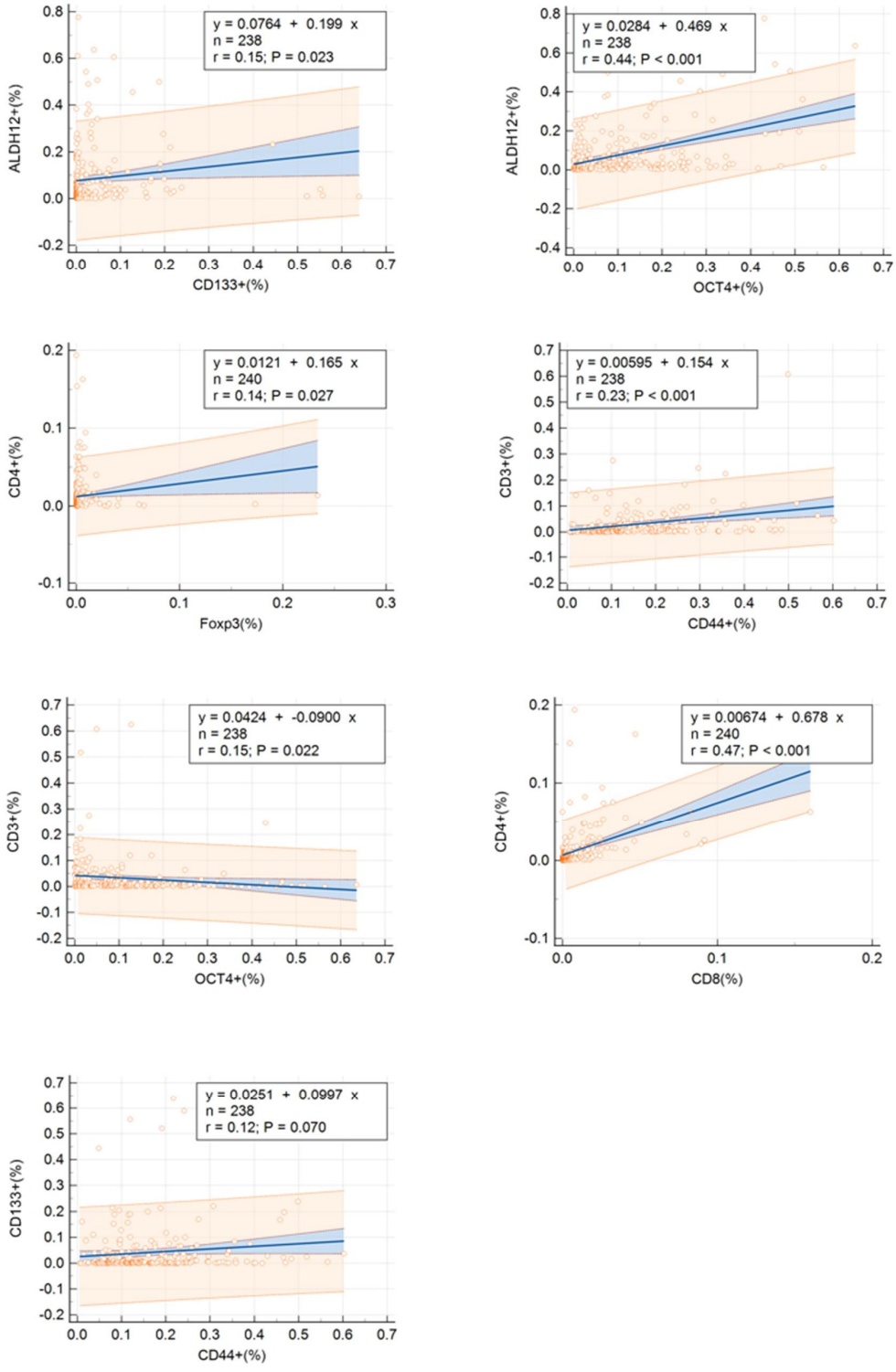

Figure 3. Regression analysis of cancer stem cell markers and immune cell markers

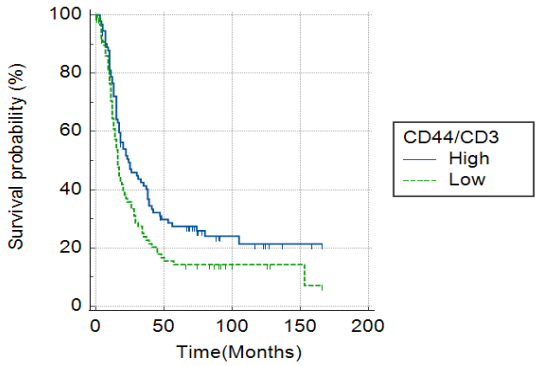

Figure 4. Relationship between markers and disease-free survival rate

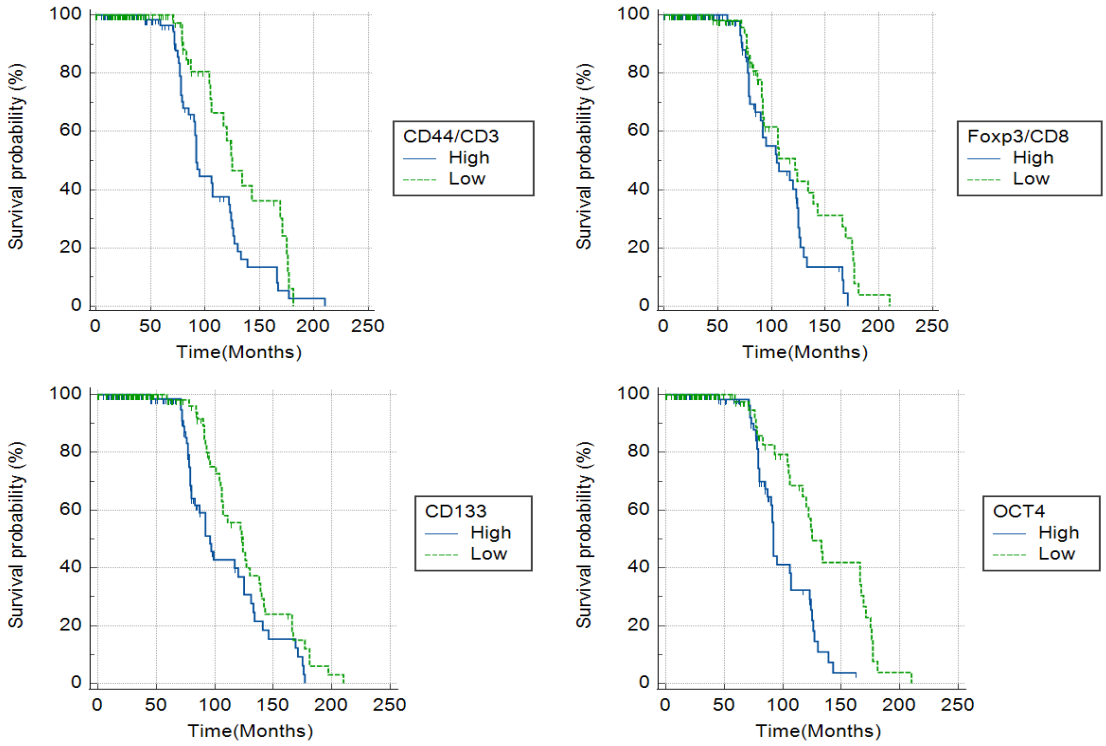

Figure 5. Relationship between markers and overall survival rate

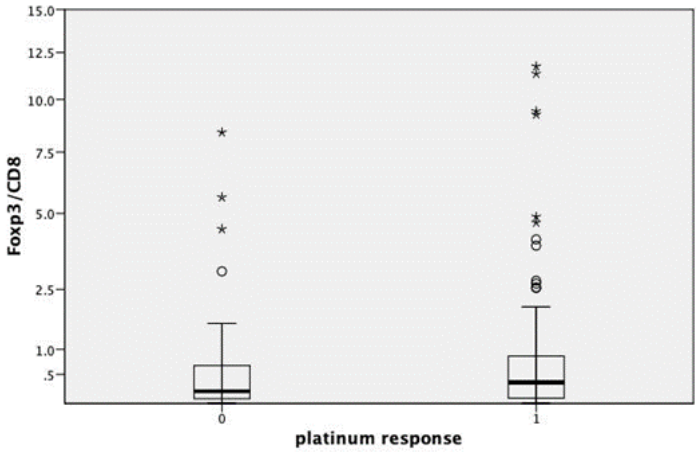

Figure 6. Relationship between markers and platinum sensitivity.

Discussion

This study aimed to investigate the association between the expression of cance r stem cell markers and immune cell markers. Previous studies have examined marker expression using immunohistochemical staining. However, simultaneously verifying multiple markers within the same tissue pose challenges. In this study, we employed immunofluorescence staining to quantitatively measure the fluoresc ence intensity of each marker, facilitating a more objective analysis.

Research findings regarding epithelial ovarian cancer have exhibited variability due to diverse testing methods, nonquantitative scoring methods for maker expre ssion assessment, variations in the composition of normal and tumor tissues, and distinctions between epithelial and stromal cells. In epithelial ovarian cancer, tum or cells and non-tumor stromal cells present a complex scenario compared to ot her cancer types, making it difficult to differentiate between invaded and non-in vaded areas in clinical settings. Tumors can invade the stroma of the ovary or f allopian tube, where epithelial ovarian cancer originates, or metastasize to abdom inal organs and the peritoneum. While it is feasible to differentiate between tum ors and normal cells, it does not explain the heterogeneity at each site. This stu dy offers insights for sue in real clinical settings by analyzing overall marker ex pression rather than distinguishing and analyzing the cells surrounding tumor cel ls.

CD56 expression confirms natural killer (NK) cells, which have been reported to be exhibit cytotoxic against cancer stem cells and tumor-initiating cells. In oral squamous cell carcinoma, the potential of NK cells is increased compared to th at in well-differentiated tumor cells, and enhanced NK cell cytotoxicity can targ et and eliminate tumor-initiating cells: in osteosarcoma[19,20]. A study non-small c ell lung cancer demonstrated a negative correlation between NK cells and CD13

3, suggesting that higher NK cell counts result in lower CD133 expression[21]. Several studies have investigated the association between CD56 expression and tumors in epithelial ovarian cancer[22,23]. However, this study did not observe such an association, indicating that NK cells may have been suppressed by other factors[24].

Other ovarian cancer studies have reported positive correlations between CD133/ALDH and CD133/CD44[17,18]. In this study, a significant positive correlation was found for CD133/ALDH, while CD133/CD44 did not exhibit significant correlation (p=0.07), although it displayed a positive correlation. A lung cancer study reported a positive correlation between CD133 and Oct4, along with their association with tumor initiation[19]. Although statistically significant results were not obtained for these two markers, in ths study, there could be a substantial correlation when considering ALDH as a mediator. Bösmüller et al. discovered that in high-grade serous ovarian cancer, the number of tumor-infiltrating lymphocytes improved prognosis, with $CD3^{high}$/$CD103^{high}$ patients exhibiting a 5-year survival rate of 90%, $CD3^{low}$/$CD103^{high}$ showing 63%, and $CD3^{low}$/$CD103^{low}$ nearly 0% (p<0.001)[25]. The CD8/PD-L1 ratio is linked to platinum sensitivity during cancer treatment and significantly impacts patient survival. CD8/FoxP3 is also associated with survival. However, such correlations were not observed in the present study.

Conclusion

This study has offered insightful observations into the association between immune cells and cancer stem cells in ovarian cancer. We have introduced a novel research methodology that curtails subjective biases from pathologists during the standard immunohistochemical staining. Unlike preceding studies that mainly focused on the correlation between cancer stem cells and prognosis in ovarian cancer, this study has broken new ground by probing the relationship between immune cells and cancer stem cells. Importantly, we have identified markers that exhibit either positive or negative correlations, offering a valuable contribution to our understanding and potentially guiding future research and treatment strategies in ovarian cancer.

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국 문 요 약

상피성 난소암에서 면역세포와 암줄기세포의 상관관계

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목적: 난소암의 치료에 있어 기존 치료에 저항을 보이는 가장 큰 이유로 생각되고 있는 암 줄기세포와 염증, 침윤 배제, 면역 사막 등으로 표현되는 암의 미세환경과의 연관성이 있을 것으로 보고되었다. 상피성 난소암에서 의미 있다고 알려진 암 줄기세포 표지자 (CD133, ALDH, CD44, Oct4)와 암세포에 대응하는 면역 세포 표지자(CD3, CD4, CD8, Foxp3, CD56), PD-L1 을 확인하여 상관관계를 파악하고자 한다.

대상 및 방법: 울산대학교 의과대학 소속 서울아산병원에서 수술적 치료를 받은 19 세 이상의 상피성 난소암 환자의 파라핀 블록을 조직미세배열 (tissue microarray, TMA)을 이용하여 슬라이드를 제작하였다. 제작된 슬라이드를 Opal multiplex immunohistochemistry assay® 를 이용하여 염색을 시행하였다. 염색한 표본을 Vectra 3.0 Automated Quantitative Pathology Imaging System® 을 이용하여 이미지 정량 분석을 시행하였으며, Opal ™을 이용하여 면역형광염색을 시행하였다. 면역형광염색된 조직을 각 표지자 사이의 상관관계를 분석하였다. 환자의 정보에 적용하여 무병생존율(Disease Free Survival)과 전체생존율(Overall Survival)과 연관성을 분석하였다.

결과: 암 줄기세포와 면역 줄기세포 간의 상관관계를 각각 비교하였으며, 피어슨 상관계수 0.2 에서 0.5 사잇값이 CD3/CD44, CD4/CD8, Oct4/ALDH12 에서 관찰되었다. 종양침윤림프구(CD3, CD8)는 CD 44 (p=0.43)와 유의한 상관관계가 관찰되었다. 각 군에 대하여 회귀분석을 시행하였으며, CD8 과 CD4 는 양의 상관관계를 가지면서 $p<0.001$ 로 매우 유의한 관계를 보였으며, CD3 과 CD44, TIL (CD3,CD4,CD8)과 CD44, Oct4 와 ALDH12, CD133 과 ALDH12, Foxp3 와 CD4, CD3 와 Oct4 에서 통

계적으로 유의함이 관찰되었다. 무병생존율은 CD44/CD3 에서 유의하였다(p=0.024). 전체생존율은 Oct4(p<0.001), CD133(p=0.044), CD44/CD3(p=0.014), CD8/Foxp3(p=0.034)로 유의하였다.

결론: 난소암에서 면역 세포와 종양 줄기세포의 연관성을 평가한 연구이며, 기존 면역조직화학염색의 경우 병리학자의 주관적 판독으로 인한 인적 요소를 최소화 할 수 있는 방법에 관한 연구이다. 난소암에서 종양 줄기세포와 예후의 연관성에 관한 문헌은 기존에 있었으나, 이와 다르게 면역 세포와 종양 줄기세포 사이의 연관성을 연구하였으며, 양의 상관관계 또는 음의 상관관계를 갖는 표지자들이 있음을 알 수 있는 연구이다.

중심 단어: 난소암, 종양 줄기세포, 면역 세포, 세포표지자