CD44+/CD24-Flow Cytometry and Immunohistochemistry on Therapy Response to the Administration of Chemotherapy in Ovarian Cancer

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Abstract

Ovarian cancer is one of the deadliest women's cancers. There is much chemoresistance to ovarian cancer standard therapy. $CD44^+/CD24^-$ have an essential role in developing chemoresistance and poor prognosis for the patients. We aimed to study $CD44^+/CD24^-$ relationship with ovarian cancer chemoresistance and its ability to predict chemoresistance. This study is an ambispective cohort study of 32 people in each group at the Obstetrics-gynecology and pathology Department of Cipto Mangunkusumo, Tarakan, Dharmais, and Fatmawati Hospital. All suspected ovarian cancer patients will undergo cytoreductive debulking and histopathological examination. Chemotherapy will be given for six series followed by six months of observation. We determine therapy response with the RECIST Criteria (Response Criteria in Solid Tumours) then classify it into chemo-resistant or chemo-sensitive groups. Our study is the first study examining $CD44^+/CD24^-$ in ovarian cancer from flow cytometry blood test and directly from ovarian cancer tissue by double immunohistochemistry. We found a significant relationship between increased levels of $CD44^+/CD24^-$ expression ($p < 0,05$) with chemoresistance of ovarian cancer from both studies while immunohistochemistry has a better multivariate analysis result. Both studies indicate that $CD44^+/CD24^-$ was significantly associated with ovarian cancer chemoresistance and $CD44^+/CD24^-$ immunohistochemistry is a better predictor.

Introduction

Ovarian cancer is the 8th deadliest of women's cancer worldwide. In 2018, there were 295.414 new cases with 184.799 deaths worldwide. In Indonesia, there were 13.310 (7,1%) ovarian cancer cases of 188.231 cancer with an incidence of 9,7 per 100.000 patients [1]. Ovarian cancer's average lifetime risk of developing cancer is 1.3% or 1 in 78 women. Only 20% of women survive more than five years after being diagnosed with ovarian cancer [2]. This is partly because the predominant type is high-grade serous carcinomas and the absence of typical symptoms at the outset and no way for early diagnosis [3].

Almost all patients experienced disease recurrence after standard therapy (cytoreductive surgery followed by chemotherapy). Standard treatment had chemosensitivity, and chemoresistance rates of 77,4% and 18,1%, respectively, with a 12-month progression-free survival (PFS) with overall survival (OS), was about 30 months [4, 5]. The low survival rate of ovarian cancer patients is due to chemotherapy resistance caused by cancer stem cells (CSCs) protein. CSC's research has made tremendous progress in recent years, and it may be used as a target for the ovarian cancer therapy [6].

CSCs are a new term that is believed to play an essential role in the initiation, tumour growths, metastasise, recurrence, and the presence of resistance. CD44 and CD24 are cell-surface transmembrane glycoproteins. CD44 and CD24 have a role in tumour formation, progression, chemoresistance, poor prognosis, and disease recurrence. CD44 overexpression was found in drug-resistance ovarian cancer cell lines and correlates with tumour recurrence following chemotherapy [6]. CD44 has higher expression in paclitaxel-resistant cell lines, while the downregulation of CD44 by miR-199a-3p correlates significantly with ovarian cancer cells' chemosensitivity to cisplatin and paclitaxel, and Adriamycin [7]. CD24 is

starting to be widely believed to be an independent prognostic factor of patient survival. CD24 could be a new genetic therapy target for the ovarian cancer [8].

Meng *et al*. flow cytometry study said that ovarian stem cell cancer with $CD44^+/CD24^-$ was 71–93% resistant to all chemotherapy agents, a recurrence rate of 83% (p = 0,003), and median PFS of 6 months [9]. Hu *et al.* found that $CD44^+$ was positively expressed in the chemotherapy-resistant ovarian cancer significantly 91,17% with p < 0,05[10]. Klemba *et al*. stated that the ovarian cancer population with high $CD44^+/CD24^-$ has high drug resistance, invasion ability and differentiation potential [2].

At first, most cancer cells are sensitive to chemotherapy, but some CSCs are not detected and develop into disease recurrence. The CSC resistance will make the cells continue to move into the mitotic and interphase (G1, S, G2), while the G0/rest phase is inactive, continuously replicating [11]. CSCs have a slow-cycling rate and are resistant to standard chemotherapy. This is a problem because the chemotherapy target is actively proliferating cells. The cell will remain in the G0 phase, so the CSCs' quiescence presents a significant issue because most treatment regimens target actively divide cells in the S or M phase [6]. This study aimed to find relationships between $CD44^+/CD24^-$ with chemotherapy response in ovarian cancer and its ability to predict ovarian cancer chemotherapy response.

Materials And Methods

Study Design

This study is an ambispective cohort (prospective and retrospective cohort) at the obstetrics-gynaecology and anatomical pathology department of Cipto Mangunkusumo Hospital, Tarakan Hospital, Dharmais Hospital, and Fatmawati Hospital from February 2018 until February 2022.

Participants

The research subjects were patients with ovarian carcinoma inclusion, stage II-IV ovarian cancer patients, and were willing to participate in the study. The sample exclusion criteria were pregnant patients and patients diagnosed with other types of cancer. The number of samples in this study was 32 people in each group with consecutive sampling methods.

Data Collection

All patients suspected of ovarian cancer will undergo cytoreductive debulking and histopathological examination. If the pathology result is malignant, chemotherapy will be given for six series followed by six months observation. We determine therapy response with the RECIST Criteria (Response Criteria in Solid Tumours) then classify it into chemo-resistant or chemo-sensitive groups. The patient will perform Flow cytometry blood tests to examine the expression of $CD44^+/CD24^-$ (prospective study), while an immunohistochemistry examination will be performed on ovarian cancer tissue (retrospective study). In addition, demographic data, cancer stage, operation type, chemotherapy response, tumour cell differentiation (cancer stage), cancer histopathology, cancer size, cancer residue, ascites, lymph node

metastasis, and serum Ca-125 levels were also taken. The staging of the disease was carried out using the FIGO criteria.

Flow cytometry method

Blood was taken from peripheral blood at five ml and centrifugated, and 50 µL was left. Their markers identified expression $CD44^+/CD24^-$. Samples were reacted with fluorescent-labeled antibody against $CD44^+/CD24^-$ (monoclonal anti-human) $CD44^+$ labeled as PerCP, $CD24^-$ labeled as APC. The reagents were removed for leukocytes with CD45 labelled pacific blue. The samples in the Falcon tube were added with 2,5 µL of CD44 marker, 2,5 µL of CD24 marker, then incubated for 15 minutes in the dark at room temperature. After incubation, cells were lysed using 300 µL of lysing solution, then set again for 15 minutes in a dark room and at room temperature. Next, 1 mL of facs flow solution was added and centrifuged at 500 g for 5 minutes, then added with 500 µL perm wash buffer and centrifuged at 500 g for 5 minutes. To be more optimal, 1mL perm wash buffer was added again and centrifuged at 500 g for 5 minutes. The last step was to add 200 µL of 1% paraformaldehyde in phosphate-buffered saline (PBS). After that, the analysis was carried out using a flow cytometer using four fluorochrome colours.

Flow cytometry cell count

Cell identification was carried out using an automated flow cytometer (*BD Facs Calibur*). CSCs were identified through the positive expression of $CD44^+/CD24^-$ markers. Protein percentage is the percentage of expression of protein markers CSCs ($CD44^+/CD24^-$) in the blood.

Immunohistochemistry slide preparations

The examination used paraffin block specimens. In each case, eight preparations were made from paraffin blocks which were cut with a microtome with a thickness of 3 µm and placed on a poly-L-lysine-coated slide, then dried at 37°C and heated on a slide warmer at 600°C for 30 minutes. Then, it deparaffinised using graded xylol (xylol I, II, and III, for 5 minutes each) and rehydrated with serial alcohol (96% and 80% alcohol, respectively, for 4 minutes), then washed with running water for 5 minutes. Furthermore, we carried a blocking method to inhibit endogenous peroxidase activity using 1.5% hydrogen peroxide in methanol for 10 minutes at room temperature. It was rewashed with running water for 5 minutes. The next step was pretreatment using *Tris EDTA* acid (pH 9.0) in a decloaking chamber at 960 degrees Celsius for 10 minutes, cooled for 45 minutes, and washed in phosphate-buffered saline (PBS) at pH 7.4. After that, we carried a blocking method to non-specific protein using Background Sniper Universal for 15 minutes.

Detection of CSC markers ($CD44^+/CD24^-$) used specific antibodies against $CD44^+$ (Monoclonal anti-human $CD44^+$ and $CD24^-$) The preparations were incubated with primary antibody $CD44^+$ and $CD24^-$ (1:500 dilution). After one hour, it was washed with PBS (pH 7.4) for 5 minutes. The painting is done in a cocktail (double staining). Each preparation was then incubated with a secondary antibody against biotin-labelled mouse immunoglobulin (Trekkie Universal Link) for 20 minutes and then washed again in PBS (pH 7.4) for 5 minutes. Next was incubation with trackAvidin-HRP labelled for 15 minutes, then

washed in PBS (pH 7.4) for 5 minutes. Then, diaminobenzene (DAB) was mixed with 1 mL of a substrate and vortexed for 15 seconds. The substrate containing DAB was dripped onto the preparation, incubated for 2 minutes, and washed with running water for 10 minutes.

Next, it was counterstained with CAT (Counterstain Kit) hematoxylin for 5 seconds and washed with running water for 5 seconds. The preparation was immersed in saturated lithium carbonate (5% in distilled water) for 5 seconds, then washed with running water for 5 minutes. The dehydration process was carried out with graded alcohol (80%, 96%, absolute, absolute) for 5 minutes each and clearing with graded xylol (xylol I, II, and III) for 5 minutes each. The preparation was closed using a mounting solution and a cover glass. Each smear included an internal positive control on the stromal tissue and a negative control without primary antibodies. Positive and negative controls were performed on the same tissue as the tumour tissue.

Double Immunohistochemistry

The immunohistochemistry preparations were observed using a Leica ICC 50 HD microscope. Positivity for the markers $CD44^+/CD24^-$ was seen in the cytoplasm of tumour cells (diffuse or atypical) so that expression calculations were carried out on cells stained in the cytoplasm. The $CD44^+$ marker was brown mainly on cell membrane staining while the $CD24^-$ marker was red, especially on cytoplasmic and membrane staining. For $CD44^+$, all cells that showed brown membrane and/or cytoplasmic were defined as positive [12]. The CD44+/CD24- marker showed a more dominant brown staining than red. High expression of $CD44^+/CD24^-$ is > 10% cells while if the percentage of $CD44^+/CD24$ < 10% cells it could be categorized as low expression.

Statistical Analysis

We analyse univariate and bivariate data. Each categorical variable was tested with the chi-square or alternative Fisher test. ROC and AUC curves were used to test the flow cytometry and immunohistochemistry $CD44^+/CD24^-$ variable as a predictor of therapy response to ovarian cancer. We performed a multivariate analysis to compare the power between $CD44^+/CD24^-$ flow cytometry and immunohistochemistry to predict ovarian cancer chemoresistance.

Ethical clearance

Research ethics approval was obtained from the Health Research Ethics Committee of the Universitas Indonesia, Cipto Mangunkusumo Hospital.

Results

Basic Participants Characteristics

The total sample in this study was 32 samples in each group. All samples had undergone chemotherapy with 32 (50%) chemoresistance and 32 (50%) chemosensitive for each flow cytometry and

immunohistochemistry study. The distribution of profiles and clinical characteristics of ovarian cancer patients can be seen in Table 1.

Table 1
Essential Clinical Characteristics of Ovarian Cancer Patient

Variable	Number (%)
• Chemoresistant	32 (50)
• Chemosensitive	32 (50)
Age (years old)	4 (6,3)
• < 40	19 (29,7)
• 40–50	41 (64,1)
• > 50	
Ca-125	30 (46,9)
• ≤35	34 (53,1)
• > 35	
Ovarian cancer stage	5 (7,8)
• Early stage: II	59 (92,2)
• Advance stage: III - IV	
Operation type:	56 (87,5)
• Optimal Debulking	8 (12,5)
• Suboptimal Debulking	
Differentiation/cancer grade	13 (20,3)
• Good	16 (25,0)
• Intermediate	35 (53,1)
• Poor	
Tumour histology type	24 (37,5)
• Serous	14 (21,9)
• High-grade serous	3 (4,7)
• Mucinous	12 (18,8)
• Endometrioid	10 (15,6)
• *Clear cell*	1 (1,6)
• Others	

Variable	Number (%)
Lymph nodes metastasize	32 (50)
• Positive	32 (50)
• Negative	
Ascites	36 (56,3)
• Positive	28 (43,7)
• Negative	
Tumour size	17 (26,6)
• 5 cm	15 (23,4)
• 5–10 cm	32 (50)
• > 10 cm	
Tumour residue	56 (87,5)
• < 1cm	8 (12,5)
• > 1cm	

Flow cytometry of Ovarian cancer

Figure 2 and 3 shows the example results of flow cytometry of ovarian cancer patients. The proportion of $CD44^+/CD24^-$ values were calculated based on the percentage of the total cells. $CD44^+/CD24^-$ was highly expressed in chemoresistance ovarian cancer patients and similar to previous studies.

Bivariate Analysis

Table 2 shows that $CD44^+/CD24^-$ flow cytometry and immunohistochemistry data, Ca-125, type of surgery, lymph node metastasize, tumour size and tumour residue have significant difference results ($p < 0,05$) with each odds ratio (OR) and relative risk (RR) values. Flow cytometry $CD44^+/CD24^-$ has OR 10.7 and 3.19 while immunohistochemistry $CD44^+/CD24^-$ has OR 134.3 and 22.6. Thus, $CD44^+/CD24^-$ immunohistochemistry has a higher OR and RR value.

Table 2
Bivariate Analysis of The Variables in Ovarian Cancer Patients.

Variable	Therapy Response		P value	OR	RR
	Chemoresistant (%)	Chemosensitive (%)			
$CD44^+/CD24^-$ flowcytometry	25 (78,1)	8 (25)	0,001*	10.7	3.19
• High (≥32.692)	7 (21,9)	24 (75)			
• Low (< 32.692)					
$CD44^+/CD24^-$ immunohistochemistry	26 (81.3)	1 (31.1)	0.000*	134.3	22.6
• High (≥10%)	6 (18.8)	31 (96.9)			
Low (< 10%)					
Ca-125 Level	2 (6,25)	28 (87.5)	0,001*	105	7,93
• ≤35	30 (93,75)	4 (12,5)			
• > 35					
Ovarian cancer stage	1 (3,13)	4 (12,5)	0,162	4.42	1.68
• Early stage: II	31 (96,87)	28 (87,5)			
• Advance stage: III - IV					
Surgery type	25 (84,4)	31 (96,87)	0,023*	8.68	4.43
• Optimal Debulking	7 (15,6)	1 (3,13)			
• Suboptimal Debulking					
Differentiation/cancer grade	6 (18,75)	7 (21,88)	0,760	1,21	1.09
• Good	26 (81,25)	25 (78,12)			
• Intermediate - Poor					
Lymph nodes metastasize	21 (65,63)	11 (34,37)	0,012*	3.65	1.91
• Positive	11 (34,37)	21 (65,63)			
• Negative					
Ascites	18 (56,25)	14 (43,75)	1,000	1	1
• Positive	14 (43,75)	18 (56,25)			
• Negative					

Note: *: p < 0,05, Significant results.

Variable	Therapy Response		P value	OR	RR
	Chemoresistant (%)	Chemosensitive (%)			
Tumour size	6 (18.8)	8 (25)	0.545	1.44	1.19
• ≤5 cm	26 (81.2)	24 (75)			
• > 5 cm					
Tumour residue	25 (84,4)	31 (96,87)	0,023*	8.6	4.43
• < 1cm	7 (15,6)	1 (3,13)			
• > 1cm					
Note: *: p < 0,05, Significant results.					

Multivariate analysis

Multivariate analysis by logistic regression results showed in Table 4. We found from this calculation that immunohistochemistry data of $CD44^+/ CD24^-$ has a significant compared with flow cytometry data of $CD44^+/ CD24^-$. Thus, $CD44^+/ CD24^-$ immunohistochemistry is a better predictor of ovarian cancer chemoresistance in this research.

Table 3

AUC analysis comparison of $CD44^+/CD24^-$ flow cytometry data and immunohistochemistry data

Variable	AUC	SD	95% *CI*	Sensitivity (%)	Specificity (%)	P value
$CD44^+/ CD24^-$ Flow cytometry	0.766	0.62	0.65–0.89	78	75	0,000*
$CD44^+/ CD24^-$Immunohistochemistry	0.891	0.45	0.80–0.98	81	96	0,000*
Note : *: p < 0,05, significant results.						

Table 4

Logistic regression of flow cytometry and immunohistochemistry data of $CD44^+/CD2$

No	Variables	Beta value (β)	Standard deviation	Wald	p value	Exp (β)	*95% CI*
X1	$CD44^+/ CD24^-$ flow cytometry	1,493	0,847	3.104	0.078	4.450	0.043–1.183
X2	$CD44^+/ CD24^-$ immunohistochemistry	4,483	1,131	15.719	0.000*	88.487	0.001–0.104
Constant		**-6.731 (β0)**	**1,103**	**11.417**	**0,001**		**-**

Note : *: p < 0,05, significant results.

ROC and AUC Curves

ROC curve in Fig. 1 and Table 3 data showed that the immunohistochemistry $CD44^+/CD24^-$ protein has the best ROC curve and AUC value. The AUC value of the immunohistochemistry $CD44^+/CD24^-$ is 0.891, which means it has a good level of accuracy with a significant value (p < 0,05). The sensitivity is 81%, and its specificity is 96% for detecting chemoresistance. Flow cytometry $CD44^+/CD24^-$ had an AUC of 0.766, which mean it has a fair level of accuracy with a significant value (p < 0,05), with a sensitivity of 78% and specificity of 75%.

Discussion

We found overexpression of $CD44^+/CD24^-$ in chemoresistance ovarian cancer patients both from flow cytometry blood study and immunohistochemistry study direct from ovarian tissue. $CD44^+$ (cluster of differentiation 44) and $CD24^-$ (set of differentiation 24) overexpression is associated with increased ovarian cancer oncogenesis and progression [9, 13]. It is related to metastasizing, recurrence, chemoresistance, and poor survival rates in the ovarian cancer [14]. $CD44^+$ overexpression has also been found in the pancreatic cancer [15], breast cancer [16], gastric cancer [17], urothelial bladder cancer [18] and colorectal cancer [19]. $CD24^-$ is a cell surface adhesion molecule frequently detected in invasive ovarian carcinoma. High $CD24^-$ expression in invasive ovarian cancer predicts shorter overall survival than low $CD24^-$ markers [20]. $CD24^-$ overexpression has been found in many tumours such as ovarian cancer, breast cancer and lung cancer field [21, 22]. Therapeutic blockade and genetic ablation of CD24 resulted in reduced tumour growth in vivo and increased the survival time [22].

$CD44^+/CD24^-$ flow cytometry and immunohistochemistry are good predictors of ovarian cancer chemoresistance. We found that the immunohistochemistry $CD44^+/CD24^-$ AUC value is 0.891 (good accuracy) with significant value (p < 0,05). The sensitivity is 81%, and its specificity is 96% for detecting

chemoresistance. Flow cytometry $CD44^+/CD24^-$ AUC is 0.766 (fair accuracy) with significant value ($p < 0,05$), with a sensitivity of 78% and specificity of 75%. Flow cytometry and immunohistochemistry of $CD44^+/CD24^-$ both are proven to be a good predictor of ovarian cancer chemoresistance.

Some of the cancer cells CSCs subpopulations had stem-cell-like properties such as self-renewal capacity, differentiation capacity, aggressiveness, migration, tumorigenesis and chemoresistance. $CD44^+/CD24^-$ previously well known in has been associated with breast cancer metastasis, chemoresistance and poor prognosis. Meng et al.'s cell lines and cell culture study found that the $CD44^+/CD24^-$ subpopulation of ovarian cancer also has the characteristics of cancer stem cells. $CD44^+/CD24^-$ in ovarian cancer correlates with aggressiveness, invasion, migration, tumorigenicity and chemotherapy resistance. Clear cell cancer type of ovarian cancer is an aggressive histology subset, and it showed 99% of $CD44^+/CD24^-$ while the serous papillary type has 66–77% of $CD44^+/CD24^-$. Ovarian cancer with high $CD44^+/CD24^-$ has shorter median progression-free survival (6 vs 18 months)[9].

Five studies with 417 cases in a meta-analysis study showed that $CD44^+$ protein was associated significantly with poorer cancer-specific survival rates in patients undergoing chemoradiotherapy. CD44 is one of the most common reported markers in many cancers. A study with 83 cases found that CD44v9 expression was linked to a worse 5-years cancer-specific survival [23]. The 5-years survival rate is 29% for the advanced stage of ovarian cancer and 14% for the advanced stage of the colorectal stage. A study by Zhang *et al.* (2019) found that CD105, $CD44^+$, and CD106 high expression is related to chemoresistance, poorly differentiated cancer cells [14], invasion properties and chemoresistance [24]. A study from 92 cases of ovarian tumours showed that $CD24^-$ expression rate is correlated positively with malignancy, clinical-stage, and metastasis [25].

A study by Li *et al.* (2017) found that high $CD44^+/CD24^-$ in breast tumours related to higher proliferation rates, metastasis, and tumourigenesis. Expression of CD44/CD24 and $ALDH1^+$ were associated with malignancy of different subtypes of breast cancer. A combination of CD44/CD24 has been used to describe the stemness of cancer cells for a long time because a single CSC marker alone was not enough to characterise the stem-cell-like properties of the cancer [26]. Yan *et al.* (2013) also found that cells with high $CD44^+/CD24^-$ expression showed higher migration and invasion properties and were the cause of chemoresistance [24].

Some previous studies also found that $CD44^+/CD24^-$ is involved in other cancers. High expression of $CD44^+/CD24^-$ in gastric carcinoma patients can predict a decreased disease-free survival. $CD44^+/CD24^-$ has a valuable prognostic combination in ovarian cancer, pancreatic cancer, and other solid cancers. Interaction between $CD44^+/CD24^-$ with osteopontin, epidermal growth factor (EGF), and fibroblast growth factor (FGF) can lead the CSCs to induce self-renewal and promotes cell invasion and metastasis. CD24 high expression has been identified in liver, pancreatic and breast cancer [27]. CD44 and CD24 expression also correlated with lower cancer-specific survival in the urothelial bladder cancer [18]. Expression of CD44 and CD24 was also found both in the membrane and cytoplasm of pancreatic cancer cells. CD44

and CD24 are upregulated in human pancreatic cancer related to the development of pancreatic cancer [28].

To our knowledge, our study is the first study examining $CD44^+/CD24^-$ in ovarian cancer directly from the blood by flow cytometry study and from the fresh ovarian cancer tissue by double cocktail immunohistochemistry. However, even though we found strong evidence from both studies that $CD44^+/CD24^-$ has correlations with ovarian cancer chemoresistance from both studies, we still need further investigations because $CD44^+/CD24^-$ ovarian cancer phenotype maybe not be the sole cause of the chemoresistance. Advance research is also required to prove that $CD44^+/CD24^-$ potentially can be used as genetic therapy target for ovarian cancer in the future.

Conclusion

We conclude a significant relationship between increased $CD44^+/CD24^-$ ($p < 0,05$) with ovarian cancer chemoresistance from flow cytometry and immunohistochemistry studies. $CD44^+/CD24^-$ immunohistochemistry is a better predictor of ovarian cancer chemoresistance.

Declarations

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Consent to participate: Informed consent was obtained from all participants included in the study.

Consent to publish: there is no personal human private data published.

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Figures

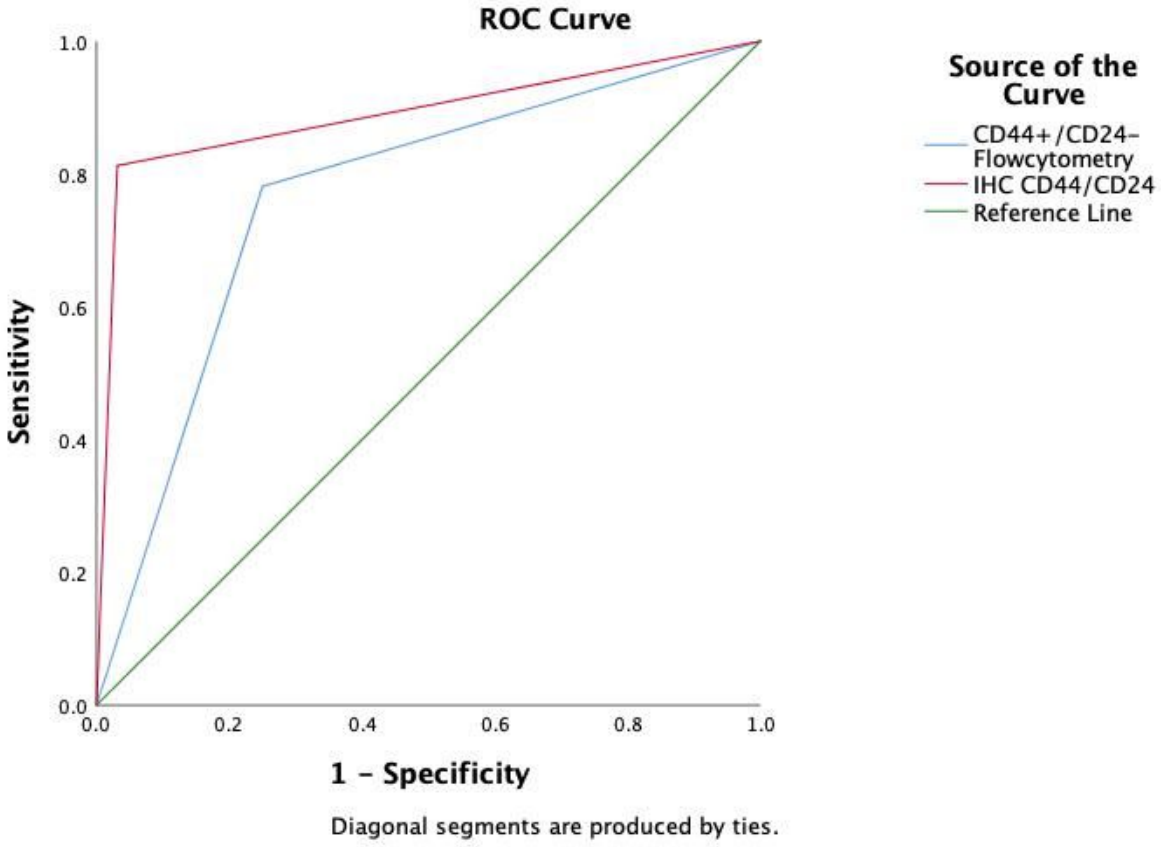

Figure 1

ROC Curve of $CD44^{+}/CD24^{-}$ flowcytometry (blue line) and immunohistochemistry (red line). $CD44^{+}/CD24^{-}$ immunohistochemistry has a better ROC curve

Figure 2

Overview of Flow cytometry Results. (A): total cells, (B): Singlet FSC, (C): CD45 labelled pacific blue, (D): UBE2A/B labelled PE-A, (E): CD44 labelled PerCP, (F): UBE2A/B labelled PE-A(G): graphic DDB2 cell count labelled FITC-A, (H): graph of UBE2A/B cell count labelled PE-A

Figure 3

Details of Flow cytometry Cell Calculation Results. $CD44^{+}/CD24^{-}$ was calculated based on the proportion of purple $CD44^{+}/CD24^{-}$ cells.

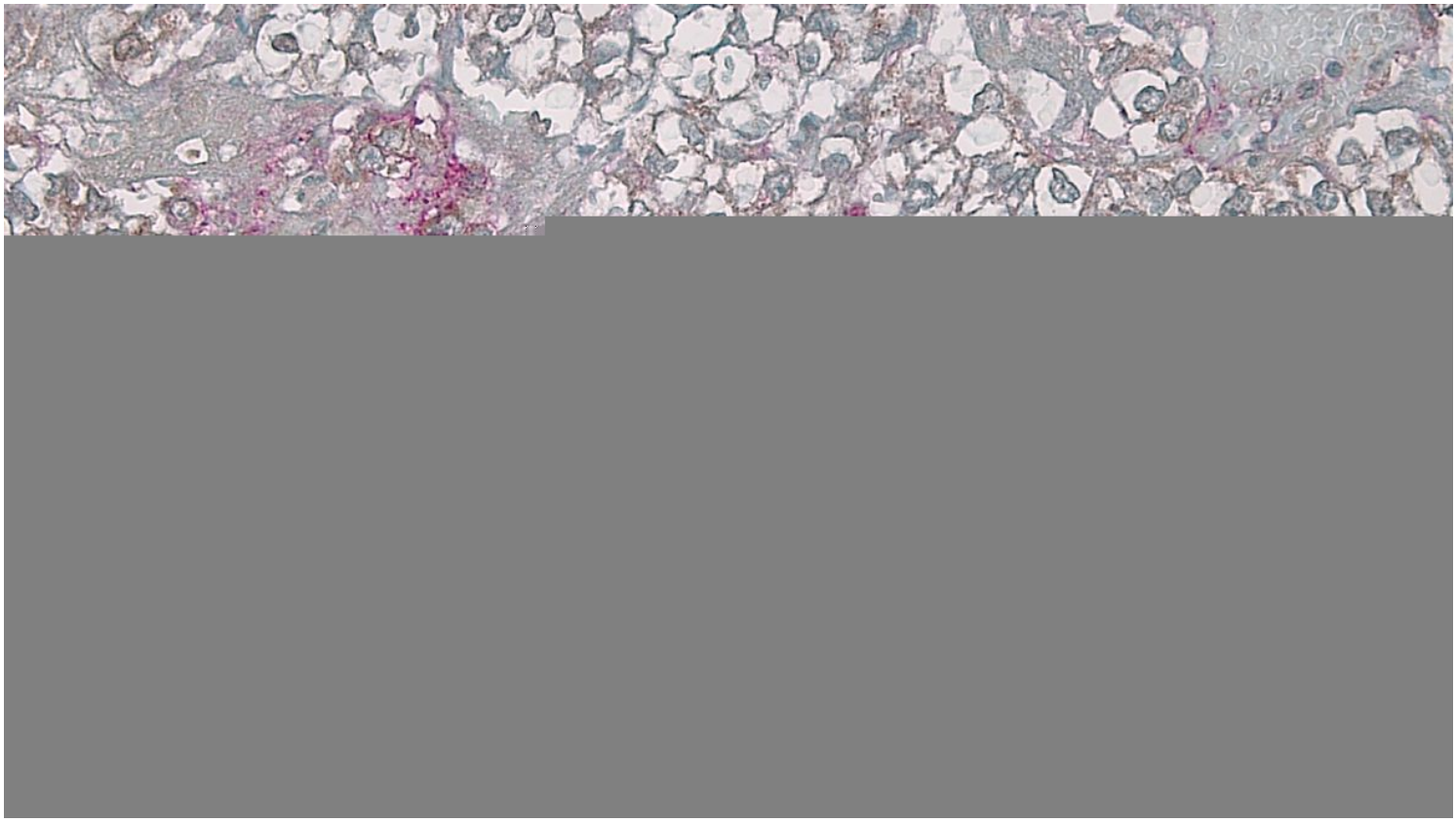

Figure 4

Overview of $CD44^+$ and CD24- expression in high-expression ovarian cancer tissue. The $CD44^+$ marker was brown, especially on cell membrane staining (green arrow), the $CD24^-$ marker was red, especially on cytoplasmic and membrane staining (yellow arrow). The $CD44^+/CD24^-$ marker showed a more dominant brown staining than red. A tumour was categorised as a high expression if the proportion of $CD44^+/CD24^-$ cells was >10% while low expression was <10%, it could be classified as low expression. [29]