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Research Report

Expression of $CD44^{+}/CD24^{-}$, RAD6 and DDB2 on chemotherapy response in ovarian Cancer: A prospective flow cytometry study

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ABSTRACT

Backgrounds: Ovarian cancer is the 8th deadliest common cancer in women around the world. Almost all ovarian cancer patients would experience chemoresistance, recurrence, and poor prognosis after cytoreductive surgery and platinum-based chemotherapy. Chemoresistant cancer cells have characteristic expressions of cancer stem cell proteins (CSCs) $CD44^{+}/CD24^{-}$, RAD6 and DDB2. The increased expression of $CD44^{+}/CD24^{-}$, RAD6, and decreased DDB2 are believed to be associated with chemoresistance, recurrence, and poor prognosis of the disease. Thus, this study's objective is to analyze the correlation between the expression of $CD44^{+}/CD24^{-}$, RAD6 and DDB2 with ovarian cancer chemoresistance.

Materials and methods: This study was conducted with a prospective cohort of 64 patients who is divided into two groups (32 patients in each group) at the Obstetrics-gynecology and pathology department of Cipto Mangunkusumo, Tarakan, Dharmais, and Fatmawati Hospital. All suspected ovarian cancer patients underwent cytoreductive debulking and histopathological examination. Chemotherapy was given for six series followed by six months of observation. After the observation, we determined the therapy's response with the RECIST Criteria (Response Criteria in Solid Tumors) and then classified the results into chemoresistant or chemosensitive groups. Flow cytometry blood tests were then performed to examine the expression of $CD44^{+}/CD24^{-}$, RAD6 and DDB2.

Results: There was a significant relationship between increased levels of $CD44^{+}/CD24^{-}$ and RAD6 ($p < 0.05$) levels with the chemoresistance of ovarian cancer. The logistic regression test showed that the $CD44^{+}/CD24^{-}$ was better marker.

Conclusions: These results indicate that $CD44^{+}/CD24$ and RAD6 expressions are significantly associated with ovarian cancer chemoresistance, and $CD44^{+}/CD24^{-}$ is the better marker to predict ovarian cancer chemoresistance.

Abbreviations: CSCs, Cancer Stem Cells; CD44, Cluster of Differentiation 44; CD24, Cluster of Differentiation 24; RECIST, Response Criteria in Solid Tumors; PFS, Progression-Free Survival; OS, Overall Survival; CD105, Cluster of Differentiation 105; CD106, Cluster of Differentiation 106; DDR, DNA Damage Response; PRR, Post Replication Repair; NER, Nucleotide Excision Repair; DDB2, DNA Damage Binding Protein 2; UBE2, E2 Ubiquitin-conjugating Enzymes; BRCA, Breast Cancer Gene; EMT, Epithelial-to-Mesenchymal Transition; ALDHA, Aldehyde Dehydrogenase; FA, Fanconi Anemia; HR, Homologous Recombination; TLS, Translesion Synthesis; SOX-2, SRY-Box Transcription Factor 2; JAK-STAT, Janus Kinase and Signal Transducer and Activator of Transcription.

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1. Introduction

Ovarian cancer is the 8th deadliest common cancer in women worldwide. In 2018, there were 295,414 new cases with 184,799 deaths. In Indonesia, of 188,231 cancer diagnoses, 13,310 (7.1%) were ovarian cancer cases with an incidence of 9.7 per 100,000 cases (Bray et al., 2018). The standard treatment for ovarian cancer is cytoreductive surgery followed by platinum-based chemotherapy. However, almost all patients would experience disease recurrence. Recent studies have stated that standard therapy had a chemosensitivity and chemoresistance rate of 77.4% and 18.1%, respectively. Furthermore, ovarian cancer patients were found to have a 12-month progression-free survival (PFS) with overall survival (OS) period of about 30 months (Vergote et al., 2010; du Bois et al., 2019). The low survival rate of ovarian cancer patients is due to chemotherapy resistance caused by cancer stem cell (CSCs) proteins.

CSCs are believed to have an association with the initiation of tumors, tumor growth, metastasis, recurrence, and the presence of resistance. Meng *et al.* stated that ovarian stem cell cancer with $CD44^{+}/CD24^{-}$ was 71–93% resistant to all chemotherapy agents, has a recurrence rate of 83% ($p = 0.003$), and a median PFS of 6 months (Meng et al., 2012). Hu *et al.* also found that $CD44^{+}$ was positively expressed in the chemotherapy-resistant ovarian cancer patients with a statistically significant value of 91.17% with $p < 0.05$ (Zhenhua Hu et al., 2013).

Attention to CSCs mechanism has focused on the DNA damage response (DDR) in the tumorigenic process. Increased DDR can prevent the formation of CSCs and chemoresistant cells. The DDR pathway consists of post replication repair (PRR), nucleotide excision repair (NER), etc. The process of NER occurs through several DNA binding proteins, such as DNA damage binding protein 2 (DDB2 or XPE) while in the PRR process and an expression of E2 ubiquitin-conjugating enzymes (UBE2) protein, such as RAD6 (Abad et al., 2020).

RAD6 is a UBE2 protein required for DNA regulation and is associated with mitotic abnormalities and cell transformation. Increased expression of RAD6 enhances stemness function, chemoresistance, progression, and metastasis. The RAD6 on DNA is associated with chemoresistance and poor clinical prognosis in ovarian cancer. Somasagara *et al.* reported that RAD6 expression < 5 and > 5 was associated with 37.5% and 70% recurrence, respectively (Somasagara et al., 2016). DDB2 is also an amino acid that plays a role in the nucleotide excision DNA repair process. Low expression of DDB2 correlates with increased chemotherapy resistance and leads to a poor prognosis (Abad et al., 2020). Han C *et al.* found that DDB2 mRNA expression levels > 0.5 had a better prognosis than values <0.5 (Han et al., 2014). This decrease in protein expression is associated with a reduction in life expectancy (Bommi et al., 2018).

Most cancer cells are sensitive to chemotherapy, but some CSCs could not be detected and would develop into disease recurrence. CSC resistance will make the cells continue to move into the mitotic and interphase (G1, S, G2), while the G0/rest phase is inactive and continuously replicating (Alberts, 2008). This study aims to find correlations between $CD44^{+}$/CD24-, RAD6, and DDB2 with chemotherapy response in ovarian cancer and the ability of the three markers to predict ovarian cancer chemotherapy response. We hypothesize that the three markers have a relationship with ovarian cancer and can be predictors of it. The results of this study can be used as proof for these markers to be used as predictors and have the potential to be developed as targets for gene therapy.

2. Materials and methods

2.1. Study design

This study used a prospective cohort at the obstetrics-gynecology and anatomical pathology department of Cipto Mangunkusumo Hospital, Tarakan Hospital, Dharmais Hospital, and Fatmawati Hospital from February 2018 until February 2022. The objective was to find correlations between $CD44^{+}/CD24^{-}$, RAD6, and DDB2 with chemotherapy response in ovarian cancer and investigate its predictive ability.

2.2. Participants

The research subjects were patients with ovarian carcinoma inclusion, stage II-IV epithelial ovarian cancer patients, and were willing to participate in the study. The exclusion criteria were pregnant patients and patients diagnosed with other types of cancer. The number of samples in this study was 64 patients who is divided into two groups (32 patients in each group) and the consecutive sampling method was used to avoid selection bias. Every patient suspected of ovarian cancer underwent cytoreductive debulking and histopathological examination. If the pathology result was malignant, chemotherapy was administered for six series followed by six months of observation. The patients who had signs of chemoresistance before 6 months of observation were completed (refractory chemoresistance) would be included in the sample.

2.3. Data collection

After the observation, we determined the therapy's response with the RECIST Criteria (Response Criteria in Solid Tumors) and then classified them into chemoresistant or chemosensitive groups. The patients were chemosensitive if there is no residual tumor lesion or pathological lymph node (complete response), and if there is a decrease of thirty percent (30%) in diameter of the longest lesion (partial response). Patients were chemoresistant if there was a twenty percent (20%) increase in the diameter of the smallest lesion and an increase of at least 5 mm in the old lesion, and then if there was a new lesion (progressive disease). Patients were also assigned chemoresistant if there was no sufficient shrinkage to meet the partial response category or was no sufficient enlargement for the disease group. We classified the therapy response by clinical examinations and CT scan or Doppler ultrasound depending on the availability of the hospital and the type of patients' insurance. The evaluation by clinical examination, ultrasound, and CT scan were carried out before chemotherapy, after chemotherapy, and after 6 months of observation.

Flow cytometry blood tests were then performed to examine the patients' expression of RAD6 and DDB2 and CSC ($CD44^{+}/CD24^{-}$). Blood was taken for a flow cytometry test after 6 months of post-chemotherapy observation was completed. if there are patients who experience chemoresistance before the observation period is over, blood will be taken directly at that time for flow cytometry examination before the 6-month observation period is completed. In addition, demographic data, cancer stage, operation type, chemotherapy response, tumor 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 conducted using the FIGO criteria.

2.4. Flow cytometry

Blood was taken from peripheral blood at five mL and then centrifugated. The supernatant was discarded, and 50 μL was left, after which the cell mixture was resuspended. The markers identified expression $CD44^{+}/CD24^{-}$, RAD6, and DDB2. The samples were reacted with fluorescent-labeled antibody against $CD44^{+}/CD24^{-}$ (monoclonal anti-human), $CD44^{+}$ was labeled as PerCP, $CD24^{-}$ was labeled as APC, RAD6 was labeled as PE, and DDB2 was labeled FITC. The four reagents were then removed for leukocytes with CD45 labeled pacific blue. The samples in the Falcon tube were then added with 2.5 μL of CD44 marker, 2.5 μL of CD24 marker, and 2.5 μL of RAD6 and DDB2. Next, they were incubated for 15 min in the dark at room temperature. After incubation, the cells were lysed by using 300 μL of lysing solution, then set again for 15 min in a dark room and at room temperature. Next, 1 mL of FACS

flow solution was added and centrifuged at 500 g for 5 min. The supernatant formed was then discarded, added with 500 μL perm wash buffer and centrifuged at 500 g for 5 min; the supernatant created was discarded. To be more optimal, 1 mL perm wash buffer was added again and centrifuged at 500 g for 5 min. The last step was to add 200 μL of 1% paraformaldehyde in phosphate-buffered saline (PBS). After that, the analysis was conducted by using a flow cytometer using four fluorochrome colors.

Next, cell identification was conducted by using an automated flow cytometer (*BD Facs Calibur*). CSCs were then identified through the positive expression of $CD44^+/CD24^-$ markers and RAD6 and DDB2 were conducted through a positive expression of RAD6 and DDB2 markers with four different colors. Protein percentage is the percentage of expression of protein markers CSCs ($CD44^+/CD24^-$), RAD6, and DDB2 in the blood.

2.5. Statistical analysis

We analyzed the univariate, bivariate, and multivariate analyses. Each categorical variable was tested with the chi-square or Fisher test while the numerical variables were tested with an unpaired *t*-test or Mann-Whitney according to the normality test result. Multivariate analysis was also performed using logistic regression.

2.6. Ethics approval

Research ethics approval was obtained from the Health Research Ethics Committee of the Universitas Indonesia, Cipto Mangunkusumo Hospital. All patients have given their informed consent to participate.

3. Results

3.1. Basic Participants' characteristics

The total sample in this study was 32 samples in each group with a total of two groups. All samples had undergone chemotherapy with 32 (50%) chemoresistance and 32 (50%) chemosensitive without any missing data or lost follow-up patients after 6 months of observation. Some patients have signs of chemoresistance before 6 months of observation and they were included in the sample. The distribution of profiles and clinical characteristics of ovarian cancer patients can be seen in Table 1. All the patients with stage II-IV epithelial ovarian cancer patients were included in the samples.

3.2. Flow cytometry of ovarian cancer

The example of flow cytometry data is described in Figs. 2 and 3. The proportion of $CD44^+/CD24^-$, RAD6, and DDB2 values were calculated based on the percentage of the total cells. $CD44^+/CD24^-$ was calculated based on the proportion of purple $CD44^+/CD24^-$ cells. RAD6 (UBE2) was calculated based on the ratio of orange, purple, and single UBE2 cells; DDB2 was calculated based on the proportion of green, orange, and white colors.

3.3. Bivariate analysis

Table 2 shows that $CD44^+/CD24^-$, RAD6, Ca-125, type of surgery, lymph node metastasis, and tumor residue have significant difference results ($p < 0.05$) with each odds ratio (OR) and relative risk (RR) value. We found that all markers have significant correlations with ovarian cancer chemoresistance ($p < 0.05$) while $CD44^+/CD24^-$ has the highest current odds ratio (OR) value (10.7) and relative risk (RR) value (3.19) in the future outcomes.

Table 1
Essential Clinical Characteristics of Ovarian Cancer Patients.

Variable	Number (%)
Therapy response	
• Chemoresistant	32 (50)
• Chemosensitive	32 (50)
Age (years old)	
• <40	4 (6.3)
• 40-50	19 (29.7)
• >50	41 (64.1)
Ca-125	
• ≤35	30 (46.9)
• >35	34 (53.1)
Ovarian cancer stage	
• Early stage: II	5 (7.8)
• Advance stage: III - IV	59 (92.2)
Operation type:	
• Optimal Debulking	56 (87.5)
• Suboptimal Debulking	8 (12.5)
Differentiation/cancer grade	
• Good	13 (20.3)
• Intermediate	16 (25.0)
• Poor	35 (53.1)
Tumor histology type	
• Serous	24 (37.5)
• High-grade serous	14 (21.9)
• Mucinous	3 (4.7)
• Endometrioid	12 (18.8)
• *Clear cell*	10 (15.6)
• Others	1 (1.6)
Lymph nodes metastasis	
• Positive	32 (50)
• Negative	32 (50)
Ascites	
• Positive	36 (56.3)
• Negative	28 (43.7)
Tumor size	
• 5 cm	17 (26.6)
• 5-10 cm	15 (23.4)
• >10 cm	32 (50)
Tumor residue	
• < 1cm	56 (87.5)
• > 1cm	8 (12.5)

3.4. ROC (Receiver operating characteristic Curve) and AUC (Area under the Curve)

The ROC curve in Fig. 1 and Table 3 data showed that the $CD44^+/CD24^-$ protein has the better ROC curve and AUC value. The AUC value of the $CD44^+/CD24^-$ is 0.783 (moderate accuracy), with significant value ($p < 0.05$, the sensitivity is 78%, and its specificity is 75%. RAD6 protein had an AUC of 0.586 (very weak accuracy), not significant ($p > 0.05$), with a sensitivity of 84% and specificity of 46%. DDB2 had an AUC value of 0.578 (very weak accuracy), not significant ($p > 0.05$), sensitivity 59%, and specificity of 53%.

3.5. Multivariate analysis

The multivariate results of Table 4 showed that $CD44^+/CD24^-$ has better value with a significant p-value ($p < 0.05$) with Exp (B) value is 12.713. It means that ovarian cancer patients with high expression of $CD44^+/CD24^-$ have a higher 12.713 times risk for chemoresistance than the patients with low $CD44^+/CD24^-$.

4. Discussion

This is the first study about the expression $CD44^+/CD24^-$, RAD6, and DDB2 in ovarian cancer patients' blood circulation directly by flow cytometry methods. Previous studies only study the expression of these proteins in the cultured cell lines. This study showed that there was an overexpression of $CD44^+/CD24^-$ and RAD6 in the chemoresistance ovarian cancer patients' blood circulation. We found that there was no

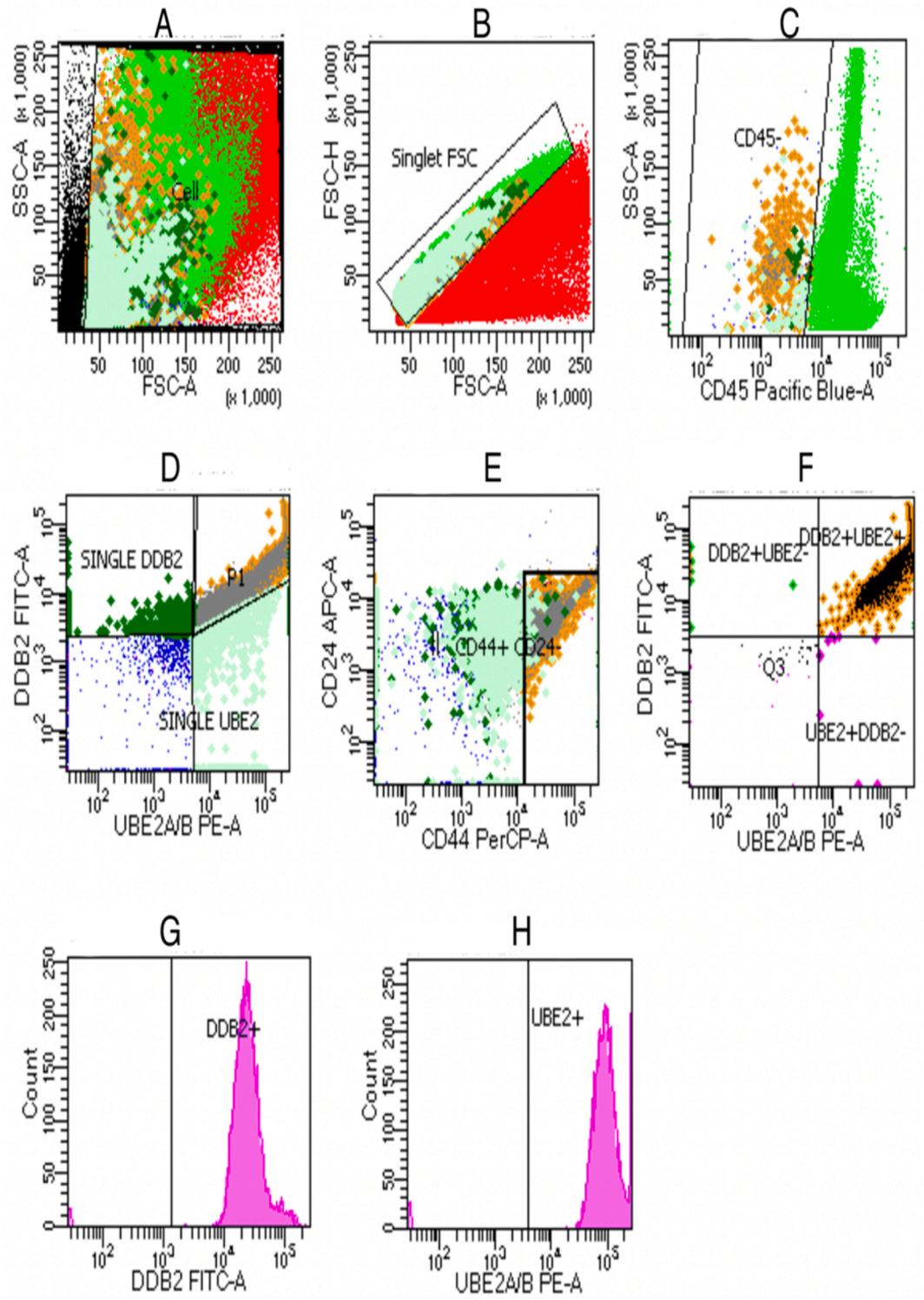

Fig. 2. Overview of Flow cytometry Results. (A): total cells, (B): Singlet FSC, (C): CD45 labeled pacific blue, (D): UBE2A/B labeled PE-A, (E): CD44 labeled PerCP, (F): UBE2A/B labeled PE-A(G): graphic DDB2 cell count labeled FITC-A, (H): graph of UBE2A/B cell count labeled PE-A.

significant difference in DDB2 expression in the chemoresistance ovarian cancer patients' blood circulation.

$CD44^+$ (cluster of differentiation 44) and $CD24^-$ expression are associated with increased ovarian cancer oncogenesis and progression (Meng et al., 2012; Yan et al., 2015). $CD44^+$ overexpression has also been found in the pancreatic cancer (Li et al., 2014), breast cancer (Hu et al., 2017), gastric cancer (Wang et al., 2014), urothelial bladder cancer (Hofner et al., 2014), and colorectal cancer (Wang et al., 2019). It is associated with metastasizing, recurrence, chemoresistance, and poor survival rates in the ovarian cancer (Zhang et al., 2019). $CD24^-$ is a cell surface adhesion molecule that is frequently detected in invasive ovarian carcinoma. High $CD24^-$ expression in invasive ovarian cancer predicts shorter overall survival than low $CD24^-$ markers (Li et al., 2018).

$CD44^+/CD24^-$ is a good predictor of ovarian cancer chemoresistance. We found that higher $CD44^+/CD24^-$ has a significant result ($p < 0.05$)

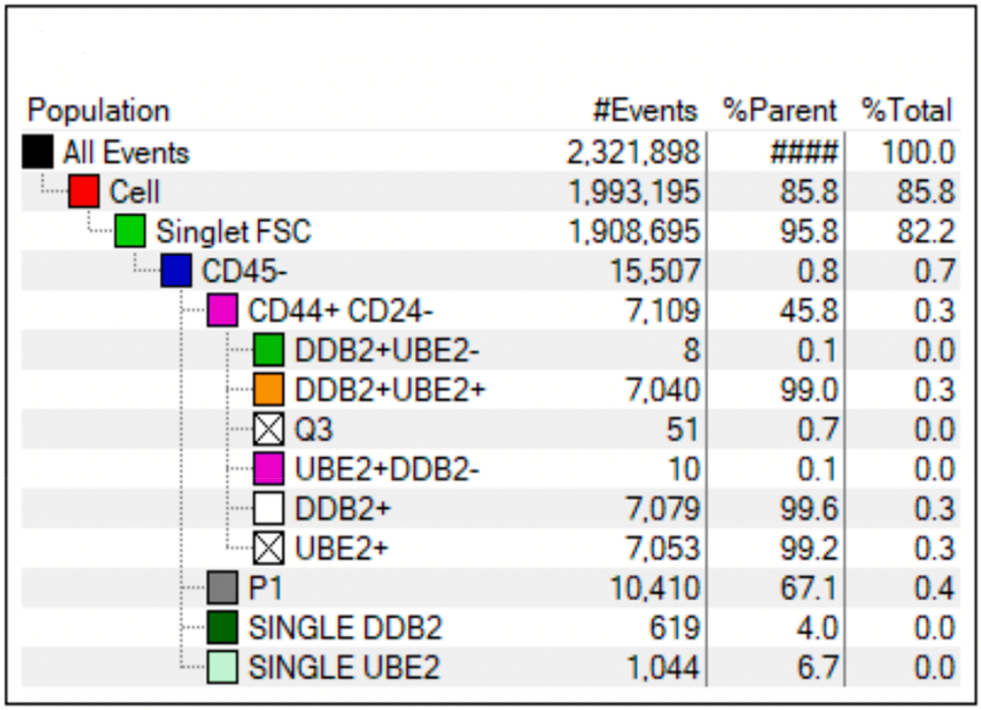

Fig. 3. Details of Flow cytometry Cell Calculation Results. $CD44^{+}/CD24^{-}$ was calculated based on the proportion of purple CD44+/CD24- cells. RAD6 (UBE2) was calculated based on the ratio of orange, purple, and single UBE2 cells; DDB2 was calculated based on the proportion of green, orange, and white colors.

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

Variable	Therapy Response		P value	OR (CI 95%)	RR (CI 95%)
	Chemoresistant (%)	Chemosensitive (%)			
$CD44^{+}/CD24^{-}$ expression	25 (78.1)	8 (25)		10.7 (3.3-34)	3.19 (1.69-6.0)
• High (≥32692)	7 (21.9)	24 (75)	0.001*		
• Low (<32692)					
RAD6 expression				4.76 (1.4-15)	2.45 (1.1-5.4)
• High (≥5846136)	15 (46.9)	5 (15.6) 27 (84.4)	0.007*		
• Low (<5846136)	17 (53.1)				
DDB2 Expression				1.457 (0.5-3.9)	1.21 (0.7-1.9)
• High (≥7370316)	18 (56.3)	15 (46.9)	0.453		
• Low (<7370316)	14 (43.7)	17 (53.1)			
Ca-125 Level			0.000*	105	7.93
• ≤35	2 (6.25)	28 (87.5)		(17-618)	(3.14-20.0)
• >35	30 (93.75)	4 (12.5)			
Ovarian cancer stage				4.42	1.68
• Early stage: II	1 (3.13)	4 (12.5)	0.162	(0.47-42)	(1.7-4.4)
• Advance stage: III - IV	31 (96.87)	28 (87.5)			
Surgery type			0.023*	8.68	4.43
• Optimal Debulking	25 (84.4)	31 (96.87)		(1.0-75.3)	(0.69-28.12)
• Suboptimal Debulking	7 (15.6)	1 (3.13)			
Differentiation/cancer grade	6 (18.75)	7 (21.88)	0.760	1,21	1.09
• Good	26 (81.25)	25 (78.12)		(0.36-4.11)	(0.62-1.96)
• Intermediate - Poor					
Lymph nodes metastasis	21 (65.63)	11 (34.37)	0.012*	3.65	1.91
• Positive	11 (34.37)	21 (65.63)		(1.29-10.2)	(1.1-3.2)
• Negative					
Ascites				1	1
• Positive	18 (56.25)	14 (43.75)	1.000	(0.37-2.68)	(0.61-1.64)
• Negative	14 (43.75)	18 (56.25)			
Tumor size	6 (18.8)	8 (25)	0.545	1.44	1.19
• ≤5 cm	26 (81.2)	24 (75)		(0.44-4.7)	(0.69-2.04)
• >5 cm					
Tumor residue				8.68	4.43
• <1cm	25 (84.4)	31 (96.87)	0.023*	(1.0-75.3)	(0.69-28.12)
• > 1cm	7 (15.6)	1 (3.13)			

Note: *: $p < 0.05$, Significant results.

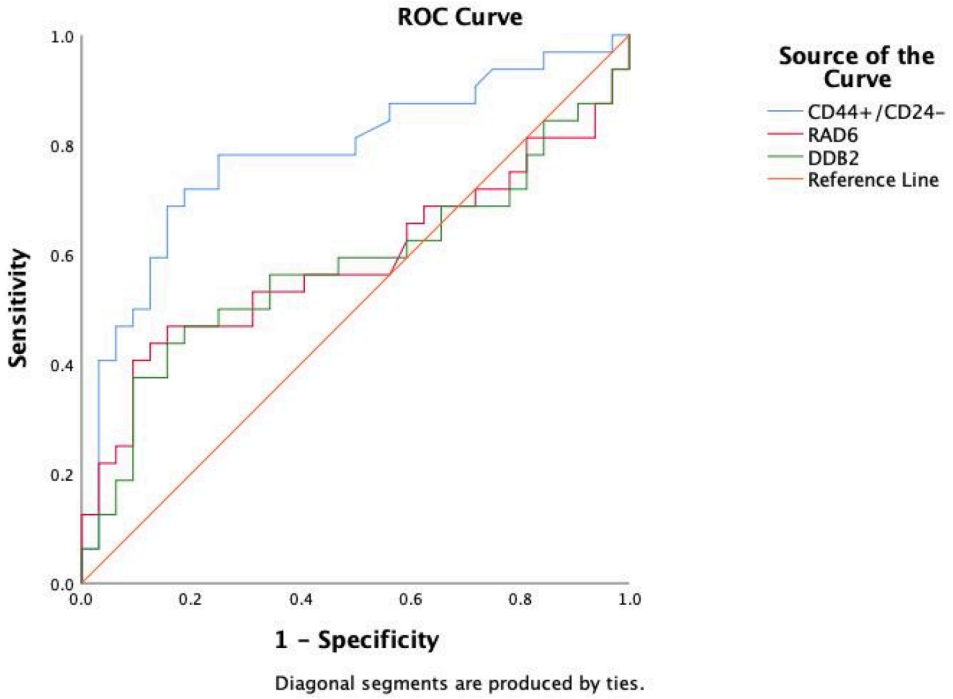

Fig. 1. ROC Curve of $CD44^{+}/CD24^{-}$, DDB2, and RAD6 with therapy response. $CD44^{+}/CD24^{-}$ is the blue line, RAD6 is the redline, DDB2 is the green line, and the reference line is orange.

Table 3
AUC analysis of $CD44^{+}/CD24^{-}$, RAD6, and DDB2 variables.

Variable	AUC	SD	95% *CI*	Sensitivity (%)	Specificity (%)	Cutoff value	P value
$CD44^{+}/CD24^{-}$	0.783	0.060	0.65–0.89	78	75	32692	0,001*
RAD6	0.586	0.074	0.44–0.73	84	46	7370316	0.237
DDB2	0.578	0.074	0.43–0.72	37.5	37.5	5607970	0.283

Note: *: $p < 0.05$, significant.

Table 4
Logistic regression of $CD44^{+}/CD24^{-}$, RAD6, and DDB2 variables.

No	Variables	Beta value (β)	Standard deviation	Wald	Exp (B)	p value	*95% CI*
1	$CD44^{+}/CD24^{-}$	2.125	0.613	11.999	8.369	0.001*	2.515–27.846
2	RAD6	0.914	0.684	1.783	2.493	0.182	9.533
Constant		**−1.239 (β0)**	**0.462**	**7.192**	**0.290**	**0.007**	–

Note: *: $p < 0.05$, significant.

with moderate accuracy (AUC 0.7–0.8) with a sensitivity of 78% and specificity of 75%. Meng et al., (Meng et al., 2012) also found that ovarian cancer cells with high $CD44^{+}/CD24^{-}$ expression have stem cell characteristics: higher aggressive, invasive, progressive, and multiplicative properties in each tumor histology type. This ovarian cancer cell also has higher chemoresistance properties, recurrence rate, and aggravating prognosis (Meng et al., 2012).

Li et al., (Li et al., 2017) found that high $CD44^{+}/CD24^{-}$ protein levels indicated breast tumor malignancy with higher rates of cell proliferation, tumorigenesis, and metastasis. It has also been found that breast cancer with CSC has resistance to chemotherapy and radiotherapy (Li et al., 2017). Furthermore, Yan et al., (Yan et al., 2013) found that cells with high $CD44^{+}/CD24^{-}$ expression showed higher migration and invasion properties and were the cause of chemoresistance (Yan et al., 2013).

Moreover, a past study has found that there was higher $CD44^{+}$ expression in paclitaxel-resistant cell lines than paclitaxel-sensitive cell lines in a mouse model that used ovarian cancer xenografts while patients with ovarian cancer showed that $CD24^{-}$ cells were more resistant to cisplatin and increased tumorigenesis ability (Gao et al., 2010). A *meta*-analysis study showed that the $CD44^{+}$ protein was associated with poorer cancer-specific survival rates in patients undergoing chemoradiotherapy (Han et al., 2019). Zhang et al.; (Zhang et al., 2019) also found that high expression of markers CD105, CD44, and CD106 are related to chemoresistance, as well as poorly differentiated and advanced-stage ovarian cancer (Zhang et al., 2019).

RAD6 has a significant role in activating several DNA repair pathways and is substantial in chemoresistance in the ovarian cancer (Spencer et al., 2016). RAD6 overexpression is associated with mitotic abnormalities and tumor progression (Clark et al., 2018). We found that there was a significant increase in RAD6 levels ($p < 0.05$) in chemoresistance patients. However, the ROC and AUC results were not significant ($p > 0.05$), and the accuracy was very weak (AUC 0.5–0.6), with a sensitivity value of 84% and specificity of 46%.

Clark *et al.*, (2018) investigated the role of RAD6 in chemoresistant ovarian cancer by inhibiting RAD6A and RAD6B in several ovarian cancer cases. These cells showed the decreased expression of CSC markers, activation of DDR protein, and concomitant sensitivity to carboplatin responses, thereby suggesting that RAD6 expression increases after chemotherapy and causes chemoresistance in cancer cells through stimulating CSC protein expression and increasing DNA repair activity (Clark et al., 2018). Somasagara et al., (Somasagara et al., 2016) found an association between chemoresistance and increased RAD6 in ovarian cancer cells through RAD6-mediated ubiquitin signaling, which led to increased DDR and CSC protein expression. In addition, a higher RAD6 (⩾5.1) was also associated with a disease recurrence rate of 70% (Somasagara et al., 2017). Another study also concluded that RAD6 is related to the severity of ovarian cancer, breast cancer, and melanoma. Additionally, RAD6 levels were significantly increased in severe ovarian cancer with platinum chemoresistance (Omy et al., 2021).

RAD6 overexpression can increase stem cell characteristics, making them more aggressive, metastasize, and relapse. The epigenetic influence of RAD6 causes the ubiquitination of some histone variants which then regulate genes related to DNA repair, cell resistance, and chemoresistance (Omy et al., 2021). RAD6 is also closely related to RAD18, a protein E3 ubiquitin ligase that regulates the DNA repair pathway in Fanconi anemia and the BRCA gene in breast cancer (Somasagara et al., 2017). RAD6 was also involved in breast cancer chemoresistance in which researchers inhibited RAD6 with a small molecule inhibitor and found an increased sensitivity to cisplatin (Haynes et al., 2020). In bladder cancer, it was also found that overexpression of enzymes from the UBE2 group, one of which was RAD6, could affect the growth of bladder cancer cells. An experiment was conducted by stopping the expression of UBE2 which caused the cells to stop growing in the G2/M phase and increase the apoptosis of these cancer cells (Gong et al., 2016).

DDB2 is a protein localized in the cell nucleus that contributes to gene transcription, cell cycle progression, and protein degradation (Gilson et al., 2019). The decrease in DDB2 also affects ovarian cancer's aggression, metastasis, and severity (Somasagara et al., 2016). This study found that DDB2 protein expression was found significantly lower in chemotherapy-resistant patients ($p < 0.05$). The ROC and AUC analysis results are $p > 0.05$ with weak accuracy (AUC 0.6–0.7) as well as a sensitivity value of 37.5% and specificity of 37.5%.

DDB2 levels are high in the chemosensitive cancer patient group because DDB2 participates in the tumor suppression process in at least three ways: promoting the nucleotide excision repair (NER) process, supporting apoptosis, and inducing cell aging after DNA damage has occurred (Stoyanova et al., 2012). Thus, the loss of DDB2 function in normal cells can lead to susceptibility to tumor growth. DDB2 gene mutation causes loss of function and gives rise to the phenotypic characteristics of Xeroderma Pigmentosum group E, which is characterized by malignant skin tumors. Mice with low DDB2 levels were found to not only be hypersensitive to UV-related carcinogenesis processes but also have a high incidence of broad-spectrum spontaneous malignant tumors from internal organs. Thus, DDB2 acts as a mediator in suppressing the p53 and BRCA1 pathways, thereby being a tumor cell suppressor. It protects against cancer by regulating the cell cycle and increasing the occurrence of apoptosis instead of being directly involved in the repair of DNA damage (Kattan et al., 2008).

Most ovarian cancer cells have a low DDB2 expression (Yang et al., 2018). Barakat *et al.* investigated the expression of DDB2 in several cisplatin-resistant ovarian cancer cell lines, and the results obtained were lower DDB2 expression than in cisplatin-sensitive cells (Barakat et al., 2010). The study also further explained that low DDB2 expression is associated with chemoresistance and poor patient prognosis (Han et al., 2014). DDB2 can reduce excess CSC protein ($CD44^+/CD24^-$) in large ovarian cancer. Overexpression of DDB2 in human ovarian cancer cells decreased the ability of these tumor cells to replicate (Han et al., 2014). Han *et al.*, (2014) also stated that low DDB2 protein expression was associated with poor prognosis in ovarian cancer patients. Studies have also found that DDB2 deficiency causes ovarian tumors to relapse due to the expansion of the CSCs population (Han et al., 2014).

Furthermore, according to Yang et al., (Yang et al., 2018), DDB2 protein expression was associated with the onset, progression, and prognosis of colorectal cancer. Increased DDB2 expression was significantly associated with a better colorectal cancer prognosis (Yang et al., 2018). DDB2 was found to be able to inhibit colon cancer metastasizing through the mechanism of decreasing gene expression, which is an activator of Epithelial-to-Mesenchymal Transition (EMT) in colon cancer, and NF-κB was found in breast cancer (Han et al., 2014). Decreased DDB2 also affects EMT activation by triggering squamous cell carcinomas in the head and neck region (Bommi et al., 2018). Moreover, a study on non-small cell lung cancer (NSCLC) found that DDB2 can facilitate the process of breaking cancer cell growth. Conversely, if DDB2 decreases, the G2 cycle will continue and tumor growth and therapy resistance will occur (Zou et al., 2016).

Ovarian cancer chemoresistance and recurrence are possibly caused by the presence of residual cancer cells that have cancer stem cells (CSCs) (Suster and Virant-Klun, 2019). The known CSC pathways include Hedgehog, JAK/STAT, Nanog, Notch, PI3K/Akt, and Wnt/β-catenin that create stemness characteristics, such as self-renewal and differentiation ability into various types of cancer cells. In addition, there may be other unknown mechanisms in the CSCs (Pieterse et al., 2019).

The overexpression of RAD6 after chemotherapy was caused by DNA damage. RAD6 can cooperate with RAD18 to activate DDR mechanisms through several pathways such as the Fanconi Anemia (FA), Homologous Recombination (HR), and the Translesion Synthesis (TLS) pathway (Somasagara et al., 2016; Clark et al., 2018). The interaction of RAD6 with RNF20/40 will cause histone monoubiquitylation and an increase of ALDHA1 and SOX-2 which causes epigenetic changes and gene transcription changes in chromatin structure. RAD6 can increase the activity of B-catenin by an unknown mechanism. The increased expression of some of the stemness factors mentioned above encourages chemotherapy resistance (Clark et al., 2018).

Multivariate analysis with logistic regression found that among the three markers, $CD44^+/CD24^-$ is better marker to be used as a predictor of ovarian cancer chemoresistance in flow cytometry study. There were no flow cytometry previous studies about RAD6 and DDB2 with ovarian cancer chemoresistance before our research. This was the first research that used flow cytometry methods to analyze correlations between $CD44^+/CD24^-$, RAD6, and DDB2 directly in the ovarian cancer patients' blood circulation. However, some shortcomings made us unable to prove that DDB2 low expression is correlated with ovarian cancer chemoresistance. Therefore, it cannot yet be used as a good predictor of ovarian cancer chemoresistance and require further research. We suggest that further research has a larger number of samples. We believe that in the future, expression of post-chemotherapy CD44+/CD24-, RAD6, and DDB2 in ovarian cancer patients' blood circulation could be used to predict chemoresistance.

5. Conclusion

We conclude that there is a significant relationship between increased levels of $CD44^+/CD24^-$ and RAD6 ($p < 0.05$) with chemoresistance in ovarian cancer. The logistic regression results also indicate that $CD44^+/CD24^-$ is better marker to be used as a predictor of ovarian cancer chemoresistance.

Ethics approval

Ethical approval was granted by the Health Research Ethics Committee of the Universitas Indonesia, Cipto Mangunkusumo Hospital No. KET-230/UN2.F1/ETIK/PPM.00.02/2021, March 15th, 2021.

Consent to participate

Informed consent was obtained from all participants included in the study.

Consent for publications

Not applicable. We have no individual person's data in the manuscript. All authors have consented to publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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