Journal on Oncology

www.journalononcology.org

Research Article

Open Access, Volume 4

RAD6 Overexpression and Ovarian Cancer Chemo-Resistance: Flow Cytometry and Immunohistochemistry

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Abstract

Objective: Ovarian cancer is one of the deadliest women's cancers in the world. Nearly all the patients have poor prognosis and recurrence after standard therapy because of chemo-resistance. The standard therapy is cytoreductive debulking surgery followed by platinum-based chemotherapy. RAD6 has an important role in chemo-resistance. RAD6 is an E2 Ubiquitin-Conjugating Enzymes (UBE2) enzyme required for DNA repair, cell proliferation, and cell mutagenesis. Increased expression of RAD6 is believed to be associated with chemo-resistance, recurrence, and poor prognosis of the disease. We aimed to study RAD6 relationship with ovarian cancer chemo-resistance and its ability to predict chemo-resistance.

Methods: 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 patients will undergo standard cytoreductive debulking and histopathological examination followed by six series of chemotherapy followed by six months of observation. After the observation, we determine therapy response with the RECIST Criteria (Response Criteria in Solid Tumors). The chemo-resistance and chemo sensitive groups will be analyzed according to the therapy response. Our study is the first study examining RAD6 in ovarian cancer from flow cytometry blood test and directly from ovarian cancer tissue by double immunohistochemistry.

Results: We found a significant relationship between increased levels of RAD6 expression (p<0,05) with chemo-resistance of ovarian cancer in both studies while immunohistochemistry has a better multivariate analysis result.

Conclusion: Both studies indicate that RAD6 is a significantly correlated and good chemo-resistance predictor for ovarian cancer chemo-resistance while RAD6 immunohistochemistry is a better predictor.

Keywords: Ovarian cancer; RAD6; Chemo-resistance; Flow cytometry; Immunohistochemistry.

Manuscript Information: *Received: Mar 04 2024; Accepted: Apr 08, 2024; Published: Apr 15, 2024*

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Citation: *Sihombing UHM, Andrijono, Purwoto G, Gandamihardja S, Harahap AR, et al. RAD6 Overexpression and Ovarian Cancer Chemo-Resistance: Flow Cytometry and Immunohistochemistry. J Oncology. 2024; 4(1): 1128.*

Introduction

One of the deadliest women's cancers is ovarian cancer. Annually, there were 295.414 new cases with 184.799 deaths in 2018 worldwide. The incidence in Indonesia of 9.7 per 100.000 [1]. Furthermore, it is hard to detect and prevent because mostly (70%) of patients present with an advanced stage [2]. Standard therapy with cytoreductive surgery followed by platinum-based chemotherapy has a high recurrence rate. It has a 77.4% chemo sensitivity rate and an 18.1% chemo-resistance rate [3] with the Progression-Free Survival (PFS) was 12 months and Overall Survival (OS) was about 30 months [4,5]. Cancer Stem Cells (CSCs) have a role in the low survival rate [6].

The chemo-resistance resulted from Cancer Stem Cells (CSCs). CSCs have an essential role in the initiation, tumor growth, metastasis, and recurrence which leads to chemotherapy resistance [7]. Studies about the DNA Damage Response (DDR) in the tumorigenic process found that DDR was correlated with the formation of CSCs and chemo-resistant cells. The DDR pathway consists of Post Replication Repair (PRR), Nucleotide Excision Repair (NER), Fanconi anemia, etc. The PRR involves several proteins such as E2 Ubiquitin-Conjugating Enzymes (UBE2) protein RAD6 [8].

RAD6 is an UBE2 protein required for DNA regulation, repair, proliferation, and mutagenesis. Cell transformation and mitotic abnormalities associated with RAD6 expression. RAD6 overexpression leads to elevated Cancer Stem Cell (CSCs) markers and signaling pathways components that enhance stemness function, chemo-resistance, metastasis, and cancer progression [9]. The RAD6 is associated with chemo-resistance 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 [9]. We want to see the expression of RAD6 in ovarian cancer patients' tissue and blood after chemotherapy which has never been conducted before. Our objective is to find relationships between RAD6 with chemotherapy response in ovarian cancer and its ability to predict ovarian cancer chemotherapy response.

Materials and methods

Study design

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

Participants

The research subjects were patients with ovarian carcinoma inclusion, stage II-IV ovarian epithelial 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 to minimize selection bias.

Data collection

Ovarian cancer patients will undergo cytoreductive debulking and histopathological examination. If the histopathology result is malignant, chemotherapy will be given for six series followed by six months of observation. After the observation, we determined therapy response with the RECIST Criteria (Response Criteria in Solid Tumors) and then classify it into chemo-resistant or chemo sensitive groups. The patient will perform Flow cytometry blood tests to examine the expression of RAD6 (prospective study), while an immunohistochemistry examination will be performed on ovarian cancer tissue (retrospective study). We also collected demographic data, cancer stage, surgery type, chemotherapy response, tumor cell differentiation (cancer stage), cancer histopathology, cancer size, cancer residue, ascites, lymph node metastasis, and serum Ca-125 levels. FIGO criteria were being used for cancer staging.

Flow cytometry method

Blood was taken from peripheral blood veins at five ml and centrifugated with 50 µL was left. Their markers identified the expression $CD44^{+}/CD24^{-}$. Samples were reacted with fluorescent-labeled antibody against RAD6 (monoclonal anti-human) labeled as PE. The reagents were removed for leukocytes with CD45 labeled pacific blue. The samples in the Falcon tube were added with 2,5 µL of RAD6 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, 1 mL 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 colors.

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 RAD6markers. Protein percentage is the percentage of expression of protein markers RAD6 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 cm 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 deparaffinized 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 RAD6 markes used specific antibodies against RAD6 (Monoclonal anti-RAD6). The preparations were incubated

with a primary RAD6 antibody (1:500 dilution). After one hour, it was washed with PBS (pH 7.4) for 5 minutes. Each preparation was then incubated with a secondary antibody against biotin-labeled 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 labeled 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 tumor tissue.

Immunohistochemistry

The immunohistochemistry preparations were observed using a Leica ICC 50 HD microscope. Positive RAD6 was seen in the staining of the cytoplasm and the nucleus of tumor cells. Immunohistochemistry assessment classified as 0: Negative expression, 1: Weak expression, 2: Moderate expression, and 3: Strong expression. Next, it is classified into low expression and high expression. The low expression has 0-1 while the high expression has a 2-3 value [10].

Statistical analysis

We conduct univariate, bivariate, and multivariate analyses. 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 RAD6 variable as a predictor of therapy response to ovarian cancer. We performed a multivariate analysis to compare the power between RAD6 flow cytometry and immunohistochemistry to predict ovarian cancer chemo-resistance. Missing data and lost follow-up patients will be discarded from the sample.

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

Result

Basic participants characteristics

We have 32 samples in each group. All samples had undergone chemotherapy with 32 (50%) chemo-resistance patients and 32 (50%) chemo sensitive patients for each flow cytometry and immunohistochemistry study. There is no missing data or lost follow-up patients after 6 months of observation. The distribution of profiles and clinical characteristics of ovarian cancer patients can be seen in Table 1.

Flow cytometry of ovarian cancer

The flow cytometry results example is presented in Figures 2 and 3. The proportion of RAD6 values was calculated based on the percentage of the total cells. RAD6 was highly expressed in chemo-resistance ovarian cancer patients like in the previous studies.

Bivariate analysis

RAD6 Flow cytometry RAD6 has OR 4.76 and 2.45, respectively while immunohistochemistry RAD6 has OR 52.2 and 6.12. Thus, RAD6 immunohistochemistry has a higher OR and RR value. The complete data is shown in Table 2. The data quietly found that chemo-resistance ovarian cancer patients have high RAD6 expression.

ROC and AUC curves

ROC curve data were presented in Figure 1 while the AUC analysis was presented in Table 3. The AUC value of the RAD6 flow cytometry is 0.656, which means it has a poor level of accuracy, but the value is significant (p<0,05). The sensitivity is 46 %, and its specificity is 84% for detecting chemo-resistance. RAD6 immunohistochemistry had a better AUC of 0.875 (good accuracy), significant (p<0,05), with a better sensitivity of 90% and better specificity of 84%. The data showed that the RAD6 immunohistochemistry has better ROC curve and AUC value.

Multivariate analysis

We conducted logistic regression and the results are shown in Table 4. We found from this calculation that immunohistochemistry data of RAD6 has a better result compared with flow cytometry data of RAD6. It means that RAD6 immunohistochemistry is a better predictor of ovarian cancer chemo-resistance in this research.

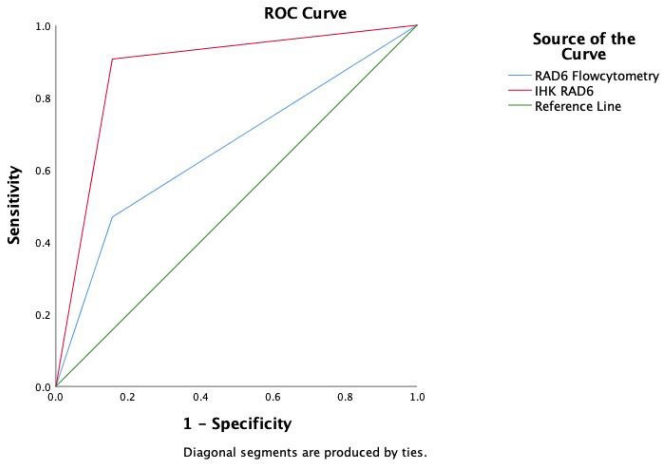

Figure 1: ROC curve of CD44+/CD24-, DD82, and RAD6 with therapy response. Red line was for RAD6 immunohistochemistry, and the blue line was for RAD6 flow cytometry.

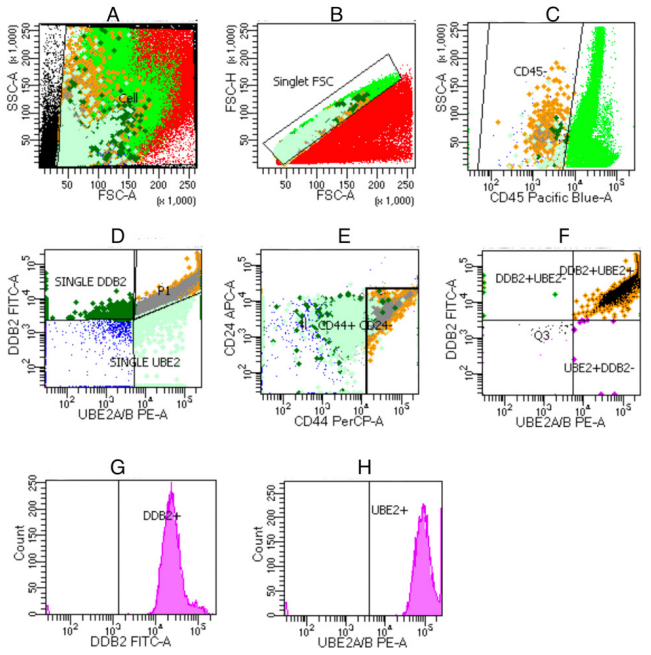

Figure 2: Overview of flow cytometry results. **(A)**: Total cells, **(B)**: Singlet FSC, **(C)**: CD45 labeled pacific blue, **(D)**: UBE2A/B labeled PE-A, **(E)**: CD44 labeled Per CP, **(F)**: UBE2A/B labeled PE-A**(G)**: Graphic DDB2 cell count labeled FITC-A, **(H)**: Graph of UBE2A/B cell count labeled PE-A.

Population	#Events	%Parent	%Total
All Events	12,603,294	####	100.0
Cell	5,053,877	40.1	40.1
Singlet FSC	4,715,069	93.3	37.4
CD45-	97,573	2.1	0.8
CD44+ CD24-	374	0.4	0.0
DDB2+UBE2-	0	0.0	0.0
DDB2+UBE2+	283	75.7	0.0
Q3	61	16.3	0.0
UBE2+DDB2-	30	8.0	0.0
DDB2+	293	78.3	0.0
UBE2+	319	85.3	0.0
P1	4,400	4.5	0.0
SINGLE DDB2	2,506	2.6	0.0
SINGLE UBE2	127	0.1	0.0

Figure 3: Details of Flow cytometry Cell Calculation Results. RAD6 was calculated based on the proportion of UBE2+ cells proportion. RAD6 (UBE2+) is calculated based on the ratio of white, orange, and purple colors.

Discussion

RAD6 is a Ubiquitin-Conjugating Enzyme E2 (UBE2), an enzyme that play a role in the occurrence of chemo-resistance in ovarian cancer. RAD6 plays a role in DNA repair and regulates gene expression through modification of histone pro-transcriptions [11]. Humans have two RAD6 proteins (RAD6A & B or UBE2A & UBE2B), which are often overexpressed in various tumor types [9].

The mechanism of RAD6 in increasing CSC gene expression is still not widely known. RAD6 is combined with several protein ubiquitin ligases to regulate DNA repair and gene transcription.

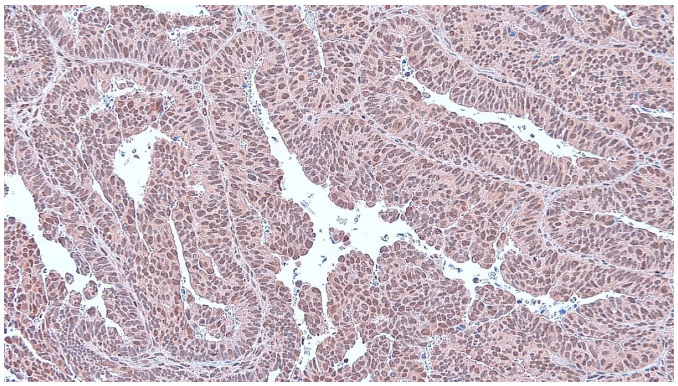

Figure 4: Overview of RAD6 expression in high-expression ovarian cancer tissue. Positive RAD6 was seen in the staining of the cytoplasm and the nucleus of tumor cells. Immunohistochemistry assessment classified as 0: Negative expression, 1: Weak expression, 2: Moderate expression, and 3: Strong expression. Next, it is classified into low expression and high expression. The low expression has 0-1 while the high expression has a 2-3 value (10).

Table 1: Essential clinical characteristics of ovarian cancer patient.

Variable	Number (%)
• 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)
Surgery 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)

Table 2: Bivariate analysis of the variables in ovarian cancer patients.

Variable	Therapy response		P value	OR (CI 95%)	RR (CI 95%)
	Chemo resistant (%)	Chemo sensitive (%)			
RAD6 flow cytometry • High (≥32.692) • Low (<32.692)	 15(46.9) 17(53.1)	 5(15.6) 27(84.4)	 0.007*	 4.76 (1.46-15.5)	 2.45 (1.11-5.43
RAD6 immunohistochemistry • High (≥10%) • Low (<10%)	 29(90.6) 3(9.4)	 5(15.6) 27(84.4)	0.000*	52.20 (11.3-239)	6.12 (2.7-13.8)
Ca-125 Level • ≤35 • >35	 2(6,25) 30(93,75)	 28(87.5) 4(12,5)	 0,001*	105 (17-618)	7,93 (3.14-20.0)
Ovarian cancer stage • Early stage: II • Advance stage: III - IV	 1(3,13) 31(96,87)	 4(12,5) 28(87,5)	 0,162	4.42 (0.47-42)	1.68 (1.7-4.4)
Surgery type • Optimal Debulking • Suboptimal Debulking	 25(84,4) 7(15,6)	 31(96,87) 1(3,13)	0,023*	8.68 (1.0-75.3)	4.43 (0.69-28.12)
Differentiation/cancer grade • Good • Intermediate - Poor	6(18,75) 26(81,25)	7(21,88) 25(78,12)	0,760	1,21 (0.36-4.11)	1.09 (0.62-1.96)
Lymph nodes metastasis • Positive • Negative	21(65,63) 11(34,37)	11(34,37) 21(65,63)	0,012*	3.65 (1.29-10.2)	1.91 (1.1-3.2)
Ascites • Positive • Negative	 18(56,25) 14(43,75)	 14(43,75) 18 (56,25)	 1,000	1 (0.37-2.68)	1 (0.61-1.64)
Tumor size • ≤5 cm • >5 cm	6(18.8) 26(81.2)	8(25) 24(75)	0.545	1.44 (0.44-4.7)	1.19 (0.69-2.04)
Tumor residue • <1cm • > 1cm	 25(84,4) 7(15,6)	 31(96,87) 1(3,13)	 0,023*	8.68 (1.0-75.3)	4.43 (0.69-28.12)

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

Table 3: AUC analysis of RAD6 flow cytometry and immunohistochemistry.

Variable	AUC	SD	95% CI	Sensitivity (%)	Specificity (%)	P value
RAD6 flow cytometry	0.656	0.069	0.521-0.792	46	84	0.032*
RAD6 immunohistochemistry	0.875	0.048	0.781-0.969	90	84	0.000*

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

Table 4: Logistic regression of RAD6 flow cytometry and immunohistochemistry.

No	Variables	Beta value (β)	Standard deviation	Wald	p value	Exp (B)	95% CI
1	RAD6 flow cytometry	2.662	1.174	5.143	0.023*	14.323	1.435-142.9
2	RAD6 immunohistochemistry	4.635	1.106	17.570	0.000*	103.077	11.79-900.5
Constant		**-8.038 (β0)**	**2.086**	**14.843**	**0,000**		-

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

The overexpression of the RAD6 protein is due to chemotherapy-induced DNA damage. RAD6 high expression affected cancer cells by cooperating with RAD18 to activate DNA repair through several pathways such as the Fanconi Anemia pathway, Homologous Recombination, and the Translation Synthesis pathway [9,11].

In the pathway of enhancing stemness, RAD6 is associated with RNF20/40 which increases stemness factors such as SOX-2, and ALDHA1 through monoubiquitinating effects on histones that cause epigenetic modifications and changes and further cause gene transcription changes in chromatin structure. RAD6 also stabilizes and promotes core localization of the B-catenin (transcription factor) unclear mechanism. B-catenin is a protein involved in the regulation and coordination of cell adhesion and gene transcription. Increased expression of stemness factors supports cancer cell survival in response to treatment with chemotherapeutic agents [11].

Clark et al., (2018) investigated the role of RAD6 in chemoresistant ovarian cancer by inhibiting RAD6A and RAD6B in several ovarian cancer. These cells showed decreased expression of CSC markers, activation of DDR protein, and concomitant sensitivity to carboplatin responses suggesting that RAD6 expression increases after chemotherapy and causes chemo-resistance in cancer cells through stimulating CSC protein expression and increasing DNA repair activity [12]. The study by Somasagara et al., (2016) found an association between chemo-resistance 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 ($\geqslant$5,1) was also associated with a disease recurrence rate of 70% [13]. Another study concluded that RAD6 is related to the severity of ovarian cancer, breast cancer, and melanoma. Rad6 levels were significantly increased in severe ovarian cancer with platinum chemo-resistance [14].

RAD6 overexpression can increase stem cell characteristics, aggressivity, metastasis, 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 chemo-resistance [14]. 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 [13], RAD6 was involved in breast cancer chemo-resistance in which researchers inhibited RAD6 with a small molecule inhibitor and found an increased sensitivity to cisplatin [15]. 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 carried out by stopping the expression of UBE2, then the cells would stop growing in the G2/M phase and increase the apoptosis of these cancer cells [16].

RAD6 is known to be weakly expressed in normal breast tissue and cells, and its overexpression is associated with breast cancer progression. RAD6 overexpression in breast cancer induces transformation and resistance to doxorubicin and cisplatin. A study found that melanoma, a skin cancer tissue, has a high expression of RAD6 and Melan-A and B-catenin by RAD6/Melan-A dual positivity [17]. Another study used OV90 and SKOV3 cell cultures with RT-PCR, and immunofluorescence staining after chemotherapy found that chemo-resistance ovarian cancer has high expression of RAD6 [13].

The epigenetic effect of RAD6 causes the ubiquitination of histone variants H2A, H2AX and H2B which then regulates genes related to DNA repair, cell resistance, and chemo-resistance. Several epigenetic molecules such as histone methylase and demethylase are known to cause the release of RAD6 against ubiquitinated histone-containing genes [14]. 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. RAD6 can cause ovarian chemo-resistance by stimulating monoubiquitylation of FANCD2 and PCNA proteins that play an important role in DNA repair and DNA Damage Tolerance (DDT) mechanisms related to platinum-based chemotherapy. RAD6 inhibition test with a Small Molecule Inhibitor (SMI) was found to decrease DNA repair signals, decrease CSC markers, and increase the sensitivity of ovarian cancer patients to chemotherapy. Another pharmacological test with RAD6-selective Small-Molecule Inhibitor (SMI) was performed on breast cancer and colon cancer. As a result, Therapy with Smi Can Increase the Sensitivity of Breast Cancer (TNBC) to cisplatin. In colon cancer, SMI also increases sensitivity to platinum-based chemotherapy [18]. Thus, RAD6 can be a target for gene therapy to treat chemo-resistance of ovarian cancer [13] RAD6 is also related to breast cancer [15], melanoma [17], and pulmonary cancer [19].

Overall, our study found that there is overexpression of RAD6 in the chemo-resistance of ovarian cancer both in flow cytometry and immunohistochemistry study. RAD6 has a significant role in activating several DNA repair pathways and is substantial in chemo-resistance in the ovarian cancer [20]. RAD6 overexpression is associated with mitotic abnormalities and tumor progression [12]. We found that there was a significant increase in RAD6 levels ($p<0,05$) in chemo-resistance patients. However, better ROC and AUC results were found in immunohistochemistry RAD6, with good Accuracy (AUC 0.875), significant ($p<0,05$), the sensitivity of 90%, and specificity of 84%.

To our knowledge, our study is the first study examining RAD6 in ovarian cancer directly from the blood by flow cytometry study and from the fresh ovarian cancer tissue by immunohistochemistry. However, even though we found strong evidence from both studies that RAD6 has correlations with ovarian cancer chemo-resistance from both studies, we still need further investigations because RAD6 ovarian cancer phenotype maybe not be the only big cause of the chemo-resistance. RAD6 is a potential gene therapy target for ovarian cancer but more research is also required to prove this.

Conclusion

We conclude that there is a significant relationship between increased levels of RAD6 expression ($p<0,05$) with ovarian cancer chemo-resistance. Logistic regression results indicate that RAD6 is significantly associated with ovarian cancer chemo-resistance and can be used as a good predictor of ovarian cancer chemo-resistance whereas RAD6 immunohistochemistry is a better predictor.

Declarations

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.

Conflict of interest: The authors declare that they have no competing interests.

Funding: The funding of the research was from all the authors. We have no support in the form of grants, equipment, drugs, etc.

Authors' contributions: The all authors' contributions are equal.

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