CD133, CD44, and ALDH1A1 as cancer stem cell markers and prognostic factors in epithelial ovarian cancer

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pISSN: 0853-1773 • eISSN: 2252-8083
https://doi.org/10.13181/mji.v28i1.2863
Med J Indones. 2019;28:63–9

Received: May 26, 2018
Accepted: February 11, 2019

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ABSTRACT

BACKGROUND Ovarian cancer is a heterogeneous disease, and most patients are diagnosed at an advanced stage. Epithelial ovarian cancer type II is characterized by rapid tumor growth and is genetically more labile than type I. This study was aimed to demonstrate the prognostic value of CSC by using the markers CD133, CD44, and ALDH1A1 in EOC.

METHODS Clinicopathological and demographic data were collected from medical records. The markers CD133, CD44, and ALDH1A1 were examined with flow cytometry and immunohistochemistry. Cancer stem cell (CSC) marker expression in patients with ovarian cancer types I and II were related to chemotherapy and survival. In multivariate analysis, the prognosis model was tested for ten months.

RESULTS The largest demographic consisted of patients aged ≥45 years, with stage I, poor differentiation, and type II, of which there were 40 samples (72.7%), 23 samples (41.8%), 30 samples (54.5%), and 16 samples (29.1%), respectively. There is a high correlation between the 10-month chemotherapy response and the 4 variables, i.e., age ≥45 years, type II, stage III–IV, and CD44, with an ROC of 80.75% and a post-test probability of 82.5%. Using the ROC curve, the highest chemoresistance score was 0.841, based on the combination of CSCs markers and clinicopathological factors, that is stage III–IV, age ≥45 years, poor differentiation, type II, negative CD133, high CD44, and high ALDH1A1.

CONCLUSIONS CSC (CD133, CD44, and ALDH1A1) markers and clinicopathological factors are prognostic of epithelial ovarian cancer.

KEYWORDS aldehyde dehydrogenase 1A1, cancer stem cells, CD133 antigen, CD44 antigen, chemotherapy response, epithelial ovarian cancer

Ovarian cancer affects 204,000 women worldwide each year, including 21,650 Americans.[1,2] Despite its relatively low incidence rate, ovarian cancer is a very deadly disease. An estimated 125,000 people globally die per year from ovarian cancer, making it the 7th leading cause of cancer death among women.[2] The 5-year survival rate for stage I ovarian cancer is more than 90%; however, most patients (75%) are diagnosed in an advanced stage (III/IV), with a 5-year survival rate of 30%.

Epithelial ovarian cancer (EOC) is divided into 2 types. Type I consists of low-grade serous carcinoma and low-grade endometrioid, clear cell, mucinous, and transitional cancer (Brenner).[3] This type of tumor is slow growing and genetically stable. In contrast, type II (high-grade serous carcinoma) is characterized by rapid and genetically labile tumor growth. In type II, there is a p53 mutation (TP53) in 80% of cases, which is rarely found in type I.[4] A study of 381 patients using multivariate analysis found that patients with low-grade serous carcinoma following macroscopically non-residual cytoreductive surgery (>1 cm) obtained significantly longer progression-free survival (PFS)

than those with high-grade serous ovarian carcinoma (type II) who had undergone optimal cytoreductive therapy (36 *versus* 16 months).

An examination of the ovarian cancer literature to date indicates that an optimal therapy does not yet exist. Therefore, more promising approaches are needed, one of which is enumeration of cancer stem cells (CSC) using CSC markers [CD133, CD44, aldehyde dehydrogenase 1A1 (ALDH1A1)]. CSC can regenerate tumors through the stem cells themselves by reforming cells and differentiating, as well as by metastasizing into various tissues; thus, stem cells play a role in the occurrence of chemoresistance.[5,6] Therefore, further studies are needed on chemoresistant CSC so that specific CSC therapy or targeted therapy can be conducted. Rizzo et al[5] showed an increase in the CSC population as a percentage of the cells in ascites fluid of recurrent ovarian cancer patients compared with cancer patients who had not previously received chemotherapy. This study was aimed to demonstrate the prognostic value of CSC by using the markers CD133, CD44, and ALDH1A1 in EOC.

METHODS

Design and research subjects

This cohort study was conducted in an ambispective (retrospective and prospective) fashion. The study was conducted in the Obstetrics and Gynecology Division, Oncology Gynecology Division of RSCM/FMUI, Department of Anatomic Pathology of RSCM/FMUI, Department of Clinical Pathology of RSCM/FMUI, Integrated Inpatient Unit Building A of RSCM/FMUI, Central Installation of RSCM/FMUI, and Medical Record of RSCM, from March 2017 to May 2018. The samples used in this study were patients with ovarian carcinoma types I and II who had previously undergone surgery and adjuvant chemotherapy. If any patient with a diagnosis of cancer other than ovarian cancer was identified, the patient was excluded from the sample. This study was approved by the Health Research Ethics Committee of the Faculty of Medicine of Universitas Indonesia and has received a certificate for passing ethical review with the number 82/UN2. F1/ETIK/2017. The patients and their families signed a letter of consent after receiving an explanation of the research procedure and possible risks.

Immunohistochemical examination was performed by using paraffin-embedded specimens. In each case, 8 preparations were made by sectioning paraffin blocks with a 3 µm microtome, followed by deparaffinization with xylol and rehydration in a graded alcohol series. The sections were then blocked to inhibit endogenous peroxidase activity and pretreated using Tris-EDTA.

In order to detect CD133, CD44, and ALDH1A1, specific mouse antibodies against CD133 (monoclonal anti-human CD133), CD44 (monoclonal anti-human CD44), and ALDH1A1 (monoclonal anti-human ALDH1A1) from Novusbio were used. For each pulse, an internal positive control in the stromal tissue and a negative control were included without primary antibody. The positive and negative controls were performed on the same tumor tissue as the test antibodies.

For the assessment of CD133, the presence of one or more well-wrapped cells was considered a positive reaction, and the absence of any wrapped cells was considered a negative one. For CD44, ≤10% was considered low and >10% was considered high cell expression. For ALDH1A1, ≤20% was considered low and >20% was considered high cell expression.[5]

Flow cytometry examination

The cell mixture in the cyst or ascitic fluid is also expected to contain CSC. To identify CSC, a panel of CSC markers consisting of CD133, CD44, and ALDH1A1 was used. On examination using flow cytometry, the cells were concentrated by centrifuging cyst fluid and ascitic fluid. The supernatant fluid was removed, leaving as much as 50 µl; afterwards, the cell mixture was resuspended.

Samples were incubated with fluorescently labeled antibodies against CD133 (phycoerythrin [PE]-labeled monoclonal anti-human CD133), CD44 (fluorescein isothiocyanate [FITC]-labeled monoclonal anti-human CD44), and ALDH1A1 (allophycocyanin [APC]-labeled monoclonal anti-human ALDH1A1). Then, cells were lysed with 1 ml fact flow fluid and discarded. The supernatant that formed was discarded. Furthermore, 2.5 µl of anti-ALDH1A1 was added, and 1 ml perm wash buffer was added and centrifuged at 500 g for 5 min. The last step was addition of 200 µl paraformaldehyde 1% in phosphate-buffered saline (PBS). Afterwards, the samples were analyzed by flow cytometry.

CSC are identified through the positive expression of all three CSC markers (CD133, CD44, and ALDH1A1). The markers CD133, CD44, and ALDH1A1 simultaneously identify the number of CSC. The percentage of cancer

cells is calculated from the percent expression of the CSC markers CD133, CD44, and ALDH1A1 in the ascetic and cystic fluid.

The results of CSC markers (CD133, CD44, and ALDH1A1) expression in patients with ovarian cancer types I and II on immunohistochemical and flow cytometry examinations will be included in a table along with chemotherapy response and survival. Data analysis was performed by using STATA software after all study recruitment phases were completed. Survival analysis was performed using the Kaplan-Meier method and the Cox proportional hazard test. Multivariate analysis was used to the model prognosis for 10 months. System scoring was created using receiver operating characteristic (ROC) curve analyses.

RESULTS

There were 55 research samples, 40 retrospective samples, and 15 prospective samples, of EOC that met the inclusion and exclusion criteria on consecutive sampling (Table 1). Retrospective samples were analyzed using immunohistochemistry (IHC) examination based on diagnosis and having undergone chemotherapy with carboplatin-paclitaxel for a minimum of 4 cycles. On the other hand, prospective samples were analyzed using IHC examination and flow cytometry based on diagnosis and the intention to undergo a minimum of 4 cycles of carboplatin-paclitaxel chemotherapy (Table 1).

In this study, a significant difference between histopathologic type and CSC markers was only seen for the marker CD44 (Table 2). The final model of 10-month chemotherapy response related to the 4 variables age ≥45 years, type II, stage III–IV, and high CD44 obtained an ROC with patients above the cut-off point of ≥25.8 being categorized as at risk of experiencing chemoresistance, and those having a score <25.8 being categorized as chemosensitive. The predictive model with a cut-off point of ≥25.8 had a sensitivity of 57.1% and a specificity of 87.8% and classified precisely 80% (Table 3). The value from the ROC was 0.7247 or 72.47%. This means that the model with the above-mentioned cut-off classified the strength of the diagnostic value of 10-month chemoresistance as 72.47%. The probability of EOC with a predictive score of chemoresistance ≥25.8 being at risk for 10-month chemoresistance increased

Table 1. Characteristics of the research subjects and CSC

Variables	n (%)
Age	
< 45 years	15 (27.3)
≥ 45 years	40 (72.7)
Parity	
< 2	26 (52.7)
≥ 2	29 (47.3)
Stage	
I	23 (41.8)
II	10 (18.2)
III	19 (34.5)
IV	3 (5.5)
Type of histology	
Type I	
Low-grade serous	6 (10.9)
Mucinous	6 (10.9)
Endometroid	7 (12.7)
Carcinoma clear cell	16 (29.1)
Seromusinosum	3 (5.5)
Mixed	1 (1.8)
Type II	16 (29.1)
High-grade serous	
Cell differentiation	
Good	19 (34.6)
Moderate	6 (10.9)
Poor	30 (54.5)
Chemotherapy response	
Chemosensitive	19 (34.5)
Chemoresistant	36 (65.5)
Surgical	
Staging	
Complete	7 (21.2)
Incomplete	26 (78.8)
Debulking	
Optimal	8 (36.4)
Suboptimal	14 (63.6)
CSCs	
CD133	
Negative	13 (23.6)
Positive	42 (76.4)
CD44	
Low	33 (60.0)
High	22 (4.0)
ALDH1A1	
Low	36 (65.5)
High	19 (34.5)

CSC=cancer stem cells; ALDH1A1=aldehyde dehydrogenase 1A1

Table 2. The relation between ovarian cancer patient characteristics and histopathology

Characteristics	Type, n (%)		p
	I	II	
Age			0.109
<45 years	14 (35.9)	2 (12.5)	
≥45 years	25 (64.1)	14 (87.5)	
Parity			0.276
<2	14 (34.9)	9 (56.2)	
≥2	25 (64.1)	7 (43.8)	
Stage			0.962
I–II	24 (61.5)	9 (56.2)	
III–IV	15 (38.5)	7 (43.8)	
Cell differentiation			1.000
Well–moderate	18 (46.2)	7 (43.8)	
Poor	21 (53.8)	9 (56.2)	
CD133			0.146
Negative	10 (25.6)	1 (6.2)	
Positive	29 (74.4)	15 (93.8)	
CD44			**0.005**
Low	19 (48.7)	15 (93.8)	
High	20 (51.3)	1 (6.2)	
ALDH1A1			0.111
Low	21 (53.8)	13 (18.2)	
High	18 (46.2)	3 (18.8)	

ALDH1A1=aldehyde dehydrogenase 1A1

to 82.5% (Table 3). Statistical analysis of chemotherapy response and survival in ovarian cancer types I and II also revealed no significant relationship (Table 4).

The final model of 10-month mortality in relation to the 3 variables type II, stage III–IV, and high CD44 found an ROC with a cut-off of ≥15.5 for the category at risk of death and a score of <15.5 as alive. The predictive model with a cut-off of ≥15.5 had a sensitivity of 83.3% and a specificity of 77.6% and classified precisely 78.2% (Table 5). The value of ROC was 86.9%, which means that the model with the cut-off point classifies the 10% strength of diagnostic value of death by 86.9%. EOC with a predictive score of death ≥15.5 was associated with an increase in the risk of 10-month mortality to 78.7% (Table 5).

The highest ROC score of death scores based on a combination of CSCs markers and clinical pathological factors, i.e., stage III–IV, age ≥45 years, poor differentiation, type II, CD133 negativity, high CD44, and high ALDH1A1, was 0.841.

Table 3. Sensitivity and specificity of chemoresistance scoring of epithelial ovarian cancer

Cut-off point	Sensitivity	Specificity	AC	LR+	LR-
≥0	100.0%	0.0%	25.5%	1.00	
≥7.1	100.0%	14.6%	36.4%	1.17	0.00
≥9.2	100.0%	34.2%	50.9%	1.52	0.00
≥9.5	92.9%	36.6%	50.9%	1.46	0.20
≥10	92.9%	46.3%	58.2%	1.73	1.15
≥16.3	92.9%	48.8%	60.0%	1.81	0.15
≥16.6	85.7%	58.5%	65.5%	2.07	0.24
≥17.1	78.1%	70.7%	72.7%	2.68	0.30
≥18.7	57.1%	80.5%	74.6%	2.93	0.53
≥19.2	57.1%	85.4%	78.2%	3.90	0.50
≥25.8	57.1%	87.8%	80.0%	4.69	0.49
≥26.3	28.6%	92.7%	76.4%	3.90	0.77
≥26.6	7.1%	100.0%	76.4%		0.93
>2.6	0.0%	100.0%	74.6%		1.00

AC=accuracy classification; LR+=positive likelihood ratio; LR-=negative likelihood ratio

DISCUSSION

EOC is the most common cause of death of all gynecologic malignancies.[1,2] EOC is a tumor originating from the ovarian epithelial surface. CSC serve in the histology and pathogenesis of EOC, known as malignant cancer cells with stem cell phenotypes. CSC subpopulations play an important role in tumor development, chemoresistance, and recurrence after first treatment.[7,8]

Whittemore et al[9] suggested that type II SC tumors have similar characteristics and degrees of differentiation to type I tumors; however, the results of SC evaluation and differentiation indicate that ovarian cancer type II has a high incidence and mortality. Characteristics of ovarian cancer type II mutations are also found in the SC subtype. These are due to the growth of precancerous lesions originating from the fallopian tube epithelium and a strong immunoreaction from the p53 mutation.[5] In ovarian cancer type I, the growth originates from the ovary surface through ovarian metaplasia or fallopian tube epithelium, endometrium, and peritoneum after an ovarian inclusion cortical cyst whose cells originate from the normal fallopian tube epithelium is formed through endosalpingiosis.[3]

There were no significant differences in the distributions of age, parity, staging, cell

Table 4. Factors related to the chemotherapeutic response of epithelial ovarian cancer

Variables	Chemotherapy response		Hazard ratio	95% CI	P
	Chemosensitive, n (%)	Chemoresistant, n (%)			
Patient age					0.087
< 45 years	4 (25.0)	12 (75.0)	5.92	0.7–45.3	
≥ 45 years	15 (38.5)	24 (61.5)			
Parity					0.211
< 2	9 (39.1)	14 (60.9)	0.50	0.1–1.4	
≥ 2	10 (31.2)	22 (68.8)			
Stage					**0.039**
I–II	5 (15.2)	28 (84.8)	3.17	1.0–9.4	
III–IV	14 (63.6)	4 (36.3)			
Type of histopathology					0.071
Type I	32 (82.1)	7 (17.9)	2.63	0.9–7.5	
Type II	9 (56.3)	7 (43.8)			
Cell differentiation					0.096
Good–moderate	3 (12.0)	22 (88.0)	4.04	7.1–20.9	
Poor	16 (53.3)	14 (46.7)			
CSCs					
CD133					0.640
Negative	11 (36.7)	19 (63.3)	1.28	0.4–3.6	
Positive	8 (32.0)	17 (68.0)			
CD44					0.374
Low	11 (30.6)	25 (69.4)	1.61	0.5–4.6	
High	8 (42.1)	11 (57.9)			
ALDH1A1					0.146
Low	4 (23.5)	13 (76.5)	3.03	0.6–13.5	
High	15 (39.5)	23 (60.5)			

CI=confidence interval; CSCs=cancer stem cells; ALDH1A1=aldehyde dehydrogenase 1A1

Table 5. Sensitivity and specificity of mortality scoring of epithelial ovarian cancer

Cut-off point	Sensitivity	Specificity	AC	LR+	LR-
≥0	100.0%	0.0%	10.9%	1.00	
≥6.2	100.0%	28.6%	36.4%	1.40	0.00
≥9.2	100.0%	42.9%	49.1%	1.75	0.00
≥10	100.0%	59.2%	63,6%	2.45	0.00
≥15.5	83.3%	77.5%	78.2%	3.71	0.21
≥16.2	50.0%	87.7%	83.6%	4.08	0.57
≥19.2	16.7%	100.0%	90.9%		0.83
>19.2	0.0%	100.0%	89.1%		1.00

AC=accuracy classification; LR+=positive likelihood ratio; LR-=negative likelihood ratio

differentiation, CD133, and ALDH1A1 between the 2 histopathological types (I and II) of ovarian cancer patients (p>0.05) (Table 2). In contrast, there was a difference in the proportion of CD44 markers between type I and type II (p=0.005). In histopathological type I, high-expression markers are more common than low-expression ones, whereas in type II, more low-expression than high-expression markers are found. A study by Sillanpää et al[10] on 307 ovarian cancer patients found high expression of CD44 in EOCs of the mantle (type I) compared with other types. CD44 is a CSC marker that is also present in non-CSC cells; therefore, this marker cannot stand alone as a CSC marker but must be combined with other markers. When used as a single marker as in this study, not only CSC but also more mature cells were measured.

The present results were also supported by Onal et al[11] on 84 patients, which stated that menopausal women older than 46 years carried a significant risk of relapse compared with those under 46 years old. The worse prognosis in older patients may be explained

by the difference in tumor biology and the immune response, as well as other comorbidities.

Apoptosis is a biological process controlled by various regulators and serves in ovarian cancer chemotherapy. Mutation of p53 in ovarian tumor type II causes inhibition of proapoptotic factors, which will induce oncogenesis and cause uncontrolled cell proliferation.[3,12] In this study, EOC type II was a factor associated with the response to chemotherapy.

Onal et al[11] found that patients with metastasis at the time of diagnosis and ascites before surgery had a statistically significant higher rate of recurrence. Similarly, patients with stage IV are more likely to relapse than those with stage III. This is associated with a chemotherapeutic response to residual tumor tissue and suboptimal surgical measure incomplete follow-up stage, as well as the patient's physical appearance status. This study shows that advanced stages of EOC are associated with the risk of resistance to chemotherapy.

The extracellular domains of CD44 interact with hyaluronan (HA), which will activate the cytoskeleton and matrix metalloproteinases (MMPs). MMPs are associated with the invasion of metastases and tumor cells. The transmembrane domain (CD44ß-like peptide) and intracellular domains serve to activate gene transcription, thereby increasing migration, invasion, angiogenesis, and metastasis.[12,13]

The clinicopathological risk factors high CD44 and high ALDH1A1 are prognostic of chemoresistance. A study by Burgos-Ojeda et al[14] using ALDH and CD133 as CSC markers determined that ALDH and CD133 positivity were associated with poorly differentiated and 4-month aggressive tumors *in vivo*, whereas ALDH and CD133 positivity are markers of good differentiation and tumors that grow within 6–12 months *in vivo.* The study by Alvero et al[15] found CD44 expression in metastatic tumors and ascitic fluid in ovarian cancer patients who had previously undergone chemotherapy. Landen et al[16] found high ALDH expression associated with worse survival in ovarian cancer. A study by Paik et al[17] suggested that a combination of CD44 and CD117 was associated with chemoresistance in isolated cancer tissue and a poor prognosis.

To determine the predictive score for chemoresistance, we used the variables age ≥45 years, type II ovarian cancer, stage III–IV ovarian cancer, and high CD44 expression. As for the predictive score for death, it was determined from the variables type II, stage III–IV, and high CD44 expression. Until now, the success of chemotherapy and survival have not been good predictors of chemoresistance. It is hoped that this scoring system will help to determine a more specific treatment in EOC patients using targeted therapy. Therefore, per the results of this study, the examination of CD133, CD44, and ALDH1A1 is recommended in determining life expectancy with attention to other clinicopathological risk factors.

Conflicts of Interest

The authors affirm no conflict of interest in this study.

Acknowledgment

I gratefully thank the principal of Cipto Mangunkusumo Hospital, Universitas Indonesia, and Universitas Hasanuddin for its support on this writing.

Funding Sources

None.

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