Co-expression of CD44/MyD88 is a poor prognostic factor in advanced epithelial ovarian cancer

Yi Zhu[1,2#], Hongtao Zhang[3#], Guonan Zhang[1], Yu Shi[1], Jianming Huang[1,4]

[1]Department of Gynaecologic Oncology, [2]Department of Ultrasound, Sichuan Cancer Hospital & Institute, Cancer Hospital Affiliated to School of Medicine, University of Electronic Science and Technology of China, Chengdu 610041, China; [3]Department of Obstetrics and Gynecology, Sichuan Jinxin Women and Children's Hospital, Chengdu 610000, China; [4]Department of Biochemistry & Molecular Biology, Sichuan Cancer Hospital & Institute, Cancer Hospital Affiliated to School of Medicine, University of Electronic Science and Technology of China, Chengdu 610041, China

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[#]These authors contributed equally to this work.

Correspondence to: Guonan Zhang; Jianming Huang. Department of Gynaecologic Oncology, Sichuan Cancer Hospital & Institute, Sichuan Cancer Center, School of Medicine, University of Electronic Science and Technology of China, No. 55, Section 4, South People's Road, Chengdu 610041, China. Email: zhanggn@hotmail.com; wesleyhuangcn2002@163.com.

Background: Cluster of differentiation 44 (CD44)/myeloid differentiation factor 88 (MyD88) is the molecular characterization of EOC stem cells. An important characteristic of CD44+/MyD88+ epithelial ovarian cancer (EOC) cells, which differentiate them from the CD44–/MyD88– EOC cells, is the presence of a functional TLR4-MyD88-NFkB pathway. The aim of our study is to investigate the clinical significance of CD44/MyD88 co-expression in EOC.

Methods: A total of 138 specimens of ovarian tissues was detected CD44 and MyD88 expression by immunocytochemistry, including EOC (N=108), borderline tumors (N=10), benign cysts (N=10) and normal ovarian tissue (N=10). The association of CD44/MyD88 co-expression with clinicopathological factors and outcomes was analyzed.

Results: The expression of CD44 was showed distinct difference in EOC (53 of 108, 49.1%), in borderline tumors (3 of 10, 30.0%), in benign cysts (2 of 10, 20.0%) and normal ovarian (2 of 10, 20.0%). A total of 41 (38.0%) cancers showed a combined expression of CD44/MyD88. The expression of CD44 and MyD88 had definitely correlativity (r=0.21, P=0.026). CD44/MyD88 co-expression was associated with tumor progression, metastasis, and recurrence in advanced EOC, and an independent prognostic factor for disease-free survival and overall survival.

Conclusions: CD44/MyD88 co-expression has been shown to contribute to EOC progression and outcome directly and has a promising as a therapeutic target in EOC.

Keywords: Ovarian cancer; cluster of differentiation 44 (CD44); myeloid differentiation factor 88 (MyD88); prognostic factors, metastasis

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Introduction

Epithelial ovarian cancer (EOC) is the leading cause of gynecologic cancer mortality worldwide (1). Due to no symptoms, EOC is usually diagnosed at a late stage. Although many tumors commonly relieve effectively after initial treatment, the survival outcome of EOC patients with metastatic and/or recurrent disease is still extremely

poor (2,3). Due to the drug resistance and the limit on the efficacy of second round chemotherapy, the 5-year survival rate for EOC is only about 30% (4). This disappointing situation strongly suggests that it is necessary to develop novel therapeutic strategies for preventing or targeting chemoresistant recurrence, to improve survival.

Tumor-initiating cells or cancer stem cells (CSCs) can self-renew and generate differentiated cells (non-stem cells) to provide a cell reservoir and maintain the tumor and may be responsible for drug resistance and the primary source of recurrence (5). CSCs are capable to survive conventional treatments, which usually target fast dividing cells, and give rise to recurrent tumors that are more chemo-resistant and more aggressive (6,7). Current evidence suggests that the molecular characterization of EOC stem cells is $CD44^+/MyD88^+$ (8).

Tumors are heterogeneous and consist of multiple types of cancer cells, which exhibit different chemo-responsiveness. An important characteristic of $CD44^+/MyD88^+$ EOC stem cells, which differentiate them from the $CD44^-/MyD88^-$ EOC cells, is the presence of a functional TLR4/MyD88 pathway. This pathway drives NF-κB activity and constitutively secretes pro-inflammatory cytokines, which confers paclitaxel resistance and plays a critical role in their own survival and tumor progression (9-12). Our previous study demonstrated that expression of TLR4 is in detected all ovarian tissues, and expression of MyD88 correlated with EOC chemoresistance and poor clinical outcome, which was detected in 77.1% of patients with EOC (10). CD44 is expressed in the majority of EOC. However, to date, investigation of CD44 has yielded conflicting results. Particularly, the role of CD44/MyD88 expressing in human ovarian cancer remains elusive.

Therefore, we focused on investigating the co-expression of CD44 and MyD88 in EOC tissues and the correlation with tumor progression, metastasis, and recurrence in patients with advanced EOC.

Methods

Tumour samples

A total of 138 patients who underwent surgery from 1999 to 2009 at the Sichuan Cancer Hospital were investigated in this study with an advanced stage EOC

A total of 138 patients who underwent surgery from 2005 to 2015 from at Sichuan Cancer Hospital & institute were investigated in this study, including EOC tissue (n=108), normal ovarian tissue (n=10), benign cysts (n=10) and borderline tumors (n=10). This study included patients with primary EOC that was at International Federation of Gynecology and Obstetrics (FIGO) IIIc–IVa. *Table 1* described the information on EOC patient age and tumor features, which was acquired from clinical and pathological data. Tumor stages were classified according to the standard proposed by the. Tissue was obtained before chemotherapy. All patients underwent 6–8 cycles paclitaxel/carboplatin (TP) combination chemotherapy after primary cytoreductive surgery. Cancers associated with germ cell tumor, sex cord-stromal tumors, or secondary tumors were excluded. The diagnosis, histological type and grade of all tumor tissues were confirmed by two pathologists. The tissue samples were obtained by ovarian resection, then fixed by formalin and embedded in paraffin for immunohistochemistry.

Ethics approval and consent to participate

Ethical approval for this project was obtained from our Internal Ethics Committee. Written informed consent was obtained from all subjects. All methods were carried out in accordance with the approved guidelines.

Immunohistochemistry

The tumor tissue (4-μm), which was continuously sliced with paraffin wax, was dewaxed in xylene and then hydrated in a series of ethanol, and the antigen was repaired by using autoclave oven technique. To quench the activity of endogenous peroxidase, the slides were then placed in a dye dish containing 0.3% H_2O_2 for 30 minutes and rinse three times with PBS. 100–200 μL of 5% BSA in PBS was added to the circumscribed areas and incubated for 20 min to avoid non-specific background. To keep the tissue or cell from drying out, the whole procedure was performed in a moisture chamber at room temperature. Next, the primary antibody [rabbit anti-human CD44, or MyD88 Abs (4–5 μg/mL); MyD88 Abs (4–5 μg/mL; Epitomics and Abcam, USA)] incubate overnight in 4 ℃, then rinse three times with PBS, and specimens with biotin-resistant rabbit IgG (5 μg/mL; SANTA, USA) and horseradish peroxidase chain mildew resistant biotin protein (4MG/ML) were incubated at 37 ℃ for 50 minutes. The DAB (2.5 mg 3,3'-two amino-benzidine in 5 mL 0.1 mol/L Tris) is used for color rendering. Add 25 μL 0.03% H_2O_2 to the color source before use. The glass slides were dyed with Hematoxylin and loaded in glycerin gel after washing with double steaming water and then

Table 1 Clinicopathological features according to CD44 and CD44/MyD88 expression

Clinicopathological factors	CD44			CD44/MyD88			
	Negative, n=55 (50.9%), No. (%)	Positive, n=53 (49.1%), No. (%)	P value	$CD44^-/MyD88^-$, n=15 (13.9%), No. (%)	$CD44^-/MyD88^+$, n=40 (37.0%), $CD44^+/MyD88^-$, n=12 (11.1%), No. (%)	$CD44^+/MyD88^+$, n=41 (38.0%), No. (%)	P value
Age (years)			0.126				0.112
≥55	36 (33.3)	27 (25.0)		11 (10.2)	33 (30.6)	19 (17.6)	
<55	19 (17.6)	26 (24.1)		4 (3.7)	19 (17.6)	22 (20.4)	
Pathology			0.029				0.352
Serous	41 (38.0)	48 (44.4)		13 (12.0)	40 (37.0)	36 (33.3)	
Other	14 (13.0)	5 (4.6)		2 (1.9)	12 (11.1)	5 (4.6)	
Histological grade			0.018				0.037
Well/moderate	23 (21.3)	11 (10.2)		9 (8.3)	14 (13.0)	11 (10.2)	
Poor/clear cell	32 (29.6)	42 (38.9)		6 (5.5)	38 (35.2)	30 (27.8)	
Malignant cells in ascites			0.001				0.017
Yes	15 (13.9)	28 (26.0)		3 (2.8)	17 (15.7)	23 (21.3)	
No	40 (37.0)	25 (23.1)		12 (11.1)	35 (32.4)	18 (16.7)	
Lymph node metastasis			0.023				0.012
Yes	10 (9.3)	20 (18.5)		2 (1.9)	10 (9.3)	18 (16.7)	
No	45 (41.7)	33 (30.6)		13 (12.0)	42 (38.9)	23 (21.3)	
Liver or lung metastasis			<0.001				<0.001
Yes	9 (8.3)	26 (24.1)		0 (0)	12 (11.1)	23 (21.3)	
No	46 (42.6)	27 (25.0)		15 (13.9)	40 (37.0)	18 (16.7)	
Residual tumor			0.002				<0.001
≥1	20 (18.5)	35 (32.4)		3 (2.8)	22 (20.4)	30 (27.8)	
<1	35 (32.4)	18 (16.7)		12 (11.1)	30 (27.8)	11 (10.2)	

CD44, cluster of differentiation 44; MyD88, myeloid differentiation factor 88.

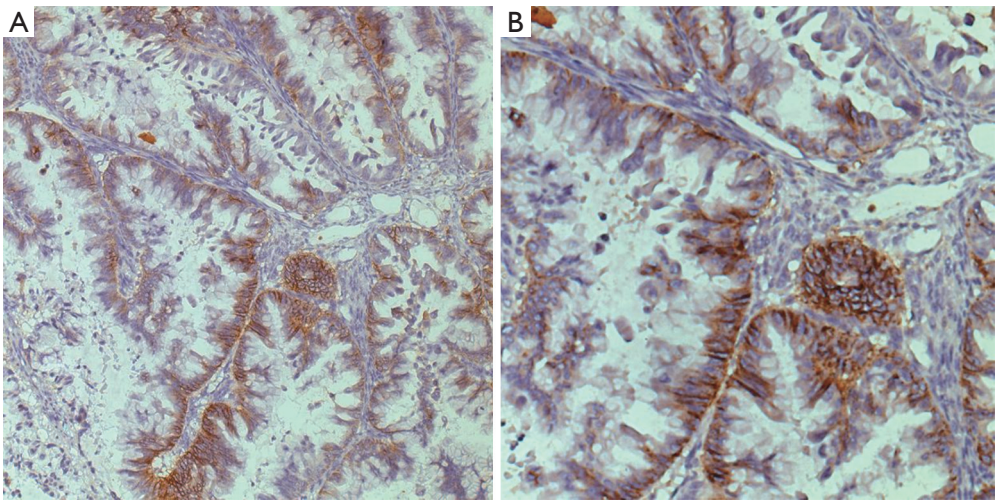

Figure 1 Detection of CD44 immunoreactivity in EOC. CD44 had a strong immunostaining localized in the membrane of EOC. Original magnification: (A) ×200; (B) ×400. EOC, epithelial ovarian cancer.

Evaluation of immunohistochemical findings

Two pathologists, who did not know the clinical and results data, independently diagnosed each slide. The use of digital cameras (Olympus IX71 inverted fluorescence microscope and analysis image capture software) captured different staining density regions of high-power fields (×400), including the higher, middle, low, and negative staining region. The photo is printed on plain paper and the grid is drawn on it. We calculated percentage of positive staining tumor cells in an average of 2,000 tumor cells per tumor (range, 1,500–2,500). Afterwards, according to the grade of 0–4 scale (0: no staining; 1+: ≤10%; 2+: 11–30%; 3+: 31–50%; 4+: >50%), to score the percentage of CD44 or MyD88 positive tumor cells. The staining intensity score: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Multiplies he percentage and staining intensity scores to produce a "combination" score: 0, negative (–); 1, 2, slight positive (+); 3, 4, 6, moderately positive (++); 9, 12, strongly positive (+++) (13).

Statistical analysis

SPSS 17.0 software was used for analysis. Use the Pearson X^2 or Fisher's exact test to compare qualitative variables. DFS was defined as the time from the date of surgery to the first day of detecting recurrence. The date of death or last follow-up was used if there was no recurrence. OS was defined as time interval between the date of the operation and last follow up or death. The median follow-up period for DFS and OS from initial surgery was 5 years. The Kaplan-Meier curve was used to estimate DFS and OS, which was compared by the log-rank test. The recurrence and death time were analyzed by cox proportional hazards model with univariate and multivariate analyses. The risk ratio (HR) between the prognostic group and its 95% confidence interval was calculated. The probability value (P) <0.05 is considered to be statistically significant.

Results

Prevalence of CD44$^+$/MyD88$^+$ cells in EOC tissues

The first purpose was to identify the prevalence of CD44$^+$/MyD88$^+$ cells in paraffin-embedded EOC tumor sections obtained before initiation of chemotherapy. The expression of CD44 varied substantially, from no expression to strong expression. Immunohistochemical staining showed that 53 of the 108 patients' samples was detected CD44 positive (*Figure 1*). There is no or very weak expression of CD44 in the normal ovarian epithelium (2 of 10, 20.0%), benign cysts (2 of 10, 20.0%), borderline tumors (3 of 10, 30.0%). In addition, 41 (38.0%) cases showed a co-expression of CD44/MyD88. In the co-expression analysis, EOC samples with CD44-positive expression frequently showed high levels of MyD88 (P=0.007). CD44 and MyD88 expression were relevant through Pearson and Spearman correlation coefficient analysis (P=0.026, P=0.023, respectively). For comparison, patients were classified into three groups, according to the prevalence of CD44 and MyD88

Table 2 Clinicopathological features, tumor markers, and patient survival (univariate analysis)

Variable	5-year DFS		5-year OS	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (≥55 *vs.* <55 years)	0.929 (0.561–1.538)	0.774	0.698 (0.373–1.305)	0.260
Pathology (serous *vs.* other)	0.665 (0.316–1.400)	0.283	1.142 (0.475–2.745)	0.766
Histological grade (poor *vs.* well/moderate)	0.950 (0.562–1.608)	0.849	1.258 (0.637–2.484)	0.508
Malignant cells in ascites (yes *vs.* no)	1.775 (1.075–2.930)	0.025	1.570 (0.831–2.968)	0.165
Lymph node metastasis (yes *vs.* no)	3.404 (2.026–5.720)	<0.001	2.620 (1.394–4.924)	0.003
Liver or lung metastasis (yes *vs.* no)	3.226 (1.950–5.336)	<0.001	2.933 (1.570–5.477)	0.001
Residual tumor (≥1 *vs.* <1)	2.721 (1.619–4.572)	<0.001	1.971 (1.007–3.859)	0.048
CD44 (negative *vs.* positive)	1.087 (0.660–1.792)	0.743	1.939 (1.015–3.706)	0.045
MyD88 (negative, low *vs.* high)	3.297 (2.185–4.976)	<0.001	3.633 (2.136–6.179)	<0.001
CD44/MyD88 (negative *vs.* positive)	2.426 (1.595–3.690)	<0.001	3.377 (1.885–6.048)	<0.001

DFS, disease-free survival; OS, overall survival; CD44, cluster of differentiation 44; MyD88, myeloid differentiation factor 88; HR, hazard ratio; CI, confidence interval.

expression in tumors: $CD44^-/MyD88^-$, $CD44^-/MyD88^+$ or $CD44^+/MyD88^-$, and $CD44^+/MyD88^+$.

Clinicopathological significance of CD44/MyD88

The relationship between the distribution of CD44/MyD88 expression and EOC clinicopathological features is shown in *Table 1*. There was significant correlation between CD44 expression and histological type, histological grade, malignant cells in ascites, liver or lung metastasis, lymph node metastasis and residual tumor (P<0.05). Furthermore, CD44/MyD88 co-expression significantly correlated with histological grade, malignant cells in ascites, liver or lung metastasis , lymph node metastasis and residual tumor (P<0.05). No significant correlation between CD44/MyD88 expression and age.

Clinicopathological parameters and patient survival in EOC

At 5-year follow-up, the recurrence rate was 58.3% (63 patients), and mortality was 37.9% (41 patients). In univariate analysis, ascites malignant cells, liver or lung metastasis, lymph node metastasis and residual tumor were important factors associated with DFS and OS (P<0.05). Patient age, pathology and histological grade had no correlation with DFS or OS (*Table 2*). Independent prognostic factors were identified through multivariate analysis. However, these clinicopathological parameters were not identified as an independent risk factor for either recurrence or death on multivariate analysis (*Table 3*).

$CD44^+/MyD88^+$ correlation with patient survival

We evaluated the effects of CD44 and CD44/MyD88 expression on the survival of patients with EOC. Compared with a negative CD44 expression, a positive expression of CD44 had no significant impact on DFS (median DFS: 18.76 *vs.* 20.96 months; log-rank P=0.743; *Figure 2A*). However, a positive CD44 expression was involved in the worse OS (median OS: 25.23 *vs.* 42.91, log-rank P=0.041; *Figure 2B*). A significantly poorer DFS (log-rank P<0.001, *Figure 2C*) and OS (log-rank P<0.001, *Figure 2D*) was found in the patients with co-expression of CD44/MyD88.

In univariate analysis of *Table 2*, there was no significant correlation between CD44 expression and DFS. MyD88 expression (HR: 3.297; 95% CI: 2.185–4.976; P<0.001) and CD44/MyD88 co-expression (HR: 2.426; 95% CI: 1.595–3.690; P<0.001) significantly influenced DFS. In addition, CD44 expression (HR: 1.939; 95% CI: 1.015–3.706; P=0.045), MyD88 expression (HR: 3.633; 95% CI: 2.136–6.179; P<0.001), and CD44/MyD88 co-expression (HR: 3.377; 95% CI: 1.885–6.048; P<0.001; *Table 2*) was particularly associated with poor OS. As the co-expression CD44/MyD88 includes both CD44 and MyD88 information, we established two models, respectively. In multivariate analysis, MyD88 expression

Table 3 Clinicopathological features, tumor markers, and patient survival (multivariate analysis)

Variable	5-year DFS		5-year OS	
	HR (95% CI)	P value	HR (95% CI)	P value
Model A				
Malignant cells in ascites (yes *vs.* no)	1.160 (0.668–2.017)	0.598	–	–
Lymph node metastasis (yes *vs.* no)	1.597 (0.767–3.322)	0.211	0.858 (0.336–2.192)	0.750
Liver or lung metastasis (yes *vs.* no)	1.786 (0.908–3.514)	0.093	2.294 (0.990–5.319)	0.053
Residual tumor (≥1 *vs.* <1)	1.823 (1.060–3.135)	0.030	1.626 (0.803–3.293)	0.177
CD44 (negative *vs.* positive)	–	–	1.120 (0.539–2.330)	0.761
MyD88 (negative, low *vs.* high)	2.623 (1.468–4.687)	0.001	3.339 (1.568–7.112)	0.002
Model B				
Malignant cells in ascites (yes *vs.* no)	1.151 (0.673–1.968)	0.608	–	–
Lymph node metastasis (yes *vs.* no)	2.331 (1.214–4.478)	0.011	1.388 (0.610–3.156)	0.434
Liver or lung metastasis (yes *vs.* no)	1.540 (0.792–2.994)	0.203	1.571 (0.689–3.585)	0.283
Residual tumor (≥1 *vs.* <1)	1.506 (0.857–2.648)	0.155	1.346 (0.671–2.698)	0.403
CD44/MyD88 (negative *vs.* positive)	1.791 (1.119–2.865)	0.015	2.729 (1.460–5.101)	0.004

DFS, disease-free survival; OS, overall survival; CD44, cluster of differentiation 44; MyD88, myeloid differentiation factor 88; HR, hazard ratio; CI, confidence interval.

particularly related to poor DFS (adjusted HR: 2.623; 95% CI: 1.468–4.687; P=0.001) and OS (adjusted HR: 3.339; 95% CI: 1.568–7.112; P=0.002) (*Table 3* model A). As *Table 3* model B shown, co-expression of CD44/MyD88 also significantly related to poor DFS and OS (adjusted HR: 1.791; 95% CI: 1.119–2.865; P=0.015; adjusted HR: 2.729; 95% CI: 1.460–5.101; P=0.004, respectively).

Discussion

CD44, as a cell surface receptor, is associated with cell signaling, differentiation, adhesion, proliferation, migration and angiogenesis, which are important properties for normal and cancerous cell function. Various epithelial malignancies, including ovarian cancer, often expressed CD44 which is a potential marker for the identification of CSCs (14-16). While CD44 is absent or very low in normal epithelial ovarian cells (17,18), CD44 has become a specific molecular marker for normal stem-like epithelial cells in the distal end of the fallopian tube (19). CD44 was frequently overexpressed in EOC play complex roles in tumor progression and metastasis. Expression of CD44 and specific isoforms in epithelial ovarian carcinoma has remained a controversial topic. Some studies have shown that CD44 expression have a significant correlation with metastasis and survival outcome (20-23), while in contrast, other studies have found no association (17,24-26). Additionally, other studies have indicated that high CD44s expression is a factor in improving prognosis of ovarian cancer (8,18,27). In this study, our data showed that CD44 may be an important molecular marker for poor prognosis, which associated with histological type and grade, residual tumor, metastasis, and 5-year survival. However, the differences of each study were not surprising because technical factors, including the use of various antibodies and detection methods, could exist a certain distinction. The other reason was that the cohorts of EOC patients examined in different studies were highly heterogeneous.

Recently, Mor *et al.* reported a distinctive phenotype of EOC stem cells characterized by $CD44^+/MyD88^+$, and confirmed the functionality of the TLR4/MyD88 pathway only in the CD44+ cell population (8). Toll like receptors (TLRs), particularly the TLR4 signaling pathway, are involved in tissue renewal and repair, the control of infection, and may correlated with tumor formation (28-30). MyD88, as a joint protein, is a critical component of TLR4 pathway. The activation of TLR4/MyD88 pathway leads to downstream activation of the NF-κB signaling pathway,

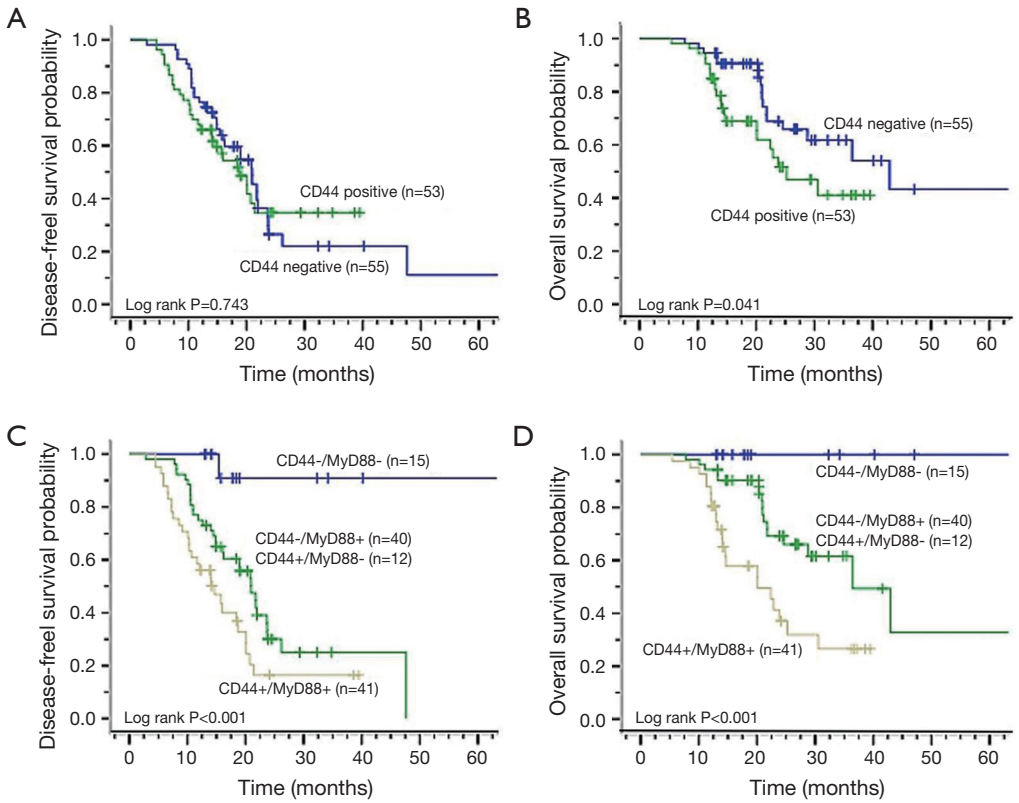

Figure 2 Kaplan-Meier survival curves of DFS and OS in patients with EOC according to CD44 and CD44/MyD88 expression. (A) CD44 expression potentially correlated to a poorer DFS (P=0.743); (B) CD44 expression significantly correlated to a poorer OS (P=0.041); (C,D) TLR4 and MyD88 co-expression significantly correlated to both poor DFS and OS (P<0.001, respectively).

cytokine production and chemo-resistance. $CD44^+$ EOC cells express the TLR4/MyD88 pathway may promote the process of repair/differentiation triggered by the CSCs. However, the investigation about the association between the co-expression CD44/MyD88 and cancer clinicopathological factors, prognostic significance, has not been done in clinical samples. It is noticed that MyD88 expression significantly decreased with the differentiation of ovarian CSC in *ex vivo* manipulation (31). This compared similarly to our findings, showing that there was significant relation between CD44 and MyD88 expression in EOC patients.

Hyaluronan (HA) induces CD44 interaction with TLR-4 signaling pathway, like "cross-talk", stimulating the production of cytokine/chemokine production in a CD44-specific and MyD88-dependent manner, resulting in the adhesion, migration, and invasion of EOC cells (32,33). Ovarian cancer tumors consist of CSCs ($CD44^+$/$MyD88^+$), progenitor cells ($CD44^-$/$MyD88^+$ or $CD44^+$/$MyD88^-$) and fast dividing cells ($CD44^-$/$MyD88^-$), which constitutes the tumor heterogeneity (27). Our findings suggested that co-expression of CD44/MyD88 promotes EOC metastasis and progression, and is an independent and significant poor prognostic factor.

Although 70% of ovarian cancers is effective for initial treatment (surgery followed with TP combination chemotherapy), but most cases frequently recur and develop chemotherapeutic resistance. It has been demonstrated that paclitaxel selectively induce cell death in $CD44^-$/$MyD88^-$ EOC cells but has a pro-survival effect and enhances self-renewal in the pleuripotent and chemoresistant $CD44^+$/$MyD88^+$ EOC stem cells (34). Fully recognize characteristic Molecular marker, like CD44/MyD88, which was associated with metastasis, recurrence and drug resistance, is helpful for designing a more strategical EOC therapeutics. Our results highlight the need to identify the EOC $CD44^+$/$MyD88^+$ patients, who should not receive paclitaxel chemotherapy. The reason is that $CD44^+$/$MyD88^+$ EOC has paclitaxel resistance, and more importantly, it can

enrich more aggressive CSCs. Moreover, it has HA-grafted particle clusters loaded with Mitomycin C as selective nanovectors for cancers, which might be suitable for future treatment of many CD44-expression tumors (35,36). Hyaluronan-CD44 antagonists provide another reasonable therapy for eliminating the characteristics of these cells (37). Our previous study reported that AO-I could significantly enhance the sensitivity of MyD88-positive EOC cells to chemotherapy of paclitaxel by blocking TLR4/MyD88 signaling. These therapeutic approaches could be a promising strategy for targeting at CD44/MyD88 co-expression EOC (38).

Taken together, $CD44^+/MyD88^+$ was an useful and important marker, which had contributed to tumor progression and poor prognosis in patients with EOC, as well as a potentially effective therapeutic target for prevention and treatment of metastasis and recurrence. Based on these data, we propose that the mode of management for EOC patients should take into consideration the tumor's molecular phenotype.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by our Internal Ethics Committee and written informed consent was obtained from all patients.

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