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Original Article

ALDH enzymatic activity and CD133 positivity and response to chemotherapy in ovarian cancer patients

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Abstract: The prognostic/predictive role of both CD133 and Aldehyde dehydrogenase (ALDH) expression in human ovarian cancer remains elusive. This is an observational study that investigated the expression of CD133 and of ALDH enzymatic activity in fresh ovarian cancer samples and their association with different clinic-pathological patient' characteristics and explored their possible predictive/prognostic role. We analyzed the expression of CD133 and ALDH enzymatic activity in 108 human ovarian cancer samples. We found that among the total patients analyzed, 13% of them was completely negative for ALDH activity and 26% was negative for CD133 staining. Both markers were variably expressed within the samples and when both studied in the same tumor sample, no statistically significant correlation between ALDH enzymatic activity and CD133 expression was found. No statistical significant correlation was found also between the percentage values of positive ALDH and CD133 cells and the number of serial passages patient's cultures underwent, suggesting that these markers do not confer by themselves a self-renewal growth advantage to the cultures. Lower levels of CD133 were associated with higher tumor grade. No correlation with response to therapy, progression free survival and overall survival was found. Our data suggest that neither ALDH enzymatic activity nor CD133 expression provide additional predictive/prognostic information in ovarian cancer patients.

Keywords: CD133, ALDH activity, ovarian carcinoma

Introduction

Ovarian cancer represents the most lethal gynecological malignancy, both due to a lack of early detection which results in diagnosis at a late stage of the disease and to the high frequency of relapse, commonly resistant to chemotherapy [1]. The identification of markers predictive of response could help in customizing therapy avoiding toxic treatments in those patients whose tumors are likely to be less responsive to a given treatment.

The recently put forward cancer stem cell hypothesis suggests that tumor might be driven and sustained by a subset of cells with characteristic of stem cell including unlimited proliferative potential and resistance to therapy [2, 3]. The existence of such cells could explain why cancers often relapse despite clinical remission with initial therapy; indeed with time few treatment-resistant stem cells could repopulate the tumor [4, 5]. A number of evidence suggests that the cancer stem cell model also applies to ovarian tumor, even if no consensus on which markers define the ovarian cancer stem cell has reached yet (for review see [6]). Among others, both CD133 and Aldehyde Dehydrogenase (ALDH) have been evocated as possible markers associated with ovarian cancer stem cells [6-11]. CD133+ cells from ovarian cancer cells generated large tumors more rapidly than CD133- cells. CD133+ cells in primary human ovarian tumor xenografts were responsible for serial tumor passage [7]. Bata and colleague [8] confirmed CD133 as a marker of tumorigenic population in ovarian cancer cell lines; they also showed that the CD133+

sorted cells were able to divide asymmetrically, to generate both CD133+ and CD133-negative cells, to be more tumorigenic *in vivo* and to be more resistant to chemotherapy. ADLH is an enzyme responsible for the detoxification of intracellular aldehydes [12]. It is responsible for tissue specific irreversible oxidation of retinal to retinoic acid, with a role in cell differentiation and proliferation [13]. In addition, it also protects cells from cytotoxic drugs [3]. ALDH isoform 1 (ALDH1) has been used to identify normal stem and progenitors cells in various tissues and recently also cancer stem cells in leukemias and solid tumors, including ovarian tumors [14-17]. ALDH1 expression and activity have been reported to be increased in chemoresistant ovarian cancer cell lines and in *in situ* primary ovarian cancer xenografts treated with platinum [9, 18].

The predictive/prognostic role of both CD133 and ALDH expression in human ovarian cancer remains elusive, as the data published to date have been conflicting. The present study investigated the expression of CD133 and ALDH enzymatic activity in fresh ovarian cancer samples, their association with different clinic-pathological patient' characteristics and explored their predictive and prognostic role.

Materials and methods

Fresh tumor samples

The Clinic of Obstetrics and Gynecology of San Gerardo Hospital (HSG) provided the human ovarian tumor samples, whose use was approved by the local scientific ethic committee with patient's written consent. The patients came to the attention for ovarian tumor mass cytoreduction. Within 24-48 hours of surgery, fresh samples were mechanically disaggregated and enzymatically digested with 2500 U/mL collagenase I (Sigma) for 1 h at 37°C and a single cell suspensions was obtained. The cell suspension was then both processed for evaluation of ALDH enzymatic activity and/or for CD133 detection. Whenever possible, the cell suspension was placed in low adherence flasks (Corning) under stem-cell conditions: *serum*-free DMEM/F12 supplemented with 5 μg/mL insulin (Sigma), 20 ng/mL human recombinant epidermal growth factor (EGF, Peprotech), 10 ng/mL basic fibroblastic growth factor (bFGF, Peprotech) and B27 Supplement (Gibco).

Ovarian cancer cell lines

Ovarian cancer cell lines (OVCAR3, OVCAR5, OVCAR8, OVCAR432, A2780, SKOV3, IGROV, OVCAR420 and OVCAR433) were obtained from American Type culture collection (ATCC, Rockville, MD, USA). Cells were grown in RPMI medium (Biowest) supplemented with 10% of FBS and 2 mM of L-Glutamine (Lonza). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Aldefluor® assay

The Aldefluor® kit (Stem Cell Technology) was used for the detection of ALDH enzymatic activity. Fresh cells obtained from the tumor digestion and cell lines were washed in PBS and adjusted to concentration of 10^6 cells/mL with assay buffer. Aldefluor® substrate was added to the sample ("test" sample), and then in half of sample the reaction was immediately blocked with the addition of the DEAB inhibitor ("control" sample). The "test" and "control" samples were incubated for 30-60 min at 37°C and then analyzed with a flow cytometer (FACS Calibur, Becton Dickinson). Each FACS analysis was performed on at least 10'000 events.

CD133 staining

Fresh cells obtained from tumor digestion and cell lines were washed in PBS and resuspended in 0.5% BSA- 2 mM EDTA buffer. Cells were stained with CD133/2 PE antibody (Miltenyi Biotech, dilution 1:50) for 10 min at 4°C. A preincubation with CD133/2 pure antibody (Miltenyi Biotech) was used in the control sample to establish background fluorescence. Samples were analyzed with a flow cytometer system (FACS Calibur, Becton Dickinson). Each FACS analysis was performed on at least 10'000 events.

Statistical methods

Demographic and pathological characteristics, markers expression and chemotherapy administered were summarized using descriptive statistics (median and range for continuous variables and absolute and percentage frequencies for categorical variables); a non parametric approach was used to detect statistical association and to estimate statistical correlation between pathological characteristics and

Table 1. Patients' characteristics

	% BIOLOGICAL MARKER POSITIVITY	
CLINICAL PARAMETERS	ALDH	CD133
Number of total patients	47	91
Age at diagnosis (years)		
Median	55	55
Range	(19-82)	(19-86)
FIGO stage (%)		
Stage I-II	13	20
Stage III-IV	33	70
n.a.	1	1
Histotype (%)		
Serous	25	41
Mucinous	8	11
Endometroid	3	9
Clear cell	1	5
Indifferentiated	2	8
Other	8	17
Tumor grade (%)		
BL*	4	6
1	5	6
2	7	8
3	30	67
n.a.	1	4
Residual tumor (%)		
<2 cm	31	61
>2 cm	3	16

*Borderline tumor. n.a.: not available.

marker expression (Wilcoxon Rank Sum Test and Spearman's rank correlation coefficient were used). Response to first-line chemotherapy was evaluated using the RECIST criteria [19]. Both progression-free survival (PFS, event: first progression of disease or death by any cause) and overall survival (OS, event: death by any cause) were calculated considering as starting point the date of diagnosis of ovarian cancer; the logistic and Cox regression models were respectively used to detect and estimate the statistical association between markers expression and treatment response or time-to-event endpoints. All tests were two-sided and a p-value less than 0.05 was considered statistically significant. Statistical analysis was done using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA); the dot and scatter plots were created using Stata Version 12.1 (Stata Corporation, TX, USA).

Results

ALDH activity and CD133 expression in fresh ovarian tumor samples

From January 2007 to December 2010, a total of 47 and 91 ovarian cancer samples were processed for ALDH enzymatic activity assay and CD133 staining respectively; in 30 cases both markers could be evaluated. Patients' characteristics are depicted in **Table 1**.

ALDH and CD133 were variably expressed (**Figure 1**, panel A and **Table 2**); 6 samples out of 47 were negative for ALDH (13%), and 24 out 91 were completely negative for CD133 (26%). The values for both markers were similar to the ones we found in different ovarian cancer cell lines (**Table 3**). In 30 cases both markers could be evaluated, but no correlation between their positivity was observed (**Figure 1**, panel B).

Both markers were not statistically associated with any clinical-pathological characteristics analyzed, except for CD133, whose levels were inversely associated with tumor grade (p=0.003) (**Table 2**). In the few patients (3 in the ALDH group and 6 in the CD133 group) who underwent neoadjuvant treatment the % of ALDH and CD133 positive cells was not higher than the median of the entire sample (**Table 2**).

Correlation of the markers with the ability to sustain low adherence cultures

For most of the samples, we seeded the tumor cell suspension obtained from fresh tumor samples in low adherence stem cell conditions, described to isolate cancer stem cells from different tumor type and that recently allowed us the isolation of the ovarian tumor initiating cells [20]. In these stem cell selective conditions, all tumors yielded floating cell aggregates; however most of the cultures did not even grow after the first passage; some grew up to seven passages but then stopped. We correlated the % values of positive ALDH and CD133 cells with the number of serial passages patient's cultures underwent. In fact, one would expect that the higher the % of positive ALDH and CD133 cells, the higher the probability of these cultures to contain cells with stem cell properties able to sustain serial low adherence passages. However, no statistically significant correlation

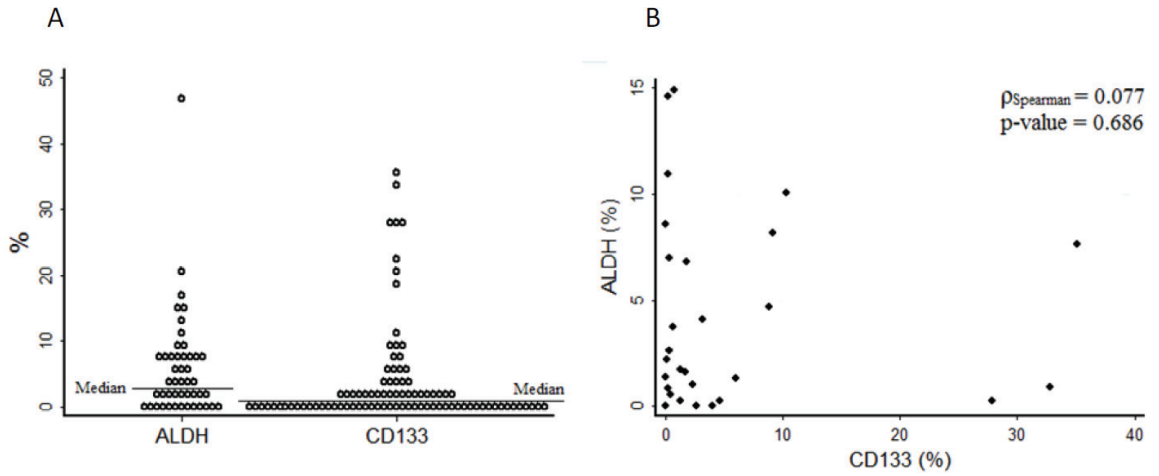

Figure 1. ADLH and CD133 expression in ovarian fresh tumor samples. A. % pattern of ALDH and CD133 positive cells in the fresh tumor ovarian samples examined. B. Correlation between the % of ALDH and CD133 positive cells in the samples in which both markers could be analyzed.

Table 2. Percentage of median values of ALDH and CD133 positive cells in patients stratified by tumor histotype, tumor grade, tumor stage and the type of chemotherapy

Patients subset	% of positive ALDH cells		% of positive CD133 cells	
	Pts no	Median (range)	Pts no	Median (range)
All patients	47	3.04 (0-46.8)	91	0.63 (0-35.15)
Tumor Histotype				
Serous Tumors	26	3.04 (0-46.8)	41	0.537 (0-27.85)
Mucinous Tumors	8	5.43 (0-19.79)	11	1.82 (0-27.36)
Endometrioid	3	7.2 (1.4-10.96)	9	0.3 (0-35.15)
Clear Cell	1	1.3	5	0 (0-6.02)
Undifferentiated	2	0.13 (0-0.26)	8	0.165 (0-32.77)
Others	7	5.5 (0-14.89)	17	1 (0-32.77)
Tumor Grade				
Borderline	4	4.99 (0-8.17)	6	7.89 (0-27.58)
Grade1-2	12	4.825 (0-19.79)	14	2.48 (0-32.77)
Grade 3	30	1.66 (0-46.8)	67	0.432 (0-27.85)
N/A	1	0.275	4	0.05
Tumor Stage				
Stage I-II	13	6.08 (0-19.79)	20	2.12 (0-27.58)
Stage III-IV	33	3.04 (0-17.92)	70	0.501 (0-35.15)
N/A	1	2.61	1	0.36
Chemotherapy* treated patients	39		81	
adjuvant	35	2.61 (0-46.8)	75	0.465 (0-35.15)
neoadjuvant	4	2.98 (0.26-14.89)	6	1 (0-2.29)

*Carboplatin/Taxol, carboplatin/gemcitabine, TIP (paclitaxel/ifosfamide/cisplatin), PEB (cisplatin/etoposide/bleomycin), PAC (cisplatin/doxorubicin/cyclophosphamide), cisplatin.

was found ($p\text{-value}_{ALDH}$ = 0.169, $p\text{-value}_{CD133}$ = 0.612; **Figure 2**, panel A and B).

Correlation of ALDH and CD133 positivity with patient outcome

The % values of both markers and selected patients clinic-pathological characteristics (tumor histological type, tumor grade tumor stage and patients age) were correlated with patient's response to chemotherapy, expressed as complete/partial response versus stable/progressive disease (**Table 4**). Complete/partial responses were respectively reached in 30/47 and 69/91 in ALDH and CD133 patient group. In these analyses only patients who underwent chemotherapy were included; specifically 8 patients in the ALDH group were

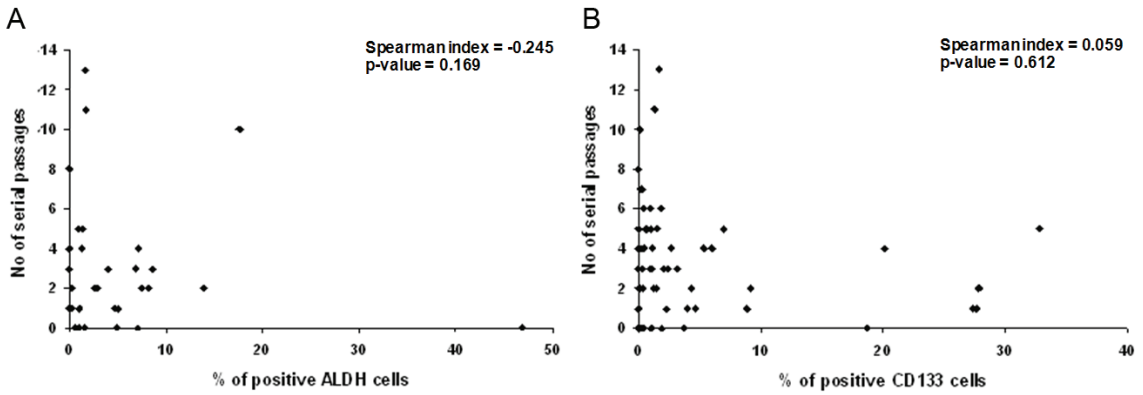

Figure 2. Correlation between the % ALDH (A) and CD133 positive cells (B) and the number of passages the tumor cultures underwent.

Table 3. Percentage of ALDH and CD133 positive cells in different cell lines

Cell line	% of positive cells	
	ALDH	CD133
OVCAR 3	18.7	2,69
OVCAR 5	6.8	0
OVCAR 8	0	1,13
OVCAR 432	0,01	4,52
A2780	0,23	3,97
SKOV 3	2,72	1,08
IGROV	8,06	1,69
OVCAR420	7,59	0,22
OVCAR433	5,89	1,78

excluded as 6 were borderline/stage I patients not requiring chemotherapy; one patient died before starting chemotherapy and the data from one patient were missing. As regards CD133 sample group, 10 were excluded as 9 were borderline/stage I patients not requiring chemotherapy and one patient died before starting chemotherapy. No correlation was found.

As for July 2012, the median follow up was 32.8 months (min-max: 10.8-62.2) and 25.1 months (min-max: 9.9-54.3) in ALDH and CD133 patient populations, 18 (46.2%) and 22 (26.2%) patients were respectively dead. The number of progression-free patients were 10/21 and 31/59 for ALDH and CD133 patient population. The possible prognostic roles of both ALDH and CD133 were examined using the percentage of positive cells as a continuous value; no association could be found between markers and progression free and overall survival (**Table 5**). Only residual tumor ≤ 2 cm correlated with both progression free survival and overall survival in both ALDH and CD133 patient populations.

Discussion

In the present paper ALDH enzymatic activity and the CD133 expression were studied in cells freshly isolated from tumor samples and correlated with the patients' clinical-pathological variables; whenever possible both markers were analyzed in the same tumor sample. We found that 13% of patients were completely negative for ALDH activity and 26% for CD133 staining. Both markers were variably expressed and when studied in the same tumor sample no correlation between ALDH enzymatic activity and CD133 expression was found.

CD133 and ALDH have been variably found to be markers of stemness in ovarian cancer; however, we found that their level of expression did not correlate with the ability to sustain serial low adherence passages of the tumor cell suspension. Considering that in these experimental conditions cultures enriched in ovarian cancer stem cells could be isolated, these data suggest that even when present, in some cases, at high percentage (49% and 30% for ALDH and CD133 respectively), these markers by themselves do not confer a self-renewal growth advantage to the cultures.

When analyzing the median % levels of expression in patients stratified for tumor histotype, grade or stage, higher % values of ALDH were found in endometrioid tumor type than in serous, as already reported [21]. However this difference did not reach a statistically signifi-

Table 4. Correlation analysis between biological markers and different clinic-pathological characteristic and response to therapy

Pts population	Characteristics		Response			
			OR	Lower 95% CI	Higher 95% CI	*p*-value
ALDH	ALDH (%)		1.017	0.906	1.142	0.772
	Histo	Seruos	1	.	.	0.761
		Other	0.772	0.145	4.105	
	Grade	1-2	1	.	.	0.304
		3	2.464	0.441	13.755	
	Stage	I-II	1	.	.	ne
		III-IV	ne	ne	ne	
	Residual tumor	≤ 2cm	1	.	.	0.118
		> 2cm	0.321	0.059	1.739	
CD133	CD133 (%)		0.978	0.899	1.065	0.615
	Histo	Seruos	1	.	.	0.209
		Other	0.343	0.065	1.820	
	Grade	1-2	1	.	.	0.352
		3	2.320	0.394	13.646	
	Stage	I-II	1	.	.	ne
		III-IV	ne	ne	ne	
	Residual tumor	≤ 2cm	1	.	.	0.085
		> 2cm	0.262	0.057	1.201	

ne: not estimable because of zero counts in the contingency table.

Table 5. Correlation analysis between biological markers and different clinico pathological characteristic and Progression free survival and Overall survival

Pts population	Characteristics		PFS				OS			
			HR	Lower 95% CI	Higher 95% CI	*p*-value	HR	Lower 95% CI	Higher 95% CI	*p*-value
ALDH	ALDH value		1.016	0.978	1.056	0.407	1.016	0.976	1.058	0.441
	Histo	Serous	1	.	.	0.118	1	.	.	0.578
		Other	0.501	0.210	1.193		0.746	0.265	2.098	
	Grade	1-2	1	.	.	0.602	1	.	.	0.705
		3	0.802	0.350	1.839		1.221	0.434	3.435	
	Stage	I-II	1	.	.	0.112	1	.	.	0.073
		III-IV	2.380	0.817	6.934		6.363	0.841	48.143	
	Residual tumor	≤ 2cm	1	.	.	0.003	1	.	.	0.008
		> 2cm	3.190	1.476	6.894		3.230	1.249	8.353	
CD133	CD133 value		0.972	0.921	1.026	0.303	1.005	0.939	1.076	0.727
	Histo	Serous	1	.	.	0.951	1	.	.	0.294
		Other	0.982	0.556	1.735		1.206	0.507	2.868	
	Grade	1-2	1	.	.	0.229	1	.	.	0.848
		3	1.697	0.717	4.017		1.076	0.312	3.708	
	Stage	I-II	1	.	.	0.018	1	.	.	0.251
		III-IV	4.105	1.272	13.249		4.384	0.586	32.812	
	Residual tumor	≤ 2cm	1	.	.	0.001	1	.	.	0.004
		> 2cm	2.523	1.458	4.576		3.252	1.338	7.902	

cant value, possibly due to the small and unbalanced sample sizes of the different categories. Interestingly, CD133 levels were inversely associated with tumor grade. No correlation with response to therapy, progression free survival and overall survival was found when considering the median values of both CD133 expression and ALDH enzymatic activity.

Data on the predictive/prognostic role of the ALDH in ovarian cancers have been recently cumulating. These data have been generally studied in retrospective cohort of patients by immunohistochemistry analysis of paraffin embedded tumors. ALDH (specifically the isoform 1-ALDH1) levels assessed by IHC have been both correlated with poor and favorable prognosis in 419, 84 and 442 cases of primary ovarian cancer [10, 22, 23]. Our data found no correlation. The discrepancies among the studies are not easily interpretable. Part of the explanation could be the ALDH detection method (immunohistochemistry-HIC- versus enzymatic activity) used, the type of tissues (paraffin-embedded and fresh tumors samples), the ALDH1 staining cut-off values and the tumor

histotype considered in the different studies. Chang et al. [23] reported high ALDH1 expression (> 20% of positive cells) to be correlated with favorable prognosis, but did not analyze the histological subtypes of ovarian tumors separately; on the contrary Deng et al [10] and Wang et al [22] found that relatively high number of ALDH1 positive cells (> 10% the former and 50% the latter) correlated with poor survival specifically in serous ovarian tumors. A lineage-specific ALDH1 expression in different histological type of ovarian tumors has been proposed. Penumatsa et al reported reduced expression of ALDH1 in serous ovarian tumors [24]; Li et al [25] reported that ALDH1 expression was repressed by histone-lysine N-methyltransferase EZH2 in high-grade serous ovarian carcinoma and Saw at al reported that ALDH1 expression was higher in the endometrioid and mucinous tumors compared with clear cell and serous tumors [21]. While our data shows a differential higher ALDH enzymatic activity in endometrioid and mucinous versus serous tumor types, we did not find any correlation (positive or negative) with both PFS and OS. Again, both the different methods (IHC versus Aldefluor assay) used and the sample size can be at the basis of the different results. The Aldefluor assay we used has lead to the isolation of leukemia stem cells based on their increased ALDH activity [14] and it was later applied to isolate ALDH+ cells with stem cell properties also from solid tumors type [15-17, 26, 27]. The enzymatic test uses a substrate recognized by different ALDH cellular isoforms. A correlation between the expression of ALHD1 and the Aldefluor enzymatic activity has been reported in ovarian cancer cell lines [10], but it cannot be ruled out the role of other ALDH isoforms. In fact, more recently, experiments with murine hematopoietic stem cells, murine progenitor pancreatic cells, and human breast CSCs indicate that other ALDH isoforms, particularly ALDH1A3, significantly contribute to Aldefluor positivity, which may be tissue and cancer specific [28].

As regards the role of CD133 expression in ovarian cancer, Ferrandina et al were the first to report that ovarian tumor samples to express CD133 [29] and that its expression did not provide any additional prognostic information for ovarian cancer patients [30] as no difference in time to progression and overall survival between cases with negative versus positive CD133 expression. On the contrary, recent data [31] have been published in a larger series of tumor ovarian samples suggesting that CD133 expression was associated with high-grade serous carcinoma, late-stage disease, with shorter disease free survival time and lack of response to chemotherapy (400 samples versus 160 of Ferrandina et al [30]). Our data agree with the ones reported by Ferrandina, even if the methodologies used are different (IHC in paraffin-embedded tissues versus FACS analysis in fresh tumor samples). Silva et al [32] reported that only the presence of ALDH+CD133+ cells in debulked primary specimens, assessed by IHC, correlated with reduced disease-free and overall survival in ovarian cancer patients, while the single marker did not have any role. In our sample population, the number of patient in which the two markers could be evaluated was too small to allow any correlation with clinical outcome.

Even if we recognize the high heterogeneity of the our sample population, the absence of a predefined statistical hypothesis to test and the lack of sample size calculation, our data suggest that neither ALDH enzymatic activity and CD133 expression provide additional predictive information in ovarian cancer patients.

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Conflict of interest statement

None declared.

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