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Automated quantitative analysis of DCC tumor suppressor protein in ovarian cancer tissue microarray shows association with β-catenin levels and outcome in patients with epithelial ovarian cancer

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Background: The deleted in colorectal cancer (DCC) protein, the product of DCC tumor suppressor gene, is frequently altered in cancer. Preclinical data demonstrate that DCC regulates β-catenin levels. Here, we sought to determine the association of DCC with β-catenin protein levels, clinicopathological parameters and patient outcome in ovarian cancer using a method of *in situ* compartmentalized protein analysis.

Methods: A tissue array composed of 150 advanced-stage ovarian cancers, treated with surgical debulking and platinum–paclitaxel (Taxol) combination chemotherapy, was constructed. For evaluation of protein expression, we used an immunofluorescence-based method of automated *in situ* quantitative measurement of protein analysis (AQUA).

Results: One hundred and twelve patients (74%) had sufficient tissue for AQUA. The median follow-up time for the entire cohort was 33 months. Patients with low nuclear DCC expression had a 3-year progression-free survival (PFS) rate of 0% compared with 33% of those with high DCC expression ($P = 0.0067$). In multivariate analysis, low nuclear DCC expression level retained its prognostic significance for PFS. Between DCC and β-catenin, a significant relationship was found, where tumors with low DCC had low β-catenin and vice versa ($P = 0.003$).

Conclusions: Low nuclear DCC levels predict for poor patient outcome in epithelial ovarian cancer. DCC may exert its antitumor function, in part, through regulation of β-catenin levels.

Key words: β-catenin, DCC, ovarian cancer, prognosis

introduction

Ovarian cancer is ranked fifth in incidence of cancers among women in the United States [1]. When the disease is confined to the ovary, the 5-year survival rate is 95%, however, fewer than one-third of the cases are detected at this stage. The current management of patients with advanced disease [International Federation of Gynecology and Obstetrics (FIGO) stages III and IV] involves surgical debulking followed by chemotherapy. The current standard chemotherapeutic approach for ovarian cancer patients includes platinum-based (plus or minus taxanes) chemotherapy. While the majority of patients respond initially, 60%–80% of them still die of the disease. Within these advanced stages, traditional clinicopathological factors do not accurately predict patients' prognosis. Therefore, considerable interest lies in identifying molecular prognostic indicators in order to predict prognosis and guide treatment decisions.

Deleted in colorectal cancer (DCC) gene is a putative tumor suppressor gene located on chromosome 18q [2]. The prognostic significance of 18q loss of heterozygosity (LOH) and loss of expression of DCC mRNA have been studied in solid tumors [3–7], predominantly in colorectal cancer, where LOH or low protein expression seems to be an adverse prognostic factor [8–11]. Data in ovarian cancer are limited. DCC loss has been reported in only 10% of adenomas and 6% of borderline tumors compared with 60% in carcinomas, suggesting an association of DCC inactivation with carcinogenesis in the ovary [12]. Genetic alterations and altered mRNA and DCC protein expression have been reported in 30%–60% of the cases [13–17], while an association with serous subtype has been suggested [16, 17]. The function of DCC has not been clarified. DCC protein has significant homology to neural cell adhesion molecules [2]. In addition, DCC protein may play a role in cell–cell or cell–substrate interactions through a functional link between DCC and N-cadherin/catenin-dependent cell adhesion complex. This link has been supported by *in vitro* data showing

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that overexpression of a truncated DCC protein resulted in decreased β-catenin expression in neuroblastoma cells [18].

Tissue microarray is a useful tool for molecular marker analysis. Positive features of this technology include studying specimens from hundreds of patients simultaneously as well as uniform handling of all specimens. In addition, a fully quantitative method of analysis for tissue microarrays that allows calculation of expression ratios has been developed [19]. This novel technology uses molecular methods to define subcellular compartments. It then quantifies the amount of protein expressed within the compartment by co-localization. Therefore, this technology permits preservation of tissue morphology while quantifying protein expression in paraffin-embedded tissue.

Here, we sought to determine the association of DCC protein levels with survival in a cohort ovarian cancer tissue microarray using a novel *in situ* quantitative method of protein expression and correlated those with β-catenin levels, clinical and pathological data.

patients and methods

patient population

Included patients should have primary epithelial ovarian cancer patients, FIGO stages III and IV. They all underwent surgical debulking in the Department of Gynecology of Alexandra University Hospital in Athens from 1996 to 2003 and treated postoperatively with paclitaxel (Taxol BMS, UK)–carboplatin chemotherapy. In all cases, an effort was made for optimal surgical cytoreduction and adequate staging, which included at least: total abdominal hysterectomy with bilateral salpingo oophorectomy, inspection and palpation of all peritoneal surfaces and retroperitoneal area, biopsies of suspect lesions for metastases, infracolic omentectomy and peritoneal washings. Gynecological examination, CA-125 assay and radiological investigations were carried out before chemotherapy initiation. During chemotherapy, CA-125 was carried out monthly, while clinical assessment of response in patients with bidimensional disease was recorded at the middle and at the end of treatment according to World Health Organization criteria [20]. Follow-up examinations were carried out every 3 months.

tissue microarray construction

A tissue microarray consisting of tumors from each patient in the cohort was constructed at the Yale University Tissue Microarray Facility. Following institutional review board approval, the tissue microarray was constructed as previously described [21], including 150 cases. Briefly, tissue cores 0.6 mm in size were obtained from paraffin-embedded formalin-fixed tissue blocks from the Alexandra University Hospital, Department of Pathology archives. Representative areas of invasive tumor to be cored were chosen by a pathologist (SM). The cores were placed on the recipient microarray block using a tissue microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with two-fold redundancy. The tissue microarray was then cut to yield 5-μm sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

quantitative immunohistochemistry

Slides were deparafinized, rehydrated and antigen retrieval was accomplished by application of proteinase K for 30 min. Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 min. Nonspecific antibody (Ab) binding was then blocked with 0.3% bovine serum albumin (BSA) for 30 min at room temperature. Slides were then incubated with primary Ab to DCC (Mouse Monoclonal Ab, Clone G97-449; BD Biosciences Pharmingen, CA, USA) at 4°C overnight, at 1 : 500 dilution in 0.3% BSA/Tris buffered saline (TBS). Primary Ab to β-catenin (clone 14; BD Transduction Laboratories, CA, USA) was used at 1 : 450 dilution in 0.3% BSA/TBS. Subsequently, slides were incubated with goat anti-mouse secondary Ab conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp., Carpinteria, CA) for 1 h at room temperature. Tumor cells were identified by the use of anticytokeratin Ab cocktail (rabbit anti-pancytokeratin Ab z0622; DAKO Corp.) with subsequent goat anti-rabbit Ab conjugated to Alexa546 fluourophore (A11035; Molecular Probes, Eugene, OR). We added 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Target (DCC and β-catenin) molecules were visualized with a fluorescent chromogen (Cy-5-tyramide; Perkin Elmer Corp., Wellesley, MA). Cy-5 (red) was used because its emission peak is well outside the green–orange spectrum of tissue autofluorescence.

automated image acquisition and analysis

Automated image acquisition and analysis using automated *in situ* quantitative measurement of protein analysis (AQUA) has been described previously [21]. In brief, monochromatic, high-resolution (1024 × 1024 pixel; 0.5-μm) images were obtained of each histo spot. We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. DAPI signal was used to identify nuclei, and the cytokeratin signal was used to define cytoplasm. Overlapping pixels [to a 99% confidence interval (CI)] were excluded from both compartments. The DCC and β-catenin signals were scored on a normalized scale of 1–255 expressed as pixel intensity divided by the target area. AQUA scores for each subcellular compartment (nuclear and cytoplasmic signal) were recorded. AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor.

Table 1. Demographic, clinical and pathological data

	n	DCC nuclear expression class		*P*
		Low	High	
Differentiation				0.799
Well	13	1	12	
Moderate	35	5	30	
Poor	63	7	56	
Not recorded	1	1	0	
Initial histology				1.000
Serous	81	10	71	
All others	31	4	27	
FIGO stage				0.566
II	7	0	7	
III	78	10	68	
IV	27	4	23	
Residual disease				0.732
≤2 cm	25	2	23	
>2 cm	87	12	75	
Clinical response to chemotherapy				0.391
PR+CR[a]	66	10	56	
All others	46	4	42	
Performance status				1.000
0	73	9	64	
≥1	39	5	34	

[a] PR, Partial response; CR, Complete response.

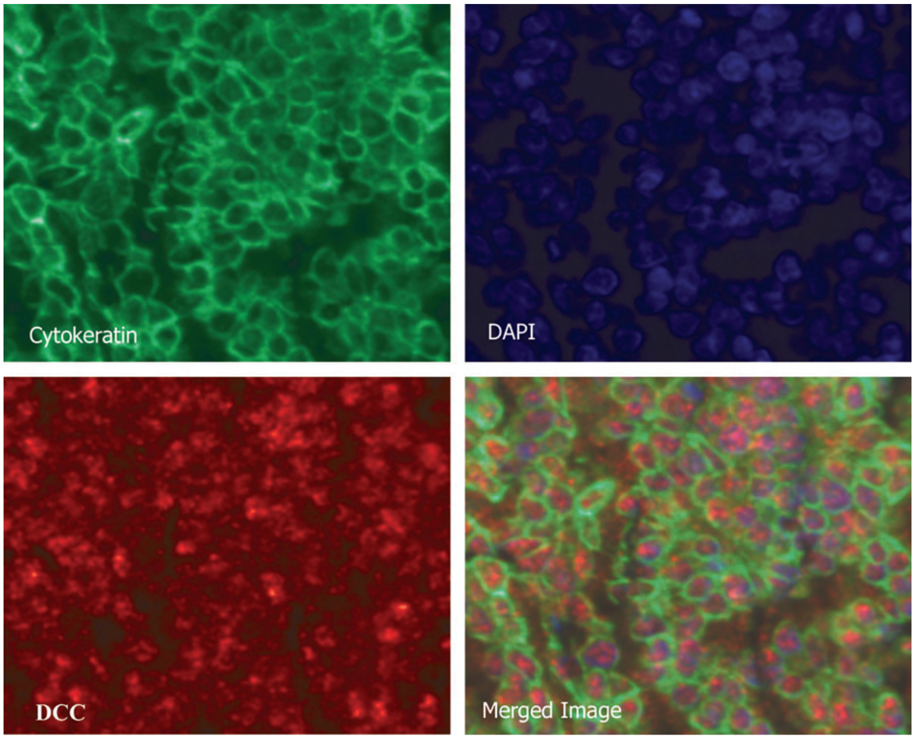

Figure 1. Protein expression of deleted in colorectal cancer (DCC) was determined using an automated quantitative method (automated *in situ* quantitative measurement of protein analysis) on the basis of immunofluorescence. Digital images of each tumor spot were captured using Cy3 anti-cytokeratin antibody to generate a tumor mask. 4′,6-diamidino-2-phenylindole was used to visualize nuclei and Cy5 was used to visualize DCC. A three-color merged image for each tumor is also shown.

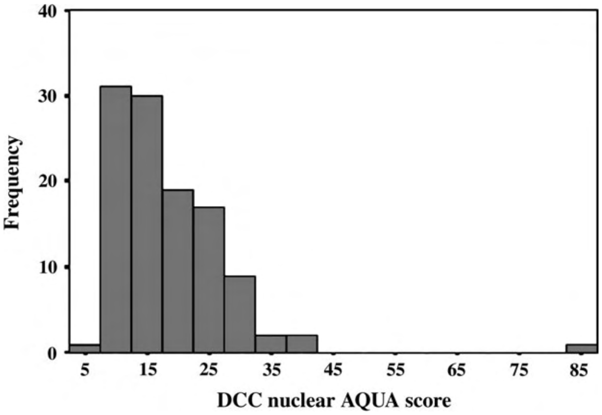

Figure 2. Deleted in colorectal cancer nuclear expression followed a skewed distribution as expected for a tissue biomarker.

statistical analysis

Histo spots containing <10% tumor as assessed by mask area (automated) were excluded from further analysis. AQUA scores represent expression of a target protein on a continuous scale from 1 to 255. It is often useful to categorize continuous variable in order to stratify patients into high versus low categories. Several methods exist to determine a cut point, including biological determination, splitting at the median and determination of the cut point which maximizes effect difference between groups. If the latter method (the so-called 'optimal *P*-value' approach) is used, a dramatic inflation of type I error rates can result [22]. A recently developed program, X-Tile, allows determination of an optimal cut point while correcting for the use of minimum *P*-value statistics [22]. As the AQUA technology is new, there are no established cut points available for quantitative protein expression. Therefore, for categorization of β-catenin and DCC expression levels, the X-Tile program was used to generate an optimal cut point. This approach has been successfully applied to AQUA data analysis. Two methods of statistical correction for the use of minimal *P*-value approach were utilized. First, the X-Tile program output includes calculation of a Monte Carlo *P* value for the optimal cut point generated. Cut points that yield Monte Carlo *P* values <0.05 are considered robust and unlikely to represent type I error. Secondly, the Miller–Siegmund minimal *P*-value correction referenced by Altman et al. [23] was utilized. This approach is accepted in the statistical literature, but relatively unknown in the medical/biological research community. Briefly, when making multiple comparisons to find the minimum *P* value using the log-rank test, the false-positive rate (i.e. the percentage of times a marker that has no true prognostic value will be found to have a $P < 0.05$) can approach 40%.

Altman's statistical adjustment generates a minimum *P* value corrected to yield a true false-positive rate of 5%. The corrected *P* value (Pcor) is calculated as follows:

$$\mathrm{Pcor} = \varphi(\xi)[\xi - (1/\xi)]\log[\mathrm{e}]\ [(1-\varepsilon)^2/\varepsilon^2] + 4\varphi(\xi)/\xi.$$

[†]Our calculations were carried out using an epsilon of 0.10. Disease-free survival (DFS) and overall survival (OS) were subsequently assessed by Kaplan–Meier analysis with log-rank for determining statistical significance, and only *P* corrected was reported. This approach has been successfully applied to AQUA data analysis. All survival analysis was carried out at 3-year

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[†] Where φ indicates the probability density function, Pmin is the minimum *P* value generated by evaluating multiple cut points, ξ is the $(1 - \mathrm{Pmin}/2)$-quantile of the standard normal distribution and ε denotes the proportion of values excluded from consideration as an optimal cut point.

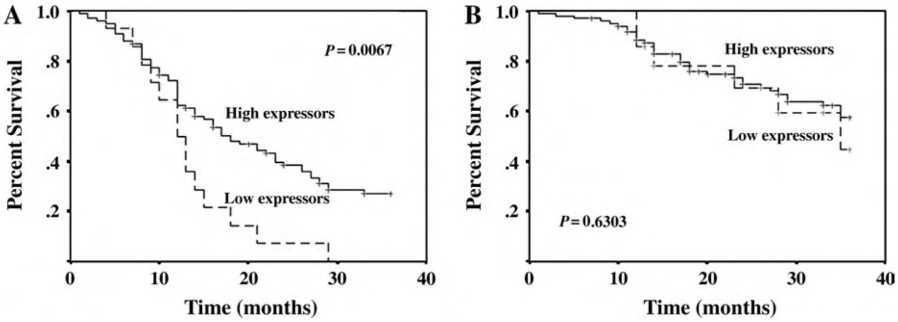

Figure 3. Kaplan–Meier estimates of 3-year disease-free (**A**) and overall survival (**B**) by nuclear deleted in colorectal cancer expression. Patients with low DCC expression demonstrated inferior disease-free survival compared to patients with high DCC expression.

Table 2. Univariate 3-year survival analysis (Kaplan–Meier log-rank)

	Mean survival (months)	Cumulative survival or recurrence (95% CI)	*P*
DFS[a]			0.0067*
High nuclear DCC	18	32.7% (21–45)	
Low nuclear DCC	12	0 (0)	
Overall survival			0.6303
High nuclear DCC	36	64.3% (53–76)	
Low nuclear DCC	35	57.1% (46–70)	

[a] One patient lacked recurrence data and was excluded from local recurrence and disease-free survival (DFS) calculations.

*Significant at the 0.01 level.

CI, confidence interval.

cut-offs. CIs were assessed by univariate and multivariate Cox proportional hazards model.OS was defined as time from first day of chemotherapy to death from any cause. DFS was defined as time from first day of chemotherapy to the first of either death from any cause or disease progression (assessed by CA 125 increase and/or imaging studies). ECOG performance status (PS) was dichotomized into '0' versus all others, and histologic type into serous versus all others. Patient population was divided into two groups according to the extent of residual disease at first surgery; ≤2 cm and >2 cm. Comparisons of DCC expression with FIGO stage and grade was made by Mantel–Haenszel chi-square test. The association between DCC and β-catenin expression (as determined by AQUA) was analyzed by Spearman's correlation. Comparisons of DCC expression with PS, histology, clinical response and residual disease were made by Fisher's exact test. Comparison of DCC expression status with age was made using Pearson correlation. All calculations and analyses were carried out with SPSS 12.0 for Windows (SPSS Inc., Chicago, IL).

results

patients

One hundred and fifty patients were included in the study. One hundred and twelve patients (74%) had sufficient tissue for AQUA. Tissues deemed insufficient had <10% tumor mask within the histo spot, as represented on the tissue microarrays. Median follow-up time for the entire cohort was 33 months (range 1–91.7). There were 85 (75%) stage III and 27 (25%) stage IV patients. Eighty-one (72%) patients had tumors of serous histology. Initial histological grade was 13 well-differentiated (11%), 35 moderately differentiated (31%) and 63 poorly differentiated (52%) and one not recorded. Residual disease after surgical debulking was distributed as follows: 25 (22%) with <2 cm and 87 (78%) with >2 cm. For clinical response to initial therapy, complete response (CR) or partial response (PR) was recorded in 66 (59%) patients and stable disease/no response in 46 (41%) patients. Demographic and clinicopathological variables for the cohort analyzed by AQUA are summarized in Table 1.

generation of optimal cut point by X-Tile analysis

As visualized by fluorescent immunohistochemistry (IHC), DCC displayed predominantly cytoplasmic and nuclear staining (Figure 1). Normalized AQUA scores were represented on a 1–255 scale. DCC expression followed a skewed distribution as expected for a cancer tissue biomarker (Figure 2).

Using the X-Tile program, an optimal cut point for DCC nuclear expression was determined at 9.96 AQUA units, with a Monte Carlo *P* value of 0.04 as determined by X-Tile. Monte Carlo *P* values <0.05 indicate robust and valid cut-point selection; patients with DCC nuclear expression ≤9.96 were classified as low expressers (n = 14) and patients with nuclear DCC expression >9.96 were classified as high expressers (n = 98). There was no association between nuclear DCC expression and any of the clinicopathological variables.

The association between DCC and β-catenin expression (as determined by AQUA) was analyzed by Spearman's correlation. A significant relationship was found, where tumors with high DCC had high β-catenin and vice versa (Spearman's $\rho = 0.278$; $P = 0.003$).

univariate survival analysis

progression-free survival. The expression status of nuclear DCC expression was evaluated for association with progression-free survival (PFS) using Kaplan–Meier survival analysis with log-rank statistic for determining significance. This analysis demonstrates that low nuclear DCC expression is associated with inferior 3-year PFS. Patients with high nuclear DCC expression had a PFS of 33% compared with 0% for patients with low DCC tumors ($P = 0.0067$) (Figure 3).

overall survival. The expression status of DCC was also evaluated for association with OS. Kaplan–Meier analysis demonstrated that there was no significant 3-year OS rate

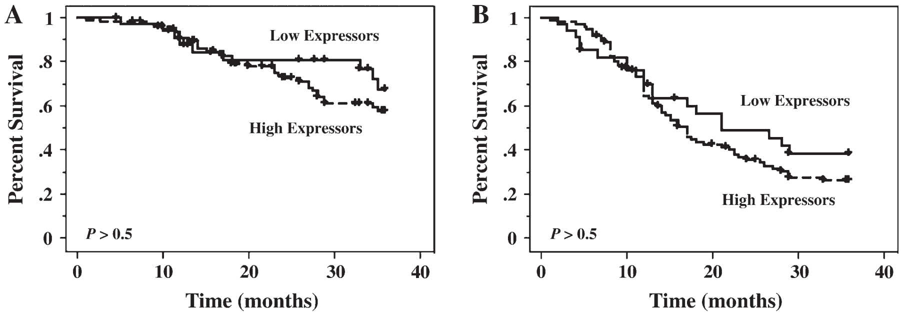

Figure 4. Kaplan–Meier estimates of 3-year overall (**A**) and disease-free survival (**B**) by β-catenin expression. No significant differences between low and high expressors were observed.

Table 3. Multivariate 3-year progression-free survival analysis by Cox regression

Variable	95% CI	*P*
DCC low	1.12–4.17	0.02
Histological grade	0.75–1.54	0.69
Residual disease	1.34–1.54	0.0001
PS	0.63–1.24	0.41
FIGO stage	0.63–0.87	0.02
Histology	0.87–1.01	0.85
Response to chemotherapy	0.593–0.958	0.86

CI, confidence interval; DCC, deleted in colorectal cancer; PS, performance status; FIGO, International Federation of Gynecology and Obstetrics.

between patients with low and high nuclear DCC expressing tumors ($P = 0.63$) (Figure 3). Results of the univariate survival analysis are summarized in Table 2.

No significant association of β-catenin expression with PFS or OS was observed (Figure 4).

multivariable analysis

Using the Cox proportional hazards model, we carried out multivariable analysis to assess the predictive value of nuclear DCC expression by AQUA for PFS. We also included the following known prognostic variables in the regression model: FIGO stage, PS, response to chemotherapy, initial histology, residual disease and tumor grade. DCC level (95% CI 1.12–4.17; $P = 0.02$) along with FIGO stage (95% CI 0.63–0.87; $P = 0.02$) and residual disease (95% CI 1.34–1.54; $P = 0.0001$) were significant predictor variables of PFS. Results of multivariable analysis are summarized in Table 3.

discussion

In the present study, using a method of *in situ* proteomic analysis on a tissue microarray, we demonstrate that decreased levels of DCC tumor suppressor protein are associated with shorter 3-year PFS in a cohort of chemotherapy-treated advanced stage ovarian cancer patients. Strikingly, all patients with low DCC protein expression relapsed within 3 years. In addition, we show that there is a direct correlation between DCC and β-catenin levels consistent with preclinical observations that DCC loss is implicated in the aberrant cellular migration through down-regulation of N-cadherin and β-catenin levels.

DCC loss is a very common genetic alteration in colorectal carcinomas. In ovarian cancer, DCC mRNA down-regulation and/or protein loss by IHC have been reported in 40% of ovarian cancers. Down-regulation of DCC mRNA was reported in 50% of serous cancers, while it was rare in other histological types. Saegusa et al. [15] analyzed immunohistochemically 124 ovarian carcinomas, 55 cystadenomas and 41 low malignant potential (LMP) tumors for DCC protein expression and compared the results with p53 protein expression, clinicopathological factors and outcome. The authors also analyzed 26 malignant, five LMP, eight benign and seven normal ovarian samples for DCC mRNA levels using RT-PCR and Southern blot hybridization. DCC mRNA and protein levels were significantly decreased in carcinomas compared with benign and LMP lesions. DCC loss was significantly associated with serous subtype, high-grade and advanced FIGO stage, whereas there was no correlation with survival or p53 protein expression.

In the present cohort, no association between DCC protein expression levels and clinical–pathological parameters was found. However, low DCC protein expression had a significant adverse impact on prognosis in this cohort of chemotherapy-treated patients with advanced ovarian cancer. Despite the high response rates of ovarian cancer patients to platinum–paclitaxel combination chemotherapy, the majority of them will relapse and die of disease. Molecular prognostic markers are needed to identify patients in need of more aggressive treatment and spare those with favorable profile, the sequelae of aggressive treatment. DCC expression status, if validated in a prospective study, may be used for patient selection for aggressive treatment protocols. DCC expression was not associated with OS, indicating that the impact on prognosis is mainly related to first-line chemotherapy. It should be mentioned that OS is determined by various factors (residual disease, PS, tumor grade, etc.), while the median follow-up was relatively short. In addition, response to subsequent lines of treatment at relapse may affect prognosis. This factor could not be studied in our cohort of patients.

The mechanism via DCC loss confers poor prognosis is not completely clear. As previously mentioned, DCC loss may be involved in the regulation of cell–cell, cell–stroma interactions.

DCC may, therefore, control cell growth, differentiation and metastatic potential. Consistent with these observations, a direct association between β-catenin and DCC levels was found in our study indicating that DCC down-regulation may confer increased metastatic potential through β-catenin loss. However, cell biology experiments are required to confirm this association.

In conclusion, we demonstrate that DCC tumor protein levels, evaluated by AQUA, determine patient outcome in ovarian cancer possibly through regulation of β-catenin levels. These results, if validated in a second cohort, may identify patients who will fail conventional chemotherapy and should receive additional therapy on experimental protocols.

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