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Original Article

Acid Ceramidase (AC)—A Key Enzyme of Sphingolipid Metabolism—Correlates With Better Prognosis in Epithelial Ovarian Cancer

Lars Christian Hanker, M.D., Thomas Karn, M.D., Uwe Holtrich, M.D., Regine Gätje, Ph.D., Achim Rody, Ph.D., Tomas Heinrich, M.D., Eugen Ruckhäberle, M.D., and Knut Engels, M.D.

Summary: Acid ceramidase (AC), a key enzyme of sphingolipid metabolism, seems to play an important role in cancer progression. The objective of this study was to explore the expression of AC in ovarian cancer and its impact on prognosis. Expression analysis of AC in n = 112 ovarian cancer patients was performed by immunohistochemical analysis of primary paraffin-embedded tumor samples. The results were scored on the basis of the staining intensity and percentage of positive tumor cells, resulting in an immunoreactive score from 0 to 12. These results were correlated to clinical and pathologic characteristics and survival. AC expression correlated significantly only with FIGO stage (0.047). In serous carcinoma, low level of AC was independently associated with reduced progression-free survival and overall survival of 12.0 mo [95% confidence interval (CI), 5.78–18.23] versus 18.1 mo (95% CI, 11.61–24.59; $P = 0.008$) and 35.7 mo (95% CI, 22.24–47.16) versus 58.7 mo (95% CI, 36.48–80.91; $P = 0.032$), respectively. In multivariate analysis, AC presents as an independent prognostic factor for progression-free survival (hazard ratio 1.88; 95% CI, 1.13–3.11; $P = 0.015$). AC is a prognostic factor in epithelial ovarian cancer. Low AC expression can be associated with tumor progression in carcinoma of the ovaries. These results are in contrast to the concept of AC as a promoter for cancer progression. Nevertheless, they are supported by the lately discovered tumor-suppressing function of sphingosine, the enzymatic product of AC. **Key Words:** Acid ceramidase—Ovarian cancer—Sphingolipid metabolism.

Epithelial ovarian cancer (EOC) is the sixth most common type of cancer in women (1). Most patients present with an advanced stage of the disease and therefore have only a limited prognosis (2). Despite high response rates to first-line chemotherapy, a frequent development of resistance to treatment is evident. Indeed, about 80% of the patients with FIGO stages II to IV EOC will progress during or after adjuvant chemotherapy (3). Therefore, the need for new therapeutic approaches and prognostic and predictive markers is evident. Identification of new prognostic factors could help to distinguish different biologic subgroups. In EOC, this is particularly

From the Department of Obstetrics and Gynecology (L.C.H, T.K., U.H., R.G., T.H., E.R.); Senckenberg's Institute of Pathology (K.E.), Goethe-University, Frankfurt; and Department of Gynecology and Obstetrics (A.R.), University of Homburg, Homburg, Germany.

E.R. and K.E. contributed equally.

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Address correspondence and reprint requests to Lars Christian Hanker, MD, Department of Obstetrics and Gynecology, Goethe-University, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany. E-mail: hanker@med.uni-frankfurt.de.

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important for the group of patients who experience recurrent disease. These patients rarely benefit from current treatment modalities and sometimes suffer intensively from the side effects of the therapy. The establishment of more targeted and individual treatment strategies in biologically distinct subgroups should be the aim of subsequent research. In this regard, the sphingolipids might play an important role in different tumor entities (4).

Sphingolipids represent a family of membrane lipids with several functions. On the one hand, they contribute to the structure of the cellular lipid bilayer membrane (5), and on the other they act as bioactive effectors to regulate many cellular functions (6). The main members are ceramide, sphingosine (SPH), and sphingosine-1-phosphate (S1P) (7). Ceramide plays a central role as a key hub in sphingolipid metabolism. It is involved in different aspects of cancer pathogenesis including apoptosis and cell proliferation (8). The enzyme acid ceramidase (AC) metabolizes ceramide to SPH and therefore holds a pivotal role in this framework. This lipid hydrolase is encoded by the human gene *ASAH1* (9). Ceramide and SPH seem to function in a partially synergistic manner and exhibit proapoptotic stimuli on cancer cells and normal tissue (10). Furthermore, an antiapoptotic impact of SPH is described (11). Another counterpart in this network is S1P, the product of sphingosine kinases. This antiapoptotic sphingolipid regulates proliferation, inflammation, angiogenesis, and resistance to apoptotic cell death (10).

Some authors have proposed the concept of a so-called sphingolipid rheostat (12). Following this concept, the dynamic equilibrium between the different sphingolipid metabolites and balanced regulation of the opposing signaling pathways is a crucial factor that determines the fate of cells. It could be shown that exogenous ceramide and ceramide analogs were able to affect this system *in vitro* and thus have therapeutic potential in various tumor types like breast cancer (13), colon cancer (14), or head and neck cancer (15).

The key enzyme AC is often overexpressed in various cancer entities (16) including head and neck cancer (17), prostate cancer (17), and melanoma (18).

In previous investigations, we have observed a prognostic impact of different sphingolipid enzymes on breast cancer (19,20). Until now, there are no data on the clinical impact of these proteins in ovarian cancer. Nevertheless, it could be shown in *in vitro* experiments that the expression of sphingosinekinase-1 decreases sensitivity to cisplatin and carboplatin in ovarian cancer cell lines, whereas expression of the dihydroceramide synthase LASS1 increases the sensitivity to platinum (21,22). Furthermore, Saad et al. (23) could previously demonstrate an association of AC upregulation and cisplatin drug resistance in primary prostate cancer tissue.

Therefore, we tested the expression of the sphingolipid key enzyme AC in n = 112 ovarian cancer patients for analyzing its impact on prognosis and platinum sensitivity in ovarian cancer.

MATERIALS AND METHODS

Patients and Treatment

The study included patients with primary EOC treated between 1995 and 2008 at the Department of Obstetrics and Gynecology at the University of Frankfurt. A total of 112 patients were retrospectively analyzed. Formalin-fixed, paraffin-embedded tissue samples were obtained from the Department of Pathology. Patient characteristics are listed in Table 1. The clinical and pathologic factors were evaluated by reviewing medical charts and pathology records. The Local Research Ethics Committee-approved studies of human tissue and samples were processed anonymously. Clinical outcome was followed from the date of surgery to the date of death or until the end of 2009. Only patients with histologically proven EOC were included. The majority of patients had advanced disease stages (FIGO III–IV) and had received primary surgery followed by a platinum-based and taxane-based chemotherapy.

Tissue Samples and Immunohistochemistry

Tissue samples were processed as described (24). Routine histopathology sections stained with hematoxylin-eosin were used for primary diagnosis and second reviewing (K.E.). Diagnosis and grading was performed according to the current World Health Organization criteria (25,26). After mounting on Superfrost Plus slides, paraffin sections (2 μm) were dewaxed in xylene and rehydrated to water through a graduated ethanol series. For antigen retrieval, sections were incubated for 20 min in a microwave oven (800 W) using EDTA buffer (10 mmol/L; pH 8.0). Following this, the sections were incubated with a monoclonal anti-*ASAH1* antibody (Biozol Diagnostica, Germany; cat. no. H00000427-M01, Clone2C9) at a 1:100 dilution for 1 hr at room temperature. For negative controls, the primary antibody (Ab) was omitted. For secondary antibody incubation, the Dako

TABLE 1. *Patient characteristics*

No. Patients (%)	112 (100)
Residual tumor after surgery	
0 mm	51 (45.5)
1–10 mm	20 (17.9)
>10 mm	40 (35.7)
Unknown	1 (0.9)
Grading	
1	6 (5.4)
2	34 (30.4)
3	72 (64.2)
Age (yr)	
Median	59.2
Mean	58.9
Range	23–83
FIGO stage	
I	20 (17.9)
II	8 (7.1)
III	70 (62.5)
IV	14 (12.5)
Histologic subtype	
Serous	87 (77.7)
Mucinous	6 (5.4)
Endometrioid	10 (8.9)
Clear cell	8 (7.1)
Transitional cell	1 (0.9)
Survival (mo)	
Progression-free survival (n = 110)	
Median	17.0
Range	13.0–21.0
Overall survival (n = 112)	
Median	52.2
Range	40.5–64.0
Platinum sensitivity	
Resistant	39 (34.8)
Sensitive	68 (60.7)
Not applicable/unknown	5 (4.5)

REAL Detection System Alkaline Phosphatase/RED (Dako, Denmark) was applied, following the instructions of the vendor. The sections were counterstained with hematoxylin. The expression levels for cytoplasmic AC were scored semiquantitatively on the basis of the product of staining intensity (SI) and percentage of positive cells (PP) using the immunoreactive score (IRS) (24,27,28): IRS = SI × PP. SI was assigned as: 0, negative; 1, weak; 2, moderate; or 3, strong. PP was defined as: 0, negative; 1, <10%; 2, 11% to 50%; 3, 51% to 80%; or 4, >80% of positive cells. All assessments were made blinded with respect to clinical patient data.

Statistical Analysis

For statistical analysis, a cutoff value was defined on the basis of the IRS, that is, a score of 0 to 2 (negative and low) was combined to define a low score and a score ≥3 was defined as a high AC expression score. The χ^2 test and the Fisher exact test were used to test for associations between AC expression of tumors and clinicopathologic parameters. For those patients with available follow-up data Kaplan-Meier curves were constructed, and the log rank test was used to determine the univariate significance of the variables. Cox regression analysis was performed to determine hazard ratios. All reported *P* values are 2 sided, and *P* values ≤0.05 were considered to indicate a significant result. All analyses were performed using the SPSS software package (SPSS, Chicago, IL) version 18.0.

RESULTS

A total of 112 patients were included in this study. All patients underwent primary debulking surgery, including a hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, appendectomy, and a systematic pelvic and para-aortic lymphadenectomy, intending the resection of all visible tumors. In the majority of patients (n = 71, 63%) optimal debulking, that is, reaching at least a so-called optimal postoperative residual tumor up to a maximum of 1 cm (according to NCCN guidelines) (29), could be achieved.

The median follow-up time was 33.9 mo (range, 18.8–57.5 mo). The median progression-free survival (PFS) and overall survival (OS) for the whole group were 17.0 mo [95% confidence interval (CI), 13.0–21.0] and 52.2 mo (95% CI, 40.5–64.0), respectively. The corresponding 5-year PFS and OS rates were 21.9% and 37.5%, respectively. Patient characteristics for the whole cohort are displayed in Table 1.

Immunohistochemistry revealed higher expression levels (IRS≥3) of AC in 58% of tumor biopsies (n = 65; Fig. 1A) and low or negative expression levels (IRS 0–2) in only 42% of the samples (n = 47; Fig. 1B).

In the cohort of 112 patients, no significant difference in AC expression based on age, platinum sensitivity, postoperative residual tumor, grading, and histologic subtype was observed. Only a significant correlation of high AC expression and low FIGO stage (*P* = 0.047) could be seen (Table, Supplemental Digital Content 1, http://links.lww.com/IJGP/A13).

In the Kaplan-Meier analysis of the whole cohort, there was a median PFS of 14.0 mo (95% CI, 9.47–18.53) in the low-expression group versus 20.7 mo (95% CI, 12.68–28.72) in the high-expression group (*P* = 0.002). Patients with low AC expression had a median OS of 37.7 mo (95% CI, 26.19–49.21) versus 58.7 mo (95% CI, 44.31–73.10) in patients with high AC expression (*P* = 0.055; Figs. 2A, B). In the

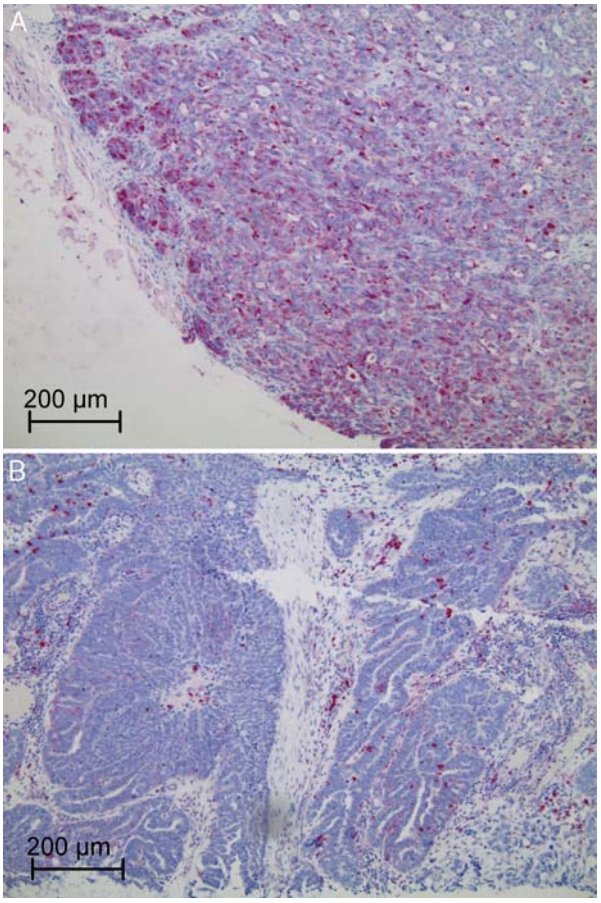

FIG. 1. AC staining of 2 ovarian cancer samples. A and B, Serial sections stained by AC with IRS≥3 (A) and IRS 0 to 2 (B), respectively. Counterstain: Mayer hematoxylin (blue). AC indicates acid ceramidase; IRS, immunoreactive score.

subgroup of platinum-sensitive patients, that is, in those with an occurrence of relapse >6 mo after the end of primary therapy, a low expression of AC was significantly associated with a PFS of 20.5 mo (95% CI, 16.61–24.38; $P = 0.004$) versus 54.9 mo (95% CI, 17.66–92.13) in the high-expression group. With regard to OS, a difference of 55.4 mo versus 82.4 mo ($P = 0.109$) was observed when comparing low versus high AC expression (Figure, Supplemental Digital Content 2, http://links.lww.com/IJGP/A14). In platinum-resistant patients, that is, those in whom relapse occurred ≤6 mo after primary therapy, this effect of AC expression on PFS and OS was not seen (OS: 19.2 vs. 19.7 mo, $P = 0.871$; PFS: 8.5 vs. 8.1 mo, $P = 0.502$) (Figure, Supplemental Digital Content 3, http://links.lww.com/IJGP/A15).

The univariate Cox regression analysis revealed a statistically significant impact on PFS for expression of AC ($P = 0.002$), FIGO stage ($P < 0.001$), grading ($P = 0.034$), and residual tumor after surgery ($P < 0.001$). Prognostic factors such as FIGO stage ($P < 0.001$), residual tumor after surgery ($P < 0.001$), and age ($P = 0.050$) were significant for OS in univariate Cox regression (Table 2A and B).

Multivariate Cox regression analysis revealed that low AC expression [hazard ratio (HR) 1.71; 95% CI, 1.08–2.72; $P = 0.023$], advanced FIGO stage (HR 2.78; 95% CI, 1.32–5.81; $P = 0.007$), and postoperative residual tumor >10 mm (HR 2.16; 95% CI, 1.33–3.53; $P = 0.002$) were significantly associated with shorter PFS (Table 3A). An advanced FIGO stage (HR 5.35; 95% CI, 1.76–16.39; $P = 0.003$) and postoperative residual tumor >10 mm (HR 2.04; 95% CI, 1.20–3.45; $P = 0.008$) show a significantly reduced OS. Low AC expression retained a trend toward shorter OS (HR 1.29; 95% CI, 0.77–2.15; $P = 0.329$) [Table 3 (B)].

Because of the distinct biologic behavior of the various histologic subtypes, the group of serous carcinoma (n = 87) was further analyzed separately. The survival analysis shows a median PFS of 12.0 mo (95% CI, 5.78–18.23) in the low-expression group versus 18.1 mo (95% CI, 11.61–24.59) in the high-expression group ($P = 0.008$). Patients with low AC expression had a median OS of 35.7 mo (95% CI, 22.24–47.16) versus 58.7 mo (95% CI, 36.48–80.91) in patients with high AC expression ($P = 0.032$; Figs. 3A, B). The Cox regression analyses revealed that low AC expression (HR 1.88; 95% CI, 1.13–3.11; $P = 0.0015$) and postoperative residual tumor >10 mm (HR 2.32; 95% CI, 1.36–3.94; $P = 0.002$) were the strongest prognostic factors for shorter PFS (Tables, Supplemental Digital Content 4, http://links.lww.com/IJGP/A16, and Supplemental Digital Content 5, http://links.lww.com/IJGP/A17). Low AC expression retained a trend toward shorter OS (HR 1.62; 95% CI, 0.92–2.84; $P = 0.092$) (Table, Supplemental Digital Content 5, http://links.lww.com/IJGP/A17).

DISCUSSION

This retrospective analysis of 112 patients with EOC shows that a reduction in AC expression correlates with disease progression. This is the first report on a prognostic impact of AC expression in this tumor entity.

There are recent data on the human AC demonstrating an important role in regulating cellular responses (10). Elojeimy et al. (30) showed that AC is overexpressed in head and neck cancer cells and

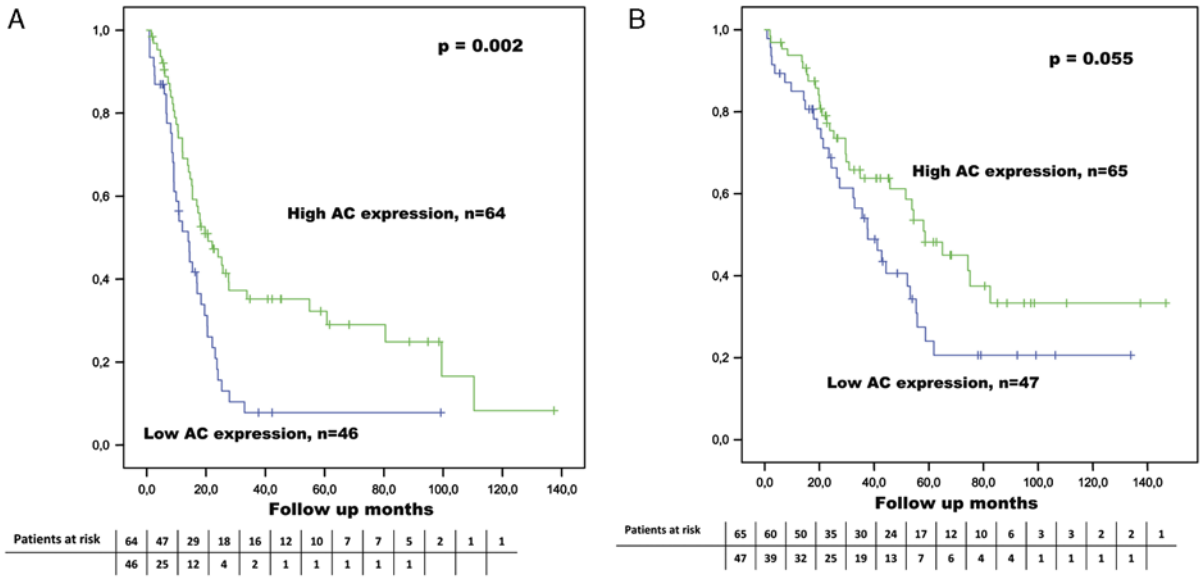

FIG. 2. Kaplan-Meier analyses of progression-free survival (A) and overall survival (B) were performed in patients with epithelial ovarian cancer on the basis of AC expression.

correlates inversely with the sensitivity of these cell lines to Fas-mediated apoptosis. In prostate cancer, Seelan et al. (31) found an overexpression of AC in 40% of the cases by using reverse transcription polymerase chain reaction, and Norris et al. (17) could point out that even up to 80% of prostate cancers had elevated AC levels compared with normal tissue. Saad et al. (23) contributed to these findings by showing that AC overexpression promotes cell proliferation and inhibits cell apoptosis in response to several apoptotic stimuli in prostate cancer. Taken together, various *in vitro* studies emphasized that AC contributes to cancer invasiveness, provides resistance against cellular death, and promotes survival (10). This effect was explained by the possible mechanism of preventing the accumulation of proapoptotic ceramides and shifting the sphingolipid balance from ceramides and SPHs toward S1P (4,10,11). Following the proposition of a sphingolipid rheostat, an increase in antiapoptotic S1P by high AC activation and expression could lead to tumor cell survival (8,10).

Nevertheless, in some studies this impact of AC could not be confirmed. For example, in an analysis of thyroid cancers, mRNA levels of AC were decreased when compared with normal tissue (32). Similarly, a study comparing enzymatic activities of AC in colorectal cancers and normal tissue did not show an increased activity of AC by using ceramidase activity assays (33). This has been called the "exception to the paradigm" by some authors (34).

Recently, our group showed a correlation between AC expression and survival data in primary breast cancer (20). However, in contrast to the above-mentioned

TABLE 2. *Univariate Cox regression analysis of standard parameters among ovarian cancers for progression-free survival (A) and overall survival (B)*

Marker	HR	95% CI	*P*
(*A*) *Progression-free survival*			
Age			
>50 vs. ≤50	1.231	0.707–2.145	0.462
Residual tumor			
>10 mm vs. 0–10 mm	2.926	1.942–4.410	<0.001
Grading			
Low (G1) vs. high (G2/3)	1.102	1.007–1.205	0.034
AC			
Low (IRS 0–2) vs. high (IRS≥3)	2.027	1.292–3.179	0.002
FIGO stage			
I–II vs. III–IV	0.229	0.117–0.449	<0.001
Histology			
Serous vs. other	1.473	0.849–2.557	0.168
(*B*) *Overall survival*			
Age			
>50 vs. ≤50	1.983	1.001–3.930	0.050
Residual tumor			
>10 mm vs. 0–10 mm	2.981	1.905–4.664	<0.001
Grading			
Low (G1) vs. high (G2/3)	1.084	0.990–1.185	0.08
AC			
Low (IRS 0–2) vs. high (IRS≥3)	1.622	0.984–2.673	0.058
FIGO stage			
I–II vs. III–IV	0.124	0.045–0.343	<0.001
Histology			
Serous vs. other	1.709	0.890–3.282	0.107

AC indicates acid ceramidase expression; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; IRS, immunoreactive score.

TABLE 3. *Multivariate Cox regression analysis of AC and standard parameters among ovarian cancers for progression-free survival (A) and overall survival (B)*

Marker	n = 109	HR	95% CI	*P*
(*A*) *Progression-free survival*				
Age				
>50 vs. ≤50	88 vs. 21	1.507	0.842–2.698	0.168
Residual tumor				
>10 vs. 0–10 mm	40 vs. 69	2.162	1.326–3.526	0.002
Grading				
Low (G1) vs. high (G2/3)	5 vs. 104	0.270	0.035–2.063	0.207
AC				
Low (IRS 0–2) vs. high (IRS≥3)	46 vs. 63	1.712	1.077–2.722	0.023
FIGO stage				
I–II vs. III–IV	27 vs. 82	0.360	0.172–0.755	0.007
Histology				
Serous vs. other	86 vs. 23	0.873	0.484–1.575	0.653
Marker	n = 111	HR	95% CI	*P*
(*B*) *Overall survival*				
Age				
>50 vs. ≤50	89 vs. 22	1.955	0.964–3.964	0.063
Residual tumor				
>10 vs. 0–10 mm	40 vs. 71	2.037	1.203–3.450	0.008
Grading				
Low(G1) vs. high (G2/3)	6 vs. 105	0.720	0.086–6.046	0.762
AC				
Low(IRS 0–2) vs. high (IRS≥3)	47 vs. 64	1.289	0.774–2.146	0.329
FIGO stage				
I–II vs. III–IV	28 vs. 83	0.187	0.061–0.576	0.003
Histology				
Serous vs. other	87 vs. 24	0.952	0.484–1.876	0.888

AC indicates acid ceramidase expression; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; IRS, immunoreactive score.

examinations, an overexpression of AC in estrogen receptor–positive breast cancer correlated with improved prognosis. In that trial, a considerable patient cohort could be examined by using large-scale affymetrix chip analysis (20).

In this current immunohistochemistry study, we defined a subset of 50 patients (42%) presenting a missing or low expression of AC. This group of patients had a worse prognosis when compared with patients with a higher expression of AC. The prognostic effect was confirmed for patients with advanced FIGO stage by analyzing the different FIGO stages separately (Figure, Supplemental Digital Content 6, http://links.lww.com/IJGP/A18). In univariate analysis, AC represents a significant prognostic marker for PFS and OS besides standard clinical markers like FIGO stage, grading, and postoperative residual tumor. Even in the multivariate analysis AC retained its remarkable prognostic impact for PFS and thus is a significant prognostic factor, independent of standard clinical markers.

In ovarian tumors it could be shown that the total content of ceramide is decreased when compared with normal ovarian tissues (35), suggesting a higher conversion rate from ceramide to SPH by higher AC expression. According to our trial, a higher AC expression results in an improved prognosis in ovarian cancer patients. This might be explained by an increase in SPH following the higher activation of AC and a simultaneously reduced conversion of SPH to S1P. In this way SPH can exhibit its apoptotic function either by reconversion to ceramide or directly without being converted (36). The latter way of action could explain our results by leading to enhanced tumor cell apoptosis. In contrast, a low activation of AC could lead to low amounts of SPH and therefore to tumor cell survival. Thus, our observation does not support the theory that a higher AC expression followed by a decrease in ceramide levels promotes the antiapoptotic behavior of tumor cells in ovarian cancer.

Ahn et al. (37) could show that SPH can have chemopreventive and chemotherapeutic effects in breast cancer. Therefore, SPH might similarly exert a protective effect of normal epithelial ovarian cells against uncontrolled proliferation analogical to an assumed effect in breast cancer.

In platinum-sensitive recurrent disease, there is a debate with regard to the benefits of platinum

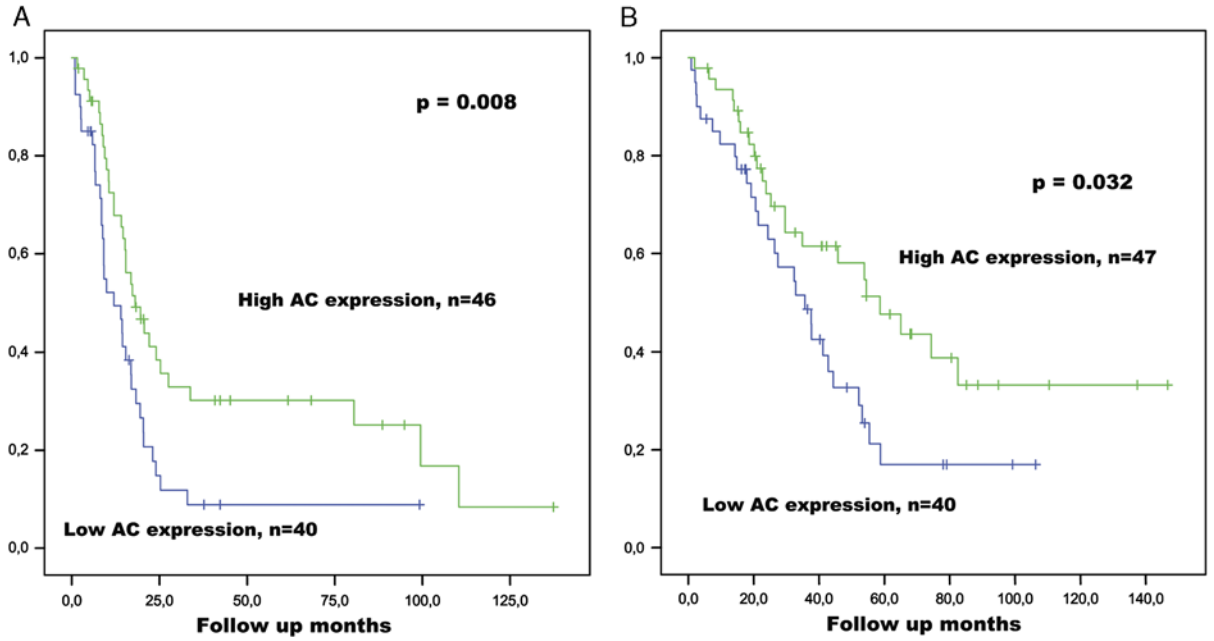

FIG. 3. Kaplan-Meier analyses of progression-free survival (A) and overall survival (B) were performed in patients with serous epithelial ovarian cancer on the basis of AC expression.

rechallenge in all women of this heterogenous cohort (38,39). We have shown that AC expression at primary diagnosis might be helpful in the identification of a biologic subgroup that gathers less benefit from reinduction chemotherapy, bearing in mind that most patients of our cohort received polychemotherapy on relapse. Although there was no association of platinum sensitivity with expression of AC (Table, Supplemental Digital Content 1, http://links.lww.com/IJGP/A13), it could be stressed that in this group a missing AC expression was associated with an unfavorable prognosis, suggesting that in these women alternative treatment strategies could be useful. In this context, AC expression might be a promising approach as a predictive factor and should be further examined.

It is well known that different histologic subtypes of ovarian cancer demonstrate different biologic behaviors (40). In our study, next to serous tumors, a small number (n = 25) of nonserous tumors were included (Table 1). Although we could not find a difference in AC expression on the basis of different histologic subtypes (Table, Supplemental Digital Content 1, http://links.lww.com/IJGP/A13), other authors have demonstrated a distinctly stronger staining of AC in mucinous ovarian carcinomas (41). Therefore, the histologic subtype of serous carcinoma was analyzed separately. These data strongly confirm the prognostic value of AC expression in serous ovarian cancer for PFS ($P = 0.008$) and OS ($P = 0.032$) (Fig. 3). Furthermore, considering the recently proposed new grading system for serous ovarian cancer (26,42), we performed a further analysis of only serous high-grade ovarian cancers and omitted the only 2 low-grade serous cancers of our cohort. This analysis also reaffirms the prognostic impact of AC. Both in the Kaplan-Meier analysis for PFS ($P = 0.021$) and in the multivariate Cox regression for PFS ($P = 0.015$) the survival benefit for higher AC expression was evident (Figure, Supplemental Digital Content 7, http://links.lww.com/IJGP/A19, and Table, Supplemental Digital Content 8, http://links.lww.com/IJGP/A20).

The limitations of our study are its retrospective monocentric nature and the fact that tumor tissue was not strictly taken from consecutive patients. This might cause a possible selection bias. However, the large number of patients in a well-characterized cohort and the established technique of immunhistochemistry are the strengths of this study.

Although the exact mechanism of the distinct effects of AC expression remains unclear, this is the first report describing an impact of AC in a clinical cohort of patients with EOC. The expression of AC could be useful as a prognostic and predictive marker. Because of the tight network of the sphingolipid metabolism, not only AC but also the other key enzymes might be helpful in the identification of new subgroups of patients with ovarian

cancer. Nevertheless, the impact of AC should be confirmed in larger trials and the underlying molecular mechanisms should be further assessed.

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