Expression of excision repair cross complementing group 1 and prognosis in adrenocortical carcinoma patients treated with platinum-based chemotherapy

Cristina L Ronchi^{1,2}, Silviu Sbiera¹, Luitgard Kraus¹, Sebastian Wortmann¹, Sarah Johanssen¹, Patrick Adam³, Holger S Willenberg⁴, Stefanie Hahner¹, Bruno Allolio¹ and Martin Fassnacht¹

¹Endocrine and Diabetes Unit, Department of Medicine I, University Hospital, University of Würzburg, Josef-Schneider-Strasse 2, 97080 Würzburg, Germany

²Endocrine Unit, Fondazione IRCCS Ospedale Maggiore Policlinico and Department of Medical Sciences, University of Milan, Milan, Italy

³Institute of Pathology, University of Würzburg, Würzburg, Germany

⁴Department of Endocrinology, Diabetes and Rheumatology, University Hospital Düsseldorf, Düsseldorf, Germany

(Correspondence should be addressed to M Fassnacht; Email: fassnacht_m@medizin.uni-wuerzburg.de)

Abstract

Therapeutic progress in adrenocortical carcinoma (ACC) is severely hampered by its low incidence. Platinum-based chemotherapies are the most effective cytotoxic treatment regimens in ACC but response rates remain <50%. In other tumor entities, expression of excision repair cross complementing group 1 (ERCC1) predicts resistance to platinum compounds. Therefore, we correlated ERCC1 protein expression and clinical outcome. We have retroactively established adrenal tissue microarrays and analyzed prospectively samples from 163 ACCs, 15 benign adrenal adenomas, and 8 normal adrenal glands by immunohistochemistry for ERCC1 protein expression. Detailed clinical data were available by the German ACC Registry. ERCC1 protein was highly expressed in all normal adrenal glands, 14 benign tumors (93%) and in 75 ACCs (47%). In ACC, no differences in baseline parameters were found between patients with and without ERCC1 expression. Detection of ERCC1 was not correlated with survival in patients who never received platinum-based chemotherapy. In platinum-treated patients ($n=45$), objective response to platinum compounds was observed in 3/21 patients (14.3%) with high ERCC1 expression and in 7/24 patients (29.2%) with low ERCC1 expression ($P=0.23$). ERCC1 expression was strongly correlated with overall survival after platinum treatment (median: eight months in patients with high ERCC1 versus 24 months in low ERCC1 expression, hazard ratio (HR) 2.95 (95% confidence interval (CI) 1.4–6.2), $P=0.004$). Multivariate analysis confirmed that high ERCC1 expression was a predictive factor for poor prognosis in platinum treated patients (HR 2.2, 95% CI 1.0–4.5, $P=0.038$). Our findings suggest that ERCC1 expression is the first factor for predicting survival in ACC patients treated with platinum-based chemotherapy.

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Introduction

Adrenocortical carcinoma (ACC) is a rare tumor with an annual incidence <2/1 million and an incompletely understood pathogenesis (Wajchenberg *et al.* 2000, Allolio & Fassnacht 2006, Koschker *et al.* 2006, Libè *et al.* 2007). This low incidence has hampered significant therapeutic progress in the past and up to

now there has been no single randomized trial published. Despite best care, the overall survival (OS) rate at 5 years is 25–50% in most series (Allolio & Fassnacht 2006, Koschker *et al.* 2006, Assiè *et al.* 2007, Libè *et al.* 2007, Fassnacht *et al.* 2008). Surgery is the treatment of choice in early to intermediate stages aiming at complete tumor removal (Schteingart *et al.*

2005, Abiven *et al.* 2006, Allolio & Fassnacht 2006), but most patients experience a disease relapse (Stojadinovic *et al.* 2002, Allolio & Fassnacht 2006). Mitotane is the only approved drug in ACC treatment but the average response rate is only about 25% (Hahner & Fassnacht 2005, Terzolo *et al.* 2007). Several cytotoxic drugs have been investigated in small case series (Ahlman *et al.* 2001, Allolio *et al.* 2004, Quinkler *et al.* 2008) and cisplatin has been the most widely used and most effective drug, either alone or in combination with other agents (Ahlman *et al.* 2001, Schteingart *et al.* 2005). The most encouraging results till date were observed by Berruti *et al.* (2005) using the combination regimen of mitotane with etoposide, doxorubicin and cisplatin (EDP) in the largest published trial including 72 patients with an objective response rate of 49%. However, this regimen has significant toxicity. Therefore, the determination of parameters that identify patients who will benefit from this cytotoxic therapy would have strong clinical implications and allow physicians to optimize patient care. However, up to date, data on prognostic factors in ACC are limited and no predictors of treatment response have been established (Schteingart *et al.* 2005).

Platinum compounds exert their cytotoxic effects by binding to DNA and forming DNA adducts leading to interstrand or intrastrand cross links that disrupt the structure of the DNA molecule. These changes eventually trigger apoptosis and cell death. However, DNA repair mechanisms, in particular nucleotide excision repair (NER) and mismatch repair, can counteract these effects and, therefore, play a key role in mediating platinum resistance in cancer patients (Reed 1998). The excision repair cross complementing group 1 (ERCC1) protein performs an essential late step in the NER pathway and plays a critical role in removing drug-induced DNA adducts (Tsodikov *et al.* 2007). Previous *in vitro* studies have linked ERCC1 expression to platinum resistance in different cancer cell lines (Chang *et al.* 2005, Cummings *et al.* 2006). Moreover, several retrospective clinical studies in different cancer types demonstrated a negative correlation between ERCC1 mRNA (Shirota *et al.* 2001, Fautrel *et al.* 2005, Joshi *et al.* 2005, Ceppi *et al.* 2006, Bellmunt *et al.* 2007) or protein expression (Olaussen *et al.* 2006, Handra-Luca *et al.* 2007, Kwon *et al.* 2007, Steffensen *et al.* 2008) and response to platinum-containing drugs (cisplatin, carboplatin, oxaliplatin). Recently, also prospective clinical trials have confirmed that assessment of ERCC1 expression may predict the subsequent response to cisplatin in patients affected by non-small cell lung cancer (Cobo *et al.* 2007, Darcy *et al.* 2007).

Our study was performed to investigate if ERCC1 expression might predict clinical outcome also in patients with ACC. We, therefore, studied ERCC1 protein expression by immunohistochemistry in a large number of ACCs and correlated the expression with baseline histopathological and clinical characteristics. In addition, we assessed the relationship between ERCC1 expression and objective response to platinum-based chemotherapy and survival.

Materials and methods

Patients and tissues

A total of 190 adrenocortical tumor tissues were collected retroactively from patients undergoing adrenalectomy due to ACC ($n=175$) or benign lesions ($n=15$), such as aldosterone-producing ($n=5$), cortisol-producing ($n=5$) or hormonally inactive adenomas ($n=5$) in 72 German hospitals between 1989 and 2007 (see Supplementary Fig. 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). Eight normal human adrenal glands derived from tumor nephrectomies were also included. A total of 143 ACC samples were derived from primary tumors, 18 from local recurrence and 17 from distant metastases. The diagnosis of ACC was based on established clinical, biochemical, and morphological criteria (Allolio & Fassnacht 2006), and all histological diagnoses were confirmed (including Weiss, Hough, and van Slooten score and Ki67 index) by the reference pathologist of the German ACC Registry (Wolfgang Saeger, Hamburg, Germany). All samples with a Weiss score of 4 or above were judged as ACC (Weiss *et al.* 1989). However, in 12 out of the 128 evaluable samples with primary surgery for ACC (9%) the diagnosis of ACC was made despite a Weiss score of 3 ($n=7$) or 2 ($n=5$). In these cases, the scores by Hough *et al.* (1979) and Van Slooten *et al.* (1985) clearly indicated malignancy. The analysis of the Ki67 index was performed by estimation of the fraction of Ki67 positive nuclei by analyzing at least 30 high-power fields.

Clinical data of ACC patients, including follow-up and survival data, were collected in a structured manner by the German ACC Registry starting (www.nebennierenkarzinom.de). The database for follow-up information was locked in August 2008. Tumor staging at the time of diagnosis was based on imaging studies, corroborated by the findings during surgery and reported according to the UICC/WHO classification 2004 (DeLellis *et al.* 2004). The secreting status was investigated by appropriate hormonal tests (Fassnacht *et al.* 2004). Surgical resection of the primary tumor

was considered complete (R0 resection) if surgical, pathological, and imaging reports did not give any evidence for remaining disease. Presence of distant metastases was evaluated at the time of diagnosis and during follow-up visits by computerized tomography of chest and abdomen. Owing to the retrospective manner of data collection, the imaging interval was not standardized. However, in patients after radical tumor resection imaging was performed usually every three to four months.

Patients gave informed consent for collecting tissue and clinical data and the study was approved by the ethics committee of the University of Wuerzburg (Germany).

Cohort of patients treated with platinum compound

A subgroup of 45 patients with ACC (18 males, 27 females, mean age at diagnosis: 46.4 ± 14.4 years, see also Supplementary Fig. 1), that received at least one cycle (median: 4; range: 1–24) of platinum compounds were investigated separately for the relationship between ERCC1 protein expression, the response to platinum-based chemotherapy and OS. A total of 36 of these patients had been treated with cisplatin, 8 with carboplatin, and 1 with oxaliplatin. The most frequently used protocols were etoposide + doxorubicin + cisplatin ($n=31$) and carboplatin + etoposide ($n=5$), cisplatin + etoposide ($n=3$), but some patients received other combinations including cyclophosphamide ($n=1$), ifosfamide ($n=1$), suramin ($n=1$), doxorubicin ($n=2$), mitomycin ($n=1$), or taxotere ($n=1$). Forty-three patients received concomitant mitotane treatment. None of the patients underwent cytotoxic chemotherapy prior surgical resection. The response to platinum-based treatment was judged according RECIST criteria (Therasse *et al.* 2000). In this group of patients, imaging for evaluation of therapy was performed every 2–3 months. In the seven patients still alive the median follow-up from the first day of platinum-based drug administration to the last visit was 19 months (range: 9–52).

Tissue samples

A total of 182 adrenocortical tumors (167 carcinomas and 15 benign lesions) and five normal adrenal samples were assembled into three tissue microarrays (TMAs). Briefly, hematoxylin and eosin stained sections of formalin-fixed and paraffin-embedded tissue blocks were re-evaluated to identify the representative areas of well-preserved morphology. The corresponding area on the block was marked for tissue punching. The TMA

was assembled using a commercially available manual tissue puncher/arrayer (Beecher Instruments, Silver Spring, MD, USA) according to the manufacturer's instructions. Five cores with a diameter of 0.6 mm were punched from each tissue block and arrayed into a recipient paraffin block at a distance of 1 mm between each core. We chose to array five punch biopsies per case to minimize the number of cases not analyzable due to tissue loss and to increase concordance rates among different cores. A total of 935 adrenocortical tissue cores were placed in three recipient blocks (70, 54, and 63 cases per block). Sections (2 μ m) were cut on silanized slides also used for conventional immunohistochemical stains. Before staining, slides were dried at room temperature for 7 days in order to minimize tissue loss. For each block, hematoxylin and eosin stained slides were cut to verify tumor cell content. Samples were only included in the analysis if two or more evaluable cores were available after sectioning and the immunohistochemical staining procedure. In addition, 11 adrenal tissue samples (8 ACCs and 3 normal adrenals glands) placed on standard slides were used to confirm homogeneous staining of ERCC1 and to increase the number of samples from patients treated with platinum-based therapy.

Immunostaining

Immunohistochemical detection was performed in all samples using an indirect immunoperoxidase technique after high temperature antigen retrieval in 0.01 mol/l citrate buffer (pH 6.0) 25 min. The primary antibody was a monoclonal mouse antibody against human full-length ERCC1 protein (clone 8F1, Neomarkers, Lab Vision Corp, Fremont, CA, USA; Olausson *et al.* 2006, 2007, Zheng *et al.* 2007). It was used at a dilution of 1:100 and was incubated overnight at 4 °C. The secondary antibody coupled with peroxidase was obtained from Biogenex (Neufahrn, Germany). The slides were incubated with the secondary antibody for 20 min and washed in PBS before diaminobenzidine as chromogen was applied. After washing in PBS and water, the slides were counterstained in hematoxylin. The negative controls were made using an IgG isotype antibody and by the omission of the primary antibody during the process of immunohistochemical staining. For positive control, endothelial cells in tonsil tissue were chosen (Olausson *et al.* 2006, Lee *et al.* 2008a,b). In addition, cells of the tumor stroma served as internal positive control, if sufficient tumor stroma cells were visible (Olausson *et al.* 2006).

Figure 1 Expression of ERCC1 protein in the adrenal tissue. Representative immunohistochemical staining of a normal adrenal gland (A) and two adrenocortical carcinomas (B and C) with a specific antibody against ERCC1. Intensity was quantified on a scale from 0 (no expression) to 3 (very strong expression); for details see Materials and methods; original magnification $\times 400$.

Microscopical analysis

Immunostaining results were analyzed using a light microscope at a magnification of $400\times$. Only samples with positive ERCC1 staining in the nucleus were judged as positive (Fig. 1). ERCC1 staining was homogeneously distributed both in the entire standard tissue slides and among the five cores from the same tissue in the TMAs. We were aiming at the analysis of >500 cancer cells. Twelve cases among those assembled in the TMAS with less than two evaluable cores were excluded from statistical analysis. In total, this resulted in 163 evaluable ACCs, 15 adrenocortical adenomas and 8 normal adrenal glands (for details see Table 1). The staining for ERCC1 protein was judged independently by two investigators (C L R and L K) without knowledge of clinical data. In case of discrepancies, a joined score was agreed on by evaluating the slides together. Staining intensity was evaluated with a grading score of 0, 1, 2, or 3, which corresponded to negative, weak, moderate, or strong

staining intensity (Fig. 1). Tonsil tissue was used as reference for an intensity grade of 2 (Olausson et al. 2006). The percentage of positive tumor cells was calculated for each specimen and scored 0 if 0% were positive, 0.1 if 1–9%, 0.5 if 10–49%, and 1 if $\geq 50\%$. A semi-quantitative H-score was then calculated by multiplying the staining intensity grading score with the proportion score as previously described (Olausson et al. 2006). In accordance with this publication an H-score > 1 was a priori chosen as the cut-off point for separating samples with high (ERCC1-high) or low ERCC1 (ERCC1-low) expression.

Statistical analysis

The primary study hypothesis that ERCC1 expression is a prognostic factor in ACC patients treated with platinum-based chemotherapy was pre-specified and the analysis was performed in a prospective manner. The comparison of clinical and histopathological characteristics was performed using an unpaired

Table 1 Excision repair cross complementing group 1 (ERCC1) protein expression in 186 evaluable samples from adrenal tissues

ERCC1 expression H-score	n	Low expression ^a		High expression ^a		P*
		0	1	2	3	
			n (%)		n (%)	
Normal adrenal glands	8	0	0	4 (50%)	4 (50%)	1.00
Adrenocortical adenomas	15	0	1 (7%)	12 (80%)	2 (13%)	
Aldosterone-producing adenoma	5	0	0	5	0	
Cortisol-producing adenoma	5	0	0	5	0	
Inactive adenoma	5	0	1	2	2	
Adrenocortical carcinomas	163	12 (7%)	76 (47%)	61 (37%)	14 (9%)	0.0007[†]
Primary tumor	132	9	65	46	12	0.006[‡]
Local recurrence	17	2	8	6	1	
Distant metastases	14	1	3	9	1	

*P value evaluated by Fisher’s exact test between low and high expression, [†]P value versus normal adrenal gland, [‡]P versus benign tumors. P values in bold represent $P<0.05$.

^aLow and high ERCC1 expression was defined as H-score ≤ 1 and > 1 respectively (for details see Materials and methods, Ref. Gossage & Madhusudan (2007)).

Student's *t*-test for continuous variables and a Fisher's exact test or a χ^2 -test for categorical variables, as appropriate. OS was defined as the time from the date of the established ACC diagnosis to death or last follow-up visit. Disease-free survival (DFS) was defined as the time from the date of complete tumor resection to the first radiological evidence of disease relapse or death. In the subgroup of patients treated

with platinum-based therapy, survival after platinum (SAP) was calculated from the first day of platinum administration to death or last follow-up visit. Kaplan–Meier survival analysis was carried out for calculating median duration (and 95% confidence interval, CI) of DFS, SAP, and OS respectively. Patients alive or disease free were censored at the time of the last visit. The comparisons of OS, DFS, and SAP between

Table 2 Relationship between excision repair cross complementing group 1 (ERCC1) protein expression and baseline clinical or pathological characteristics of 128 patients with adrenocortical carcinoma (only tumor samples derived from primary surgery).

Variable	ERCC1 expression		<i>P</i>
	Low (<i>H</i> -score ≤ 1)	High (<i>H</i> -score > 1)	
<i>N</i>	71	57	
Age at diagnosis – years			0.59
Median (range)	48 (17–77)	52 (18–77)	
Sex – <i>n</i> (%)			0.71*
Male	25 (39)	18 (32)	
Tumor stage (UICC/WHO 2004) – <i>n</i> (%)			0.50†
Evaluated patients (<i>n</i>)	70	56	
I	2 (3)	4 (7)	
II	27 (39)	24 (43)	
III	17 (24)	9 (16)	
IV	24 (34)	19 (34)	
Hormonal status – <i>n</i> (%)			
Evaluated patients (<i>n</i>)	65	50	
Secreting tumors	49 (75)	37 (74)	1.00*
Non-secreting tumors	16 (25)	13 (26)	
Tumor size (cm)			0.72
Median (range)	11.0 (4.5–20.0)	11.5 (4.5–23.0)	
Surgical resection			0.28†
Evaluated patients	66	54	
R0	32 (48)	19 (35)	
R1/RX	15 (23)	18 (33)	
R2	19 (29)	17 (32)	
Medical treatment during follow-up			
Mitotane (<i>n</i>)	50 (70)	29 (51)	0.42
Cytotoxic chemotherapy	20 (28)	22 (39)	0.26
Weiss score			0.78
Evaluated patients (<i>n</i>)	67	54	
Median (range)	5 (2–9)	5 (2–8)	
MIB1 (Ki67) index (%)			0.04
Evaluated patients (<i>n</i>)	57	53	
Median (range)	5 (0–60)	10 (0–50)	
Distant metastases at diagnosis			
Evaluated patients (<i>n</i>)	63	50	0.40*
Affected patients (<i>n</i> (%))	15 (24)	16 (32)	0.48
Number of tumoral organs			0.20
Median (range)	1.0 (1–2)	1.5 (1–2)	
Number of tumoral lesions			
Median (range)	7.0 (1–15)	7.5 (2–18)	
Follow up (months)			
Evaluated patients (still alive)	25	33	
	37 (6–200)	37 (6–150)	0.53

n, number; secreting tumors tumor-induced excess of glucocorticoids, androgens, mineralcorticoids, estrogens and/or steroid precursor; R0, complete resection, R1/RX, microscopically incomplete or uncertain resection; R2, macroscopically incomplete resection. *P* evaluated by Student's *t*-test; **P* evaluated by Fisher's exact test; †*P* evaluated by χ^2 -test. *P* values in bold represent $P < 0.05$.

groups were made with log-rank test. Multivariate regression analysis was performed by the Cox proportional hazards regression model to identify those factors that might have significantly influenced survival in platinum-treated patients. For this analysis, all the variables with $P < 0.1$ in the univariate analysis were included as continuous or dichotomized variables, as appropriate. The statistical analysis was performed with SPSS (version 15 for Windows, SPSS Inc., Chicago, IL, USA) software. All tests were two-sided and a $P < 0.05$ was considered as statistically significant for each analysis.

Results

Expression of ERCC1 protein in adrenocortical tissue

As shown in Fig. 1 and as expected, ERCC1 staining was localized to the nucleus. As in all ERCC1 positive specimens the percentage of positive tumor cells was $> 50\%$ (proportion score = 1), the H -score (for definition see Materials and methods) corresponded to the respective staining intensity grading score (Table 1). A high ERCC1 nuclear staining intensity (H -score > 1) was observed in all normal adrenal glands, in 14/15 benign adenomas (93%), and in 75 of ACCs (46%, $P = 0.0007$ versus normal adrenals and

$P = 0.006$ versus benign tumors, Table 1). Twelve of 163 ACC samples but none of the adenomas and normal adrenal glands were completely negative for ERCC1 staining. There were no significant differences in the proportion of low or high ERCC1 stained samples among ACC primary tumors, local recurrences or distant metastases.

Correlation of ERCC1 with histopathological and clinical characteristics and prognosis

To evaluate the relation of ERCC1 to histopathological and clinical parameters in ACC, patients were only included in the analysis, when tumor tissue from the primary surgery and complete histological and clinical parameters were available ($n = 128$, 47 males, 81 females, mean age at diagnosis: 50.0 ± 16.1 years). Parameters are summarized in Table 2. No statistically significant differences in age, sex, tumor stage, tumor size, presence of distant metastasis (including number of tumoral organs or metastatic lesions), hormonal status, and Weiss score were found between patients with low and high ERCC1 expression (Table 2). Only the mean MIB1 (Ki67) index was higher in ERCC1-high compared with ERCC1-low samples (13.4 ± 14.6 vs $8.2 \pm 11.9\%$, $P = 0.04$, Table 2). In 17/53 samples with high ERCC1 and in 8/57 samples with low ERCC1 Ki67 index was $> 10\%$ ($P = 0.039$).

Figure 2 Impact of ERCC1 expression (high = H -score > 1 ; low = H -score ≤ 1) on overall survival (OS) and disease-free survival (DFS) in patients with adrenocortical carcinoma never treated with platinum compounds. (A and B) All patients ($n = 91$); (C and D) patients with tumor stages I and II ($n = 66$). The analysis of DFS was performed only in patients after radical resection.

Considering altogether the 128 evaluable primary operated ACC patients with full clinical annotation, the OS did not differ significantly between those with low or high ERCC1 expression (Supplementary Fig. 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). For further analysis, patients treated with platinum-based therapy were analyzed separately. In the group of patients who had never received platinum-based regimes ($n=91$), the median follow-up period from diagnosis to last visit was 47 months (range: 1–200) in the 47 patients still alive. Accordingly, no significant impact of ERCC1 expression was found for both DFS and OS in patients after complete resection and OS in these 91 patients (Fig. 2A and B). Similar results were obtained considering only patients with limited tumor stages (WHO 1 and 2, $n=66$) at the time of diagnosis (Fig. 2C and D).

ERCC1 expression and response to platinum-compounds

In the 45 patients treated with platinum-based chemotherapy, 21 samples (47%) showed high ERCC1 expression. No statistically significant

differences were observed in clinical and histological characteristics at the time of the diagnosis between patients with low or high ERCC1 staining (Table 3). Types and doses of different platinum-based drugs were comparable between patients with high or low ERCC1 expression (Table 3). Ten patients showed an objective tumor response to treatment (22.2%), while seven patients (15.6%) had a stable and 28 a progressive disease. In particular, three ERCC1-high patients had a partial response (14.3%), while seven ERCC1-low patients (29.2%, $P=0.23$) experienced an objective response (partial response in six and complete response in one). ERCC1-high patients had considerably shorter 1-year and 2-years survival rate after platinum therapy (14 vs 65% and 5 vs 37% respectively, both $P<0.001$). Accordingly, high ERCC1 expression was significantly associated with poor SAP therapy with a hazard ratio (HR) of 2.95 (95% CI 1.41–6.17; median SAP: 8 months in patients with high versus 24 months in patients with low ERCC1 expression, Fig. 3A and Table 4). Age over 44 years and Ki67 index $>10\%$ were also associated with a shorter SAP (HR: 2.08 (95% CI 1.04–4.18) and 2.10 (95% CI 1.02–4.34))

Table 3 Clinical and pharmacological data from the 45 patients with adrenocortical carcinoma treated with platinum-compounds

Variables at diagnosis	Low ERCC1 expression (H-score ≤ 1)	High ERCC1 expression (H-score > 1)	P
N	24	21	
Age – years (median (range))	47 (22–70)	44 (21–73)	0.43
Sex – n males (%)	11 (46)	6 (29)	0.36
Tumor stage – WHO 2004 (median (range))	4 (1–4)	4 (1–4)	0.94
Hormonal status – n secreting tumors (%)	20 (83)	17 (81)	1.00
Tumor size – cm (median (range))	12.0 (3–19)	11.2 (4–25)	0.78
Weiss score (median (range))	5.0 (3–8)	6.0 (4–8)	0.14
MIB1 (ki67) index (median (range))	9.0 (0–50)	20.0 (0–50)	0.10
Distant metastasis – n (%)	8 (33)	8 (38)	0.76
<i>Information on treatment regimen</i>			
Type of platinum compound (n)			0.31
Cisplatin ^a	21 (87.5)	15 (71)	
Carboplatin ^b	3 (12.5)	5 (24)	
Oxaliplatin	0	1 (5)	
Scheme of chemotherapy (n)			0.89
Cisplatin+etoposide+doxorubicin (EDP)	17 (71)	14 (67)	
Carboplatin+etoposide	2 (8)	3 (14)	
Cisplatin+etoposide	2 (8)	1 (5)	
Other schemes ^c	3 (13)	3 (14)	
Duration of treatment – n cycles (median (range))	5.5 (1–24)	3.0 (1–9)	0.02
Follow up – months (median (range)) ^d	11 (4–22)	29 (6–52)	0.19
Concomitant mitotane – n (%)	24 (100)	19 (90)	0.12

P evaluated by Student's *t*-test or Fisher's exact test or χ^2 -test, as appropriate.

^aMedian cumulative dose of cisplatin per cycle was 80 mg/m².

^bMedian cumulative dose of carboplatin per cycle was 400 mg/m².

^cOther schemes included: cyclophosphamide ($n=1$); ifosfamide ($n=1$); suramin ($n=1$); mitomycin ($n=1$) or taxotere ($n=1$).

^dOnly in patients still alive ($n=9$).

Figure 3 Impact of ERCC1 expression on survival in 45 patients with advanced adrenocortical carcinoma treated with platinum-based chemotherapy. Survival time was calculated from first day of platinum administration to death or last follow-up visit. (A) Patients were divided according to low (H -score ≤ 1) or high (H -score > 1) ERCC1 expression. (B) Patients subdivided for graded ERCC1 expression (H -score: 0=absent; 1=weak; 2=moderate; 3=strong).

respectively (Table 4). A multivariate logistic regression, including age, Ki67 index and ERCC1 expression (overall model fit, $P=0.013$ by χ^2 test), confirmed that high ERCC1 expression was an independent prognostic factor for poor SAP with an adjusted HR of 2.16 (95% CI 1.04–4.46; $P=0.038$, Table 4). When including in the analysis only patients treated with EDP ($n=31$), again patients with high ERCC1 expression had a considerably shorter median survival after EDP (9.5 vs 30.0 months; HR of 2.56 (95% CI 1.03–6.34), $P=0.043$).

Finally, we analyzed survival according to ERCC1 staining intensities (H -score 0, 1, 2, 3). We observed a correlation between H -score and clinical outcome: all patients with an H -score of three died early after platinum treatment (5/5 patients died within 10 months), whereas the two patients with H -score 0 (no ERCC1 staining) are still alive at 19 and 35 months respectively, after therapy ($P=0.0011$ by log-rank test for trend, Fig. 3B).

Discussion

In this study, we have demonstrated for the first time that low ERCC1 protein expression in ACC tumor cells is associated with improved SAP-based chemotherapy. Platinum-based chemotherapies are currently the most frequently used cytotoxic regimens in this very rare malignancy. However, as it is also highlighted in this paper, only a minority of patients responds to this treatment, which is associated with significant toxicity. Thus, our results may have important implications for both individual patients and future treatment concepts. Indeed, ERCC1 is the first molecular parameter associated with prognostic value in advanced ACC and multivariate regression analysis, including all parameters with potential impact in this cohort, confirmed the independent prognostic value of ERCC1 expression on outcome after platinum-based therapy. Moreover, a very strong or an absent ERCC1 expression was even more strikingly correlated with poor or favorable outcome after platinum treatment respectively.

Our results are in accordance with several other studies in different cancer types demonstrating that high ERCC1 expression is associated with poor clinical outcome after administration of platinum-based drugs (Shirota *et al.* 2001, Fautrel *et al.* 2005, Joshi *et al.* 2005, Ceppi *et al.* 2006, Olaussen *et al.* 2006, Azuma *et al.* 2007, Bellmunt *et al.* 2007, Handra-Luca *et al.* 2007, Kwon *et al.* 2007, Lee *et al.* 2008a,b, Steffensen *et al.* 2008). As ERCC1 has a crucial role in the nuclear excision repair pathway, its involvement in the resistance to platinum treatment is very plausible (Gazdar 2007, Gossage & Madhusudan 2007) and most likely responsible for the effects seen in our study, although we cannot fully exclude a confounding role of co-medication like other cytotoxic drugs or mitotane.

We did not find a statistically significant effect of ERCC1 expression on the objective response rate to treatment. However, there was a twofold higher proportion of patients with an objective response to platinum-based therapy in the ERCC1-low group in comparison with the ERCC1-high group suggesting that with a larger sample size this difference may become statistically significant. Of note, some lung cancer studies did also find no significant differences in response rate but in SAP treatment (Azuma *et al.* 2007, Lee *et al.* 2008a,b). Therefore, ERCC1 expression may retard the proliferation rate of tumor cells in patients treated with platinum-based therapy independent of objective tumor response. Furthermore, tumor response may not necessarily correlate with survival in all tumor entities (Gollob & Bonomi 2006).

Table 4 Predictive factors for overall survival after platinum based therapy in 45 patients with advanced adrenocortical carcinoma (from the first administration of platinum-compounds)

Variable	<i>n</i>	Median survival (months)	HR	95% CI	<i>P</i> *
<i>Univariate analysis</i>					
Age at diagnosis			2.08	1.04–4.18	0.039
≤44 years	20	8.0			
>44 years	25	17.0			
Sex			1.19	0.60–2.35	0.63
Male	17	17.0			
Female	28	10.0			
Weiss score			0.74	0.33–1.54	0.39
Evaluated patients	40				
≤6	30	10.0			
>6	10	10.0			
Ki67 index			2.10	1.02–4.34	0.045
Evaluated patients	40				
≤10%	20	14.5			
>10%	20	8.5			
Distant metastasis					
Affected patients	43				
Tumoral organs ^a			1.82	0.85–3.88	0.12
≤2	27	13.0			
<i>n</i> >2	16	9.5			
Tumoral lesions ^a			0.94	0.44–2.05	0.89
Evaluated patients	34				
≤6	16	11.0			
<i>n</i> >6	18	8.0			
ERCC1			2.95	1.41–6.17	0.004
Low (<i>H</i> -score ≤1)	24	24.0			
High (<i>H</i> -score >1)	21	8.0			
Variable			HR	95% CI	<i>P</i> [†]
<i>Multivariate analysis</i>					
Age at diagnosis			0.98	0.96–1.00	0.14
Ki67 index			1.80	0.87–3.72	0.15
ERCC1 expression			2.16	1.04–4.46	0.038

The median value for age, Weiss score, Ki67 index, tumoral organs and lesions was used as cut-off values to discriminate the groups. HR, hazard ratio; CI, confidence interval; *n*, number; ERCC1, excision repair cross complementing group 1. **P* evaluated by log-rank test. †*P* evaluated by Cox proportional hazards regression model (including age as a continuous variable, Ki67 index, and ERCC1 expression as dichotomized variables). *P* values in bold represent *P*<0.05.

^aIncluding lesions in the adrenal gland.

Our study has several limitations, mainly the presumably low number of patients, the heterogeneity of platinum-based treatment regimens and the fact that the collection of tumor samples was not performed in a prospective manner. However, the main study hypothesis was pre-specified and the analysis was performed prospectively. In comparison to more common malignancies, the total sample size of 163 ACC patients and 45 evaluated patients after platinum based therapy seems to be small, but for this rare disease it is exceptionally high. No phase III trial for ACC has been published and the largest published phase II trial in ACC enrolled 72 patients (Berruti *et al.* 2005). Furthermore, the largest series analyzing specific

immunohistological parameters in ACC included 124 patients, but detailed analysis was performed only in a subset of 67 and 38 patients respectively (Stojadinovic *et al.* 2002). Therefore, our study represents the largest series investigating the association of a specific immunohistological marker with clinical outcome. After the publication by Berruti *et al.* (2005), the EDP regimen is by far the most accepted platinum-based regimen in ACC. Therefore, it is of note, that ERCC1 also predicts outcome in the subgroup of patients treated according to the Berruti protocol (*n*=31). An additional limitation of our study is related to the use of TMAs for immunohistochemical analysis. In only about fifty percent of tumor samples, a significant

number of tumor stroma cells were visible to serve as internal positive control for ERCC1 staining.

In several but not all tumor entities, ERCC1 was also of prognostic value in patients not treated with platinum-based therapy (Lee et al. 2008a,b), especially in patients with limited disease (Zheng et al. 2007). However, in our series ERCC1 expression was not related to clinical outcome in patients who never received platinum drugs. This was true for the entire cohort but also for patients with limited disease. Moreover, with the exception of Ki67, we did not find any difference in terms of baseline histopathological or clinical characteristics between ERCC1-low and -high ACC patients. In contrast to ERCC1, Ki67 was not of predictive value for outcome after platinum-based treatment.

The high expression of ERCC1 in benign adrenocortical adenomas and normal adrenals confirms findings in other normal tissues (Gazdar 2007, Gossage & Madhusudan 2007). Therefore, it has been postulated that a reduced DNA repair capacity may constitute a risk factor for cancer development (Friedberg 2001, Cheng et al. 2002). However, it is currently not clear if low ERCC1 expression is cause or consequence of malignancy.

In conclusion, our findings provide first evidence that ERCC1 protein expression represents a novel independent prognostic factor useful for predicting survival in patients with advanced ACC in whom platinum-based chemotherapy is planned. Thus, it may provide critical information for individualized treatment and optimized patient care in this rare malignancy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research.

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