Predictive Biomarkers and Personalized Medicine

Ribonucleotide Reductase Large Subunit (RRM1) Gene Expression May Predict Efficacy of Adjuvant Mitotane in Adrenocortical Cancer

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Abstract

Purpose: Mitotane is the most broadly used systemic therapy for adrenocortical carcinoma (ACC), but its mechanism of action and possible predictors of treatment response are currently poorly defined. Our aim was to evaluate the gene expression of ribonucleotide reductase large subunit 1 (RRM1) and excision repair cross-complementation group 1 (ERCC1) in ACC as potential biomarkers for clinical outcome and response to mitotane.

Experimental Design: Forty-five and 47 tissue samples from two cohorts (Orbassano, Italy; Wuerzburg, Germany) of completely resected ACC were centrally analyzed using real-time PCR for RRM1 and ERCC1 expression. Fifty-four patients received surgery alone and 38 received adjuvant mitotane after surgery. Clinical and pathologic features were highly comparable in the two series. H295R and SW-13 ACC cell lines were also used for pharmacologic tests.

Results: ERCC1 gene expression was not associated to clinical outcome. In contrast, high RRM1 gene expression was associated to shorter disease-free survival (DFS) and overall survival at both univariate and multivariate analysis. In patients with low RRM1 gene expression, adjuvant mitotane was associated with improved DFS, whereas this effect was lost in cases with high RMM1 expression. In vitro mitotane induced strong up regulation of RRM1 transcription (up to 25-fold increase) in mitotane-insensitive SW-13 but not in mitotane-sensitive H295R cells. Furthermore, RRM1 silencing in SW-13 cells induced sensitivity to mitotane.

Conclusion: Our in vitro and in vivo data indicate that RRM1 gene expression is functionally associated to mitotane sensitivity and support a possible role of RRM1 determination as a novel molecular biomarker predicting response to adjuvant mitotane in ACC. Clin Cancer Res; 18(12); 3452–61. ©2012 AACR.

Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive malignant tumor (1, 2). Surgery is the mainstay of therapy (3, 4), however medical treatment has also an important role because a large proportion of ACC patients presents with metastatic disease, and most of the radically resected patients relapse after surgery, often with metastases (5).

Mitotane, either as monotherapy or in combination with cytotoxic chemotherapy, is the reference drug in the management of advanced ACC (6, 7).

Mitotane has also been used in an adjuvant setting. A large retrospective multicentric study carried out at several referral centers in Italy and Germany has shown that adjuvant mitotane therapy prolonged recurrence-free and overall survival (OS) in radically resected ACC patients (8), and a panel of international experts recently recommended the administration of adjuvant mitotane in all patients at high risk of relapse (9). However, this recommendation based on retrospective data has a low level of evidence, whereas a prospective randomized controlled trial on adjuvant mitotane treatment is currently under way (www.adiuvo-trial.org).

The risk of relapse or death in ACC patients may be assessed by several parameters, including pathologic features, such as mitotic index (10–12), molecular alterations, such as TP53 mutations and β-catenin dysregulation (13), or immunohistochemical markers including matrix metalloproteinase type 2; ref. 14), glucose transporter 1 (15), and steroidogenic factor-1 (16). In addition, a gene signature
Translational Relevance

Mitotane is the reference drug in the management of adrenocortical carcinoma (ACC) and is increasingly prescribed in an adjuvant setting. Serum mitotane levels are the only known predictive factor of efficacy. In this article, we observed in 2 independent series of radically resected ACC patients that in patients with low ribonucleotide reductase large subunit 1 (RRM1) gene expression adjuvant mitotane was associated with improved disease-free survival, whereas this effect was lost in cases with high RRM1 expression. Moreover, this observation was supported by data in adrenal cancer cell lines, showing that low responsiveness to mitotane was associated to upmodulation of RRM1 gene and that selective RRM1 silencing can restore mitotane sensitivity in vitro. The present data are of translational relevance because they generate the hypothesis that RRM1 gene expression is a genetic marker predicting mitotane efficacy that may be used to select patients who can benefit from adjuvant mitotane therapy.

profile has been shown to correlate more closely with prognosis than clinical data (17, 18). However, in contrast to the availability of prognostic parameters, very few factors predicting the efficacy of treatment have been identified. A single study reported that a low immunohistochemical expression of excision repair cross-complementation group 1 (ERCC1) correlated to objective response and OS in ACC patients treated with platinum-based chemotherapy (19).

With regard to mitotane treatment, attainment of drug levels in the target range of 14 to 20 mg/L is the only factor predicting efficacy that has been convincingly shown (20–22).

Because most ACC patients are at high risk of recurrence after primary surgery applying current prognostication methods (9), selection of patients for adjuvant therapy with mitotane based upon predictive factors for drug efficacy may represent a superior approach than selection based on prognostic markers. Such an approach is particularly appealing because mitotane therapy is a cumbersome and quite toxic treatment that has to be maintained for years and needs frequent drug monitoring and a complex regimen of steroid replacement and management of adverse events (5, 23).

We designed this study stemming from the previous work of our research group on the role of genes involved in DNA repair or synthesis as predictors of response to chemotherapy in non–small cell lung cancer (NSCLC), neuroendocrine tumors, and mesothelioma (24–26) and aimed to test the expression of ribonucleotide reductase large subunit 1 (RRM1) and ERCC1 genes in a cohort of ACC patients and to correlate gene expression with clinical outcome. The rationale for investigating RRM1 and ERCC1 genes as potential biomarker in ACC was based on their prognostic relevance in other cancer types (27) and on the sequential use of platinum- and gemcitabine-based therapy in ACC (28). In addition, we analyzed the predictive role of these 2 markers in patients treated with adjuvant mitotane and strengthened the in vivo results by in vitro analysis on ACC cell lines.

Patients and Methods

Patients

Ninety-two patients with ACC radically resected between 1989 and 2007 at the University of Turin at Orbassano (Turin), Italy (45 patients), and 35 centers in Germany all coordinated by the German ACC Registry (47 patients) were included according to the following: (i) age of 18 years or older; (ii) histologically confirmed diagnosis of ACC after revision (MV, MP); (iii) stage I to III disease, (iv) complete tumor resection, (v) availability of follow-up information, (vi) availability of representative paraffin-embedded tissue block(s). All patients fulfilling the inclusion criteria were included in the study. All German patients, during follow-up were transferred to the center in Würzburg, Germany. The diagnosis of ACC was based on established clinical and biochemical parameters (29) and the pathologic Weiss score (30). Parameters recorded included age, sex, hormone secretion (according to ENSAT recommendations at www.ensat.org/acc.htm), ENSAT stage at diagnosis (31), initial therapeutic options including primary surgery, disease-free survival (DFS), defined by the time elapsing from diagnosis to either disease relapse or patient death, OS, calculated from diagnosis till death, Weiss score, mitotic count, and sites of metastases at the time of progression. Relapse was ascertained by computerized tomography (or MRI) of chest and abdomen every 3 to 6 months. Disease relapse was defined as the appearance during follow-up of local recurrence or metastatic disease at imaging techniques. Adjuvant mitotane was offered to patients considered at high risk of relapse, in presence of the following criteria: (i) stage III ACC; (ii) high mitotic index [the value of mitotic index prompting treatment ranged between 10 and 20 mitoses per 50 high-power field (HPF) among centers]. When a postoperative adjunctive measure was deemed necessary, a monitored mitotane treatment aiming at plasma concentrations between 14 and 20 mg/L (20, 21) was used. In the absence of intolerability to mitotane, treatment was scheduled for at least 2 years or till ACC recurrence. All the patients received the same mitotane formulation (Lysodren, 500 mg tablets) that was purchased by Bristol-Meyers Squibb till 2003 and thereafter by Laboratoire HRA Pharma. Mitotane was given orally starting with 1 to 2 g daily followed by progressive dose increments according to local protocols and patient compliance with the aim to reach concentrations between 14 and 20 mg/L. When such or even higher concentrations were attained, doses were tapered with further individual dose adjustments guided by the results of mitotane measurement and toxicity assessment. In the event of unacceptable side effects, the patients were allowed to return to a lower dose or discontinue mitotane temporarily restarting with a lower dose.
Follow-up protocols were similar among the different centers including imaging of both chest and abdomen at baseline and thereafter every 3 to 6 months until disease progression or end of the study period. Follow-up procedures did not vary according to whether the patients received adjuvant mitotane or not. At each visit, the patients underwent physical examination, routine laboratory evaluation, and hormonal work-up. Monitoring of mitotane concentrations was done in treated patients. For recurrent disease, radical surgery was carried out if complete resection seemed feasible. In case of nonresectable disease, mitotane-naive patients were treated with mitotane alone or in combination with cytotoxic drugs. In patients with recurrence during mitotane treatment, chemotherapy was added. Patients gave informed consent for collecting tissue and clinical data, and the study was approved by the ethics committees of both centers.

Cell culture, siRNAs, and pharmacologic assays
NCI-H295R and SW-13 cell lines were supplied from the American Type Culture Collection (ATCC). The H295R cells were cultured in a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 Nutrient mixture (DMEM/F12; Sigma) supplemented with 1% l-glutamine (Sigma) and 2.5% of Nu-Serum (Becton Dickinson), and enriched with 1% of ITS+Premix (Becton Dickinson); whereas SW-13 cells were cultured in DMEM (Sigma) supplemented with 10% FBS (ATCC). Three human NSCLC cell lines (2 from adenocarcinoma—NCI-H520 and NCI-H1395, and one from squamous cell carcinoma—SKMES), also purchased from ATCC, were used as controls; all cells were maintained in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mmol/L-glutamine, penicillin (25 units/mL), and streptomycin (25 μg/mL, all from Sigma-Aldrich). For RRM1 silencing experiments in SW-13 cells, on-Target plus Smart Pool siRNAs targeting RRM1, on-Target plus Smart Pool siRNAs nontargeting, and Silgo-Transfection indicator siRNA were used (Dharmacon). INTERFERin siRNA transfection reagent was purchased from Polyplus transfection. Transfection of SW-13 was INTERFERin siRNA transfection reagent introduced with INTERFERin, by flow cytometry using FACS Canto II (Becton Dickinson). Briefly, using a reverse transfection, siRNAs (at a final concentration of 100 nmol/L) and INTERFERin were used (Dharmacon). INTERFERin siRNA transfection reagent was purchased from Pluspolus transfection. Transfection of SW-13 was first optimized by measuring the level of Silgo-green transfection indicator introduced with INTERFERin, by flow cytometry using FACS Canto II (Becton Dickinson). Briefly, using a reverse transfection, siRNAs (at a final concentration of 100 nmol/L) and INTERFERin were used (Dharmacon). INTERFERin siRNA transfection reagent was purchased from Pluspolus transfection. Transfection of SW-13 was first optimized by measuring the level of Silgo-green transfection indicator introduced with INTERFERin, by flow cytometry using FACS Canto II (Becton Dickinson). Briefly, using a reverse transfection, siRNAs (at a final concentration of 100 nmol/L) and INTERFERin were used (Dharmacon). INTERFERin siRNA transfection reagent was purchased from Pluspolus transfection. Transfection of SW-13 was first optimized by measuring the level of Silgo-green transfection indicator introduced with INTERFERin, by flow cytometry using FACS Canto II (Becton Dickinson). Briefly, using a reverse transfection, siRNAs (at a final concentration of 100 nmol/L) and INTERFERin were used (Dharmacon). INTERFERin siRNA transfection reagent was purchased from Pluspolus transfection. Transfection of SW-13 was first optimized by measuring the level of Silgo-green transfection indicator introduced with INTERFERin, by flow cytometry using FACS Canto II (Becton Dickinson). Briefly, using a reverse transfection, siRNAs (at a final concentration of 100 nmol/L) and INTERFERin were used (Dharmacon). INTERFERin siRNA transfection reagent was purchased from Pluspolus transfection. Transfection of SW-13 was first optimized by measuring the level of Silgo-green transfection indicator introduced with INTERFERin, by flow cytometry using FACS Canto II (Becton Dickinson). Briefly, using a reverse transfection, siRNAs (at a final concentration of 100 nmol/L) and INTERFERin were used (Dharmacon). INTERFERin siRNA transfection reagent was purchased from Pluspolus transfection. Transfection of SW-13 was first optimized by measuring the level of Silgo-green transfection indicator introduced with INTERFERin, by flow cytometry using FACS Canto II (Becton Dickinson).

RNA isolation from paraffin-embedded tissues and cell lines
Representative tumor areas were dissected under stereomicroscopic assistance from 10-μm sections of paraffin-embedded tissue in RNase-free conditions. RNA isolation was carried out by commercially available RNA extraction kits designed for paraffin material according to the manufacturer’s instructions (High Pure RNA Paraffin Kit; Roche Applied Science). Four samples of normal paraffin-embedded adrenal tissue collected from 1- to 10-year-old blocks were also analyzed in parallel and compared with a cDNA obtained from a commercial total RNA (Stratagene). Total RNA from cell lines lyastes was extracted using QIAzol lysis Reagent (Qiagen). Complementary DNA was transcribed using 500 μg/mL oligodT (Roche Applied Science) and 500M-MLV RT (200 U/μL; Invitrogen) according to standard protocols.

Quantitative real-time PCR
Relative cDNA quantification for RRM1, ERCC1, and an internal reference gene (b-actin) was done in duplicate using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System—TaqMan; Applied Biosystems) using primers and probes previously published (26, 32). To analyze target gene expression in individual tumors, the relative gene expression levels were expressed as ratios (differences between the C values) between 2 absolute measurements (genes of interest/internal reference gene). Then, the ΔΔC values were calculated subtracting ΔC values of each case to the value of the case with the lowest expression, and converting the ratio by the 2ΔΔC formula; cases were considered of low or high expression according to the median expression level obtained.

Statistical analysis
Correlation between the expressions of the 2 genes was tested using the Spearman method, differences of categorical variables were analyzed using the χ² test. DFS and OS survival curves were computed using the Kaplan–Meier method and compared using the log-rank test. Hazard ratios (HR) for disease progression and patient death were estimated using the Cox proportional hazard model. Multivariate analyses were carried out adjusting for patient age, sex, ACC stage, mitotic count, and cortisol secretion. Cox models were also used to assess the presence of heterogeneity in the effect of marker expression in the different patient subgroups, defined by the covariates, by including in the model the appropriate treatment/covariate interaction term(s). In vitro efficacy of mitotane in nonsilenced and RRM1 siRNA SW-13 cells was measured by means of the F test. All P values reported are the result of 2-sided tests. Statistical analyses were carried out by using the SPSS for windows software (version 17).

Results
Patients
Both cohorts (Table 1) were comparable in terms of age, sex proportion, stage of disease, presence of clinical
syndromes, and tumor characteristics. Adjuvant mitotane therapy was administered to 18 Italian and 20 German patients, respectively; the remaining patients did not receive any postoperative treatment. Target mitotane levels were reached in less than 40% of cases. The median follow-up was 66.3 months in all patients, and 80 and 62.8 months in the Italian and German cohorts, respectively. During follow-up, 29 patients of the Italian series (67.4%) and 35 of the German series (74.5%) developed disease recurrence, whereas 28 (54.9%) and 23 (48.9%) patients, respectively, died of ACC progression. Mitotane-treated patients had a longer DFS than patients followed up only: median 22.5 months [95% confidence interval (CI), 1.8–43.1] versus 13.2 months (95% CI, 6.2–20.2), HR, 0.70 (95% CI, 0.43–1.16; \( P = 0.17 \)), and longer OS; median 154 months (95% CI, 65.1–242.9) versus 53 months (95% CI, 22.6–83.4), HR, 0.63 (95% CI, 0.34–1.16; \( P = 0.14 \)).

### Correlation between RRM1 and ERCC1 gene expression levels and patient/tumor characteristics

**ERCC1** gene expression in normal adrenal tissues was rather low as compared with a commercially available total RNA pool, whereas **RRM1** gene expression levels were comparable with reference total RNA. Moreover, the difference in **RRM1** gene expression levels among normal tissues collected from paraffin blocks of different ages was in the range of 1-fold change (Fig. 1), therefore showing that the time frame of sample collection did not influence gene expression analysis. **RRM1** and **ERCC1** gene expression levels were reciprocally correlated in the patients’ study population (Spearman correlation test: \( R = 0.4425, P = 0.0021 \)). When dichotomized at the median value, both genes did not show any significant relationship with pathologic features, except for the higher RRM1 expression levels

### Table 1. Patients’ characteristics

<table>
<thead>
<tr>
<th></th>
<th>Italian series</th>
<th>German series</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>46 (20–85)</td>
<td>49 (18–77)</td>
</tr>
<tr>
<td>Sex, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (44.4)</td>
<td>19 (40.4)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (55.6)</td>
<td>28 (59.6)</td>
</tr>
<tr>
<td>ENSAT Disease stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (15.6%)</td>
<td>4 (8.5%)</td>
</tr>
<tr>
<td>II</td>
<td>26 (57.8%)</td>
<td>23 (48.9%)</td>
</tr>
<tr>
<td>III</td>
<td>12 (26.6%)</td>
<td>20 (42.6%)</td>
</tr>
<tr>
<td>Secreting tumor, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsecreting tumor</td>
<td>22 (48.9)</td>
<td>28 (59.6)</td>
</tr>
<tr>
<td>Cortisol ± androgens</td>
<td>15 (33.3)</td>
<td>12 (25.5)</td>
</tr>
<tr>
<td>Androgens</td>
<td>4 (8.9)</td>
<td>4 (8.5)</td>
</tr>
<tr>
<td>Estradiol</td>
<td>—</td>
<td>3 (6.4)</td>
</tr>
<tr>
<td>Mineralocorticoids</td>
<td>4 (8.9)</td>
<td>—</td>
</tr>
<tr>
<td>Adjuvant mitotane, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18 (40.0)</td>
<td>20 (42.5)</td>
</tr>
<tr>
<td>No</td>
<td>27 (60.0)</td>
<td>27 (57.5)</td>
</tr>
<tr>
<td>Patients with recurring ACC, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weiss score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>6 (4–9)</td>
<td>5 (3–9)</td>
</tr>
<tr>
<td>Mitoses in 50 HPF, no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>12 (26.7)</td>
<td>16 (34.0)</td>
</tr>
<tr>
<td>6–10</td>
<td>15 (33.3)</td>
<td>6 (12.8)</td>
</tr>
<tr>
<td>&gt;10</td>
<td>18 (40.0)</td>
<td>25 (53.2)</td>
</tr>
</tbody>
</table>

### Table 2. Prognostic role of ERCC1 and RRM1 expression in the series of 92 ACC

<table>
<thead>
<tr>
<th></th>
<th>Univariate HR (95% CI)</th>
<th>( P )</th>
<th>Multivariate HR (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High ERCC1</td>
<td>1.31 (0.81–2.13)</td>
<td>0.27</td>
<td>0.97 (0.57–1.64)</td>
<td>0.91</td>
</tr>
<tr>
<td>High RRM1</td>
<td>2.60 (1.57–4.30)</td>
<td>&lt;0.0001</td>
<td>2.31 (1.36–3.95)</td>
<td>0.002</td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High ERCC1</td>
<td>1.25 (0.71–2.21)</td>
<td>0.44</td>
<td>0.98 (0.52–1.85)</td>
<td>0.96</td>
</tr>
<tr>
<td>High RRM1</td>
<td>3.19 (1.73–6.00)</td>
<td>&lt;0.0001</td>
<td>3.59 (1.85–6.96)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**NOTE:** ERCC1 and RRM1 expression was categorized at the median value.
in cases with a high mitotic count (≤10 as compared with >10 mitoses in 50 HPF, \(P = 0.035\)).

**Relationship between RRM1 and ERCC1 gene expression and survival**

ERCC1 gene expression was not associated with DFS and OS neither at univariate nor multivariate analysis. Conversely, high RRM1 expression levels were associated with shorter DFS both in univariate (\(P < 0.0001\)) and multivariate analysis (\(P = 0.002\)). Similar results were seen for OS (univariate: \(P < 0.0001\); multivariate: \(P < 0.0001\)) [Table 2]. Dividing patients according to each population, the prognostic role of RRM1 was more evident in German series than the Italian series (interaction test \(P = 0.079\)), but similar results have been obtained in the 2 series in terms of survival (interaction test \(P = 0.99\)).

**Relationship between RRM1/ERCC1 gene expression and outcome of adjuvant mitotane treatment**

Patients attaining the target mitotane levels were similarly distributed between patients showing different RRM1 and ERCC1 expression. To test the predictive role of RRM1 and ERCC1 gene expression in the adjuvant setting, patients treated with adjuvant mitotane or simply followed up were analyzed and stratified according to marker expression (dichotomized at the median value). In patients with low RRM1 expression, mitotane administration was associated with a significantly longer DFS than that of patients undergoing follow-up only (Fig. 2). The prognostic role of adjuvant mitotane therapy in patients with low RRM1 expression was maintained in multivariate analysis after adjustment for sex, age, stage, and mitosis status [HR of recurrence, 0.31 (95% CI, 0.13–0.74); \(P = 0.009\)], whereas no effect of mitotane treatment on DFS was observed in patients with high RRM1 levels [adjusted HR of recurrence, 0.87 (95% CI, 0.43–1.79); \(P = 0.72\); Fig. 2]; the interaction test was close to attain the statistical significance (\(P = 0.098\)). The HRs of recurrence in mitotane-treated patients compared with untreated patients with low RRM1 expression did not vary in the 2 patient cohorts considered separately: HR, 0.36 (95% CI, 0.12–1.44; \(P = 0.08\)) in the Italian series and HR, 0.40 (95% CI, 0.11–1.47; \(P = 0.17\)) in the German series (interaction test: \(P = 0.83\)). The relationship between the prognostic role for DFS of adjuvant

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**Figure 2.** Influence of adjuvant mitotane on DFS and OS of ACC patient population segregated according to RRM1 expression levels, evaluated using the Kaplan-Meier curve estimation and univariate survival analysis (log-rank test).
mitotane therapy and RRM1 expression was also explored dividing patients according to tertile distribution of RRM1. A step-wise increase in the HR of adjuvant mitotane versus follow-up was observed: HR, 0.36 (95% CI, 0.12–1.09; \( P = 0.07 \)) in the first tertile, HR, 0.47 (95% CI, 0.19–1.13; \( P = 0.09 \)) in the second tertile and HR, 1.33 (95% CI, 0.63–2.79; \( P = 0.45 \)) in the third tertile. These HRs were significantly different at the interaction test (\( P = 0.034 \)). Conversely adjuvant mitotane failed to be associated with OS either in patients with low RRM1 or in those with high RRM1 (Fig. 2), adjusted HR, 0.50 (95% CI, 0.16–1.60; \( P = 0.25 \)) and 0.56 (95% CI, 0.25–1.29; \( P = 0.17 \)), interaction test (\( P = 0.98 \)). As far as ERCC1 is concerned, adjuvant mitotane therapy failed to be significantly associated with DFS in patients with low or high ERCC1 expression (Fig. 3); in multivariate analysis, however, adjuvant mitotane therapy was significantly associated with lower risk of relapse HR, 0.40 (95% CI, 0.18–0.90; \( P = 0.026 \)) in patients with low ERCC1 expression, but not in those with high ERCC1 expression, HR, 0.65 (95% CI, 0.31–1.37; \( P = 0.26 \)); these 2 HRs were not different at the interaction test (\( P = 0.79 \)). OS of mitotane-treated patients was not significantly different than patients followed up only either in patients with low ERCC1 expression (Fig. 3), adjusted HR, 0.47 (95% CI, 0.18–1.24; \( P = 0.13 \)) or in patients with high ERCC1 expression, adjusted HR, 0.63 (95% CI, 0.26–1.51), interaction test (\( P = 0.94 \)).

RRM1 and ERCC1 modulation and interplay with mitotane responsiveness in vitro

The possible functional interaction between RRM1 and ERCC1 gene expression and mitotane responsiveness was tested in vitro in 2 established ACC cell line models, the H295R and SW-13 cells. Baseline ERCC1 gene expression was comparable in the 2 cell lines tested and was not modulated under mitotane administration (Supplementary Fig. S1). RRM1 gene expression levels were similar in H295R and SW-13 cells (data not shown). Mitotane treatment induced a dose-dependent reduction of H295R cell viability and the H295R cells did not show significant changes in their RRM1 gene expression levels. Conversely, SW-13 cell viability was unaffected by mitotane, but SW-13 cells displayed an impressive increase in RRM1 gene expression levels (up to 25 times over baseline in cells incubated with...
25 μmol/L mitotane; Fig. 4). By contrast, control NSCLC cell lines were not sensitive to mitotane treatment nor showed significant upmodulation of RRM1 gene under mitotane treatment (Fig. 5).

Thus, we inhibited RRM1 expression by RNA-specific silencing (Fig. 6). Efficiency of transfection measured by means of flow cytometry was optimal (not shown) and RRM1 mRNA expression in SW13 cells was knocked down by RRM1 siRNA to less than 10% of control cells transfected with nontargeting siRNAs. Silencing of RRM1 gene in SW13 did not affect cell viability under basal conditions but induced mitotane sensitivity, indicating a direct link between RRM1 gene expression and antiproliferative activity of mitotane in this cell model.

Discussion

Mitotane is the reference drug for treatment of advanced ACC and is increasingly adopted in an adjuvant setting (33–37). Demonstration of the adrenolytic effect of mitotane dates back to the fifties of the last century when studies showed that administration of the drug was able to destroy the adrenal glands in animal models (38, 39) and to inhibit steroidogenesis at different enzymatic steps (23). However, the precise mechanism of action of mitotane remains still largely unknown, and it is generally thought that mitotane cytotoxicity is mediated through binding of the reactive acyl-chloride to mitochondrial proteins and subsequent oxidative damage through generation of free radicals (23, 37). Recently, mitotane has been shown to sensitize H295R and SW-13 ACC cells to ionizing radiations by attenuating DNA repair and interfering with cell proliferation (40), data suggesting that mitotane, in addition to the assumed binding to proteins and phospholipids, may interact also with DNA, as was previously shown in vitro (41).

Moreover, it is known that metabolic activation is required for mitotane biologic activity (37), but its metabolic pathway and the effective role of its metabolites, as well as its target molecules in tumor cells are far from being identified.

For the first time, in this study we provide in vitro and in vivo evidence of a relationship between RRM1 enzyme and the antineoplastic activity of mitotane in ACC. RRM1 is an enzyme involved in the synthesis of deoxyribonucleotides for DNA synthesis and represents the cellular target for gemcitabine, being its mRNA expression and genetic variants predictive of response to gemcitabine treatment in patients with different types of cancer (27).

This study includes 2 independent series of radically resected ACC patients recruited in 2 European countries (35). In the overall patient population, low RRM1 gene expression was significantly associated to longer DFS and OS and this contrasts with other tumor models, such as NSCLC, in which low RRM1 expression in patients treated with surgery is associated with reduced survival, whereas improved survival is observed in metastatic disease in gemcitabine-treated patients (42). However, it should be noted that RRM1 may not be a direct target of mitotane, and possible alternative molecular or metabolic interactions between mitotane and RRM1 are to be further evaluated to explain this apparent discrepancy. Moreover, in vitro analysis of control NSCLC models showed that the antineoplastic mitotane efficacy as well as its effect in RRM1 gene modulation is specific for ACC cancer cell models (see also below). Subsequently, we tested whether RRM1 gene expression affects the efficacy of adjuvant mitotane treatment, analyzing the DFS of patients treated with mitotane in the low and high RRM1 expression groups, respectively, in comparison with patients left untreated after surgery. Our results clearly indicate that mitotane efficacy was confined
to the patients with low RRM1-expressing tumors, and this effect was evident also when the Italian and German cohorts were analyzed separately. Despite the significant DFS prolongation in patients with low RRM1-expressing tumors, mitotane therapy was not able to significantly influence the OS. Survival is affected by therapies administered upon progression and patients not receiving mitotane therapy in adjuvant setting received the drug at disease relapse, and this could have influenced the OS.

These data suggested a predictive role of RRM1 gene expression of mitotane efficacy in ACC and prompted a subsequent in vitro investigation to assess possible functional pharmacologic interactions. Two established ACC cell lines were, therefore, analyzed for RRM1 gene expression levels at baseline and under mitotane treatment. Mitotane sensitivity in H295R cells was associated with a lack of RRM1 gene modulation, whereas SW-13 cells were not sensitive even at high doses of mitotane, showing a significant and dose-dependent increase in RRM1 transcription following mitotane treatment. This observation was consistent with the data we obtained in vivo and supports the hypothesis that high RRM1 expression might impair the antineoplastic activity of mitotane. As a further step to establish a direct relationship between RRM1 and mitotane, RRM1 silencing was done, which sensitized SW-13 cells to mitotane, thus showing that low RRM1 expression is of critical importance for mitotane antitumor efficacy. The lack of available tumor specimens sampled after mitotane treatment in the patient cohort prevented a subsequent tissue analysis to speculate the modulation of RRM1 in vivo; therefore the issue whether baseline RRM1 gene expression levels or rather its upmodulation under treatment are predictive of response to mitotane remains to be ascertained.

ERCC1 gene expression levels were neither prognostic nor predictive of response to mitotane in our study population. The trend to significance of greater efficacy of mitotane treatment in patients with low ERCC1 expression is, in our opinion, most probably justified by the significant correlation between RRM1 and ERCC1 gene expression levels.

Our study has certain limitations and strengths. The retrospective nature of the study, the absence of randomization between mitotane-treated and nontreated patients, and apparently, relative small sample size might lead to unknown biases. However, when compared with the
literature, our cohort seems to be quite representative for patients with stage I–III ACC and we believe—acknowledging the rarity of the disease—that the sample size is one of the strengths of this study. Moreover, the inclusion of 2 independent cohorts from different countries favors the generalization of the present results. Although it might be surprising that less than half of the patients have been treated with mitotane in this cohort, this may be explained by the fact that more than 80% of patients had surgery before the results of our adjuvant mitotane study have been published (43). Another strength of this study is the consistency of data on human tumors and cell culture in vitro experiments, providing new data that may help understanding the mechanism of action of mitotane and its target molecules.

In conclusion, the present data represent the first evidence that RRM1 gene expression levels predict response to mitotane treatment in an adjuvant setting and are functionally associated to mitotane sensitivity. These findings suggest that the determination of RRM1 gene expression may be of potential clinical utility to select patients for adjuvant mitotane therapy.

Disclosure of Potential Conflicts of Interest
R. Allolio is a consultant and a member of advisory board at Boehringer Ingelheim and HRA Pharma. M. Fassnacht is a consultant and a member of advisory board at HRA Pharma and OSI Pharma; M. Terzolo has received commercial research grant from HRA Pharma.

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Figure 6. Influence of RRM1 gene expression levels on mitotane sensitivity. In mitotane-insensitive SW-13 cells, RRM1 RNA silencing (RRM1 siRNA) reduced target gene expression but did not influence cell viability in the absence of mitotane; by contrast, an increase in mitotane sensitivity was induced by RRM1 interference (F test comparing IC50 doses of RRM1 siRNA and nontargeting siRNA control cells); for simplicity, mitotane values are represented in the X-axis of the logarithmic curve as the effective concentrations.
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