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ERCC1 as predictive biomarker to platinum-based chemotherapy in adrenocortical carcinomas.

Valeria Laufs[1], Barbara Altieri[1,2], Silviu Sbiera[1], Stefan Kircher[3,4], Sonja Steinhauer[1], Felix Beuschlein[5,6], Marcus Quinkler[7], Holger S. Willenberg[8], Andreas Rosenwald[3,4], Martin Fassnacht[1,4], Cristina L. Ronchi[1].

[1]Division of Endocrinology and Diabetes, Department of Internal Medicine I, University Hospital, University of Wuerzburg, Wuerzburg, Germany.

[2]Division of Endocrinology and Metabolic Diseases, Institute of Medical Pathology, Catholic University of the Sacred Heart, Rome, Italy.

[3]Institute of Pathology, University of Wuerzburg, Wuerzburg, Germany.

[4]Comprehensive Cancer Center Mainfranken, University of Wuerzburg, Germany.

[5]Medizinische Klinik and Poliklinik IV, Ludwig-Maximilians University, Munich, Germany.

[6]Klinik für Endokrinologie, Diabetologie und Klinische Ernährung, Universitätsspital Zürich, Zürich, Switzerland.

[7]Endocrinology in Charlottenburg, Berlin, Germany.

[8]Division of Endocrinology and Metabolism, Rostock University Medical Center, Germany.

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Corresponding author:

Cristina L. Ronchi (MD, PhD)

Division of Endocrinology and Diabetes, Department of Internal Medicine I

Oberduerrbacher-Str 6

University Hospital of Wuerzburg

97080 Wuerzburg (Germany)

Ronchi_C@ukw.de

Abstract

Objective: Platinum-based chemotherapy (PBC) is the most effective cytotoxic treatment for advanced adrenocortical carcinoma (ACC). Excision repair cross complementing group 1 (ERCC1) plays a critical role in the repair of platinum-induced DNA damage. Two studies investigating the role of ERCC1 immunostaining as a predictive marker for the response to PBC in ACC had reported conflicting results. Both studies used the ERCC1-antibody clone 8F1 that later turned out to be not specific. The aim of this study was to evaluate the predictive role of ERCC1 with the new specific antibody in a larger series of ACC.

Design and Methods: 146 ACC patients with available FFPE slides were investigated. All patients underwent PBC (median cycles=6), including cisplatin (n=131) or carboplatin (n=15), in most cases combined with etoposide (n=144), doxorubicin (n=131) and mitotane (n=131). Immunostaining was performed with the novel ERCC1-antibody clone 4F9. The relationship between ERCC1 expression and clinico-pathological parameters, as well as best objective response to therapy and progression-free survival (PFS) during PBC was evaluated.

Results: High ERCC1 expression was observed in 66% of ACC samples. During PBC, 43 patients experienced objective response (29.5%), 49 stable disease (33.6%), 8 mixed response (5.5%) and 46 progressive disease (31,5%) without any relationship with the ERCC1 immunostaining. No significant correlation was also found between ERCC1 expression and progression-free survival (median 6.5 vs 6 months, *P=0.33*, HR=1.23, 95%CI=0.82-2.0).

Conclusion: ERCC1 expression is not directly associated with sensitivity to PBC in ACC. Thus, other predictive biomarkers are required to support treatment decisions in patients with ACC.

Introduction

Platinum-based chemotherapy (PBC) is the most effective cytotoxic treatment for advanced adrenocortical carcinoma (ACC), mostly in combination with etoposide and doxorubicin plus mitotane in the EDP-M regime[1]. However, the best objective response rates remain below 30% and the impact on overall survival is not satisfying as shown in the phase III clinical trial FIRM-ACT [2]. Similarly, other possible cytotoxic drugs such as streptozotocin[2] or gemcitabine did not show a better effectiveness[3] and no effective targeted therapies have emerged for ACC patients with advanced disease[4-6]. Finally, PBC as other chemotherapeutic combinations is associated with relevant toxicity. Thus, it is obvious that there is an urgent need of biomarkers that may serve to predict the response to PBC.

Excision repair cross complementing group 1 (ERCC1) is an important member of the nucleoside excision repair pathway, which plays a critical role in the DNA repair by removing DNA covalent helix-distorting adducts caused by platinum compounds[7]. ERCC1 has been demonstrated to be a predictive biomarker for platinum treatment in several cancers, such as non-small cell lung cancer, testicular germ cell tumor, bladder cancer, pancreatic carcinoma and gastric cancer[8-12]. Two previous studies, one from our group[13] and one from France[14] investigated ERCC1 immunostaining in relationship with the response to PBC in a relatively small series of ACC patients (n=45 and n=33, respectively). These two studies described a similar overall response rate to PBC (25-30% of cases), but reported conflicting results regarding the influence of ERCC1 on sensitivity to PBC, being significant only in the first study. All the previous studies on ERCC1 immunostaining, including those on ACC, have been performed by using the monoclonal anti-mouse antibody clone 8F1. However, already some years ago, it had been suggested that this clone might be not specific, being ERCC1 not the principal antigen recognized by the 8F1 antibody[15, 16]. In fact, more recently, it has been demonstrated that the clone 8F1 immunoglobulin recognizes also the choline phosphate cytidyltransferase 1 alfa (PCYT1A), an unrelated nuclear membrane protein, involved in the metabolism of phosphatidylcholine biosynthesis[17]. These findings raise doubts on previously published data using the clone 8F1 to investigate ERCC1 as a predictive marker to PBC in several solid tumors. Finally, a new highly specific clone 4F9 has been identified and then validated [17-19].

Thus, the aim of the present study was to evaluate ERCC1 immunostaining with the new highly specific clone 4F9[17, 18] in a larger series of ACC and to correlate it with the response to PBC.

Subjects and methods

Patients and treatment regimen

Inclusion criteria were age of at least 18 years, histopathologic diagnosis of ACC, available formalin-fixed paraffin embedded (FFPE) specimens and treatment with PBC. We identified a total of 153 patients that fulfilled these criteria and were treated with PBC in our centers between 2004 and 2015. Seven of these patients received only one cycle of PBC and were then excluded from further analysis. Thus, the final series included 146 patients with advanced ACC (F:M=90:56, median age 48 years). None of these patients were already included in our previous paper on ERCC1[13], while 49 participated in the FIRM-ACT study[2]. Specifically, 127 samples derived from primary surgery, 6 from local recurrences, 4 from biopsies (patients not operable) and 9 from distant metastasis. The baseline clinical parameters, such as sex, age at initial diagnosis, tumor size, biochemical evaluation, tumor stage according to the European Network for the Study of Adrenal Tumors (ENSAT) classification[20], Weiss score, Ki67 proliferation index, presence and number of distant metastases, and previous local and/or pharmacological treatments are given in ***Table 1*****.** All baseline data were collected through the ENSAT Registry (www.ens@t.org/registry).

The treatment regimen included cisplatin (n=131) or carboplatin (n=15) and was in most cases administered as combination therapy (see details ***Table 1***). The median number of PBC cycles was 6 ranging from 2 to 15. Treatment was discontinued in cases of unacceptable toxicity, patient's refusal or evidence of disease progression. A total of 131 patients (90% of cases) were treated with concomitant mitotane (target plasma concentration: 14-20 mg/L). 114 patients received PBC as first-line cytotoxic treatment (78% of cases), while the remaining 32 patients were treated with PBC as second- or third- line therapy, with a history of failed streptozotocin[2] or gemcitabine + capecitabine[3] (***Table 1***). All patients had undergone regular and standard follow-up visits with clinical, biochemical, and radiological (abdominal and thoracic CT scan with contrast agent) evaluation with a staging interval usually every 8 weeks. The sensitivity to PBC was evaluated as progression-free survival

during treatment and as best overall objective response. For this evaluation, according to our clinical practice, all radiological images were reviewed by the local expert radiologists and discussed in our multidisciplinary tumor board meetings to determine a final consensus response (progressive disease, stable disease, partial or complete response). Clinical benefit was defined as stable disease or treatment response for a minimum of 4 months.

The collection of the clinical data and the biomaterial for this retrospective study was approved by the ethics committee of the University of Wuerzburg (No. 93/02 and 88/11) according to the Declaration of Helsinki. Written informed consent was obtained from all patients.

Immunohistochemistry

A total of 146 FFPE adrenocortical tissues on standard full slides were evaluated by immunohistochemistry. In brief, sections were deparaffinized and immunohistochemical detection was performed using an indirect immunoperoxidase technique after high temperature antigen retrieval in 10 mM citric acid monohydrate buffer (pH 6.5) in a pressure cooker for 13 min. Blocking of unspecific protein-antibody interactions was performed with 20% human AB serum in PBS for 1h at room temperature. Primary antibody for ERCC1 was the new highly specific monoclonal anti-mouse antibody (mAb) clone 4F9 (UM500008, dilution 1:100) that was purchased from OriGene Technologies, Inc (Rockville, USA). A mouse negative control was used (Dako North America Inc., Carpinteria, USA). The slices were incubated overnight at 4°C. Signal amplification was achieved with En-Vision System Labeled Polymer-HRP Anti-Mouse (Dako) for 40 min and developed for 10 min with DAB Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Nuclei were counterstained with Mayer's hematoxilin for 2 min. For positive controls, sections of colon adenocarcinoma, renal cell carcinoma, breast cancer, hepatocellular carcinoma and normal tonsil were chosen, while cells of the tumor stroma served as internal negative control.

All slides were analyzed independently by two investigators blinded to clinical information (V.L. and S.S.) Nuclear staining intensity was graded as negative (0), low (1), medium (2), or strong (3). The percentage of tumor cells with positive nuclei was calculated for each specimen and scored 0 if 0% were positive, 0.1 if 1–9% were positive, 0.5 if 10–49% were positive and 1 if 50% or more were

positive. A semiquantitative H-score was then calculated by multiplying the staining intensity grading score with the proportion score as described previously [13]. In case of discrepant results, staining intensities were jointly assessed by both investigators, forming the final score by consensus. Inter-observer agreement was investigated via Pearson's correlation coefficients 0.72 (95%CI: 0.63-0.79).

Comparison between anti-ERCC1 antibody clone 8F1 vs clone 4F9

We also intended to re-evaluate our old results obtained with the mAb against ERCC1 clone 8F1 (old batch)[13] with the new high specific mAb clone 4F9. To this aim, we re-stained 38 ACC samples out of the 45 previously published and re-investigated the relationship between ERCC1 expression and the response to PBC in terms of both progression-free survival (PFS) and disease-specific survival (DSS) after treatment. Moreover, the specificity of the currently available clone 8F1 has been shown to be altered from the old clone 8F1[21, 22]. In addition, we also evaluated a subgroup of 21 out of the 146 samples in our present series with the current clone 8F1 (new batch) in addition to the new clone 4F9.

Statistical analysis

The Fisher's exact or the Chi-square tests were used to investigate dichotomic variables, while continuous variables were investigated with a two-sided *t* test (or non-parametric test). A non-parametric Kruskal-Wallis test, followed by Dunn's test, was used for comparison among several groups for non-normal distributed variables. Correlations and 95% confidence intervals (95%CI) between different parameters were evaluated by linear regression analysis. PFS was defined as the time from the date of first administration of PBC to the first radiological evidence of disease progression or death, as appropriate. DSS was defined as the time from the first administration of PBC to disease-specific death or last follow-up. All survival curves were obtained with Kaplan-Meier estimates, and the differences between survival curves were assessed by the log-rank (Mantel-Cox) test. For the calculation of hazard ratios (HR), two ACC-groups with low or high protein expression were considered (high expression: H-score ≥ 2). A multivariate regression analysis was performed via a Cox proportional hazard regression model, aiming to identify factors that might independently influence survival. Statistical analyses were made using GraphPad Prism (version 6.0, La Jolla, CA,

USA) and SPSS Software (PASW Version 21.0, SPSS Inc., Chicago, IL, USA). P values *<0.05* were considered as statistically significant.

Results

Efficacy of platinum-based chemotherapy

The data about efficacy of PBC in the current series of 146 patients with advanced ACC are summarized in ***Table 2***. Concerning the best objective response during PBC, one patient experienced complete response (0.7%) and 42 patients partial remission (28.8%), 49 stable disease (33.5%), 8 mixed response (5.5%) and 46 progressive disease (31.5%), respectively. The median PFS during PBC was 6 months, ranging from 2 to 18, while the median DSS was 17 months, ranging from 1.5 to 127. Additionally, we observed a clinical benefit defined as at least a stable disease for a minimum of 4 months in 84 patients (58%) with a median PFS in this group of 6 months (range: 4-18). Only one patient died unrelated to ACC during follow up. Thus, overall survival was more or less identical to DSS (data not shown).

ERCC1 expression and baseline clinical characteristics in ACC

Nuclear ERCC1 immunostaining was homogeneous in individual ACC samples with a median percentage of positive cells of 80% (> 50% in 135/146 samples, 92.5%). Tissue samples exemplifying the range of staining intensity are shown in the ***Figure 1***. ERCC1 expression was low (H-score 0-1) in 50 samples (34.2% of cases) and high (H-score 2-3) in 96 samples (65.7%). We did not observe any significant differences in ERCC1 immunostaining among primary tumors, local recurrences and/or distant metastasis. No significant correlation was also observed between the nuclear ERCC1 expression and the ENSAT tumor stage at the time of diagnosis, the Weiss score or the Ki67 proliferation index.

Predictive role of ERCC1 expression on sensitivity to platinum-based chemotherapy

Considering the potential predictive role of ERCC1 immunostaining on the objective response to PBC, no significant differences were observed between the groups with high and low nuclear ERCC1

expression (***Table 2***). Similarly, no differences were found in terms of both PFS (median 6.5 *vs* 6 months, respectively, *P=0.33*, HR=1.23, 95%CI=0.82-2.0) and DSS (median 17 *vs* 16.5 months, respectively, *P=0.87*, HR=1.03, 95%CI=0.70-1.53) (***Figure 2A-B***).

Comparison between anti-ERCC1 antibody clone 8F1 vs clone 4F9

We re-stained 38 out of 45 ACC samples of our previously published series (stained with the 8F1 clone old batch) with the new clone 4F9. Not unexpected, ERCC1 expression in terms of H-score corresponded in only 49% of cases. As a consequence, ERCC1 nuclear expression did not longer significantly correlate with response to PBC in terms of both PFS (data not shown) and DSS (Supplementary Figure 1A and B).

Furthermore, we stained 21 out of the present 146 samples with the currently available clone 8F1 (new batch) additionally to the clone 4F9. Two representative examples are shown in the Supplementary Figure 2. Comparing the ERCC1 immunostaining results we observed here a correspondence between the two antibodies in 81% of cases.

Discussion

We evaluated the potential role of ERCC1 nuclear expression as predictive biomarker to PBC in the largest series of ACC patients up to date (n=146) by using for the first time the new high ERCC1-specific monoclonal antibody clone 4F9. To note, ERCC1 has been previously demonstrated to be a predictive biomarker for platinum treatment in several cancers, such as non-small cell lung cancer (NSCLC), testicular germ cell tumors, bladder cancer, pancreatic carcinoma and gastric cancer[8-12]. In ACC, we previously demonstrated in a relatively small series of patients that ERCC1 immunostaining was significantly correlated with overall survival during PBC[13]. Another study, however, did not confirm this finding[14]. Nevertheless, several concerns about the reliability of the ERCC1 immunohistochemical analysis have been raised recently. First, it has been demonstrated that the clone 8F1 used in all the reported studies is not specific for ERCC1[15-17]. Specifically, the anti-ERCC1 antibody clone 8F1 has been identified to stain also the PCYT1A, a phospholipid synthesis enzyme regulated by RAS[17, 23] with no known clinical implication in platinum drug resistance. PCYT1A has

also been confirmed to play a role as prognostic biomarker in both lung and head and neck squamous cell carcinomas[23].

Moreover, the batch of the clone 8F1 in use since 2011 seems not to be identical with the batch in use in 2006[19], thus rendering new data about NSCLC not comparable with previous ones[22]. According to this new information, important previous results on the role of ERCC1 in the treatment of NSCLC [8] have been revised by the same group[21]. Furthermore, this year the first randomized trial to evaluate ERCC1 prospectively in 648 patients with NSCLC (ET trial) has been published definitively demonstrating that selecting chemotherapy using the commercially available ERCC1 antibodies (clone 8F1) does not confer any additional survival benefit[24].

In parallel, a new highly ERCC1-specific clone 4F9 has been recently proposed and validated [17-19]. For all these reasons, we decided to use the clone 4F9 to investigate a new large series of ACC samples in order to re-evaluate our previous results on ERCC1 as predictive marker of sensitivity to PBC. Most importantly, we could not confirm the previous results and our data now indicate that ERCC1 itself is probably not the main factor involved in the response to PBC in ACC patients. In addition, we were able to demonstrate that the current version of the clone 8F1 significantly differs from the old one that we used for our pilot study[13] and we were not able to reproduce the earlier results using now the same tumor samples.

One reason that could explain the lack of correlation between ERCC1 and PBC, independently from the issues with immunohistochemistry, is that ERCC1 works together with the XPF protein, codified by *ERCC4*. ERCC1–XPF complex is a two subunit structure-specific endonuclease that plays a key role during the nucleotide excision repair (NER) process[7, 25]. Thus, XPF itself might be involved in the sensitivity to the response to PBC[26, 27]. However, the ET trial demonstrated that XPF expression is not predictive for response to 648 patients with NSCLC[24]. Moreover, the ERCC1–XPF complex makes incisions on the damaged DNA strand on the 5′ side and acts in cooperation with several other proteins, like XPC–RAD23B, XPA, RPA, TFIIH and XPG, during the NER process[28, 29]. Thus, although ERCC1 plays a major role in the NER, several other proteins and mechanisms could influence the response to PBC.

Another explanation, why ERCC1 expression and clinical outcome in our and other series did not correlate could be the fact that virtually all patients have received in parallel to the platinum derivate 1-3 other additional cytotoxic drugs (mostly doxorubicin, etoposide and mitotane) diluting the hypothesized correlation. Other potential biomarker could for instance be involved in the prediction of response to these concomitant treatments (i.e. TOP2A[30]). Finally, one potential limitation in our study as well as in several others might be that ERCC1 was assessed on tumor specimens obtained months or even years before the start of chemotherapy. Nevertheless, we did not observe any significant differences in ERCC1 immunostaining among primary tumors, local recurrences and/or distant metastasis, thus suggesting that the ERCC1 levels remain quite stable over the time and tumor progression.

More generally, the search for predictive biomarkers to conventional cytotoxic chemotherapy has been proven challenging due to frequent discrepant and non-replicable findings. And this is true not only for protein expression where issues with antibodies and immunohistochemical analysis are common, but also for gene expression. Thus, if a plethora of biomarkers predicting chemotherapy efficacy have been evaluated also in the clinical setting, none of them is ready for clinical implementation yet[31]. Considering that most mechanisms of resistance or sensitivity to chemotherapy are multifactorial, a combinatorial approach and further efforts are required[32].

Concerning the response rate to PBC in general, we observed an objective partial response in 29.5% of cases and a stable disease in further 33.5%, thus confirming that PBC is the currently most effective cytotoxic therapy for advanced ACC. These data are generally superimposable to those reported in the FIRM-ACT study on EDP-M[2].

In conclusion, ERCC1 expression as detected by immunostaining is not directly associated with sensitivity to PBC in ACC. Thus, the search for predictive biomarkers in this devastating disease with poor response to medical therapy has to continue.

Declaration of interest

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

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Figure legends

Figure 1. Representative examples of nuclear ERCC1 immunostaining in adrenocortical tissue samples using the monoclonal ERCC1 antibody clone 4F9. A) Normal adrenal gland; B) Adrenocortical carcinoma with high intensity and high percentage of positive cells (H-score 3). C) Adrenocortical carcinoma with intermediate intensity and high percentage of positive cells (H-score 2). D) Adrenocortical carcinoma with low intensity and low percentage of positive cells (H-score 0,5). Magnification 1x10.

Figure 2. Relationship between ERCC1 expression and response to platinum-based chemotherapy in 146 patients with adrenocortical carcinoma (ACC). Progression-free survival (A) and overall survival (B) during treatment (Kaplan-Meyer curves and log-rank test) in ACC patients with high (H-score $\geq$2) and low staining (H-score $\leq$1) of ERCC1.

Supplementary data

Supplementary Figure 1. Re-evaluation of the overall survival in the old series of 38 patients with adrenocortical carcinoma treated with platinum-based chemotherapy [13]. (A) ERCC1 immunostaining with the 8F1 clone (old batch) (B) ERCC1 immunostaining with the new specific 4F9 clone.

Supplementary Figure 2. Direct comparison between ERCC1 antibodies 4F9 (A) and C) and 8F1 clone (new batch) (B) and D)) in one normal adrenal gland (A) and B)) and in one adrenocortical carcinoma (C) and D)). Magnification 1x20.