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

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29   **Abstract**

30   **Objective:** Platinum-based chemotherapy (PBC) is the most effective cytotoxic treatment for

31   advanced adrenocortical carcinoma (ACC). Excision repair cross complementing group 1 (ERCC1)

32   plays a critical role in the repair of platinum-induced DNA damage. Two studies investigating the role

33   of ERCC1 immunostaining as a predictive marker for the response to PBC in ACC had reported

34   conflicting results. Both studies used the ERCC1-antibody clone 8F1 that later turned out to be not

35   specific. The aim of this study was to evaluate the predictive role of ERCC1 with the new specific

36   antibody in a larger series of ACC.

37   **Design and Methods:** 146 ACC patients with available FFPE slides were investigated. All patients

38   underwent PBC (median cycles=6), including cisplatin (n=131) or carboplatin (n=15), in most cases

39   combined with etoposide (n=144), doxorubicin (n=131) and mitotane (n=131). Immunostaining was

40   performed with the novel ERCC1-antibody clone 4F9. The relationship between ERCC1 expression

41   and clinico-pathological parameters, as well as best objective response to therapy and progression-free

42   survival (PFS) during PBC was evaluated.

43   **Results:** High ERCC1 expression was observed in 66% of ACC samples. During PBC, 43 patients

44   experienced objective response (29.5%), 49 stable disease (33.6%), 8 mixed response (5.5%) and 46

45   progressive disease (31,5%) without any relationship with the ERCC1 immunostaining. No significant

46   correlation was also found between ERCC1 expression and progression-free survival (median 6.5 vs 6

47   months, *P=0.33*, HR=1.23, 95%CI=0.82-2.0).

48   **Conclusion:** ERCC1 expression is not directly associated with sensitivity to PBC in ACC. Thus, other

49   predictive biomarkers are required to support treatment decisions in patients with ACC.

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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].

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85 Thus, the aim of the present study was to evaluate ERCC1 immunostaining with the new highly

86 specific clone 4F9[17, 18] in a larger series of ACC and to correlate it with the response to PBC.

87

88 **Subjects and methods**

89 *Patients and treatment regimen*

90 Inclusion criteria were age of at least 18 years, histopathologic diagnosis of ACC, available formalin-

91 fixed paraffin embedded (FFPE) specimens and treatment with PBC. We identified a total of 153

92 patients that fulfilled these criteria and were treated with PBC in our centers between 2004 and 2015.

93 Seven of these patients received only one cycle of PBC and were then excluded from further analysis.

94 Thus, the final series included 146 patients with advanced ACC (F:M=90:56, median age 48 years).

95 None of these patients were already included in our previous paper on ERCC1[13], while 49 participated

96 in the FIRM-ACT study[2]. Specifically, 127 samples derived from primary surgery, 6 from local

97 recurrences, 4 from biopsies (patients not operable) and 9 from distant metastasis. The baseline

98 clinical parameters, such as sex, age at initial diagnosis, tumor size, biochemical evaluation, tumor

99 stage according to the European Network for the Study of Adrenal Tumors (ENSAT) classification[20],

100 Weiss score, Ki67 proliferation index, presence and number of distant metastases, and previous local

101 and/or pharmacological treatments are given in *Table 1*. All baseline data were collected through the

102 ENSAT Registry (www.ens@t.org/registry).

103 The treatment regimen included cisplatin (n=131) or carboplatin (n=15) and was in most cases

104 administered as combination therapy (see details *Table 1*). The median number of PBC cycles was 6

105 ranging from 2 to 15. Treatment was discontinued in cases of unacceptable toxicity, patient's refusal

106 or evidence of disease progression. A total of 131 patients (90% of cases) were treated with

107 concomitant mitotane (target plasma concentration: 14-20 mg/L). 114 patients received PBC as first-

108 line cytotoxic treatment (78% of cases), while the remaining 32 patients were treated with PBC as

109 second- or third- line therapy, with a history of failed streptozotocin[2] or gemcitabine + capecitabine[3]

110 (*Table 1*). All patients had undergone regular and standard follow-up visits with clinical, biochemical,

111 and radiological (abdominal and thoracic CT scan with contrast agent) evaluation with a staging

112 interval usually every 8 weeks. The sensitivity to PBC was evaluated as progression-free survival

113 during treatment and as best overall objective response. For this evaluation, according to our clinical

114 practice, all radiological images were reviewed by the local expert radiologists and discussed in our

115 multidisciplinary tumor board meetings to determine a final consensus response (progressive disease,

116 stable disease, partial or complete response). Clinical benefit was defined as stable disease or

117 treatment response for a minimum of 4 months.

118 The collection of the clinical data and the biomaterial for this retrospective study was approved by the

119 ethics committee of the University of Wuerzburg (No. 93/02 and 88/11) according to the Declaration

120 of Helsinki. Written informed consent was obtained from all patients.

121

122 *Immunohistochemistry*

123 A total of 146 FFPE adrenocortical tissues on standard full slides were evaluated by

124 immunohistochemistry. In brief, sections were deparaffinized and immunohistochemical detection was

125 performed using an indirect immunoperoxidase technique after high temperature antigen retrieval in

126 10 mM citric acid monohydrate buffer (pH 6.5) in a pressure cooker for 13 min. Blocking of

127 unspecific protein-antibody interactions was performed with 20% human AB serum in PBS for 1h at

128 room temperature. Primary antibody for ERCC1 was the new highly specific monoclonal anti-mouse

129 antibody (mAb) clone 4F9 (UM500008, dilution 1:100) that was purchased from OriGene

130 Technologies, Inc (Rockville, USA). A mouse negative control was used (Dako North America Inc.,

131 Carpinteria, USA). The slices were incubated overnight at 4°C. Signal amplification was achieved

132 with En-Vision System Labeled Polymer-HRP Anti-Mouse (Dako) for 40 min and developed for 10

133 min with DAB Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's

134 instructions. Nuclei were counterstained with Mayer's hematoxilin for 2 min. For positive controls,

135 sections of colon adenocarcinoma, renal cell carcinoma, breast cancer, hepatocellular carcinoma and

136 normal tonsil were chosen, while cells of the tumor stroma served as internal negative control.

137 All slides were analyzed independently by two investigators blinded to clinical information (V.L. and

138 S.S.) Nuclear staining intensity was graded as negative (0), low (1), medium (2), or strong (3). The

139 percentage of tumor cells with positive nuclei was calculated for each specimen and scored 0 if 0%

140 were positive, 0.1 if 1–9% were positive, 0.5 if 10–49% were positive and 1 if 50% or more were

141 positive. A semiquantitative H-score was then calculated by multiplying the staining intensity grading

142 score with the proportion score as described previously [13]. In case of discrepant results, staining

143 intensities were jointly assessed by both investigators, forming the final score by consensus. Inter-

144 observer agreement was investigated via Pearson's correlation coefficients 0.72 (95%CI: 0.63-0.79).

145

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

147 We also intended to re-evaluate our old results obtained with the mAb against ERCC1 clone 8F1 (old

148 batch)[13] with the new high specific mAb clone 4F9. To this aim, we re-stained 38 ACC samples out of

149 the 45 previously published and re-investigated the relationship between ERCC1 expression and the

150 response to PBC in terms of both progression-free survival (PFS) and disease-specific survival (DSS)

151 after treatment. Moreover, the specificity of the currently available clone 8F1 has been shown to be

152 altered from the old clone 8F1[21, 22]. In addition, we also evaluated a subgroup of 21 out of the 146

153 samples in our present series with the current clone 8F1 (new batch) in addition to the new clone 4F9.

154

155 *Statistical analysis*

156 The Fisher's exact or the Chi-square tests were used to investigate dichotomic variables, while

157 continuous variables were investigated with a two-sided *t* test (or non-parametric test). A non-

158 parametric Kruskal-Wallis test, followed by Dunn's test, was used for comparison among several

159 groups for non-normal distributed variables. Correlations and 95% confidence intervals (95%CI)

160 between different parameters were evaluated by linear regression analysis. PFS was defined as the

161 time from the date of first administration of PBC to the first radiological evidence of disease

162 progression or death, as appropriate. DSS was defined as the time from the first administration of PBC

163 to disease-specific death or last follow-up. All survival curves were obtained with Kaplan-Meier

164 estimates, and the differences between survival curves were assessed by the log-rank (Mantel-Cox)

165 test. For the calculation of hazard ratios (HR), two ACC-groups with low or high protein expression

166 were considered (high expression: H-score ≥ 2). A multivariate regression analysis was performed via

167 a Cox proportional hazard regression model, aiming to identify factors that might independently

168 influence survival. Statistical analyses were made using GraphPad Prism (version 6.0, La Jolla, CA,

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

170 considered as statistically significant.

171

172 **Results**

173 *Efficacy of platinum-based chemotherapy*

174 The data about efficacy of PBC in the current series of 146 patients with advanced ACC are

175 summarized in *Table 2*. Concerning the best objective response during PBC, one patient experienced

176 complete response (0.7%) and 42 patients partial remission (28.8%), 49 stable disease (33.5%), 8

177 mixed response (5.5%) and 46 progressive disease (31.5%), respectively. The median PFS during PBC

178 was 6 months, ranging from 2 to 18, while the median DSS was 17 months, ranging from 1.5 to 127.

179 Additionally, we observed a clinical benefit defined as at least a stable disease for a minimum of 4

180 months in 84 patients (58%) with a median PFS in this group of 6 months (range: 4-18). Only one

181 patient died unrelated to ACC during follow up. Thus, overall survival was more or less identical to

182 DSS (data not shown).

183

184 *ERCC1 expression and baseline clinical characteristics in ACC*

185 Nuclear ERCC1 immunostaining was homogeneous in individual ACC samples with a median

186 percentage of positive cells of 80% (> 50% in 135/146 samples, 92.5%). Tissue samples exemplifying

187 the range of staining intensity are shown in the *Figure 1*. ERCC1 expression was low (H-score 0-1) in

188 50 samples (34.2% of cases) and high (H-score 2-3) in 96 samples (65.7%). We did not observe any

189 significant differences in ERCC1 immunostaining among primary tumors, local recurrences and/or

190 distant metastasis. No significant correlation was also observed between the nuclear ERCC1

191 expression and the ENSAT tumor stage at the time of diagnosis, the Weiss score or the Ki67

192 proliferation index.

193

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

195 Considering the potential predictive role of ERCC1 immunostaining on the objective response to PBC,

196 no significant differences were observed between the groups with high and low nuclear ERCC1

197   expression (**Table 2**). Similarly, no differences were found in terms of both PFS (median 6.5 *vs* 6

198   months, respectively, *P=0.33*, HR=1.23, 95%CI=0.82-2.0) and DSS (median 17 *vs* 16.5 months,

199   respectively, *P=0.87*, HR=1.03, 95%CI=0.70-1.53) (**Figure 2A-B**).

200

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

202   We re-stained 38 out of 45 ACC samples of our previously published series (stained with the 8F1

203   clone old batch) with the new clone 4F9. Not unexpected, ERCC1 expression in terms of H-score

204   corresponded in only 49% of cases. As a consequence, ERCC1 nuclear expression did not longer

205   significantly correlate with response to PBC in terms of both PFS (data not shown) and DSS

206   (Supplementary Figure 1A and B).

207   Furthermore, we stained 21 out of the present 146 samples with the currently available clone 8F1 (new

208   batch) additionally to the clone 4F9. Two representative examples are shown in the Supplementary

209   Figure 2. Comparing the ERCC1 immunostaining results we observed here a correspondence between

210   the two antibodies in 81% of cases.

211

212   **Discussion**

213   We evaluated the potential role of ERCC1 nuclear expression as predictive biomarker to PBC in the

214   largest series of ACC patients up to date (n=146) by using for the first time the new high ERCC1-

215   specific monoclonal antibody clone 4F9. To note, ERCC1 has been previously demonstrated to be a

216   predictive biomarker for platinum treatment in several cancers, such as non-small cell lung cancer

217   (NSCLC), testicular germ cell tumors, bladder cancer, pancreatic carcinoma and gastric cancer[8-12]. In

218   ACC, we previously demonstrated in a relatively small series of patients that ERCC1 immunostaining

219   was significantly correlated with overall survival during PBC[13]. Another study, however, did not

220   confirm this finding[14]. Nevertheless, several concerns about the reliability of the ERCC1

221   immunohistochemical analysis have been raised recently. First, it has been demonstrated that the clone

222   8F1 used in all the reported studies is not specific for ERCC1[15-17]. Specifically, the anti-ERCC1

223   antibody clone 8F1 has been identified to stain also the PCYT1A, a phospholipid synthesis enzyme

224   regulated by RAS[17, 23] with no known clinical implication in platinum drug resistance. PCYT1A has

8

225   also been confirmed to play a role as prognostic biomarker in both lung and head and neck squamous

226   cell carcinomas[23].

227   Moreover, the batch of the clone 8F1 in use since 2011 seems not to be identical with the batch in use

228   in 2006[19], thus rendering new data about NSCLC not comparable with previous ones[22]. According to

229   this new information, important previous results on the role of ERCC1 in the treatment of NSCLC [8]

230   have been revised by the same group[21]. Furthermore, this year the first randomized trial to evaluate

231   ERCC1 prospectively in 648 patients with NSCLC (ET trial) has been published definitively

232   demonstrating that selecting chemotherapy using the commercially available ERCC1 antibodies (clone

233   8F1) does not confer any additional survival benefit[24].

234   In parallel, a new highly ERCC1-specific clone 4F9 has been recently proposed and validated [17-19]. For

235   all these reasons, we decided to use the clone 4F9 to investigate a new large series of ACC samples in

236   order to re-evaluate our previous results on ERCC1 as predictive marker of sensitivity to PBC. Most

237   importantly, we could not confirm the previous results and our data now indicate that ERCC1 itself is

238   probably not the main factor involved in the response to PBC in ACC patients. In addition, we were

239   able to demonstrate that the current version of the clone 8F1 significantly differs from the old one that

240   we used for our pilot study[13] and we were not able to reproduce the earlier results using now the same

241   tumor samples.

242   One reason that could explain the lack of correlation between ERCC1 and PBC, independently from

243   the issues with immunohistochemistry, is that ERCC1 works together with the XPF protein, codified

244   by *ERCC4*. ERCC1–XPF complex is a two subunit structure-specific endonuclease that plays a key

245   role during the nucleotide excision repair (NER) process[7, 25]. Thus, XPF itself might be involved in the

246   sensitivity to the response to PBC[26, 27]. However, the ET trial demonstrated that XPF expression is not

247   predictive for response to 648 patients with NSCLC[24]. Moreover, the ERCC1–XPF complex makes

248   incisions on the damaged DNA strand on the 5′ side and acts in cooperation with several other

249   proteins, like XPC–RAD23B, XPA, RPA, TFIIH and XPG, during the NER process[28, 29]. Thus,

250   although ERCC1 plays a major role in the NER, several other proteins and mechanisms could

251   influence the response to PBC.

252   Another explanation, why ERCC1 expression and clinical outcome in our and other series did not

253   correlate could be the fact that virtually all patients have received in parallel to the platinum derivate

254   1-3 other additional cytotoxic drugs (mostly doxorubicin, etoposide and mitotane) diluting the

255   hypothesized correlation. Other potential biomarker could for instance be involved in the prediction of

256   response to these concomitant treatments (i.e. TOP2A[30]). Finally, one potential limitation in our study

257   as well as in several others might be that ERCC1 was assessed on tumor specimens obtained months

258   or even years before the start of chemotherapy. Nevertheless, we did not observe any significant

259   differences in ERCC1 immunostaining among primary tumors, local recurrences and/or distant

260   metastasis, thus suggesting that the ERCC1 levels remain quite stable over the time and tumor

261   progression.

262   More generally, the search for predictive biomarkers to conventional cytotoxic chemotherapy has been

263   proven challenging due to frequent discrepant and non-replicable findings. And this is true not only for

264   protein expression where issues with antibodies and immunohistochemical analysis are common, but

265   also for gene expression. Thus, if a plethora of biomarkers predicting chemotherapy efficacy have

266   been evaluated also in the clinical setting, none of them is ready for clinical implementation yet[31].

267   Considering that most mechanisms of resistance or sensitivity to chemotherapy are multifactorial, a

268   combinatorial approach and further efforts are required[32].

269   Concerning the response rate to PBC in general, we observed an objective partial response in 29.5% of

270   cases and a stable disease in further 33.5%, thus confirming that PBC is the currently most effective

271   cytotoxic therapy for advanced ACC. These data are generally superimposable to those reported in the

272   FIRM-ACT study on EDP-M[2].

273   In conclusion, ERCC1 expression as detected by immunostaining is not directly associated with

274   sensitivity to PBC in ACC. Thus, the search for predictive biomarkers in this devastating disease with

275   poor response to medical therapy has to continue.

276

277   **Declaration of interest**

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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 ≥2) and low staining (H-score ≤1) of ERCC1.

433 **Supplementary data**

434

435 **Supplementary Figure 1. Re-evaluation of the overall survival in the old series of 38 patients**

436 **with adrenocortical carcinoma treated with platinum-based chemotherapy** [13]. (A) ERCC1

437 immunostaining with the 8F1 clone (old batch) (B) ERCC1 immunostaining with the new specific 4F9

438 clone.

439

440 **Supplementary Figure 2. Direct comparison between ERCC1 antibodies 4F9 (A) and C) and 8F1**

441 **clone (new batch) (B) and D))** in one normal adrenal gland (A) and B)) and in one adrenocortical

442 carcinoma (C) and D)). Magnification 1x20.

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