

CLINICAL STUDY

Mutational analyses of epidermal growth factor receptor and downstream pathways in adrenocortical carcinoma

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Abstract

Background: Adrenocortical carcinoma (ACC) is a rare disease with a poor prognosis and limited therapeutic options. Mitotane is considered the standard first-line therapy with only 30% of the patients showing objective tumour response. Defining predictive factors for response is therefore of clinical importance.

The epidermal growth factor receptor (EGFR) has been implicated in the development of one-third of all malignancies. EGFR pathway members in ACC have been investigated, however, without available clinical data and relation to survival.

Methods: In this study, mutation status of EGFR and downstream signalling pathways was evaluated in 47 ACC patients on mitotane using direct sequencing, a TaqMan allele-specific assay and immunohistochemistry. Archival formalin-fixed paraffin-embedded tumour tissue was used for all analyses. Patient data were obtained anonymously, after coupling with the collected tumour tissue.

Results: One *BRAF*, two *EGFR* TK domain (c.2590G>A, p.864A>T) and 11 *TP53*, but no *PIK3CA* or *KRAS*, mutations were found. No relationship was found between mutation status, immunostaining and mitotane response or survival.

Conclusion: In conclusion, our data suggest that the role of EGFR tyrosine kinase inhibitors in ACC is limited. Treatment with EGFR monoclonal antibodies on the other hand might be beneficial for a larger group of patients. The possible efficacy of this therapy in ACC should be evaluated in future trials.

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Introduction

Adrenocortical carcinoma (ACC) is considered one of the most aggressive solid tumours with a poor prognosis. Fifty years after its first use, mitotane (o,p'-DDD) is still considered the most effective drug for ACC. Nonetheless, a number of limitations for this treatment exist including poor bioavailability, side effects and a narrow therapeutic range. Furthermore, the mechanism of action is not well known (1). Although mitotane treatment has its limitations, response to mitotane monotherapy has been associated with a better prognosis (2, 3, 4). As only 30% of patients on mitotane monotherapy show objective tumour response (3, 4, 5), defining these patients would be of clinical importance.

Until now, poor understanding of ACC pathogenesis has hampered the development of new therapies. Gene expression profiling has been successfully used to improve this understanding. With this technique, subgroups of ACC with different gene expression profiles

and clinical outcomes have been identified (6, 7). P53 and β -catenin, for example, were shown to be poor prognostic factors in ACC (8). In addition to molecular profiling, the search for new therapies has resulted in the investigation of several potential candidates (9).

Targeted tailor-made therapy for individual patients has been applied in advanced stages of several cancers (10). Most of these treatments are directed towards molecular pathways in the tumour cells that are constitutively activated in these cancers (based on the so-called oncogene addiction theory). An important therapy target is the epidermal growth factor receptor (EGFR) pathway. EGFR is involved in the regulation of many cell processes such as cell growth, proliferation and apoptosis, and it has been implicated in the development of 30% of all cancers (11). As a result, two anti-EGFR therapies have been developed, tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (MABs), both potential new therapies in ACC. EGFR pathway involvement in ACC has been investigated in a

few studies; however, only limited clinical data are available while the relationship with survival is unknown.

In order to improve prediction of tumour response to mitotane treatment and further study the involvement of EGFR pathway in the tumour genesis of ACC, we determined the mutation status of EGFR and downstream effector molecules, stratifying also for TP53 mutation status as well as for β -catenin expression in a Dutch cohort of ACC patients treated with mitotane monotherapy.

Materials and methods

Tumour specimens and clinical data

Archival formalin-fixed paraffin-embedded tumour tissue (FFPE) of 47 ACC patients treated with mitotane monotherapy, in four Dutch hospitals, i.e. Leiden University Medical Centre, Erasmus MC – University Medical Centre Rotterdam, Amsterdam Medical Centre, Amsterdam, and Maxima Medical Centre Eindhoven, was collected. Clinical data were obtained from each patient. Follow-up was available for all patients from date of diagnosis until January 2010. After collection of the tumour tissue blocks, histopathological slides of each case were reviewed by two pathological experts in adrenal disease. In all cases, the diagnosis of ACC was confirmed as all samples had van Slooten scores > 8 (12) and Weiss scores > 3 (13). Furthermore, malignant nature was confirmed by the presence of stage IV disease or recurrent disease in all patients. After coupling of the clinical data to the pathology specimen, both patient information and tumour tissue were anonymised.

Definitions

Mitotane monotherapy was defined as treatment with mitotane (Lysodren, HRA Pharma, Paris, France) tablets as single treatment. Patients with concomitant chemotherapy or radiation therapy were excluded from the study. Response to mitotane treatment was assessed by CT-examination, at least every 2–3 months. Evaluation of tumour response was based on the RECIST criteria version 1.0 (14). Responders were defined as patients with radiological partial (PR) or complete (CR) tumour response to mitotane therapy. Non-responders were defined as patients with progressive disease during mitotane treatment. Staging was performed using the ENS@T staging criteria (15). According to ENS@T staging, the stages are defined as follows: stage I, tumour size < 5 cm without any risk factor (T1N0M0); stage II, tumour size > 5 cm but still without risk factors (T2N0M0); stage III, tumour of any size with at least one of the following factors: tumour infiltration in surrounding tissue (T3), tumour invasion into adjacent organs or thrombus in the vena cava or renal vein (T4), positive lymph node (N1), but no distant

metastasis (M0) and stage IV, the presence of distant metastases irrespective of tumour size or lymph node status (T1–T4 N0–N1M1).

For determination of serum levels at the time of radiological response, the sample closest to date of response or progression was selected. Serum levels were defined as < 14 or ≥ 14 mg/l.

Somatic mutation analysis

Sections of the stained slides, which contained $> 80\%$ tumour cells, were selected for DNA isolation and mutation analysis. From the selected sections, tissue-punches were taken with a tissue microarrayer (TMA; Beecher, Sun Prairie, WI, USA). DNA was isolated from three tissue cores using the Nucleospin Tissue kit (Marcherey-Nagel, Bethlehem, PA, USA) according to manufacturer's instructions with modifications; tissue cores were deparaffinised once in 1 ml xylene and washed in 100% ethanol, proteinase-K digestion was performed overnight and DNA was eluted in water. DNA concentrations were measured using Nanodrop 1000 (Isogen, De Meern, The Netherlands).

After DNA isolation, EGFR, BRAF, KRAS and PIK3CA somatic mutation analysis was performed using an allele-specific quantitative real-time PCR (qPCR) with hydrolysis probes (16) for the mutation hotspots: BRAF c.1799T $>$ A (p.V600E), KRAS (c.34G $>$ A, p.G12S/c.34G $>$ C, p.G12R/c.34G $>$ T, p.G12C/c.35G $>$ A, p.G12D/c.35G $>$ C, p.G12A/c.35G $>$ T and p.G12V/c.38G $>$ T, p.G13V) and PIK3CA (c.3140 A $>$ G, p.H1047R/c.1624G $>$ A, p.E542K) and EGFR c.2573T $>$ G (p.L858R) and exon 19 deletions were detected as previously described (26, 27). Oligonucleotide and probe sequences are available upon request.

Sanger sequencing of EGFR exons 18–21 and TP53 exons 5–8 was performed using PCR and direct DNA sequencing, in order to find additional mutations. Sanger sequencing was performed at the Leiden Genome Technology Centre using both forward and reverse M13 primers. Data were analysed with Mutation Surveyor DNA analysis software version 3.2.4 (Softgenetics LLC, State College, PA, USA).

AKT, phosphorylated AKT, ERK, pERK, β -catenin and PTEN immunohistochemistry

A tissue microarray was constructed containing three representative tissue cores (of 0.6 cm in diameter) from each tumour (17). Four-micrometer tissue sections were cut and transferred to glass slides using a tape-transfer system (Instrumedics, Hackensack, NJ, USA). A standard indirect immunohistochemistry (IHC) procedure was performed including citrate antigen retrieval (0.01 M, pH 6.0), blockage of endogenous peroxidase activity and diaminobenzidine development. The following primary antibodies were used: rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)

(1:50, #9101, Cell Signalling Technology, Danvers, MA, USA), mouse monoclonal 3A7 anti-p44/42 MAPK (Erk1/2) (1:1000, #9107), Cell Signalling Technology, rabbit monoclonal (D9E) anti-phospho-Akt (Ser473, 1:25, #4060), Cell Signalling Technology, mouse monoclonal (40D4) anti-Akt (pan, 1:500, #2920), monoclonal mouse anti-human PTEN (1:400, 6H2.1, Dako, Glostrup, Denmark) and purified mouse monoclonal anti β -catenin (1:250, #610154, BD Transduction Laboratories, Bedford, MA, USA).

AKT, phosphorylated AKT (pAKT), ERK, pERK and PTEN were assessed by the presence or absence of cytoplasmic and/or nuclear staining. β -Catenin was assessed by the presence of nuclear staining as an indication of activation. Scoring was performed according to prevalence and intensity of cytoplasmic/nuclear staining. Tumours were divided into four groups. Group 1, negative staining (-); group 2, weak positive staining (+/-); group 3, positive staining (+) and group 4, strong positive staining (++)

Statistical analysis

Clinical characteristics of the patients were presented as mean with s.d. or median with range as appropriate. Survival curves were calculated using Kaplan–Meier method. The influence of different clinicopathological parameters on survival was evaluated using Mantel–Cox log-rank test.

Mitotane-related overall survival (MROS) was calculated from the date of mitotane initiation. Only patients with at least 3 months of mitotane monotherapy were included in the MROS analysis. Overall survival (OS) was calculated from the date of diagnosis. Differences in survival according to clinicopathological parameters, mutation status and protein expression were analysed using Cox-regression analysis. Differences in patient and tumour characteristics between responders and non-responders were analysed using Pearson χ^2 test and Fisher’s exact test. Differences in age, van Slooten index, tumour weight and tumour size were calculated using the Student’s *t*-test. A *P* value <0.05 was considered significant. All analyses were performed using SPSS version 15.0.

Results

Patient characteristics

Tumour samples of 47 ACCs (30 primary ACCs and 17 recurrences), obtained from a series of 47 patients, were used for IHC and mutation analysis. Patients presented with stage II in 42.6% of cases (*n*=20), stage III in 14.9% (*n*=7) and stage IV in 42.6% (*n*=20). Median OS was 44 months. Mitotane was given as monotherapy for locally advanced or metastatic disease. In 42.2% of the cases, mitotane was given for primary ACC (*n*=19;

patients 1–7, 9, 11–13, 15, 20, 22–24, 36, 39 and 43; Table 1), in 51.1% (*n*=16, patients 16–19, 21, 25–27, 29, 31, 33–36, 41, 42 and 47; Table 2) for first recurrence and in 6.67% (*n*=3, patients 38, 40 and 46; Table 2) for the second recurrent disease.

Serum levels and response to mitotane

Of the 47 included patients, 37 patients were available for evaluation of mitotane response. Two patients were excluded from the mitotane response analysis as they did not receive monotherapy but adjuvant mitotane therapy. Another eight patients died before first CT evaluation; therefore, these patients could not be classified as a responder or non-responder.

Objective response was seen in 14 patients, stable disease in four patients and progressive disease in 19 patients. Mitotane serum levels ≥ 14 mg/l were seen in 13 responders (92.9%) and in three (15.8%) non-responders. Levels ≥ 14 mg/l were significantly associated with treatment response (χ^2 *P*=0.00). MROS of responders was significantly longer than survival observed for non-responding patients (median 51 vs 7 months, *P*=0.01), even after correction for age, gender, functionality and stage at diagnosis (*P*=0.02) (Fig. 1). After correction for mitotane serum levels < or ≥ 14 mg/l, the survival benefit disappeared (*P*=0.71). No relationship was found between the presence of hormonal excess and response to mitotane treatment (*P*=0.32).

Mutation analysis of EGFR, KRAS, BRAF and PIK3CA

Hotspot mutation analysis of *EGFR* exon 19 deletions (del 19) and *EGFR* 21 (c.2573T>G, p.L858R) was

Table 1 TP53 mutations found in 46 adrenocortical carcinoma samples.

Patient	Exon	Mutation	
1	5	c.430C>T, p.Q44X	Nonsense
9		c.437G>A, p.W146WX	Nonsense
11		c.452C>T, p.P151PL, c.453C>T, p.P151P	Missense
13		c.524G>A, p.R175H	Missense
17		c.446_447delCC	Deletion
38		c.381C>T, p.S127SS	Silent
46		c.464C>T, p.T155L	Silent
4	6	c.606T>A, p.R202RR	Silent
7		c.667C>T, p.P223PS	Missense
10		c.578A>T, p.H193L	Missense
17		c.636del T	Silent
24		628_634delAACACTT	Deletion
37		c.626G>A, p.R209RK	Missense
17	7	c.746G>A, p.R249RK	Missense
8	8	c.855G>A, p.E285EE	Silent
43		c.833C>A, (c.833C>A), p.P278H	Missense

NA, not available.

Table 2 Immunohistochemical results for primary and recurrent ACC.

Pt n=47	ACC	P53 mut n=46	EGFR n=46	PIK3CA/ KRAS		BRAF n=45	AKT n=45	pAKT n=45	ERK n=45	pERK n=45	VERK n=45	β-Catenin n=43	PTEN loss n=45
				n=46	n=46								
1	Pr	n.m	0	0/0	0	+	+	+/-	+/-	-	+	No	
2	Pr	0	0	0/0	0	+	+/-	+	+/-	-	+	No	
3	Pr	0	0	0/0	0	-	-	+/-	-	-	+	No	
4	Pr	s.m	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
5	Pr	0	s.m	0/0	0	+	+/-	+/-	-	-	-	No	
6	Pr	0	0	0/0	0	+	+/-	+	+/-	+	+	No	
7	Pr	m.m	0	0/0	0	+	-	+	-	-	+	No	
8	Pr	s.m	s.m	0/0	0	+	-	+	+/-	+	+	No	
9	Pr	n.m	s.m	0/0	0	+/-	+/-	+	+	-	-	No	
10	Pr	m.m	0	0/0	0	+	+	+	+	-	+	No	
11	Pr	m.m	0	0/0	0	+	+/-	+	+/-	-	+	No	
13	Pr	m.m	0	0/0	0	+	+/-	+/-	+	+/-	-	No	
14	Pr	NA	0	0/0	0	+	-	+	-	+/-	+	No	
15	Pr	0	0	0/0	0	+	+	+	+/-	+/-	NA	No	
16	Pr	NA	0	0/0	0	+	+	+	+	+/-	NA	No	
17	Pr	del; m.m	s.m	0/0	0	+	+/-	+	+	+	+	No	
19	Pr	NA	0	0/0	0	+	+	+	+	-	+	No	
21	Pr	NA	0	0/0	0	NA	NA	NA	NA	NA	NA	NA	
22	Pr	0	0	0/0	0	+	+/-	+	+/-	-	-	No	
24	Pr	del	0	0/0	0	+	+/-	+	-	-	-	No	
26	Pr	0	m.m; s.m	0/0	0	+	+	+	-	-	-	No	
27	Pr	0	0	0/0	0	+	+/-	+	-	-	-	No	
28	Pr	0	0	0/0	0	+	-	+	-	-	+	No	
29	Pr	0	0	0/0	0	+	-	+	-	-	+	No	
31	Pr	NA	0	0/0	0	+	+/-	+	+/-	+	-	No	
37	Pr	m.m	0	0/0	0	+	+	-	+/-	-	+	No	
38	Pr	s.m	0	0/0	0	+	+	+	-	+/-	-	No	
41	Pr	0	0	0/0	0	+	+/-	+	-	+/-	-	No	
42	Pr	0	m.m	0/0	+	+	+/-	+	-	-	-	No	
44	Pr	0	0	0/0	0	+	+	-	+/-	+/-	+	No	
12	R	NA	0	0/0	0	+	+/-	+/-	-	0/0	-	No	
18	R	0	0	0/0	0	+	-	+	+/-	-	-	No	
20	R	0	0	0/0	0	+	+	+	+/-	+/-	+	No	
23	R	NA	0	0/0	0	+	-	+	-	-	-	No	
25	R	0	0	0/0	0	-	-	+	-	-	+	No	
30	R	0	0	0/0	0	+	-	+/-	+/-	+/-	-	No	
32	R	0	0	0/0	0	+	+/-	+	+/-	-	+	No	
33	R	NA	0	0/0	0	+/-	-	+/-	+/-	-	-	No	
34	R	NA	0	NA	NA	+/-	-	+	-	-	-	No	
35	R	0	0	0/0	0	+	+	+	-	+/-	-	No	
36	R	0	0	0/0	0	+	+	+	+/-	+/-	+	No	
39	R	0	0	0/0	0	+	+/-	+	+/-	-	+	No	
40	R	0	0	0/0	0	+	+	+	+	+/-	+	No	
43	R	m.m	s.m	0/0	0	+	+	+	++	+/-	+	No	
45	R	0	0	0/0	0	+	+/-	+	+/-	-	-	No	
46	R	s.m	m.m	0/0	0	+	+/-	+	+/-	-	-	No	
47	R	NA	0	0/0	0	+	+/-	+	-	+	+	No	

Pt, patient; Pr, primary tumour; R, recurrence; -, negative staining; (+/-), weak positive staining; (+), positive staining; (++) , strong positive staining; NA, not available; n.m, non-sense mutation; m.m, missense mutation; s.m, silent mutation; del, deletion; 0, no mutation.

successfully performed in 45 samples using a hydrolysis probe. No mutations were found. Additional DNA sequencing of *EGFR* revealed ten mutations, of which seven were silent.

One of the three remaining mutations was found to be a single nucleotide polymorphism (SNP) (exon 20, c.2271G>A, p.787A>T, patient 42). Two missense mutations were found in exon 21 (c.2590G>A, p.864A>T, patient 26, c.2765 G>GA, p.866E>EK, patient 46) (Fig. 2). One activating *BRAF* mutation

(c. 1799T>A, p V600E) was found. No mutations were found in *KRAS* or *PIK3CA*.

TP53 mutation analysis

DNA sequencing of TP53 exons 5–8 could be obtained in the FFPE DNA of 46 ACC samples. In 37 tumours, DNA sequencing of all exons was successful. Sequencing analysis revealed 16 mutations (Table 1). TP53 mutations were more common in patients presenting

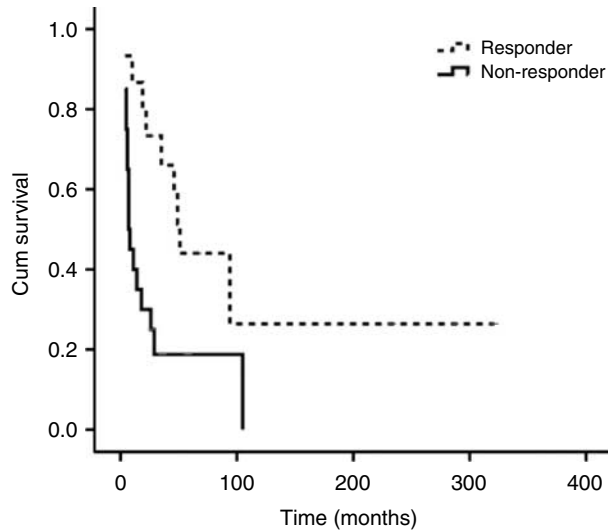


Figure 1 Overall survival from date of mitotane initiation (MROS) for mitotane responders ($n=14$) and non-responders ($n=19$). Responders showed a significantly longer OS (median 51 months, range 42–70 months) compared with non-responders (median 7 months, range 5–9 months, $P=0.01$).

with stage IV tumours. The median survival of patients with a *TP53* mutation was 11 vs 51 months for patients without a *TP53* mutation ($P=0.19$).

PTEN, AKT, phospho-AKT, ERK and phospho-ERK IH

IHC data were available for 45 tumours (see Table 2 for an overview of the immunohistochemical scoring for each tumour). None of the tumours showed loss of PTEN immunoreactivity. AKT expression was also positive overall. pAKT was detected in 31% of tumours (Fig. 3). Median OS of 30 months was observed in patients with pAKT-negative tumours, 44 months in patients with pAKT weak positive tumours and 95 months in patients with pAKT-positive tumours ($P=0.163$). A shorter median survival was found for patients with tumours without pAKT staining compared with patients with pAKT-positive tumours (+ and +/-) (30 and 59 months respectively, $P=0.06$).

ERK staining was positive in the majority of samples (80%). In 60% of the samples, phosphorylation of ERK was observed. Seven of these samples (26%) showed strongly positive nuclear staining (Fig. 4). Median survival for patients with perk-negative tumours was 73 months, for patients with perk-positive tumours 23 months and for patients with pERK strongly positive tumours 14 months. A trend towards shorter survival was observed for patients with perk-positive tumours ($P=0.08$) compared with patients with perk-negative tumours. No difference in ERK or pERK staining was observed between primary ACC and recurrences (χ^2 , $P=0.10$, $P=0.28$ respectively).

A remarkable positive pERK staining of endothelial cells of blood vessels of 18 ACC samples (40%) was observed. Five samples even showed a very strong endothelial staining (Fig. 4). No significant survival difference was seen between patients with tumours with negative, positive and strongly positive endothelial staining ($P=0.09$).

β -Catenin IH

β -Catenin staining results were successfully obtained for 43 samples. Positive nuclear staining was observed in 23 samples (53.5%). Median survival of patients with tumours with nuclear immunostaining was 22 vs 59 months for patients with tumours with negative staining ($P=0.30$). In the group of primary ACCs ($n=30$), a trend towards shorter survival was revealed (10 vs 59 months, $P=0.06$).

Correlation of mutation status, IHC and mitotane response

No relationship was found between the presence of an *EGFR* mutation and immunostaining of any of the downstream effectors. Samples harbouring an *EGFR* mutation showed negative or weak positive staining of pERK and weak positive or positive staining of pAKT. No relationship was found between mutation status, IH results and mitotane response.

Discussion

We were not able to find a relationship between the *EGFR* pathway and mitotane response. We did find two

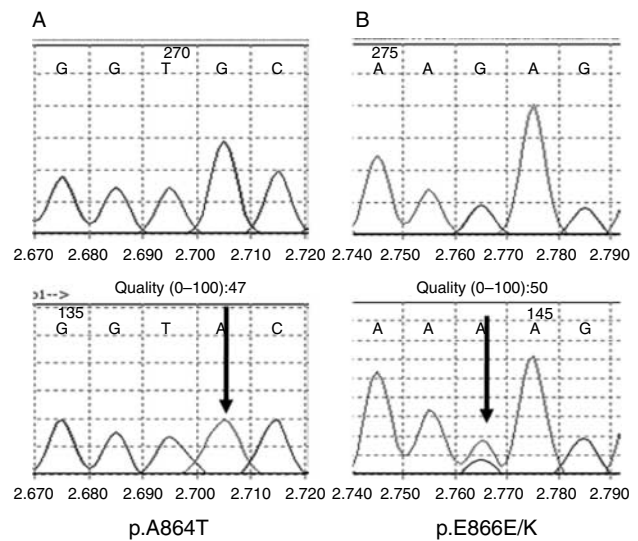


Figure 2 EGFR mutation analysis by direct sequencing. The wild-type sequence is shown above, the corresponding mutated sequence is shown below. (A) c.2590G>A, p.A864T. (B) c.2765G>A, p.E866E/K.

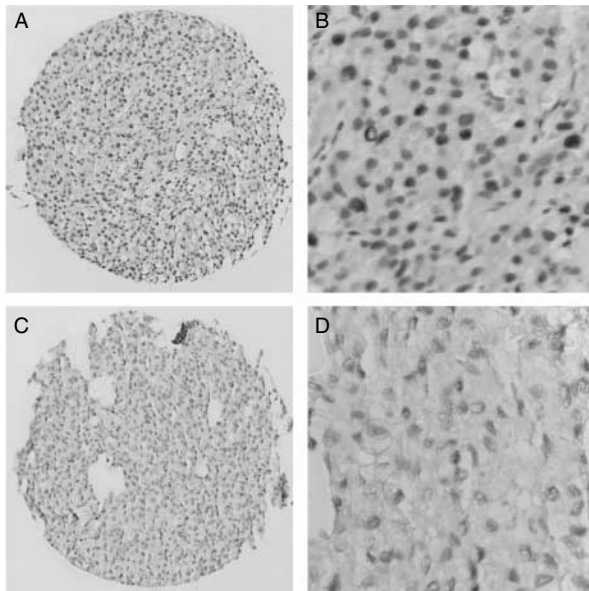


Figure 3 pAKT IH in a TMA of ACC tumours (ACC-TMA). ACC 1 shows positive nuclear staining (A, magnification $\times 125$; B, magnification $\times 500$). ACC 3 shows negative staining for pAKT (C, magnification $\times 125$; D, magnification $\times 500$).

mutations of the *EGFR* TK domain. The c.2590G>A, p.864A>T (patient 26, Table 2 and Fig. 2) *EGFR* mutation was described previously in a non-small-cell lung carcinoma and was suggested to respond to *EGFR* TKI (18). For the other *EGFR* TK domain mutation (p.866E>K) found in our study, unresponsiveness upon *EGFR* TKI treatment was described (19).

We also identified *BRAF* (c. 1799T>A, p.V600E) mutation in one ACC. This patient also had an SNP on *EGFR* exon 20 (c.2271G>A, p787A>T), probably unresponsive to *EGFR* TKI treatment. Specific targeting of *BRAF*, however, may be an option in this patient as *BRAF* targeting has recently been shown to be effective in melanomas (20).

Until now, only four mutations of the *EGFR* TK domain have been reported in ACC (21, 22). Kotoula *et al.* described four activating *EGFR* mutations, three missense (c.2168T>C, exon 18, c.2354C>A, exon 20, and c.2533G>A, exon 21) and one non-sense mutation (c.2193G>A, exon 19) in 35 ACCs. The observed mutations were also described earlier in lung and colon carcinoma, but the response to *EGFR*-TKI therapy was not reported (23, 24, 25). Recently, Adam *et al.* (22) found no mutations in 30 ACCs.

The expected incidence of the *EGFR* mutation in ACC is rather low, $\sim 5\%$ of patients. The role of the *EGFR* TKIs is therefore expected to be minor. Furthermore, the majority of patients harbouring an *EGFR* mutation will be treated with mitotane, which is known to interact with *EGFR*-TKIs (26).

A second result of potential clinical relevance is that in the remaining ACCs without activating *EGFR*

mutations, no downstream mutations were present in *KRAS*, *BRAF* or *PIK3CA* and preserved *PTEN* expression was seen.

MABs like cetuximab and panitumumab have been used in colorectal carcinoma without mutations of *EGFR* or downstream effectors. Patients are selected for MABs based on *EGFR* protein expression on the membrane of the tumour (27). Since the majority of ACCs have neither *EGFR* nor downstream mutations of *KRAS*, *BRAF* or *PIK3CA*, MABs could be of interest, especially since *EGFR* protein expression is observed in the majority of ACC (22). However, before the initiation of a clinical trial, the *EGFR* gene status should be determined as *EGFR* protein expression does not correlate with response to the *EGFR* MABs (28, 29).

The presence of *BRAF* and *KRAS* mutations in ACC was reported in the literature to be present in only 1.6% (1/59) and 6.7% (1/15) of ACC respectively (21, 30). The presence of oncogenic *PIK3CA* mutations in ACC has not previously been studied.

From this study, we concluded that the mutation status of *EGFR*, *KRAS*, *BRAF*, *PIK3CA* and *TP53* did not correlate with mitotane response. The same holds true for AKT, pAKT, ERK, pERK and β -catenin expression.

In addition to the relationship of *EGFR* and downstream pathways with mitotane response, we also studied the relationship with OS. Phosphorylation of pERK and pAKT and downregulation (loss) of *PTEN* have been associated with disease progression as well as shorter disease-free and OS in multiple malignancies.

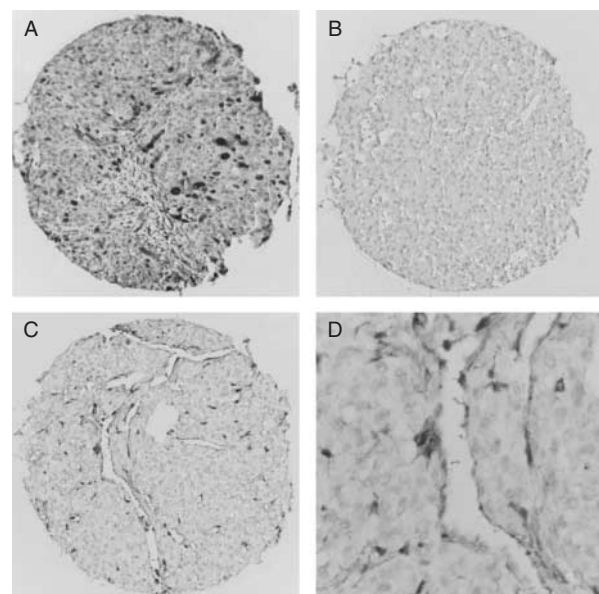


Figure 4 PERK IH of ACC-TMA. ACC 17 (A) shows strong nuclear staining in a fraction of ACC cells. Furthermore, endothelial cell positivity can be observed. In ACC 7 (B), no positivity of ACC or endothelial cells is seen. In ACC 35 (C, magnification $\times 125$; D, magnification $\times 500$), only positive nuclear and cytoplasmic staining of endothelial cells is observed.

The role of these molecules in ACC has not yet been established.

Overexpression of ERK, as well as aberrant phosphorylation of ERK, has been associated with poor prognosis in several tumours (31). We were the first to study the expression of both markers in ACC and we found a trend towards shorter survival for pERK-positive samples. However, this survival benefit was not found to be significant, probably because of the small sample size. Furthermore, we found a remarkable pERK staining in endothelial cells of blood vessels of unknown biological significance in 18 ACCs.

Another remarkable finding was the observation of a trend towards shorter survival in pAKT-negative samples ($P=0.06$). This is a rather unexpected finding as pAKT positivity has been associated with shorter survival in other malignancies (32). As pAKT and pERK have not yet been extensively studied in ACC, future studies are needed in order to demonstrate involvement and relevance of this pathway in ACC.

β -Catenin and *TP53* are frequently mutated in ACC. Both have been suggested as markers of malignancy (33, 34). Furthermore, nuclear β -catenin staining has recently been shown to be associated with a poor prognosis. In our study, we were not able to confirm these findings as only a trend towards shorter survival was observed in primary ACCs with positive nuclear β -catenin. Furthermore, no relationship was found between the presence of a *TP53* mutation and survival. However, due to the nature of the FFPE DNA, some mutations might have been missed in the current study.

Mitotane remains one of the most effective treatments for ACC; however, limitations do exist so that progress in the development and understanding of mechanism of action of this drug is highly important. In order to improve prediction of tumour response to mitotane treatment, we determined the mutation status of *EGFR* and downstream signalling pathways. Although we were not able to demonstrate a relationship between the *EGFR* pathway and mitotane response, we were able to confirm the relevance of the therapeutic threshold of 14 mg/l as serum levels ≥ 14 mg/l were significantly associated with objective response. Furthermore, significantly longer MROS was seen in responding patients. The observation of disappearance of the survival benefit after correction for serum level even strengthens the importance of the therapeutic threshold. As mitotane is the most active treatment available, further progress regarding predictors of response is still needed. Although our study has limitations, such as the use of FFPE material, the use of primary as well as recurrent ACC, we feel that it contributes to the improvement of insight into the pathogenesis and the behaviour of ACC.

In conclusion, no relationship was found between the *EGFR* pathway and mitotane response. Our data suggest that the role of *EGFR* TKIs in ACC is limited. Treatment with *EGFR* MABs on the other hand might be beneficial for a larger group of patients. The possible efficacy of this

therapy in ACC should be evaluated in future trials. Furthermore, our data confirm the importance of the 14 mg/l cut-off level of o,p'-DDD in the therapeutic management of ACC patients as improved survival was demonstrated for patients with serum levels ≥ 14 mg/l independently of the occurrence of response.

Declaration of interest

The 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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Author contribution statement

I G C Hermesen prepared the manuscript, collected the patient data, prepared the TMA, carried out the DNA isolation and mutational analysis and drafted the manuscript. H R Haak participated in the design of the study and drafting of the manuscript. T M A Kerkhofs helped collect patient data in SPSS file and helped perform the statistical analysis. R R de Krijger, W W de Herder, H W Wilmsink, J W A Smit and H J Gelderblom contributed to acquisition of patient data. N F C C de Miranda carried out and interpreted the immunoassays. R van Eijk participated in the sequence alignment (Taqman). T van Wezel R van Eijk participated in the sequence alignment and interpretation of somatic mutational analysis. JM was involved in design, drafting and revising the manuscript critically. All authors read and approved the final manuscript.

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