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An Analysis of Potential Surrogate Markers of Target-Specific Therapy in Archival Materials of Adrenocortical Carcinoma

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Abstract Adrenocortical carcinoma (ACC) is a rare neoplasm but some of the cases are highly malignant. Clinical outcome of the patients with advanced ACC still remained poor or dismal despite recent development of aggressive antitumor therapies. Target-specific therapies have been developed in a number of human malignancies and resulted in therapeutic benefits in some cancer patients. However, these therapies are only effective in the cases in which corresponding targets are expressed in tumor tissues. Therefore, we evaluated expression of potential surrogate markers using immunohistochemistry in archival materials of adrenocortical carcinoma in order to explore the potential application of target specific therapies in ACC in this study. We immunolocalized ten established or potential surrogate markers of target-specific therapies, located in the Ras/extracellular signal-regulated kinase and phosphatidylinositol-3 kinase/Akt pathways, in 41 ACC cases, 54 adrenocortical adenoma (ACA) cases, and five nonpathological adrenal glands and correlated the findings with clinicopathological factors of the patients. Among these markers examined, only epidermal growth factor receptor (EGFR) was significantly more abundant in ACC than in ACA ($P<0.01$). These findings suggest that the agents which specifically inhibit signal transductions through EGFR such as monoclonal antibodies against EGFR are considered to be worthwhile to be attempted in future clinical studies.

Keywords adrenocortical carcinoma · target-specific therapy · EGFR · Ras/ERK pathway · PI3K/Akt pathway · immunohistochemistry

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Introduction

Adrenocortical carcinoma (ACC) is a rare tumor with a reported annual incidence of 0.5 to 2 cases per million [1].

The great majority of the patients with ACC present symptoms related to hormonal activity. Complete surgical excision is still considered the most effective therapy for ACC [1]. However, gradual clinical onset and/or development of these endocrine-related symptoms may be unrecognized for a long time and many of the patients present with advanced clinical stages including distant metastasis. Chemotherapy in combination with mitotane, an anti-adrenocortical agent, has been frequently utilized with varying response rates in these patients [2]. It is, however, true that clinical outcome or prognosis in these cases is still markedly poor or dismal despite development of these aggressive therapies above.

The detailed study of molecular pathways related to tumor growth, metastasis, and infiltration resulted in the development of target-specific therapy. This therapy inhibits or suppresses the proliferation of tumor cells by interfering with specific molecules expressed in the tumor cells, rather than by nonspecifically interfering with rapidly proliferating cells as in conventional chemotherapy. Target-specific therapy has been reported to be more effective than current chemotherapy and less harmful to normal cells in several cancers [3, 4]. However, the presence of these specific targets is prerequisite or mandatory for these agents to exert any therapeutic effects and it is necessary to evaluate the presence or absence of these in tumor tissues, especially in archival tissue materials in cases of recurrent or metastatic cases. The most studied signaling targets in the context of current drug development which may provide candidates of potential therapeutic agents include erbB family, vascular endothelial growth factors (VEGFs) and its receptors, and cytoplasmic kinases lying on Ras/extracellular signal-regulated kinases (ERK) and phosphatidylinositol-3 kinase (PI3K)/Akt pathway [5, 6]. Therefore, in this study, we immunolocalized ten specific molecules related to the Ras/ERK and PI3K/Akt pathway in 41 ACC cases, 54 adrenocortical adenoma (ACA) cases, and five nonpathological adrenal glands and determined which markers were more abundant in ACC than ACA or normal adrenals as a first step toward exploring the possibility of target-specific therapy in the patients with ACC. These markers include vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor receptor 2, epidermal growth factor receptor (EGFR), human EGFR-related 2, extracellular signal-regulated kinases 1/2, Akt, mammalian target of rapamycin (mTOR), p70S6 kinase, S6 ribosomal protein, and 4E binding protein.

Materials and Methods

Adrenals

The total of 95 cases of adrenocortical neoplasms (41 ACC cases, 54 ACA cases) and five nonpathological adrenal glands were retrieved from surgical pathology and autopsy files including consultation materials between 1976 and 2007 from Department of Pathology, Tohoku University Hospital (Sendai, Japan). Clinicopathological features of the cases examined are summarized in Table 1. The data on survival and mutation or copy number are not shown because the information about consultation cases is restricted. All of the specimens had been fixed in 10% formalin at room temperature and embedded in paraffin wax. The diagnosis of ACC was made according to Weiss' criteria of adrenocortical malignancy [7]. In brief, the criteria consist of histological scoring systems evaluating multiple histological parameters obtained from evaluation in hematoxylin–eosin stained histological slides. A tumor is defined as ACC when three or more of the following criteria are met: (1) nuclear grade III or IV, (2) mitotic rate six or more per 50 high power fields, (3) atypical mitosis, (4) clear cells less than 25%, (5) a diffuse architecture pattern in greater than one third of the tumor, (6) confluent necrosis, (7) venous invasion, (8) sinusoidal invasion, and (9) capsular invasion. The criteria are relatively straightforward and considered the most effective standard for diagnosis of adrenocortical malignancy [8]. Research protocols for this retrospective study were approved by the Ethics Committee of Tohoku University School of Medicine (2007-360).

Surrogate Markers

The erbB family and VEGF receptors (VEGFRs) or receptor tyrosine kinases are cell surface transmembrane proteins, which activate intracellular signaling pathways with or without bindings of ligands, resulting in induction or stimulation of cell proliferation, migration, invasion, and other biological activities [9, 10]. The erbB family receptors include epidermal growth factor receptor (erbB1), human EGFR-related 2 (HER2; erbB2), HER3 (erbB3), and HER4 (erbB4) [11, 12]. VEGF family, a putative target of bevacizumab, includes six secreted glycoproteins referred to as VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, and placenta growth factors 1 and 2. VEGFA is a mitogen and survival factor for vascular endothelial cells [13],which interacts with two receptor tyrosine kinases including VEGFR1 and VEGFR2 [10]. VEGFR2 is the major mediator of the effects of VEGFA [13].

ERK1/2 and Akt represent potential biomarkers to assess the clinical activity of lapatinib in human breast adenocarcinoma cell line, BT474 [14–16]. ERK1/2, also known as p42/p44 mitogen-activated protein kinases (p42/p44MAPK), is one of the factors related to the cell proliferation and differentiation [17]. Akt, also referred to as protein kinase B, plays an important role in controlling the balance between survival and apoptosis of the cells [18]. Increased phosphorylation of both Akt and p70S6 kinase (p70S6k) was

Table 1 The summary of clinicopathological findings in all the cases

	ACC	ACA	Nonpathological adrenal gland
Case	41	54	5
Age	0 years 7 months–69 years	21–83 years	0 years 6 months–77 years
Median	37 years	47 years	63 years
Gender			
Male	12 (29%)	20 (37%)	5 (100%)
Female	29 (71%)	34 (63%)	0 (0%)
Cushing syndrome	23	14	
Pre-Cushing syndrome	0	14	
Aldosteronism	5	25	
Virilization	11	0	
Nonfunctioning	5	1	
DOC producing	2	0	

DOC deoxycorticosterone

especially reported in Kaposi's sarcoma, which was most likely the result of the activation of VEGF receptors [15]. mTOR is a sensor for adenosine triphosphate and amino acids and is involved in cellular growth and homeostasis [19]. p70S6k is a direct target of mTOR and plays a role in tumor invasiveness, motility, and angiogenesis. The effect of p70S6k on mRNA translation is indirect via intermediates such as S6RP [20]. In addition, 4E binding protein (4EBP) is the other direct target of mTOR [20]. 4EBP normally binds eukaryotic translation initiation factor 4E. Phosphorylation of 4EBP by mTOR resulted in the activation of cap-dependent translation.

These ten molecules above are therefore considered to represent potential surrogate markers for target-specific therapy at least in advanced ACC cases at this juncture. Therefore, in this study, we immunolocalized ten specific molecules related to the Ras/ERK and PI3K/Akt pathway. No clinical trials with agents targeted these molecules have been reported in the literature at this juncture to the best of our knowledge.

Antibodies for Immunohistochemistry

The properties of antibodies employed in immunohistochemistry were summarized in Table 2. Antibodies for the cytoplasmic molecules detect only activated forms of the protein.

Immunohistochemistry

Immunohistochemical procedures were performed employing polymer signal amplification system with EGFR pharmDx kit

Table 2 The properties of antibodies employed in this study

Product name	Company		Catalog number	IgG typing	Dilution	Positive control
VEGF (A-20)	Santa Cruz Biotechnology, Inc.	(Santa Cruz, CA, USA)	sc-152	Polyclonal rabbit	1/500	Breast carcinoma
VEGF Receptor 2	Abcam Ltd.	(Cambridge, UK)	ab2349	Polyclonal rabbit	1/100	Angiosarcoma
EGFR pharmDx Kit	DakoCytomation	(Glostrup, Denmark)	K1492	Monoclonal mouse	1/1	Control cell slide
c-erbB-2	Invitrogen Corporation	(Carlsbad, CA, USA)	08-1203	Monoclonal mouse	1/1	Breast carcinoma
Phospho-p44/42 MAPK (Thr202/Tyr204)	Cell Signaling Technology, Inc.	(Danvers, MA, USA)	#4376	Monoclonal rabbit	1/100	Colon carcinoma
Phospho-Akt (Ser473)	Cell Signaling Technology, Inc.	(Danvers, MA, USA)	#4051	Monoclonal mouse	1/200	Lung carcinoma
Phospho-mTOR (Ser2448)	Cell Signaling Technology, Inc.	(Danvers, MA, USA)	#2971	Polyclonal rabbit	1/50	Lung SCC
Phoapho-p70 S6 Kinase (Thr389)	Cell Signaling Technology, Inc.	(Danvers, MA, USA)	#9206	Monoclonal mouse	1/1,000	Breast carcinoma
Phospho-S6 Ribosomal Protein (Ser240/244)	Cell Signaling Technology, Inc.	(Danvers, MA, USA)	#2215	Polyclonal rabbit	1/100	Breast carcinoma
Phospho-4E-BP1(Thr70)	Cell Signaling Technology, Inc.	(Danvers, MA, USA)	#9455	Polyclonal rabbit	1/50	SCCHN

SCC squamous cell carcinoma, *SCCHN* squamous cell carcinoma of head and neck

(DakoCytomation, Glostrup, Denmark) for EGFR, the streptavidin–biotin amplification method using an EnVision + kit (DakoCytomation) for mTOR, and a Histofine kit (Nichirei, Tokyo, Japan) for the others. Three-micron slices obtained from paraffin-embedded specimens were deparaffinized. Antigen retrieval was performed by heating the slides placed on glue coated glasses in a microwave at 500 W for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) for VEGFR2, ERK 1/2, Akt, mTOR, p70S6k, S6RP, and 4EBP and then allowed to cool down for 1 h at room temperature. Dilutions of the primary antibodies used in this study were determined based on the results of previously reported studies as well as the manufactures' recommendations (Table 2) [21–28]. The antigen–antibody complexes were visualized with 3, 3′-diaminobenzidine solution [1 mM 3, 3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6)] and counterstained with hematoxylin. Normal rabbit or mouse immunoglobulin G was also used in place of the primary antibodies as a negative control. Tissues used as a positive control were summarized in Table 2.

The cells were considered positive, when immunoreactivity was detected in the cytoplasm for VEGFA, ERK 1/2, Akt, mTOR, p70S6k, S6RP, and 4EBP and on cell membrane for VEGFR2, EGFR, and HER2. Immunoreactivity was evaluated by semiquantitative grading according to the percentage of immunopositive cells, and all of specimens were tentatively categorized into six groups (0, 0%; 1, 1% to 5%; 2, 6% to 25%; 3, 26% to 50%; 4, 51% to 75%; 5, 76% to 100%). The findings were evaluated by two of the authors (M.N. and H.S.) and both inter- and intraobserver differences were less than 5%.

Statistical Analysis

The data obtained in this study were assessed by Z test for the statistical comparison between ACC and ACA cases. The possible correlations between the status of immunoreactivity and ages at diagnosis or scores of Weiss' criteria of adrenocortical malignancy in ACC were examined by Spearman's rank correlation. The possible correlations between the status of immunoreactivities and other clinicopathological features in ACC were examined using χ^2 test. All statistical analyses in this study were performed using StatMate III (ATMS, Tokyo, Japan). Statistical significance was determined as less than 0.05 level of probability ($P<0.05$).

Results

Results were summarized in both Fig. 1 and Table 3.

Immunohistochemistry in Nonpathological Adrenal Glands

VEGFA, VEGFR2, ERK1/2, Akt, mTOR, and S6RP immunoreactivities were abundant in adrenocortical parenchymal

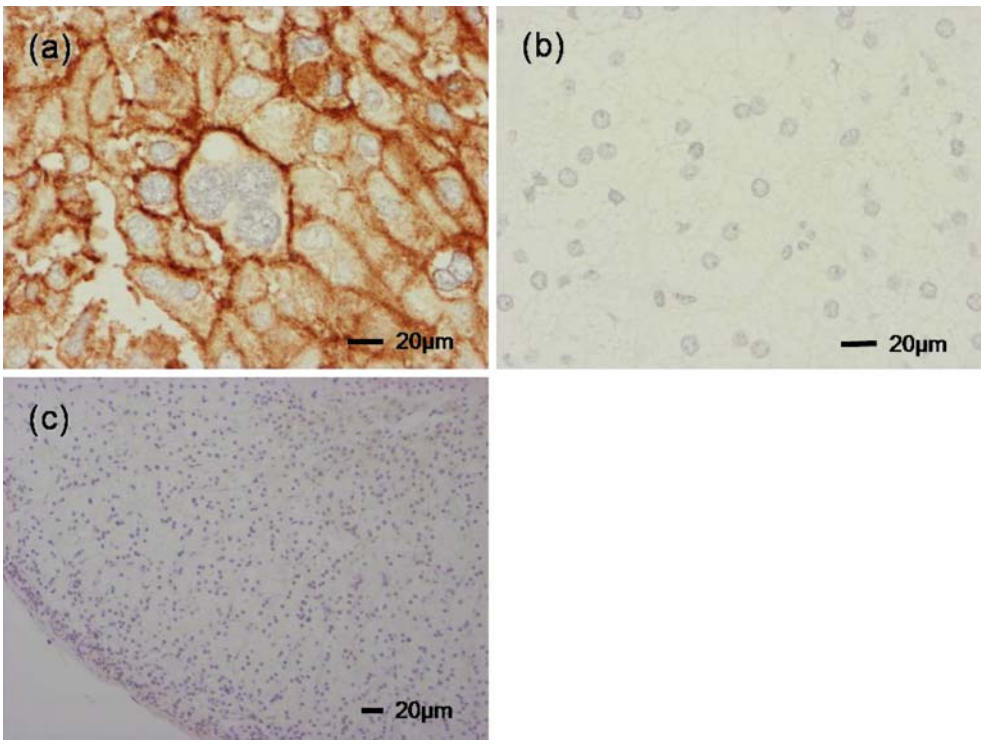

Fig. 1 Immunohistochemistry for EGFR. Expression of EGFR was significantly higher in ACC (**a**) than ACA (**b**) or nonpathological adrenal gland (**c**). Original magnifications **a**, **b** ×400, **c** ×100

Table 3 Mean ± standard deviation of expression in the specimens

	ACC	ACA	Nonpathological adrenal gland
VEGFA	2.05±1.22	2.15±1.51	5.00±0.00
VEGFR2	0.39±0.97	0.57±0.90	3.40±2.19
EGFR	2.44±1.98	1.43±1.22	0.30±1.30
HER2	0	0	0
ERK1/2	0.15±0.36	0.26±0.52	2.80±1.64
Akt	0.56±0.92	0.52±1.02	4.20±1.30
mTOR	0.41±1.00	0.74±1.10	4.60±0.55
p70S6k	0.15±0.42	0.28±0.45	1.80±1.30
S6RP	0.34±0.69	0.46±0.66	4.60±0.89
4EBP	0.22±0.57	0.28±0.49	1.20±1.64

cells. EGFR, p70S6k, and 4EBP immunoreactivities were sporadically detected in adrenocortical parenchymal cells. HER2 immunoreactivity was not detected at all in all the cases examined. Only ERK1/2 expression was detected in adrenal medulla in one case and all other immunoreactivities examined in this study were negative in adrenal medullar.

Immunohistochemistry in ACC and ACA

EGFR immunoreactivity was significantly more abundant in ACC than ACA or nonpathological adrenal gland ($P<0.01$; Fig. 1a–c). There were no significant differences in the status of immunoreactivities of other molecules examined between ACC and ACA.

In the cases of ACC, the correlation between the status of immunoreactivity of these molecules and age at diagnosis, gender, hormonal features, or score of Weiss' criteria were examined, respectively. VEGFA immunoreactivity was significantly more abundant in the cases with higher score of Weiss' criteria ($P<0.01$; Fig. 2d, e) and in the cases with positive findings in mitotic rate, atypical mitosis, or venous invasion, respectively ($P<0.05$, $P<0.05$,

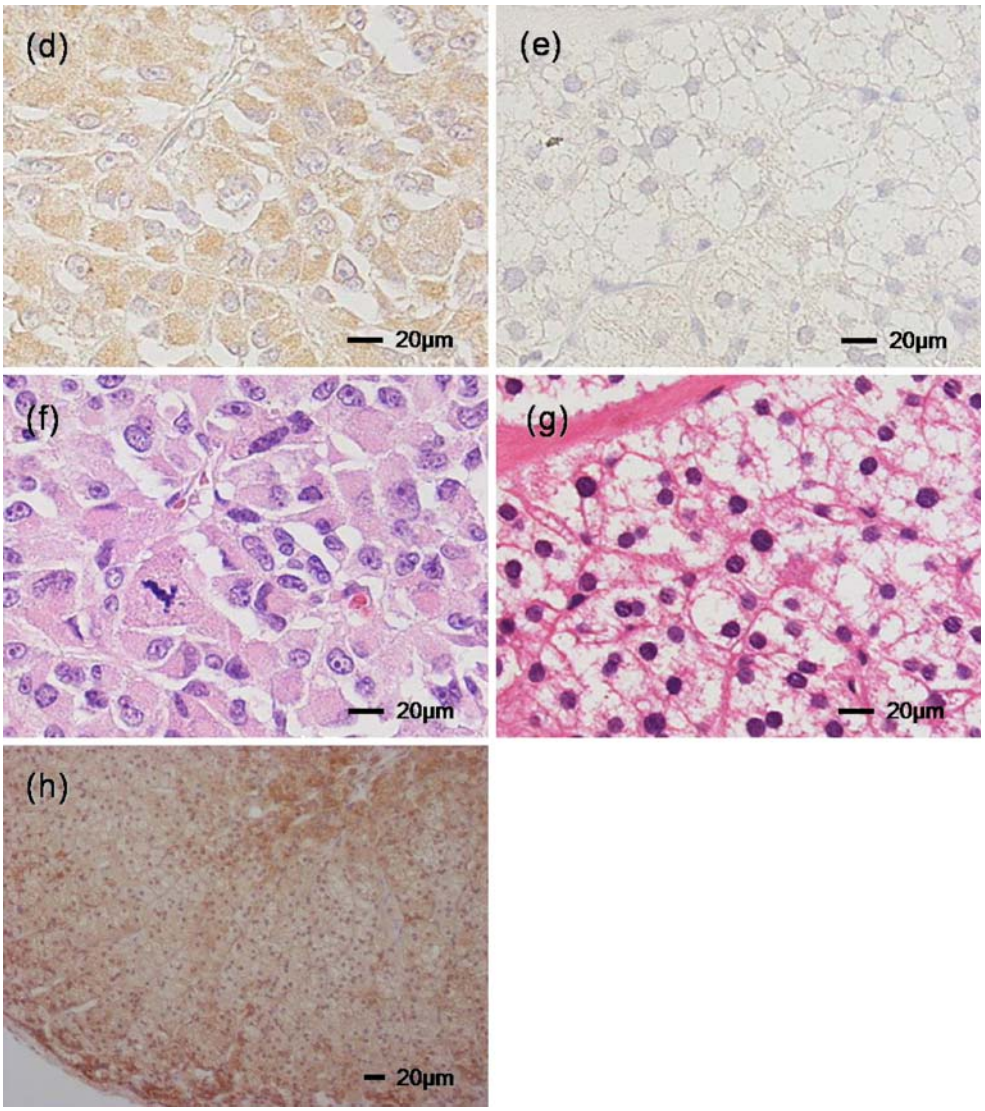

Fig. 2 Immunohistochemistry for VEGFA (**d**, **e**, **h**) and hematoxylin–eosin staining (**f**, **g**). **d**, **f** A case of ACC with Weiss' criteria of 9. **e**, **g** A case of ACC with Weiss' criteria of 3. Expression of VEGFA was significantly higher in cases with high score of Weiss' criteria (**d**) than in cases with low score (**e**). But high expression of VEGFA was detected in nonpathological adrenal cortex (**h**). Original magnifications **d**–**g** ×400, **h** ×100

$P<0.01$). No significant correlations were detected between the status of other molecules and the features above.

Discussion

In this study, we immunolocalized the specific molecules located on the Ras/ERK and PI3K/Akt pathway in adrenocortical neoplasms. The status of EGFR immunoreactivity was significantly more abundant in ACC than ACA or nonpathological adrenal gland. EGFR expression was also reported to be markedly elevated in ACC [29], which is consistent with results of our present study. Cetuximab, gefitinib, and erlotinib, agents targeting on EGFR, are clinically available and have been reported to be administered in the patients with cancer, like nonsmall cell lung cancer [4]. The relative abundance of EGFR in ACC suggests that these agents may provide clinical benefits. However, recent studies also demonstrated that, in contrast to the status of HER2 expression in the tumor cells, EGFR expression itself is by no means a robust predictor of clinical response to target therapies against EGFR, because some patients with EGFR overexpressing tumors have been reported to be refractory to EGFR inhibitors, while on the other hand, patients with a low degree of EGFR expression still respond to EGFR antagonists [5]. In addition, EGFR gene mutations have been reported in the patients with nonsmall cell lung cancer and the status of these mutations may be correlated with the clinical responses to the tyrosine kinase inhibitors such as gefitinib [30]. In addition, recent results of CRYSTAL phase III trial also demonstrated that k-ras wild-type status increased progression-free survival and response rate in patients with metastatic colorectal cancer receiving cetuximab therapy or anti-EGFR antibody. It is also interesting to note that results of previous studies of k-ras mutations demonstrated the absence of mutations in the case of ACC [31]. Lin et al., however, reported that mutations of the k-ras gene were detected in adrenocortical tumor but their study did not include any patients of ACC [32]. These findings all suggest the potential response to the treatment with cetuximab in the patients with ACC. It is true that further investigations are required to apply these tyrosine kinase inhibitors for the therapy toward the ACC patients associated with EGFR abnormalities in the carcinoma cells including point mutations or other genetic abnormalities of EGFR itself. However, at least, results of our present study suggest that anti-EGFR therapies for the patients with adrenocortical carcinoma are considered worthwhile to pursue further investigations.

The criteria of Weiss are a universally well-established or validated histopathological scoring system to make a definitive final diagnosis of ACC in surgically resected specimens of adrenocortical tumors. VEGFA expression in the tumor cells was positively correlated with scores of Weiss' criteria. The patients with ACC were reported to be associated with markedly high circulating VEGF levels, significantly higher than the patients with other adrenocortical disorders [33]. In our present study, the status of VEGFA immunoreactivity was positively correlated with some score of Weiss' criteria. In particular, the status of VEGFA immunoreactivity in carcinoma cells was significantly higher in the cases associated with venous invasion, high mitotic rate, or atypical mitosis. Weiss reported that among the nine histological factors in his criteria, mitotic activity, especially with atypical forms, and venous invasion were correlated best with metastasizing or recurring behavior of the resected adrenocortical tumors [7]. Therefore, the presence of VEGFA in carcinoma cells is considered to be associated with aggressive biological behavior in the patients with ACC. However, the difference of VEGFA immunoreactivity between ACC and ACA was not necessarily statistically significant, and its relatively abundant expression was also detected in nonpathological adrenal cortical parenchymal cells and it awaits further investigations for clarification.

There were no significant differences in the status of immunoreactivities between ACC and ACA or among the different histopathological parameters in ACC in other molecules studied. Abundant immunoreactivity for HER2, ERK1/2, Akt, mTOR, and p70S6k has been reported in several types of cancer cell lines or tissues [34, 35]. The status of the immunoreactivity for HER2 and ERK1/2 was correlated with the presence of mutations in some malignancies [36, 37]. Therefore, the analysis of abnormalities of these molecules at the DNA levels may provide further information regarding the possibility of potential targets in the patients with ACC.

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Conflict of Interest The authors indicated no potential conflict of interest and no financial interests relating to the material in the manuscript.

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