How good are the current models of adrenocortical carcinoma for novel drug discovery?

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1. Introduction

During the last decade, significant advances have been made in the definition of the molecular mechanisms underlying the onset and progression of adrenocortical carcinoma (ACC). A better understanding of ACC tumorigenesis has come from the identification of several genetic and molecular drivers of this malignancy thanks to an extensive profiling analysis of ACC tumors. Unfortunately, the development of novel therapeutic options has been hampered by the lack of *in vitro* and *in vivo* preclinical models recapitulating the entire spectrum of ACC heterogeneity, molecular features, tumor microenvironment and sensitivity to the available treatments. The recent establishment and implementation of novel ACC cell lines, genetically engineered mouse models, patient-derived ACC xenografts (PDX) in mice and emerging preclinical *in vivo* models offer novel experimental possibilities for drug discovery.

2. Preclinical research models

2.1 *In vitro* cell lines

Until recently, the NCI-H295 and its H295R subclone were the only differentiated human ACC cell line available [1]. Their ability to produce steroids from different zones of the adrenal cortex after stimulation with effective agonists made them as important models for ACC research. Those cell lines were exploited to dissect the molecular mechanisms underlying adrenocortical tumorigenesis, whose understanding is necessary for novel drug discovery [2].

In 2016, Hantel and coworkers first successfully established an ACC PDX model (see section 2.2). In order to broaden the existing toolbox of ACC cell lines they also explanted xenografts pieces for *in vitro* culturing. The established MUC-1 cells expressed adrenocortical markers and maintained *in vitro* hormonal activity [3]. MUC-1 displayed drug resistance against the clinical gold standard therapy etoposide,

doxorubicin, cisplatin and mitotane (EDP-M), which was not observed for the NCI-H295R model [3].

In 2018, two new *bona fide* PDX-derived ACC cell lines, called CU-ACC1 and CU-ACC2, have been established [4]. CU-ACC1 derived from a sporadic ACC metastasis to the perinephric region, whereas CU-ACC2 originated from an ACC liver metastasis in a patient with Linch syndrome. CU-ACC1 cells secreted cortisol but not aldosterone, while CU-ACC2 were no-secretory and exhibit a loss of *MSH2* (consistent with the known germline mutation of the patient causing Lynch syndrome) [4]. They have been both already used to explore novel therapeutic targeted therapies in ACC [5].

The latest ACC cell line developed is the JIL-2266. Of note, those cells have been transferred directly to the cell culture, without a previous passage as xenograft in nude mice nor the presence of feeder cells [6]. Comparing to NCI-H295R, JIL-2266 cells revealed a low steroid secretion and are significantly less sensitive to mitotane [6]. They present a high mutational burden associated with a germline mutation in the *MUTYH* gene encoding a protein involved in DNA glycosylase-initiating base-excision repair. JIL-2266 cells thus represent a novel pre-clinical ACC model to study adrenal oxidative DNA damage.

2.2 *In vivo* models

The early genetically engineered mouse models used in modeling ACC were generated with either activation of Wnt/ß catenin signaling cascade or overexpression of IGF2, two important pathways activated in human ACC [7-9]. However, in all those models the genetic alterations in both pathways failed to trigger malignant adrenocortical tumorigenesis. More recently, Batisse-Lignier *et al.* have developed a transgenic mice model with adrenocortical specific expression of SV40 large T-antigen (AdTAg mice) to evaluate the oncogenic potential of p53/Rb inhibition in the adrenal gland [10]. All mice developed large adrenal carcinomas with metastasis to the liver and lungs [10]. The use of this model has allowed to prove the efficacy of rapamycin dependent mTORC1 inhibition on tumor development [10].

NCI-H295R cell line xenografts have been established and solid tumors were locally measurable, however no development of metastasis was observed in those tumor-bearing mice (reviewed in [11]). Of note, subcutaneous NCI-H295R xenografts have been employed upon genetic modification for preclinical testing of already existing therapies and to implement new pharmacological approaches (reviewed in [11]). Recently, a mouse xenograft model of metastatic ACC established by intrasplenic injection of NCI-H295R cells followed by splenectomy has been shown to produce liver metastasis, representing a new preclinical model for drug screening in the advanced-stage disease [12].

PDX models in athymic mice that reflected the diversity and heterogeneity of ACC patients' tumors have been recently described. The first PDX of pediatric ACC (SJACC3) has been developed by Pinto *et al.* [13] via the implantation in immunocompromised mice of an adrenal mass incidentally found and resected from an 11-year-old boy bearing the germline *TP53* p.G245C mutation. The screening of this xenograft for drug responsiveness showed that cisplatin had a potent antitumor effect, whereas etoposide, doxorubicin and a panel of commonly employed chemotherapeutics had little or no antitumor activity [13]. Of note, the topoisomerase inhibitor topotecan showed cytostatic effects with tumor growth impairment, indicating this drug as a potential new treatment for pediatric ACC [13]. Unfortunately, it was not possible to establish a cell line for complementary *in vitro* experiments from this xenograft.

The first adult ACC PDX model MUC-1 along with the corresponding cell line (see 2.1), derived from a metastatic ACC neck lesion of an adult patient [3]. MUC-1 tumor analysis revealed highly vascularized, proliferating and SF-1 positive xenografts [3].

Two further PDX models, CU-ACC1 and CU-ACC2 and the corresponding cell lines (see 2.1) have been recently established via subcutaneous implantation of patient tumor tissues in nude mice [4]. Their adrenocortical origin has been confirmed and the immunohistological features of the derived tumor tissues matched with those of patients' tumor [4].

Recently, Lang *et al.* described the first humanized CU-ACC2-M2B ACC PDX mouse model to examine the *in vivo* effects of the PD-1 inhibitor pembrolizumab on tumor growth as well as the changes in infiltrating lymphocytes and immune cells in the peripheral lymph organs respect to the matching patient [14]. Mice treatment with pembrolizumab showed a significant tumor inhibition respect to the controls, which correlated with an increased tumor infiltrating lymphocyte activity [14]. Those effects were paralleled by a remarkable response of the CU-ACC2 patient to the treatment, with a reduction in the size of target lesions and no new metastatic lesions. Overall, these data suggest that humanized ACC PDXs represent a useful model to define mechanisms and biomarkers involved in the response and the resistance to immunotherapy.

New non-mammalian based models have also emerged as novel preclinical tools in ACC research. Chicken embryo chorioallantoic membrane (CAM) models have been used for the evaluation of the metastatic potential of NCI-H295R cells overexpressing SF-1 in a doxycycline-dependent fashion [15]. Moreover, zebrafish embryos xenografted with ACC cells have been recently exploited as a model to study the *in vivo* cytotoxicity of abiraterone acetate, zebrafish emerging as a useful tool for drug screening in human ACC [16].

Expert opinion

ACC is a rare disease that results in heterogeneous clinical phenotypes and molecular genotypes, with no curative treatments available up to date and a poor survival rate at five years. Unfortunately, the paucity of preclinical research models has severely limited the development of targeted therapies for ACC patients. The disappointing results concerning the clinical translation of novel therapeutic approaches for ACC patients have highlighted the inadequacy of the currently employed tumor models, which poorly represent ACC heterogeneity, being thus non-predictive of the clinical suitability of the novel pharmacological strategies.

Recent studies have given deeper insight into the genomic and the genetic landscape of ACC, revealing specific molecular subtypes of ACC tumors with high mutational rates, are predictive of a very poor prognosis [17]. With the enlarged classification of ACC tumor subtypes and the heterogeneity of the disease, the need of models to shed light on the molecular pathways leading to malignant transformation and to test the efficacy of novel drugs has become more urgent.

Indeed, thanks to novel *in vitro* techniques based on the use of ROCK inhibitors and feeder cells and the growing experience in establishing PDXs, novel ACC cell lines and matched PDX models have been developed providing new tools to investigate the molecular mechanisms underlying ACC pathogenesis and to evaluate patient-specific therapeutic options.

The development of ideal preclinical models that mimic the heterogeneity of patients' tumors, defined at an "omics" or at a "multi-omics" single-cell level, represents a challenge for the future of personalized ACC therapies. Moreover, further advances in ACC PDX humanized mouse models will allow to better understand the tumor microenvironment and the response to immunotherapy and to guide more personalized clinical treatment decisions. The functional analysis of tumor drug responses will need a fine interconnection between the choice of the so-called cancer "avatar" models and the time, scale and cost of drug screening to hope for a precision targeted therapy for ACC patients. The availability of multiple preclinical models such as ACC tumor cell lines, mice PDXs, emerging zebrafish PDXs and so far unexplored Patient Derived Organoids models will offer valuable tools for *in vitro* screening and *in vivo* testing of novel drugs to treat ACC.

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