Are we failing in treatment of AdrenoCortical Carcinoma? Lights and shadows of molecular signatures.

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Abstract

Adrenocortical carcinoma (ACC) is a rare disease whose prognosis is reported to be invariably poor mainly because of lack of effective and durable treatments, which makes this cancer an orphan disease.

Recent studies of functional genomics and, in particular, the results from The Cancer Genome Atlas (TCGA) project have shed new light on the molecular biology of ACC while improving our comprehension of potentially targetable alterations.

This review is focused on the shift of methodology in preclinical pharmacology and drug selection for ACC patients which is rapidly moving from a heuristic approach to a dynamic, deterministic one.

Introduction

Adrenocortical carcinoma (ACC) is a rare tumor derived from the adrenal cortex, with an estimated incidence between 0.7 and 2.0 per million population per year. To date, radical surgery at experienced centers still remains the only potential curative treatment for those patients with early stage disease and for patients with locally advanced ACC responding after neoadjuvant treatment. However, 30%-70% of radically operated patients will recur within two years and the expected overall survival in this patient population is less than 20% at 5 years [1]. These data explain why, in published literature, ACC is largely and invariably referred to as a poor prognosis neoplasm.

The disappointing outcomes of ACC are in part the consequence of intrinsic biological features that translate into specific clinical patterns such as endocrinological syndromes, metabolic alterations, immune function defects, that complicate the picture of the primary tumor and secondary metastases [2]. However, some of these features are also shared with other human cancers and cannot fully justify the poor prognosis of ACC. In a recent survival analysis from the TCGA-PanCancer project, ACC displayed an intermediate prognosis compared to several other neoplasms [3]. The main reason of the general belief of "failure" in the ACC management is mostly linked with an overall low response to current medical therapies including molecular target therapy [2] and immunotherapy [4, 5], that makes ACC an orphan disease.

Recent comprehensive genomic studies and, in particular, the results of the Cancer Genome Atlas (TCGA) project have transformed our knowledge of ACC pathobiology and have identified cellular programs that can be pharmacologically targeted.

Thus, this review is focused on two main issues: 1 - the methodological limits of previous and current therapeutic approaches; 2 – new issues in preclinical therapeutic target identification and drugs testing as a result of functional genomics studies.

Historical evolution of medical treatment of ACC

Mitotane

Clinical experience with mitotane was corroborated with studies from the early 60' indicating that the drug had specific adrenolytic activity in the zona reticularis and zona fasciculata of the adrenal cortex and that it could therefore control primary and secondary Cushing syndrome by reducing cortical steroidogenesis [6]. Sbiera et al. recently demonstrated that the mitotane adrenolytic effect is mediated by a down-regulation of sterol-O-acyl-transferase 1 (SOAT1) leading to increase of toxic intracellular free cholesterol, oxysterols and fatty acids [7]. Evidence of an antiproliferative effect of mitotane on adrenocortical cells [8,9] and the observation that mitotane could in part revert P-glycoprotein-mediated chemoresistance [10] further contributed to the clinical development of this drug whose exact mechanism of action is yet not fully understood and remains object of active investigation [11]. Although the bulk of clinical evidence relies on retrospective studies [12,13], mitotane remains the only FDA/EMA-approved drug for the treatment of ACC in either adjuvant or metastatic settings. Ongoing prospective randomized studies are evaluating the role of mitotane in improving disease-free survival of radically operated patients with high versus low risk of relapse [14].

Chemotherapy

Chemotherapy of ACC during the 90' was essentially based on an empiric selection of available antiblastic agents often used in other neoplasms and applied by analogy in ACC [15]. For example, the doublet cisplatin and etoposide, the reference regimen for small cell lung cancer (SCLC) [16], was also tested in ACC with similar schedule. From this process it emerged clearly the role in ACC patients of drugs with alkylating activity such as cisplatin [17-18] and streptozotocin [19,20] or drugs interfering with DNA-topoisomerase activity such as etoposide, teniposide, irinotecan [21,18,22,23] and doxorubicin [20,24-26]. All of these classes of antineoplastic agents were used alone or in poly-

chemotherapy schedules with or without mitotane [20, 23,26,27]. The EDP-M (Etoposide, Doxorubicin, Cisplatin and Mitotane) regimen was introduced in 1992 [28] and subsequently developed in Italy in the following 13 years [29, 30]. This scheme was designed empirically by simply including all the drugs that had demonstrated some activity against ACC at the time when the regimen was ideated. In the randomized phase 3 clinical trial FIRM-ACT in advanced ACC, EDP-M obtained a progression-free survival advantage and a greater objective response rate with respect to the comparator streptozotocyn plus mitotane [20,26]. On the basis of the results of the FIRM-ACT trial the EDP-M regimen represents the first-line treatment for advanced ACC currently recommended by international guidelines [1]. Other cytotoxic agents included analogues of DNA bases such as gemcitabine, fluorouracil and capecitabine [31,32] or taxanes [33] or trofosfamide [34] (see Table 1 for most important studies of cytotoxic chemotherapy involving more than 10 ACC patients in first- or following-line of treatment).

Lessons learned from these early trials can be summarized as follows:

- ACC is characterized by a medium-low sensititvity to alkylating drugs (cisplatin, streptozotocin), drugs interfering with DNA-topoisomerase (etoposide, doxorubicin, irinotecan) or analogues of DNA bases (gemcitabine, capecitabine);
- Objective response rates are in the range of <10%-48% but are short-lived: in advanced ACC, PFS is invariably less than 6 months; this reflects intrinsic and acquired chemoresistence mediated by P-glycoprotein overexpression;
- There are not validated markers predictive of chemosensitivity: Ki67 index [35], topoisomerase-2α and thymidylate synthase [36] are putative markers of different sensitivity to chemotherapy. However, identification of a precise cut-off level, robust standardized analytical methodology and validation studies are lacking;

Target therapy

The paradigm of target therapy at the beginning of the 2000' was represented by imatinib in gastro-intestinal stromal tumors (GIST) and chronic myeloid leukemia (CML) [37]. In a simplified view, the inhibition of a specific molecular pathway over-functioning in a single disease as a result of oncogenes constitutive activation, was the underlying methodology for molecular target therapy and opened the path to personalized therapy. Thus, the knowledge of mutations or amplifications at the single gene level and overexpression of a single protein at tissue level was the rational prerequisite for target therapies. Following this methodology, at the beginning of 2000s the three most frequent molecular alterations in ACC were the Insulin-like Growth Factor 2 (IGF-2) pathway, overexpressed in >90% of ACC [38,39], the Wnt/beta catenin pathway overexpressed in 40%-66% of cases [40] and the p53 apoptosis/Rb pathway affected in 20%-45% of cases [41,42].

While therapeutic strategies against p53/Rb and Wnt/beta catenin were not available at that time, most of the efforts were addressed toward inhibition of EGFR, VEGFRs, IGFRs with monoclonal antibodies (mAb) or multi-target tyrosine kinase inhibitors (TKi). In ACC, this approach gained at best minor responses and disease stabilization in most trials. The most striking example of failure of this kind of approach to target therapy in ACC is represented by studies involving the IGF2/IGF-1R signaling pathway.

In preclinical studies, targeting IGF-1R with pharmacological agents resulted in the growth inhibition of both ACC cell lines and human ACC xenograft models [43-45]. Furthermore Given the body of evidence from initial molecular studies and from preclinical models, targeting the IGF-2/IGF receptor signaling pathway appeared a promising strategy. However, in the phase III GALACCTIC trial, the largest trial of target therapy in advanced ACC with linsitinib, an oral small molecule inhibitor of IGF-1R, disease control (including 3 partial responses) at 3 months was obtained in only 14 of 90 linsitinib-treated patients (15.6%) and the median PFS was less than two months [46].

Lessons learned from target therapy trials can be summarized as follows:

- Overexpression of a molecular pathway as a result of a single genetic alteration is a minimum but not sufficient requirement for making a good therapeutic target;
- Early target therapy trials lacked identification of targets linked to the multiple driver molecular alterations observed in ACC;
- In the context of an overall non-responding setting there are few responding patients indicating inter-patient heterogeneity;
- The possible interference with previous or concomitant mitotane therapy could have influenced the negative results obtained by molecular target agents in ACC. Thus, negative trials could have erroneously stopped the development of potentially active drugs in this disease.

Functional genomics applied to ACC

In less than five years, multiplatform comprehensive genomic studies have revolutionized the knowledge of the pathobiology of ACC by integrating data from recurrent somatic mutations with derived molecular programs [47-49]. It is beyond the scopes of this review to discuss in detail the bulk of data from those works and we invite interested readers to consult some excellent reviews that have recently been published on this issue [50-52].

The TCGA project used a common algorythm of ten analysis working groups (AWG) for all of the 33 different neoplasms included. These working groups analyzed important biological, clinical and therapeutical aspects in the oncogenic process including: cell-of-origin analysis, somatic and germline mutations and their interactions, pathogenetic driver genes, aneuploidy, recurrent fusions, new tools for imaging interpretation, immune-response programs and clinical endpoints analysis. Thus, results from each TCGA substudy can be compared with those of other neoplasms and re-analyzed into a broader pan-cancer context (namely the PanCancer Atlas Project) [53].

The ACC-TCGA study included data from 91 patients with primary ACC for whom clinical annotations were available. According to the above-cited schema, results from the ACC-TCGA study are summarized in Table 2.

The ACC-TCGA study extended results from the ENSAT study by Assié et al. [47] and improved the prognostic stratification of ACC patients identifying three different molecular subtypes paired with distinct survival outcomes. These breakthroughs also started the process of matching clinical/molecular profiles of ACC patients with specific therapeutic programs and suggest possible explanations of therapeutical failures in the past. For example, the ACC-COC1 is characterized by a low grade of aneuploidy, better survival outcome and high expression of the IGF2 pathway. Patients falling in this category would likely be more responsive to anti IGF2/IGF-1R compounds. On the opposite, patients in the COC3 group characterized by mutations involving the cell cycle and DNA damage repair machinery would probably better respond to polychemotherapy.

One important conceptual issue is if metastases from ACC share the same genomic alterations of the corresponding primary tumor and, more importantly, if their genotype confers different sensititvity or resistance to therapy. A recent study from Gara et al. investigated genomic heterogeneity in 33 metastatic ACCs. Investigators demonstrated that a significant inter-patient but not intra-patient heterogeneity exists. Of note, metastatic ACC has a 2.8 times higher mutation rate compared to the primary ACC and the Ingenuity® pathway analysis identified novel signaling pathways deregulated in metastatic but not in primary ACC, including four targets potentially druggable with available drugs (ERBB4, retinoic acid receptor, GPCR, PDGFR) [54].

In a recent study by Lippert et al., researchers attempted to perform prognostic stratification of 107 patients by means of multiplatform genomic analyses. This study represents the first attempt of generating molecular prognostic classes from routinely available clinical samples and underlines the

importance of integrating molecular information with clinical data such as the GRAS (Grade, R status, Age and Symptoms) parameters [55].

New topics in preclinical anticancer drug selection

Lessons learned from treatment evolution in ACC indicate that most patients only transiently respond or do not benefit at all from chemotherapy, target therapy and immunotherapy because of a limited knowledge of candidate essential targets. Therefore, the drawing of a cancer dependency map appears as a necessary element in order to identify tumor vulnerabilities and enable precision medicine [56]. As functional genomic studies are expanding the scale of candidate alterations (in terms of 1,000-10,000 per experiment), researchers will have to deal with very large datasets and synthetize relevant information. Thus, analytical resources enabling to prioritize therapeutic targets will become increasingly important in the process of drug selection and development. Among the screening techniques, the CRISPR-Cas9 genome editing appears to be promising particularly in identification of mutations that confer drug resistance [57,58]. In a recent study, CRISPR-Cas9 technology was used to create genome-scale screens in 324 human cancer cell lines from 30 cancer types and to prioritize cancer therapeutic targets matching them with commercially available drugs [59]. Despite rising enthusiasm on functional genomics, a note of caution should be addressed. In the French study PROFILER, that aimed at molecular profiling of patients with a variety of solid neoplasms, only 6% of them received the matched target therapy and 0.9% obtained an objective response [60]. It is probable that the design of future clinical trials in ACC will have to change as well to deal with large datasets coming from multiplatform genomic analyses .Some of the emerging issues of this methodology shift include identification of new predictive rather than prognostic factors, adaptive rather than static clinical trials and incorporation of liquid biopsies to deal with temporal heterogeneity [61, 62].

Closing remarks

Functional genomics revealed the high degree of biologic heterogeneity acting on ACC in terms of number of driver molecular alterations and in terms of evolution of these alterations over time. Discrepancies between overexpressed pathways and driver molecular alterations can, in part, explain previous failure of target therapy in this disease and highlight the limits of early clinical trials. Many of these constraints will hopely be overcome by emerging genomic techniques to identify cancer dependencies and select priority drug candidates. However, many of these techniques are not yet ready for clinical routine use. In the next future we will have to concentrate on interpreting molecular analyses in the light of clinical patterns that remain, to date, the most reliable picture of each single ACC patient.

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Table 1. Summary of published clinical trials of cytotoxic chemotherapy in patients with advanced ACC. Clinical outcome is reported as best response observed and median progression-free survival (PFS) from treatment. Trials are ordered by year of publications.

Author/year	No. of patients	Treatment	Best response (%)	Median PFS (months)	Ref.
Decker, 1991	15	Doxorubicin +/- Mitotane	PR+SD 19	14	22
Schlumberger, 1991	13	5-Fluorouracil, Doxorubicin, Cisplatin	CR 7; PR 21.4; SD 14.2	7.6	24
Bukowski, 1993	36	Cisplatin + Mitotane	CR+PR 30	11.8	17
Bonacci, 1998	17	Cisplatin + Etoposide	CR 16.6; PR 16.6; SD 11	13.5	21
Williamson, 2000	45	Cisplatin + Etoposide	PR 11; SD NR	10	18
Khan, 2000	12	Streptozocin + Mitotane	PR 25; SD 8.3	9	19
Baudin, 2002	12	Irinotecan	SD 25	<3	63
Abraham, 2002	36	Doxorubicin + Vincristin + Etoposide	CR 2.8, PR 20, SD NR	13.5	25
Kahn, 2004	10	Vincristine + Teniposide + Cisplatin + Cyclophosphamide	PR 18, SD 63	22	23
Berruti, 2005	71	Etoposide + Doxorubicin + Cisplatin + Mitotane	CR 7; PR 13.8; SD 27.8	18	26
Sperone, 2010	28	Gemcitabine + Capecitabine/5-Fluorouracil	CR 3.5; PR 3.5; SD 27.8	5.3	31
Hermsen, 2011	91	Mitotane and different cytotoxic drug	CR 1.1; PR 17.6; SD 27.5	<25	27
Fassnacht, 2012	125	Streptozocin + Mitotane	PR 11; SD 34	2.2	20
Fassnacht, 2012	127	Etoposide + Doxorubicin + Cisplatin + Mitotane	PR 29; SD 53	5.6	20
Urup, 2013	17	Cisplatin + Docetaxel	PR 21; SD 32	3	33
Kroiss, 2016	21	Trofosfamide	SD 14	21	34
Henning, 2017	145	Gemcitabine + Capecitabine	PR 4.9; SD 25	3	32

Table 2. Descriptive summary of key findings from the ACC-TCGA study [41-44]. Data are presented as result of the single analysis working groups (AWG).

Type of analysis in TCGA project	**Results in ACC**		**Comment**
Aneuploidy (copy number changes)	Quiet pattern (9%): diploid genome, no focal gains or losses; no WGD (no TERT overexpression). Better prognosis.		ACC genome is frequently hypodiploid compared to other tumors (ACC 31% vs others 1%). WGD is a marker of tumor progression.
	Chromosomal pattern (61%): highest frequency of chromosome gains and losses; WGD in 51% of cases (TERT overexpression in WGD+ cases). Intermediate prognosis.		
	Noisy pattern (30%): high frequency of arm breaks, losses and gains; WGD in 68% of cases (TERT overexpression in WGD+ cases). Worse prognosis.		
Somatic mutations in putative driver genes (frequency >10%)	No recurrent mutations (≈20%).		In the integrated genomic landscape of ACC the most frequently altered genes mutated gene were: *TP53 (21%), ZNRF3 (19%), CDKN2A (15%), CTNNB1 (16%), TERT (14%), PRKAR1A (11%)*
	Mutations leading to cell cycle/apoptosis dysregulation (≈45%): *TP53, CDKN2A, RB1, CDK4, CCNE1, MDM2*		
	Mutations leading to Wnt/beta-catenin activation (≈40%): *CTNNB1, ZNRF3, APC, MEN1*		
	Mutations leading to histone/chromatin remodeling (≈20%): *MLL, MLL2, MLL4, ATRX, DAXX, MEN1, SETD2, TET1, SMARCA4, TERT, TERF*		
	Inactivating somatic mutations of *PRKAR1A* (8%)		Germline loss of function of *PRKAR1A* is observed in Carney complex and in PPNAD. In PRKAR1A mutants MEK and BRAF proteins are overexpressed.
Gene expression (transcriptome)	Methylation profiles (CIMP)	Low	This analysis identified hyperthylation and gene silencing as a hallmark of aggressive ACC.
		Intermediate	
		High	
	miRNA profiles	Steroid phenotype low + Immune score high	This analysis combined expression of steroidogenesis enzymes (including steroidogenic factor (SF1) with expression of proliferation marker (MKI67) to create an Adrenal Differentiation Score (ADS). High steroidogenesis inversely correlated with immune score. Almost all ACC cases present IGF2 protein overexpression.
		Steroid phenotype high + Immune score low	
		Steroid phenotype high + proliferation high + Immune score low	

DNA damage repair (DDR)	High frequency of MMR pathway silencing (MLH1, MSH2, MSH6). Less frequent alterations of BER (ERCC1), HR (BRCA2, RAD52), NER and FA pathways.	High mutational burden of DDR genes is predictive of worse PFS.
ACC Cluster of clusters (CoC)	COC1: Genome profile quiet or chromosomal type, rare WGD; CIMP-low; steroid phenotype high + Immune score low; frequent mutations of MMR genes; high expression of IGF2; better clinical outcome (median EFS not reached).	Integrative analysis of genomic and clinical data with prognostic impact and therapeutic implications
	COC2: Genome profile chromosomal type +/- WGD; activation of WNT pathway; CIMP-intermediate; steroid phenotype high +/- proliferation high; intermediate clinical outcome (median EFS≈48 months).	
	COC3: Genome profile noisy + WGD; TERT overexpression; activation of cell cycle, WNT and chromatin remodeling genes; CIMP-high; steroid phenotype high + proliferation high + Immune score low; poor clinical outcome (median EFS≈12 months).	

Legend: WGD: whole genome doubling; PRKAR1A: protein kinase cAMP-dependent regulatory type I alpha; PPNAD: primary pigmented nodular adrenocortical disease; CIMP: CpG island methylator phenotype; COC: Cluster of clusters; IGF2: insulin-like growth factor-2; MMR: mismatch repair; BER: base excision repair; HR: homologous recombinational repair; NER: nucleotide excision repair; FA: Fanconi anemia; PFS: progression-free survival; EFS: event-free survival.