REVIEW

The next step: mechanisms driving adrenocortical carcinoma metastasis

Enzo Lalli^{1,2,3,4} and Michaela Luconi⁵¹Université Côte d'Azur, Valbonne, France²CNRS UMR7275, Valbonne, France³NEOGENEX CNRS International Associated Laboratory, Valbonne, France⁴Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France⁵Department of Experimental and Clinical Biomedical Sciences 'Mario Serio', University of Florence, Florence, ItalyCorrespondence should be addressed to E Lalli or M Luconi: ninino@ipmc.cnrs.fr or michaela.luconi@unifi.it

Abstract

Endocrine tumors have the peculiarity to become clinically evident not only due to symptoms related to space occupation by the growing lesion, similarly to most other tumors, but also, and most often, because of their specific hormonal secretion, which significantly contributes to their pathological burden. Malignant endocrine tumors, in addition, have the ability to produce distant metastases. Here, we critically review the current knowledge about mechanisms and biomarkers characterizing the metastatic process in adrenocortical carcinoma (ACC), a rare endocrine malignancy with a high risk of relapse and metastatization even when the primary tumor is diagnosed and surgically removed at an early stage. We highlight perspectives of future research in the domain and possible new therapeutic avenues based on targeting factors having an important role in the metastatic process of ACC.

Key Words

- ▶ adrenocortical carcinoma
- ▶ metastasis
- ▶ biomarkers
- ▶ liquid biopsy

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An introduction to the metastatic process

Primary and metastatic cancer

Endocrine tumors have the peculiarity to become clinically evident not only due to symptoms related to space occupation by the growing lesion, similarly to most other tumors, but also, and most often, because of their specific hormonal secretion, which significantly contributes to their pathological burden. Malignant endocrine tumors, in addition, have the ability to produce distant metastases. Metastasis is the result of the dissemination through the bloodstream of an aggressive tumor beyond its organ of origin, resulting in colonization of other tissues and organs. This is a complex multistep process that is the eventual cause of the overwhelming majority of deaths due to cancer (Valastyan & Weinberg 2011, Vanharanta & Massagué 2013). Therefore, it is

quite paradoxical that metastases stem from an extremely inefficient process for which several sequentially occurring events are required, similar to an obstacle race: from the initial local invasion of tumor cells through the surrounding extracellular matrix to the subsequent steps of entering the microcirculation (intravasation), being transported through the vasculature, arresting at distant organ sites, migrating into the tissue parenchyma (extravasation), initially surviving in the new, potentially hostile, environment forming micrometastases and finally growing into clinically detectable macroscopic neoplastic masses. All this while being forced to continuously decoy the surveillance of the immune system and to play a tug-of-war game with the local microenvironment, which can

act in favor or against the metastatic growth (Valastyan & Weinberg 2011, Lambert *et al.* 2017). It has been calculated that the rate of attrition through all these steps exceeds 99% of the initial pool of cells entering the first steps of the metastatic process (Luzzi *et al.* 1998, Valastyan & Weinberg 2011). The complexity of the metastatic process and the small chance for a single metastatic event to reach completion probably accounts for the often long interval between the diagnosis of the primary tumor and of the metastases, even if, unfortunately, notable exceptions are represented by lung and pancreatic adenocarcinomas.

Molecular determinants and markers of the first steps in the metastatic process

Most of the knowledge about the molecular mechanisms driving metastatization and biomarkers of metastasis derives from the study of carcinomas, which are derived from epithelia, with breast cancer, colon cancer and melanoma often representing the paradigms. Concerning the initial steps of the metastatic process, several studies have revealed the importance of collective invasion by cohesive multicellular units as well as of individual tumor cell invasion in its amoeboid or mesenchymal types (Clark & Vignjevic 2015). In carcinomas, the importance of epithelial-to-mesenchymal transition (EMT) is well recognized as an important mechanism to drive metastatic invasion (Lambert *et al.* 2017, Liao & Yang 2017). Briefly, EMT consists in a process of loss of *adherens* and tight junctions in epithelial cells, characterized by downregulation of E-cadherin expression and accompanied by a loss in cell polarity. All these events favor acquisition of a mesenchymal phenotype making epithelial tumor cells more prone to migration. EMT programs, which are physiologically very important during embryonic development, are orchestrated by a core of coordinately regulated transcription factors and miRNAs (Nieto *et al.* 2016). However, in the case of endocrine tumors, it is presently uncertain whether expression of classical EMT markers also indicates tendency to local invasion because of their distinct embryonic origin compared to epithelial tumors (see below). Important roles in the initiating steps of the metastatic process are also played by factors involved in extracellular matrix degradation, like matrix metalloproteinases (MMPs), by stromal cells in the local microenvironment and by tumor induction of local inflammation and angiogenesis (Valastyan & Weinberg 2011, Vanharanta & Massagué 2013). In addition, the up- and downregulation of specific sets of miRNAs have

been associated with metastatization in several types of endocrine cancers through a variety of mechanisms (Lima *et al.* 2017).

Metastasis in adrenocortical carcinoma (ACC): do paradigms apply?

ACC is a rare endocrine malignancy with a high risk of relapse and metastatization even when the primary tumor is diagnosed and surgically removed at an early stage (Else *et al.* 2014, Creemers *et al.* 2016). Current treatments for ACC have only a limited efficacy when disease is at an advanced stage and rely upon use of the adrenolytic agent mitotane (Terzolo *et al.* 2007) and of combination chemotherapy in patients with progressive disease (Fassnacht *et al.* 2012). Classically, malignancy of adrenocortical tumors (ACTs) has been assessed based on evaluation of specific histopathological parameters. Recent molecular and genomic studies have allowed to make considerable advances in the identification of the biological bases of ACC and of the aggressive behavior of a subset of histologically malignant tumors.

Histopathological evaluation of malignancy in ACT: around and beyond the Weiss score

For adrenal tumors, the pathological diagnosis of malignancy cannot rely on single morphological parameters but rather on the presence of a combination of multiple features each one indicative, even if not sufficient by itself, of an aggressive behavior. The most widely used approach in routine pathological practice is the scoring method by Weiss, which takes into account an ensemble of nine different histopathological features related to tumor structure (presence of necrosis, diffuse architecture, clear cells <25%), tumor cell properties (nuclear atypia, mitotic index >5/50 high-power fields, atypical mitoses) and tumor invasion (sinusoidal, venous and capsular invasion), respectively (Weiss 1984). A binary score of either 0 (absence) or 1 (presence) is attributed to each parameter. A given tumor is usually considered malignant when its cumulative score is equal to or higher than 3 taking into account all nine parameters. Suggestions for modifications of the Weiss scoring system have been given by Aubert and coauthors, who decreased the number of criteria to five and attributed higher weight to the number of mitoses and to a percentage of clear cells <25% (Aubert *et al.* 2002). While the Weiss score is very useful as a general indicator of malignancy in clinical practice, it may have a limited value as a prognostic

indicator in individual patients, especially in cases with borderline features (up to 10% of cases according to Papotti *et al.* (2011)), in children (Wieneke *et al.* 2003) and in histological subtypes of ACT as the oncocytic, myxoid and sarcomatoid tumors (Papotti *et al.* 2011). For these reasons, a new scoring system (Helsinki score) was recently introduced, which is based on the combined evaluation of morphological parameters (necrosis and mitotic index >5/50 high-power fields) and of the Ki67 labeling index (LI) (see below). This system was shown to be able to predict the metastatic potential of ACC with very high sensitivity and specificity (Pennanen *et al.* 2015). Another very recent study extended and confirmed the prognostic validity of the Helsinki score on a larger series of ACC cases (Duregon *et al.* 2017).

Another independent simplified diagnostic algorithm has been proposed for ACT (Volante *et al.* 2009) and further validated (Duregon *et al.* 2013a). This method takes into account the integrity of the reticulin network in the tumor, as visualized by silver-based histochemical staining, in ACT. Disruption of that network plus the presence of at least one of the three additional histopathological parameters (mitotic index >5/50 high-power fields, necrosis and vascular invasion) showed 100% sensitivity and specificity to recognize malignant tumors, as assessed using the Weiss system.

Immunohistochemical markers improve prognostic assessment in ACC

The most common marker used in clinical pathology is the Ki67 LI. Ki67 is a sensitive marker of the proliferative compartment in a cell population. The MIB1 antihuman Ki67 monoclonal antibody labels cell nuclei in phases of the cell cycle other than G₀ (Gerdes *et al.* 1983). The Ki67 protein, encoded by the *MKI67* gene, has a direct role in the mitotic process (Cuylen *et al.* 2016). Many studies have demonstrated the prognostic power of the Ki67 LI in different ACC series (McNicol *et al.* 1997, Terzolo *et al.* 2001, Morimoto *et al.* 2008, Duregon *et al.* 2014a) (Table 1). A recent large multicentric ENS@T study has shown that the Ki67 LI is a very reliable parameter to predict disease recurrence (disease-free survival, DFS) and overall survival (OS) in ACC patients after complete tumor resection (Beuschlein *et al.* 2015). Furthermore, Ki67 LI integration with morphological parameters in the newly introduced Helsinki score makes it to outperform the Weiss system as a prognostic indicator in ACC (see above) (Pennanen *et al.* 2015, Duregon *et al.* 2017). However, a limitation of the Ki67 LI consists in

difficulties in its standardization and reproducibility when applied to many tumor types, including ACC (Papathomas *et al.* 2016). These data indicate that other markers are needed to complement the Ki67 LI in the assessment of malignancy in ACC. So far, a substantial number of immunohistochemical markers have been shown to have a prognostic value in ACC:

- genomic studies (see below) have shown that in ACC, the Wnt pathway is the most frequently altered molecular pathway by gene mutations causing constitutive activation (Assié *et al.* 2014, Juhlin *et al.* 2015, Zheng *et al.* 2016). Canonical Wnt signaling regulates the translocation of the membrane protein beta-catenin to the nucleus, where it controls key gene expression programs through interaction with Tcf/Lef and other transcription factors. In many types of cancer, the gene encoding beta-catenin (*CTNNB1*) is mutated in those nucleotides that encode phosphorylation sites targeting the protein for destruction (El Wakil & Lalli 2011). Tissier and coworkers first reported the presence of activating *CTNNB1* mutations both in benign and malignant ACTs (Tissier *et al.* 2005). Further studies showed that in ACC, *CTNNB1* and *TP53* mutations, which are both associated with aggressive disease, are in large part mutually exclusive (Ragazzon *et al.* 2010). Beta-catenin nuclear staining is associated with shorter DFS and OS in ACC (Gaujoux *et al.* 2011). The important role of activated beta-catenin in driving ACC cell proliferation and maintaining viability was shown by studies using pharmacological inhibitors (Doghman *et al.* 2009) and RNA interference *in vitro* and in mouse H295R xenografts (Gaujoux *et al.* 2013, Salomon *et al.* 2015). In the study of Salomon and coworkers, *CTNNB1* knockdown reportedly induced the reversal of 'EMT' in H295R cells, monitored through the expression of the putative markers vimentin, N-cadherin and Slug. However, the mesodermal embryologic origin of the adrenal cortex makes ACT cells different from epithelial cells, for which the EMT process has been defined and demonstrated to play an important role in metastatic dissemination. In fact, in contrast to epithelial cells, mesoderm-derived tissues such as the adrenal cortex constitutively express vimentin as the characteristic intermediate filament, while N-cadherin was reported to be downregulated in ACC compared to adrenocortical adenomas (ACAs) (Khorram-Manesh *et al.* 2002). In mouse models, constitutive adrenocortical beta-catenin activation, obtained using different transgenic strategies, leads to tumorigenesis

Table 1 Immunohistochemical prognostic markers in ACC.

Marker	Biological pathways	Number of patients studied	Outcome	References
Ki67	Cell proliferation	40	Expression >3% associated with shorter DFS	McNicol <i>et al.</i> (1997)
		11	Inverse correlation between Ki67 LI and OS	Terzolo <i>et al.</i> (2001)
		17	Expression $\geq 7\%$ associated with shorter DFS	Morimoto <i>et al.</i> (2008)
		52	Expression $\geq 22\%$ associated with shorter DFS	Duregon <i>et al.</i> (2014a,b)
		319 + 250 (validation cohort) patients with R0 resection	Ki67 LI <10%, 10–19% and $\geq 20\%$ associated with different classes of DFS and OS	Beuschlein <i>et al.</i> (2015)
		56 pediatric patients	Ki67 LI $\geq 15\%$ and <i>MKI67</i> overexpression associated with shorter DFS	Pinto <i>et al.</i> (2017)
Beta-catenin	Cell proliferation and adrenocortical tumorigenesis	79	Nuclear staining associated with shorter DFS and OS	Gaujoux <i>et al.</i> (2011)
Cyclin E	Cell proliferation	49	LI $\geq 5\%$ associated with shorter DFS	Tissier <i>et al.</i> (2004)
ERCC1	DNA repair and resistance to cisplatin	163	H-score >1 associated with shorter OS in patients who received platinum chemotherapy	Ronchi <i>et al.</i> (2009)
FATE1	ER-mitochondria contacts; resistance to apoptosis and chemotherapeutic drugs	141	H-score >1 associated with shorter OS i	Doghman-Bouguerra <i>et al.</i> (2016)
GLUT1	Glucose transporter	167	High expression associated with shorter OS	Fenske <i>et al.</i> (2009)
		78	High expression associated with shorter DFS and OS	Pinheiro <i>et al.</i> (2015)
MCT1	Monocarboxylate transporter	78	High expression associated with shorter OS	Pinheiro <i>et al.</i> (2015)
MCT2	Monocarboxylate transporter	78	High expression associated with increased OS	Pinheiro <i>et al.</i> (2015)
CAIX	Carbonic anhydrase (pH regulator)	78	High expression associated with shorter OS	Pinheiro <i>et al.</i> (2015)
JAG1	Notch ligand	178 + 77 (validation cohort)	High expression associated with longer DFS and OS	Ronchi <i>et al.</i> (2015)
MMP-2	Breakdown of extracellular matrix	50	Expression >20% associated with shorter DFS and OS	Volante <i>et al.</i> (2006)
Phospho-histone H3	Cell proliferation	52	Expression >9% marginally associated with shorter OS	Duregon <i>et al.</i> (2014a,b)
SF-1	Steroidogenesis and adrenocortical cell proliferation	130 + 58 (validation cohort)	High expression associated with shorter DFS and OS	Sbiera <i>et al.</i> (2010)
		75	High expression associated with shorter OS	Duregon <i>et al.</i> (2013a)
SGK1	Growth factor – regulated protein kinase	203	Low expression associated with shorter OS	Ronchi <i>et al.</i> (2012)
VAV2	Cytoskeletal remodeling and tumor cell invasion	105	H-score >1 associated with shorter DFS and OS	Ruggiero <i>et al.</i> (2017)
		171	H-score >1 associated with shorter DFS and OS; combined assessment of VAV2 expression and Ki67 LI improves prognostic power	Sbiera <i>et al.</i> (2017)

DFS, disease-free survival; NA, not available; OS, overall survival.

that only in rare cases has the features of malignancy (Berthon *et al.* 2010, Heaton *et al.* 2012);

- high expression of the G1 cyclin E, which is involved in cell proliferation, is associated with shorter DFS (Tissier *et al.* 2004);
- high levels of excision repair cross-complementing group 1 (ERCC1) protein, involved in nucleotide excision repair of DNA, correlate with reduced OS selectively in ACC patients who received platinum-based chemotherapy (Ronchi *et al.* 2009);
- FATE1 is a cancer–testis antigen localized at the interface between the endoplasmic reticulum and mitochondria that modulates calcium-mediated apoptosis in adrenocortical cells. High FATE1 levels are associated with reduced OS (Doghman-Bouguerra *et al.* 2016);
- high expression of the glucose transporter GLUT1 is associated with reduced OS (Fenske *et al.* 2009, Pinheiro *et al.* 2015) and reduced DFS (Pinheiro *et al.* 2015). In the latter study, high levels of the monocarboxylate transporter (MCT) MCT1, which allows lactate to enter the cell, and the pH regulator carbonic anhydrase IX (CAIX) were also associated with shorter OS, while a high expression of MCT2, which pumps lactate outside the cell, was associated with increased OS. These results suggest that an increased glycolytic metabolism has a

role in the determination of an aggressive phenotype in ACC;

- following binding of ligands belonging to either the Jagged or the Delta-like families to Notch transmembrane receptors, the integral membrane γ -secretase complex cleaves Notch and releases the NICD (cleaved Notch intracellular domain). This translocates to the nucleus to interact with constitutively DNA-bound CSL transcription factors, this way allowing recruitment of the Mastermind-like (MAML) coactivator and stimulating transcription of Notch target genes, such as the HES (hairy enhancer of split) family of transcription factors (Bray 2006). The Notch ligand JAG1 is upregulated in ACC compared to normal adrenal adenomas and ACAs and has an important role in regulating mouse Y1 adrenocortical cell proliferation (Simon *et al.* 2012). Conversely, high JAG1 expression correlates with longer DFS and OS in ACC (Ronchi *et al.* 2015);
- matrix metalloproteinase type 2 (MMP-2) contributes to mediate the process of extracellular matrix degradation by cancer cells. Overexpression of this enzyme involved in metastatic invasion is associated with reduced DFS and OS (Volante *et al.* 2006);
- staining for phospho-histone H3, a marker for mitotic cells, was shown to have a borderline correlation with

Figure 1

VAV2 overexpression induces cytoskeleton remodeling in H295R cells and is a marker of malignancy in ACC. (A) Cytoskeletal morphology revealed by phalloidin staining (red) in H295R/TR SF-1 cells transfected with vectors encoding either GFP or GFP-VAV2 (green). Scale bars, 5 μ m. Reproduced, with permission, from Ruggiero C, Doghman-Bouguerra M, Sbiera S, Sbiera I, Parsons M, Ragazzon B, Morin A, Robidel R, Favier J & Bertherat J (2017) Dosage-dependent regulation of VAV2 expression by Steroidogenic actor-1 drives adrenocortical carcinoma cell invasion, *Science Signaling*, volume 10, eaal2464. Copyright 2017 American Association for the Advancement of Science. (B) OS in ACC patients with both high VAV2 expression (H -score ≥ 2) and high Ki67 LI ($\geq 20\%$) group (red line) vs all other patients (green line) $P < 0.001$, Kaplan–Meier method. Reproduced, with permission, from Sbiera S, Sbiera I, Ruggiero C, Doghman-Bouguerra M, Korpershoek E, de Krijger RR, Haak H, Volante M, Papotti M, Reimondo G, *et al.* (2017) Assessment of VAV2 expression refines prognostic prediction in adrenocortical carcinoma, *Journal of Clinical Endocrinology and Metabolism*, volume 102, pages 3491–3498. Copyright 2017 Oxford University Press.

OS in ACC, which was less significant than mitotic count (Duregon *et al.* 2014a);

- low expression of serum glucocorticoid kinase 1 (SGK1), an ubiquitous serine/threonine kinase regulated by growth factors, is correlated with shorter OS in ACC (Ronchi *et al.* 2012);
- Steroidogenic Factor-1 (SF-1) is a transcription factor, belonging to the nuclear receptor superfamily, that has an essential role in the development of adrenal glands and gonads (Lalli 2010). SF-1 expression is a hallmark of ACT and its higher expression levels in ACC are associated with reduced DFS and OS (Sbiera *et al.* 2010, Duregon *et al.* 2013b). In ACT cells, SF-1 regulates different sets of target genes according to its dosage (Doghman *et al.* 2007a, 2013). Some of these dosage-dependent SF-1 target genes have a direct role in decreasing apoptotic death (NOV, FATE1; Doghman *et al.* 2007b, Doghman-Bouguerra *et al.* 2016) and increasing invasion of tumor cells (VAV2; Ruggiero *et al.* 2017). VAV2 is a guanine nucleotide exchange factor regulating cytoskeleton remodelling (Fig. 1A). A recent multicentric study showed that its overexpression is correlated with shorter DFS and OS and that the combined assessment of VAV2 expression and Ki67 LI improves prognostic prediction in ACC (Sbiera *et al.* 2017) (Fig. 1B). An advantage of VAV2 scoring in prognostic assessment may be its higher intratumoral homogeneity compared to the Ki67 LI.

Molecular markers reveal disease mechanisms and improve patient stratification and prognostication in ACC

Great progress has been made in our understanding of the pathogenesis of ACC and in the improvement of its prognostic assessment, thanks to the molecular analysis of tumors and biological fluids from patients. The majority of the published studies that identified the molecular profiles of ACC has been performed in cohorts including a limited number of patients, mostly because of the rarity of the disease. Nevertheless, molecular assays derived from those studies are in the process of being transferred into routine analysis and it is easy to predict that in the near future, they will become essential in the diagnostic and prognostic workups of ACC (Armignacco *et al.* 2017).

The foundation of genomic studies in adult ACC was laid by the analysis of Giordano and collaborators who compared gene expression profiles of 11 ACC, 4 ACA, 1 macronodular hyperplasia and 3 normal adrenal cortices using Affymetrix microarrays (Giordano *et al.* 2003).

This seminal study identified a cluster of about 90 genes that was differentially expressed in ACC compared to benign tumors, among which a prominent place was occupied by genes related to cell proliferation. In particular, this study confirmed the previously known association of ACC with overexpression of the insulin-like-growth-factor-2 (IGF2). An almost constant finding in ACC is the loss of heterozygosity of the imprinted 11p15 region with a deletion of the maternal allele and a consequent overexpression of *IGF2* (Boulle *et al.* 1998, Gicquel *et al.* 2001). The gene expression profiling study by de Fraipont and coworkers confirmed that overexpression of a cluster of genes including *IGF2* was a predictor of malignancy in ACC, while high expression of a cluster of genes linked to steroidogenesis was a favorable prognostic indicator (de Fraipont *et al.* 2005). *IGF2* overexpression also characterizes pediatric ACT, but with no association with malignancy (West *et al.* 2007, Rosati *et al.* 2008). Further molecular studies showed that gene expression profiles can stratify histologically confirmed adult ACC in 2 subgroups with very different clinical outcomes: one group (C1A), enriched in genes involved in transcription and cell cycle progression, consists of aggressive tumors, while another group (C1B), enriched in genes involved in cell metabolism, intracellular transport, apoptosis and cell differentiation, has mostly an indolent course (de Reyniès *et al.* 2009, Giordano *et al.* 2009). In addition, de Reyniès and coworkers showed that the combined expression of the *BUB1B* and *PINK1* transcripts evaluated by RT-qPCR, an indicator derived from transcriptome analysis, is a stage-independent predictor of OS in ACC (de Reyniès *et al.* 2009). This two-gene molecular predictor has been validated in an independent cohort (Fragoso *et al.* 2012). Other mRNA markers that have been shown to have prognostic values in ACC are as follows:

- Ribonucleotide reductase large subunit 1 (*RRM1*) encoding a protein involved in DNA synthesis. High *RRM1* expression levels are associated with reduced DFS and OS (Volante *et al.* 2012);
- Topoisomerase II alpha (*TOP2A*) is an enzyme responsible for transcription, replication and chromosome condensation and segregation during cell division. High *TOP2A* expression is correlated with longer time for progression after etoposide – doxorubicin – cisplatin – mitotane (EDP-M) treatment (Roca *et al.* 2017).

MicroRNAs (miRNAs) are a class of evolutionarily conserved, small non-coding RNA molecules which regulate gene expression at the post-transcriptional

level. They are implicated in virtually every biological process, from development to viral infection, and are also associated with oncogenesis. Several studies have shown a differential expression of a distinct subset of miRNAs in ACC compared with benign ACT (Cherradi 2016, Hassan *et al.* 2017). Although a certain discrepancy exists about the outcome of different studies, the most consistent modulations of miRNA abundance in ACC compared to benign ACT were reported to be the upregulation of miR-483-5p/3p, miR-210 and miR-503 and the downregulation of miR-195, miR-335 and miR-375. In the study by Soon and coworkers, high miR-483-5p and low miR-195 expressions were significantly correlated with shorter DFS (Soon *et al.* 2009).

RNA studies in clinical specimens are difficult to perform because of the need for frozen, well-preserved tissue for analysis. For this reason, many efforts have been made to identify other more resilient prognostic markers such as DNA markers. Tumor DNA analysis has shown that a cluster of specific genomic alterations has a significant relationship with OS in ACC (Barreau *et al.* 2012). Another important prognostic molecular feature in ACC is DNA methylation. Aggressive tumors have a common CpG island methylator phenotype (CIMP) that can be further divided into two subgroups, each one showing different levels of methylation (CIMP-high and CIMP-low), with CIMP-high tumors having the less favorable prognosis (Rechache *et al.* 2012, Fonseca *et al.* 2012, Barreau *et al.* 2013). An important step toward the introduction of DNA methylation as a clinical prognostic marker is represented by a recent study that showed a robust correlation in ACC patients between DFS/OS and DNA hypermethylation, measured by only 4 probes using a methylation-specific multiplex ligation-dependent probe amplification assay that is amenable to implementation in routine analysis (Jouinot *et al.* 2017).

Recently, it has been possible to integrate multiple genomic data to obtain deeper insights into the pathogenetic mechanisms of ACC and to show a consistent correlation pattern of molecular aberrations and patient prognosis. A European study including 45 cases and validated in an independent cohort of 77 ACC has been the first to show that tumors in the C1A cluster, which is associated with a worse prognosis, have distinct methylation, mRNA and miRNA expression patterns, more frequent mutations in driver genes and higher global mutation rate than tumors belonging to the C1B cluster, characterized by more favorable prognosis (Assié *et al.* 2014). These findings were confirmed by another study performed on 91 ACCs from worldwide centers

(Zheng *et al.* 2016). In addition to the Wnt pathway (see above), an integrative genomic analysis of ACC revealed recurrent somatic alterations in *TP53*, 11p15, cell cycle regulation, telomere maintenance, chromatin remodeling and PKA pathway genes (Assié *et al.* 2014, Juhlin *et al.* 2015, Zheng *et al.* 2016, Armignacco *et al.* 2017). Interestingly, PKA pathway upregulation due to *PRKACA*-activating mutations is a hallmark of a subset of cortisol-secreting ACAs (Beuschlein *et al.* 2014, Cao *et al.* 2014, Goh *et al.* 2014, Sato *et al.* 2014). These findings suggest that at least in some cases, ACC may be derived from ACAs (Ronchi *et al.* 2013). Furthermore, it is well known that germline mutations in tumor suppressor genes (e.g. *TP53*, *MEN1*) may predispose to adrenocortical tumorigenesis (Else 2011).

While all those reports were focused on the analysis of nucleic acids, only a few studies performed proteomic analysis of markers able to discriminate between benign and malignant ACTs. Yang and coworkers identified calreticulin and prohibitin as overexpressed in ACC compared to ACA and showed that calreticulin overexpression was significantly associated with increased tumor stage (Yang *et al.* 2013). Kjellin and coworkers confirmed that the IGF2 is overexpressed at the protein level in ACC compared to benign ACT as well as aldolase A, an enzyme involved in glycolysis, while several proteins belonging to complex I in the mitochondrial respiration chain are downregulated (Kjellin *et al.* 2014). These findings further suggest (see the study by Pinheiro *et al.* mentioned earlier) that a metabolic shift toward aerobic glycolysis (Warburg effect) takes place in malignant ACT. More recently, another study confirmed that ACCs harbor significant changes in their glucose metabolic pathways and in addition identified fascin-1 as overexpressed in ACC compared to normal adrenal (Poli *et al.* 2015). This protein has an important role in cytoskeletal organization and is associated with malignancy in a variety of cancers (Hashimoto *et al.* 2011). Fascin-1 is an attractive new therapeutic target since specific small molecule inhibitors have been developed and shown to impair metastatic dissemination in preclinical cancer models (Chen *et al.* 2010, Huang *et al.* 2015).

Markers of malignancy are different in pediatric ACT compared to adult

Pediatric ACTs differ from adult tumors under several aspects related to their pathogenesis, clinical presentation, molecular signatures and prognosis (Lalli & Figueiredo 2015). In addition, histopathological (Weiss score) and molecular (*IGF2* overexpression, *BUB1B/PINK1* expression)

parameters linked to malignancy in adult tumors do not have the same predictive value in pediatric ACT (Wieneke *et al.* 2003, Rosati *et al.* 2008, Dehner & Hill 2009, Papotti *et al.* 2011, Fragoso *et al.* 2012, Lalli & Figueiredo 2015). In the comprehensive study from the International Pediatric Adrenocortical Tumor Registry based on the analysis of 254 patients, the major parameters influencing the risk of recurrence were age, stage at diagnosis and tumor weight (Michalkiewicz *et al.* 2004). After analysis of a series of 83 patients, Wieneke and coworkers proposed a set of 9 macroscopic and microscopic criteria for the diagnosis of malignancy in pediatric ACT (Wieneke *et al.* 2003), further validated in other series (Magro *et al.* 2012, Das *et al.* 2016). Transcriptome analysis of pediatric ACT revealed that HLA class II gene expression was downregulated in tumors classified histologically as carcinomas (West *et al.* 2007). A subsequent immunohistochemical study validated those data showing that MHC class II expression was significantly higher in benign compared to malignant ACT and was associated with longer DFS. Among HLA class II antigens, HLA-DPA1 expression was most significantly associated with DFS after adjustment for tumor weight and stage (Pinto *et al.* 2016). Integrative analysis of genomic alterations of pediatric ACT identified a high-risk group characterized by concomitant *TP53* and *ATRX* mutations, massive structural variations and frequent background mutations and higher expression of genes associated with chromosome instability and cell cycle (Pinto *et al.* 2015). A recent study in pediatric ACT showed that an elevated KI67 LI and *MKI67* overexpression are associated with reduced DFS. Interestingly, a correlation was reported between *MKI67* overexpression and low *HLA-DPA1* expression (Pinto *et al.* 2017).

The 'liquid biopsy' and circulating markers of malignancy

As in other solid tumor types, it would be extremely important for the clinician to count on minimally invasive markers of malignancy in patients with ACC to improve their stratification for prognostic and therapeutic purposes. In particular, markers that can be obtained by sampling from minimally invasive and easily accessible biological fluids, such as blood, urine and saliva, would be pivotal not only for diagnostic and prognostic purposes but also for continuous monitoring of patients during follow-up. The liquid biopsy is referred to a simple blood draw that can provide material released from the tumor into the bloodstream, such as circulating tumor cells (CTCs), miRNAs, exosomes and cell-free DNA of tumor

	CTCs	miRNAs	Exosomes	ctDNA
Tumor origin	Y	Y/N	Y/N	Y
Tumor heterogeneity	Y	Y	Y	Y
Tumor clonal evolution	Y	N	Y	Y
Somatic mutation and CNVs	Y	N	Y	Y
Genetic profile	Y	N	Y	Y
Methylome analysis	Y	N	Y	Y
Expression profile	Y	Y	Y	N
Proteomic profile	Y	N	Y	N
Morphological analysis	Y	N	Y	N
Functional assessment	Y	Y	Y	N
In vitro culturing	Y	N	N	N
Xenograft studies	Y	N	N	N

Figure 2

The liquid biopsy consists of a blood draw containing CTCs, miRNAs, exosomes and ctDNAs deriving from the tumor mass. Head-to-head comparison between the applications of the different entities that can be isolated from the liquid biopsy is shown in the table. Y, yes; N, no.

origin (ctDNA) (Fig. 2). This is a novel and minimally invasive technique developed for solid tumors and recently also applied to ACC (Chabre *et al.* 2013, Szabó *et al.* 2014, Creemers *et al.* 2017, Batth *et al.* 2017, Bardelli & Pantel 2017) to identify potential markers to assess malignancy in ACC, even if the predictive power needs to be confirmed on larger cohorts of patients. In addition to its diagnostic/prognostic value, the liquid biopsy represents a unique tool that can provide informative material to be analyzed and compared to the primary lesion for longitudinal surveillance of clonal evolution and progression of the tumor, also in response to anticancer treatments. All this information is pivotal for the development of personalized medicine approaches (Siravegna *et al.* 2017). In particular, both clonal analysis and tumor monitoring during follow-up cannot be effectively achieved using the currently available tumor tissue specimens.

CTCs: metastatic cells caught in the act?

The presence of CTCs of adrenal origin has recently been characterized in patients affected by ACC compared to benign adenomas (Pinzani *et al.* 2013). These cells are characterized by specific morphological traits, such as nucleocytoplasmic ratio $\geq 50\%$, irregular nuclear shape, hyperchromatic nucleus, basophilic cytoplasm and cell size $\geq 16 \mu\text{m}$ (Pinzani *et al.* 2013). In particular, the last trait enables their trapping through blood filtration on specific size-selective filter devices (ScreenCell).

This technique is particularly useful in ACC (Fig. 3A and B), since CTC sorting on the basis of epithelial cell adhesion molecule (EpCAM) surface exposure is tricky and might lead to underestimation in this cancer, which has been demonstrated to be negative for EpCAM (Went *et al.* 2004). Conversely, the enrichment by size associated with the positivity for the adrenal-specific marker SF-1 (Fig. 3C) resulted in finding of a significant correlation between the number of CTCs/mL and the tumor diameter or the tumor stage (Pinzani *et al.* 2013), suggesting that CTCs may be a useful marker in aggressive/metastatic ACC. CTCs may be isolated or arranged in groups of 3–20 cells (circulating tumor microemboli, CTMs) which are detached together from the primary lesion. CTMs seem to be more prone to metastasize than single CTC and their presence/number is a poor prognostic factor in solid tumors (Hou *et al.* 2012, Chang *et al.* 2016).

Meta-analyses of studies identifying CTCs in breast cancer (Zhang *et al.* 2012), colorectal cancer (Rahbari *et al.* 2010) and melanoma (Mocellin *et al.* 2006) revealed a robust prognostic value of CTCs in those tumors. Interestingly, CTCs, despite decreased in number, can be detectable in the blood long time after tumor removal, indicating that a reservoir could be present in the bone marrow, as suggested for breast cancer (Wiedswang *et al.* 2006). Thanks to the recent progress made in the genetic analysis of single cells, the genomic profile of even the few CTCs recovered from the bloodstream could be obtained and compared with that of the primary lesion and of the eventual metastasis in order to gain insight into the heterogeneity of ACC. This heterogeneity is the basis for the selection of those specific clones able

to enter the bloodstream, and among those CTCs, heterogeneity further drives the ones with potential of metastasizing (Salvianti & Pinzani 2017, Siravegna *et al.* 2017). Moving forward from the initial preliminary studies, several ongoing clinical trials assessing CTCs in different tumors have been designed to validate in large patient cohorts the use of CTCs for diagnostic/prognostic disease-monitoring purposes (see Wang *et al.* (2017) for a review). In rare cancers such as ACC, an effort to set up multicenter studies is urgently needed in order to assess a CTC prognostic value in metastatic tumors.

Circulating miRNAs

It is now well known that miRNAs produced by cancer cells are passively and/or actively shedded into the circulation and that their serum/plasma levels may be correlated with malignancy in different tumor types, including ACC (Cherradi 2016, Hassan *et al.* 2017).

Among the different circulating miRNA profiles characterized in ACC (Szabó *et al.* 2014), miR-483-5p is the recurrent one in almost all the studies and the most suggestive one as it maps in the second intron of the *IGF2* gene, whose hyperexpression is a recurrent tract of aggressive ACC (Giordano *et al.* 2009). Indeed, miR-483-5p has been demonstrated to be overexpressed both in the primary tumor (Soon *et al.* 2009, Patterson *et al.* 2011, Duregon *et al.* 2014b) and the bloodstream (Chabre *et al.* 2013, Patel *et al.* 2013, Szabó *et al.* 2014). Chabre and coworkers showed that high circulating serum levels of miR-483-5p and low circulating levels of miR-195 in 21 ACCs stratified into non-aggressive and aggressive cancers

Figure 3

Microscopic evaluation of CTCs enriched by size of specific filter devices. (A and B) Hematoxylin/eosin staining identifies CTCs as large cells with a high nucleus/cytosol ratio; the arrow indicates a polymorphonuclear leukocyte for comparison; micropores of the ScreenCell™ filter devices (black holes) have a diameter of 8 µm. (C) Immunohistochemistry on CTC shows positivity for nuclear SF-1, indicating adrenal origin. Reproduced, with permission, from Pinzani P, Scatena C, Salvianti F, Corsini E, Canu L, Poli G, Paglierani M, Piccini V, Pazzagli M, Nesi G, *et al.* (2013) Detection of circulating tumor cells in patients with adrenocortical carcinoma: a monocentric preliminary study, *Journal of Clinical Endocrinology and Metabolism*, volume 98, pages 3731–3738. Copyright 2013 Oxford University Press.

were associated with both shorter DFS and OS (Chabre *et al.* 2013). Salvianti and coworkers went further, as they developed an absolute method to measure plasma miR-483-5p levels that could be easily transferred to the routine analysis (Salvianti *et al.* 2017). A cut-off value of 221 ng/mL equivalent is able to significantly discriminate between aggressive (stages 3 and 4) and non-aggressive (stages 1 and 2) ACCs in a cohort of 27 ACCs, predicting DFS and OS in a Kaplan–Meier analysis (Salvianti *et al.* 2017). Notably, miR-483-5p levels significantly correlate with CTC number in the same blood sample, suggesting a direct release of these cells from the tumor (Salvianti *et al.* 2017). Importantly, standardization of pre-analytical procedures and absolute/relative analysis are mandatory to use information derived from circulating miRNA for ACC prognostication.

Exosomes and tumor microenvironment in ACC progression

Exosomes are 40–100 nm diameter particles actively released in extracellular space and body fluids by exocytosis from almost all cell types, including tumor cells. As they contain proteins, lipids, DNA, mRNA and miRNAs, they have been suggested to play a pivotal role in exchanging information between cells, in particular between tumor cells and their microenvironment. Indeed, the tumor cells ‘educate’ the surrounding stromal cells in the microenvironment (fibroblasts, endothelial cells, preadipocytes) to modify their functions and metabolism in order to support tumor progression (Cirri & Chiarugi 2012, Chiarugi & Cirri 2016). On the other hand, the surrounding stroma responds and interacts with the tumor cells exchanging pivotal factors through these exosomal cargoes. In particular, exosomes have been demonstrated to support the metastatic process both locally and at distance by affecting the potential metastatic organs. Exosomes released by tumor cells and environmental stroma appear critical for supporting processes involved in metastasis, such as angiogenesis, tumor invasion, extracellular matrix digestion, cell motility and immunomodulatory escape activity of the tumor (Milane *et al.* 2015, Suchorska & Lach 2016). Exosomes are recruited at the plasma membrane of tumor cell invadopodia, the tumor cell structures which release metalloproteinases for extracellular matrix degradation (Hoshino *et al.* 2013, Mu *et al.* 2013). Transfer of exosomes extracted from tumor provided with invadopodia induces invadopodia formation in non-invading cells (Hoshino *et al.* 2013). Moreover, the ability of the cancer-released

exosomes to influence the stromal ‘premetastatic’ niche in the potential target metastatic organs has recently been hypothesized. Through organ-specific delivery of these so-called metastasomes, cancer cells from the primary tumor or CTCs would prepare the soil in the target organs for the metastatic colonization (Ghasemi *et al.* 2013).

Notably, Wnt5A signaling has been shown to induce active metastasis-driving exosome formation in melanoma (Ekström *et al.* 2014). This mechanism could also be relevant in ACC, where Wnt signaling activating mutations have been described to be the most frequent tumor mutations (Assié *et al.* 2014, Zheng *et al.* 2016).

Extracellular vesicle-associated miR-483-5p isolated from plasma samples of a cohort of 18 ACCs and 16 ACAs has been recently reported to have a high significant diagnostic power (Perge *et al.* 2017), further indicating the relevance of miR-483-5p in ACC; however, further studies are required to assess exosome miR-483-5p importance in metastatic ACC.

Circulating cell-free DNA (cfDNA) and cell-free tumor DNA (ctDNA)

Finally, the liquid biopsy can also provide circulating cfDNA, derived from non-tumoral and tumoral cells (ctDNA). The exact mechanism by which the tumor releases DNA into the bloodstream is still obscure, although necrosis and apoptosis, often occurring in the tumoral mass, might be involved. The circulating levels of ctDNA largely depend on the tumor type, diameter, disease stage and therapeutic response (Bettegowda *et al.* 2014). Being essentially a waste product, the quality of this DNA is very poor and together with its extremely low levels makes the analysis of genomic profile and methylation state quite difficult (Perdigones & Murtaza 2017). For this reason, the development of highly sensitive and high-throughput technologies, such as digital PCR and Next Generation Sequencing, only recently enabled to obtain ctDNA profiling and compare it with the profile of the primary lesion to gain insight into the heterogeneity and clonal evolution of the tumor (Ignatiadis *et al.* 2015, Perdigones & Murtaza 2017, Salvianti & Pinzani 2017, Siravegna *et al.* 2017). For ACC, the knowledge of single mutations in driver genes and copy number variations (CNVs) identified in the primary lesion will be pivotal to guide the analysis of ctDNA in ACC patients. Creemers and coworkers were the first to report the presence of circulating ctDNA in plasma of a metastatic ACC patient following mutation identification in the primary tumor (Creemers *et al.* 2017). However, the same authors were

not able to detect in ctDNA the same mutations already characterized in the primary tumor, suggesting that mutation detection in ctDNA using the current approach may be variable between ACC patients.

Taking together these findings, among the variety of tumor material that can be isolated by a non-invasive liquid biopsy, only CTCs and ctDNA can be univocally linked to the primary lesion. In fact, for CTCs, organ-specific markers or the presence of mutation/CNVs also detected in the primary lesion can demonstrate the origin of these circulating cells (Fig. 3). Conversely, for exosomes, it depends on their content, while for miRNAs, their production by cells different from the tumor cannot be ruled out. CTCs also enable us to study not only the genetic and methylomic profiles of the tumor cells entering the circulation, but also gene and protein expressions, thus providing useful information to understand the metastatic process and the tumor cell factors involved (Fig. 2). Moreover, although very few attempts have been made so far, CTCs could be cultured *in vitro* (Yu *et al.* 2014, Cayrefourcq *et al.* 2015) and used to derive patient-personalized xenograft models (Baccelli *et al.* 2013, Khoo *et al.* 2016) (Fig. 2) which can allow to screen novel, more efficient drugs selective for metastatic tumors rather than the primary lesion.

Animal models of metastatic ACT

Up to a very recent time, mouse models of metastatic ACT have been lacking. At best, only sporadic local dissemination and virtually no distant metastases were observed in the available mouse models (Basham *et al.* 2016, Leccia *et al.* 2016). However, the situation changed in 2017: a breakthrough has been made by the publication of the first mouse model developing differentiated ACC able to produce metastases with high frequency (Batisse-Lignier *et al.* 2017). In those mice, the SV40 large T antigen, which sequesters and inactivates p53 and Rb, is produced in the adrenal cortex under the control of the 0.5-kb *Akr1b7* promoter/intron regulatory sequences, whose expression is restricted to the adrenal *zona fasciculata* (AdTag mice). This transgenic strategy targets two critical tumor suppressors frequently mutated in human ACC (Assié *et al.* 2014, Zheng *et al.* 2016). AdTag mice present adrenal nodular lesions that develop morphological and histopathological characteristics of malignancy worsening with age and are associated with primary hypercorticism. Importantly, at 8 months of age, metastases are found at high frequency in the lungs, liver and other tissues. Therefore, AdTag mice represent a unique and invaluable

tool to study the molecular mechanisms involved in ACC pathogenesis in an immunocompetent host and to test new pharmacological treatments for disseminated ACC. Interestingly, Batisse-Lignier and coworkers also showed that long-term mice treatment with rapamycin (an inhibitor of mTOR signaling) significantly reduced tumor size and hormonal production. These data are consistent with previous studies showing the efficacy of mTOR and dual PI3K/mTOR inhibitors to impair H295R cell proliferation both *in vitro* and grown as xenografts in nude mice (Doghman *et al.* 2010, Doghman & Lalli 2012, Poli *et al.* 2016).

Xenografts in immunodeficient mice are the only available method to study the ability of human ACC cells (both cell lines and patient-derived primary cells) to form tumors and their drug sensitivity in preclinical studies (Luconi & Mannelli 2012, Hantel & Beuschlein 2015). The most widely used cell line for xenograft experiments is H295R (Logié *et al.* 2000), even if the recently described SJ-ACC3 (only growing as xenografts; Pinto *et al.* 2013) and MUC-1 (xenografts and cell line; Hantel *et al.* 2016) hold promise to enlarge the representation of the spectrum of heterogeneity present in human tumors. We need to underline here that the SW-13 cell line that has been used in several studies, derived from a metastasis of a small cell carcinoma in the adrenal cortex, does NOT express markers of adrenocortical differentiation and as such is not a valid model for human ACC. Important progress has recently been made in the domain of xenograft models for ACC with the report of a method to induce metastatic dissemination of H295R cells. Since subcutaneous H295R xenografts in nude mice show no tendency to metastasize, Morin and coworkers tested several alternative methods to obtain liver metastases. The most efficient method resulted from tumor cell injection in the spleen followed by splenectomy to avoid local cell growth and to allow enough time for cells to form metastases detectable by bioluminescence (Morin *et al.* 2017). Bypassing the stages of local invasion and intravasation of tumor cells, this method has limitations. Nevertheless, it genuinely represents the later stages of the metastatization process (see 'An introduction to the metastatic process' section above), which are often rate-limiting (Luzzi *et al.* 1998). For these reasons, this model appears valuable for studies concerning the human disease and to test new therapies for metastatic ACC.

Perspectives

Current therapies are only marginally efficient against metastatic ACC. The cornerstone for the treatment of

metastatic disease is mitotane (o,p'-DDD), a drug provided with both antihormonal and antitumoral activities (Terzolo *et al.* 2007, Else *et al.* 2014, Creemers *et al.* 2016). This compound is commonly associated with EDP polychemotherapy in patients with rapidly progressive disease, but this regimen does not substantially improve OS of patients (Fassnacht *et al.* 2012). Alternative therapies to treat metastatic ACC are therefore urgently needed. Initial hopes in IGF-1R antagonists, spurred by data indicating the importance of IGF2-IGF-1R signaling in controlling ACC cell proliferation, were dashed by the results of a study which on the whole showed no improvement in either DFS or OS in patients with locally advanced or metastatic ACC treated with the oral IGF-1R inhibitor linsitinib (OSI-906) (Fassnacht *et al.* 2015). However, a partial response was observed in a small group of patients treated with the drug. These findings further highlight the molecular heterogeneity that characterizes ACC and affects the results obtained by a single therapy approach. In addition, they suggest that a comprehensive molecular characterization of the tumor might identify those cases that could be predicted to be responsive to IGF-1R inhibitors or other targeted therapies. But what about non-responders? Future therapies for metastatic ACC should probably be oriented to target already-disseminated tumor cells and impair their growth into clinically relevant macroscopic metastases, since this is the limiting step in the metastatization process for most carcinomas (Valastyan & Weinberg 2011). To this purpose, it will be essential to obtain an extensive molecular and genomic characterization of metastases as compared to their primary tumors in order to identify potential genetic and epigenetic drivers of the metastatic process. According to the findings so far presented, the prognostic classification of ACC based on integrated histopathological and genomic parameters of primary tumors seems to suggest that ACCs *ab initio* possess features able to influence their spreading and metastasizing ability. However, at this stage of knowledge, acquisition of further alterations in the context of a milieu predisposing the tumor cells toward a metastatic fate cannot be ruled out. Another important aspect to be considered is the intratumor heterogeneity. It is presently unknown to which extent heterogeneity exists within an individual ACC, which may reflect tumor evolution and give rise to subclonal populations with different functional properties, influencing resistance to therapy and metastasizing ability. Heterogeneity occurring in ACC is strongly supported by the observation that Ki67 labeling may vary widely from one area of the tumor to another (Duregon *et al.* 2015). This is likely determined

by intratumoral differences in genetic and/or epigenetic profiles. Accordingly, histopathologic analysis of ACC often reveals areas with distinct cellular features, including nuclear pleomorphism, cytoplasmic aspect and tumor architecture. Altogether, these data suggest that tumor heterogeneity plays an important role in ACC biology, with a potential direct impact on its metastatic ability and response to treatment. Immunotherapy will probably also have an important role to boost the immune system to hold a grip on tumor cells disseminated in peripheral organs. Another relevant therapeutic avenue to be explored will be the modulation of the microenvironment in the primary organ (from which subclones with metastatic properties may evolve) as well as the microenvironment in metastasis-prone organs, with the purpose to make them more hostile to thriving of tumor cells. Finally, after elucidating the nature of RNA and DNA alterations involved in ACC pathogenesis, research now needs to move on the analysis of the aberrations in the ACC proteome and metabolome, which may reveal important new therapeutic targets. The new animal models today available for studying metastatic ACC will undoubtedly provide essential tools both to understand the molecular mechanisms underlying metastatic dissemination and growth and to test novel targeted therapies in the era of precision medicine.

Declaration of interest

The author declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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