Transcriptome analysis of adrenocortical cancers: from molecular classification to the identification of new treatments

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Abstract

Transcriptome analysis has been successfully used to study the gene profile expression of adrenocortical tumors (ACT) for 7 years. The various studies reported to date have produced an abundance of new information on adrenocortical cancer (ACC), underlying the validity of this approach to study the molecular genetics and pathogenesis of these tumors. The gene expression profile of ACC clearly differs from that of benign adrenocortical adenomas (ACA). Interestingly, transcriptome analysis has the ability to establish a subclassification of ACC based on the gene expression profile. In particular, it is able to identify two groups of tumors with different outcomes (i.e. good prognosis and poor prognosis). This approach has been used to develop molecular markers for ACC diagnosis and prognostication. An IGF2 cluster of genes up-regulated in ACC has been identified. Transcriptome analysis has shown that, in comparison with ACA, IGF2 is indeed the gene most overexpressed in ACC. By contrast, genes associated with steroidogenesis are down-regulated in ACC. Genes controlling the cell cycle are dysregulated in ACC, and several are dramatically overexpressed. Analysis regarding the level of expression of Wnt/β-catenin and p53 signaling has shown alterations, in keeping with the known molecular somatic genetic defects of these pathways that are observed in ACC. This review summarizes the main findings of studies reporting ACC transcriptome analysis, demonstrating its power for ACT classification, and examines the resulting progress in understanding the pathogenesis of ACC. The potential for both ACC diagnosis and the identification of new therapeutic targets will be discussed.

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Introduction

Unilateral adrenocortical tumors (ACT) are either benign (adenomas) or malignant (cancers). Adenomas are rather common, and are frequently found as incidental tumors (Grumbach et al. 2003). The clinical manifestations of ACTs may be consequences of steroid oversecretion, or malignant progression in the case of adrenocortical cancer (ACC). In contrast with adrenocortical adenomas (ACA), ACC is rare, with an estimated prevalence between 4 and 12 per million in adults. The prognosis of ACC is very poor, with a 5-year survival rate below 35% in most series (Luton et al. 1990, Allolio et al. 2004, Abiven et al. 2006, Libe et al. 2007a). As is often the case with endocrine neoplasms, the diagnosis of malignancy in an ACT can be a difficult task. The Weiss score, which analyzes nine items of histopathologic alterations, is most commonly used (Lau & Weiss 2009). However, this score suffers limitations as it depends on the expertise of the pathologist, and requires careful and time-consuming examination of the tumor. Although the overall prognosis of ACC is poor, it varies greatly among patients. The tumor stage at initial diagnosis, stratified by the MacFarlane stage, was recently revised by the European Network for the Study of Adrenal Tumors (Fassnacht & Allolio 2009); it is an obvious and well-validated prognostic factor for overall survival.
The rarity of ACC is a limiting factor in the progress to discern the pathophysiology of this tumor. Knowledge gained from the study of the inherited syndromes that increase ACC risk, coupled with the recent advances in genomic and expression profiling, is important to more completely understand the molecular mechanisms underlying ACT development.

High-throughput methods to assay genome-wide expression (transcriptome) have been developed over the last decade. These methods have become very potent and reliable. Current DNA chips used for microarray analysis allow researchers to study the expression level of virtually every gene known in the human genome. Microarray analysis has been successfully used for tumor classification and prognosis assessment in various neoplasms (Paik et al. 2004, Wang et al. 2005, Potti et al. 2006, Chen et al. 2007, Pollack 2007). This method has also been used to advance the understanding of tumorigenesis through the identification of genes, or groups of genes, involved in tumor development.

ACTs are among the various tumors that have been studied by transcriptome analysis. Over the last 7 years, several studies have been published that allow for a better understanding of the pathophysiology of ACTs. Interestingly, these studies have also resulted in the description of genes that could be used for the classification of ACTs. The most recent of these studies revealed a new classification of ACC based on gene profiling.

This review will summarize the various ACT transcriptome analyses reported to date, focusing on the genes implicated in ACT development, as well as the classification of ACT that could be discussed in view of these studies.

**Methods**

A PubMed search from 1995 to March 2010 was performed to identify all published studies involving transcriptome analysis of ACTs. The key words transcriptome, gene profiling, adrenal, and tumors were utilized.

Thirteen studies were identified (Table 1). Analysis of these studies was performed with two aims: 1) to analyze the tumor classification revealed by gene profiling (i.e. a gene expression profile that could be specific to a tumor group), and 2) to identify genes dysregulated in ACC that could play a role in tumorigenesis.

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<th>Study</th>
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<td>Giordano et al. (2003)</td>
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Thirteen studies from 2003 to 2010 compare expression profiles of adrenocortical carcinomas, adrenocortical adenomas and for few, normal adrenals. ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma.

²Study compares expression profiles of pediatric tumors.
²Meta-analysis.

**Molecular landmarks of malignancy in ACTs**

The malignancy signature is the strongest transcriptome feature of ACTs.

In clinical practice, ACTs are mainly classified according to their secretion and malignancy status. When ACTs are classified according to their transcriptome resemblance, two distinct groups emerge, corresponding to ACCs and ACAs, irrespective of their secretion (Giordano et al. 2003, 2009, de Fraipont et al. 2005, Velazquez-Fernandez et al. 2005, Slater et al. 2006, West et al. 2007, Fernandez-Ranvier et al. 2008a,b, Laurell et al. 2009, de Reynies et al. 2009, Soon et al. 2009, Tombol et al. 2009).

In our series of 92 unilateral ACTs, unsupervised hierarchical clustering based on tumor transcriptome classifies 98% of clinically identified benign tumors in one cluster, and 96% of clinically identified malignant tumors in the other cluster (de Reynies et al. 2009; Fig. 1).

The clear discrimination between ACC and ACA, which occurs when using unsupervised methods applied to raw transcriptome data, demonstrates that a large number of genes are differentially expressed in ACC versus ACA (Table 2). A recent meta-analysis underlined the consistency of these differences across the major studies published to date (Szabo et al. 2010). The following paragraphs will detail how these numerous differentially expressed genes can be clustered in functional pathways with either pathophysiological or clinical relevance.
The drastic overexpression of cell cycle regulators in ACCs

Various major alterations in the expression of genes involved with both cell proliferation and the cell cycle have been described for several tumors. In ACC, transcriptome analysis also shows the expression alterations of genes involved in G₁/S and G₂/M transition.

In an analysis of adrenocortical microarray data (Szabo et al. 2010) from three studies (Giordano et al. 2009, de Reynies et al. 2009, Tombol et al. 2009), some cyclin genes were dysregulated in ACC (Szabo et al. 2010). Overexpression of G₁ cyclins (CCNE1, CCNE2) was found in several studies (Table 2 and Szabo et al. 2010). Other regulators of G₁/S, including cell division protein kinases (CDK2 and CDK4), were overexpressed in ACC (Bourcigaux et al. 2000, Szabo et al. 2010).

Several genes involved in G₂/M transition were overexpressed. These included CCNB2, CDK7, the cell division control 2 (CDC2), cell division cycle 25 homolog B (CDC25B) genes, the topoisomerase II alpha (TOP2A) gene, ubiquitin C (UBC), and Mdm2 p53-binding protein homolog (MDM2; Szabo et al. 2010). In our microarray study, we have found additional genes overexpressed: CCNB1 for G₂/M transition and CCNA2 for S/G₂ transition (de Reynies et al. 2009, Assie et al. 2010).

The loss of steroidogenesis differentiation in ACC

Several genes involved in steroidogenesis are down-regulated in ACC, when compared to ACA (Table 2). In our microarray study, either the melanocortin 2 receptor or ACTH receptor (MC2R) is down-regulated in ACC. Moreover, three genes involved in...
steroidogenesis are down-regulated in ACC (Fig. 2): cytochrome P450, family 11, subfamily B, polypeptide 1 (CYP11B1); hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (HSD3B2); and aldo-keto reductase family 1, member B1 (aldo-keto reductase) (AKR1B1). AKR1B1 is regulated by cAMP, and was previously shown to be strongly decreased in ACC (Lefrancois-Martinez et al. 2004).

MC2R is a seven transmembrane G-protein-coupled receptor. It belongs to a family with five members. Its intronless gene is located at 18p11.2. Loss of allele at the MC2R locus has been reported in ACC. It seems that this alteration is rare in cortisol-secreting ACAs. Furthermore, MC2R expression is up-regulated in these adenomas. By contrast, down-regulation of the receptor is observed in non-secreting ACAs and in ACC (Reincke et al. 1997, 1998). Moreover, amino-glutethimide, an inhibitor of glucocorticoid synthesis, induces profound MC2R down-regulation in the human H295 ACC cell line (Fassnacht et al. 1998). This suggests a role for MC2R in cellular differentiation (Reincke et al. 1997).

It is likely that the decreased expression of MC2R in ACC could take part in the down-regulation of genes involved in steroidogenesis, the dedifferentiation of these aggressive tumors, and dysregulation of the cAMP

<table>
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<th>Table 2 The malignancy signature</th>
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<td><strong>Gene symbols</strong></td>
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<td><strong>Up in ACC</strong></td>
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<td>Growth factors and receptors</td>
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Adrenocortical carcinomas (ACC) differ from adrenocortical adenomas (ACA) by their increased expression of genes involved in growth, cell cycle, and DNA replication and by their decreased expression of genes involved in steroidogenesis and metabolism.
pathway that plays an important role in the physiology of the adrenal cortex (Rosenberg et al. 2003).

A steroidogenesis cluster containing 14 genes was identified to be down-regulated in ACC by de Fraipont et al. (2005). Six of these genes are directly involved in steroid biosynthesis: steroidalogenic acute regulatory protein (STAR); cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1); hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1); CYP11B1; cytochrome P450, family 21, subfamily A, polypeptide 2 (CYP21A2); and cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1). This cluster also contains cAMP-responsive element modulator (CREM), retinoblastoma 1 (RB1); protein phosphatase, Mg2+/Mn2+ dependent, 1A (PPM1A); non-metastatic cells 1, protein (NM23A) (NME1); transforming growth factor, beta receptor III (TGFBR3); S100 calcium binding protein B (S100B); glypican 3 (GPC3); and inhibin, alpha (INHA).

The tumors re-clusterized with the expression profiles of these steroidogenesis cluster genes were separated into two groups. Of the tumors in the group with low expression, 81% were ACC, and 93% of the tumors in the group with high expression were ACA.

Importance of the insulin-like growth factor 2 pathway

In the various transcriptome analyses comparing ACC and ACA, insulin-like growth factor 2 (IGF2) is the gene most overexpressed in ACC (Giordano et al. 2003, 2009, Bourdeau et al. 2004, de Fraipont et al. 2005, de Reynies et al. 2009, Soon et al. 2009). De Fraipont et al. (2005) described an IGF2 cluster containing eight genes that is overexpressed in ACC: two growth factors (IGF2 and TGFB2), two fibroblast growth factor receptors (FGFR1 and FGFR4), the macrophage stimulating 1 receptor (MST1R), the TGFβ receptor type I (TGFBR1), KCNQ1 overlapping transcript 1 (KCNQ1OT1 or LIT1) located in the IGF2 locus, and glyceraldehyde-3-phosphate dehydrogenase.

The tumors re-clusterized with expression profiles of these IGF2 cluster genes were separated into two groups. Ninety percent of the group with low expression was ACA, and in the group with high expression, 75% was ACC.

IGF2 involvement in ACC was initially considered because of Beckwith–Wiedemann syndrome (BWS). BWS causes macrosomia, macroglossia, organomegaly, and the development of embryonic tumors (Wilms’ tumor, hepatoblastoma, rhabdomyosarcoma, and ACC). The BWS locus at 11p15 (Henry et al. 1989) contains IGF2, H19 imprinted maternally expressed transcript (H19), and cyclin-dependent kinase inhibitor C (CDKN1C or p57/kip2). Because of parental imprinting, IGF2 is expressed from the paternal allele, and H19 and CDKN1C are expressed from the maternal allele. In BWS, various genetic and epigenetic alterations are associated with the overexpression of IGF2, as well as with the low expression of CDKN1C and H19 (Lam et al. 1999). IGF2 is
mostly expressed in embryonic tissues. Studies performed before the era of microarray have shown that ~90% of ACC and 8.5% of ACA overexpress IGF2 (Iivesmaki et al. 1993, Gicquel et al. 1994, 1997, 2001).

Several mechanisms may explain IGF2 overexpression. The most frequent is loss of the maternal allele with duplication of the paternal allele (paternal isodisomy). More rarely, a loss of imprinting occurs, leading to IGF2 expression by both parental genes (Ogawa et al. 1993, Rainier et al. 1993, Gicquel et al. 1997). Interestingly, it has been previously demonstrated that IGF2 expression could be used as a molecular marker for the diagnosis of ACC (Gicquel et al. 2001).

The IGF signaling pathway plays an important role in cell proliferation, and participates in the development of several tumors. Overexpressed IGF2 is thought to act in a paracrine manner through the IGF1 receptor (IGF1R), sustaining tumor and cell proliferation (Boule et al. 1998, Logie et al. 1999, Slater et al. 2006). It appears that the IGF1R, which mediates the trophic effects of IGF2, is expressed at the same level in benign and malignant tumors (Boule et al. 1998).

Regarding other cell types, Logie et al. (1999) showed in the ACC cell line H295R, the IGF2 effect on proliferation is dependent of IGF1R.

Six IGF-binding proteins (IGFBPs) regulate and modulate the effects of IGF1 and IGF2. In ACC, the expression of some IGFBPs was altered (Boule et al. 2001, Slater et al. 2006, Giordano et al. 2009, de Reynies et al. 2009). IGFBP2, which is strongly expressed in H295R cells and ACC, was particularly affected. IGFBP2 expression correlates with tumor mass (Boule et al. 2001).

The IGF system is an attractive therapeutic approach for ACC, and IGF1R antagonists are currently being tested in clinical trials.

Other growth factors

Several other growth factors or receptors are overexpressed in ACC, but their functional relevance remains to be determined. For example, various microarray studies have demonstrated the overexpression of FGFR1 and FGFR4 (de Fraipont et al. 2005, Velazquez-Fernandez et al. 2005, Slater et al. 2006, Giordano et al. 2009, de Reynies et al. 2009). These receptors may participate in tumorigenesis through their role in proliferation and vascularization.

Abnormal activation of the Wnt signaling pathway in ACCs

Several microarray analyses have shown an up-regulation of targets of the Wnt pathway in ACC. These have included baculoviral IAP repeat-containing 5 (BIRC5), ectodermal-neural cortex 1 (ENC1), pituitary tumor-transforming 1 (PTTG1), and twist homolog 1 (TWIST1; de Fraipont et al. 2005, Velazquez-Fernandez et al. 2005, Slater et al. 2006, Giordano et al. 2009, de Reynies et al. 2009). Moreover, in our microarray study (de Reynies et al. 2009), gene set enrichment analysis showed that the Wnt pathway was enriched in ACC versus ACA. These observations suggest activation of the Wnt pathway as a major alteration in ACC pathogenesis.

The Wnt proteins form a secreted growth factor family that is highly conserved, regulating several cellular processes. Abnormalities of the Wnt pathway have been described in the development of several cancers (Laurent-Puig et al. 2001, Chiang et al. 2002, Clements et al. 2002), including colorectal cancer and those associated with familial adenomatous polyposis (FAP) syndrome.

The regulation of β-catenin accumulation in the cytoplasm, with subsequent translocation into the nucleus, is the central intracellular event regulating the canonical Wnt pathway. In the absence of Wnt stimulation of its receptor, the AXIN–adenomatous polyposis coli (APC)–glycogen synthase kinase 3β complex binds and phosphorylates β-catenin, resulting in its ubiquitylation and degradation by proteosomes (Kikuchi 2003).

When the Wnt ligand activates intracellular signaling, β-catenin enters the nucleus and interacts with the lymphoid enhancer-binding factor 1/T cell-specific transcription factor family of transcription factors. This activates transcription of Wnt target genes.

Mutation of various actors of the Wnt signaling pathway that lead to a stimulation of Wnt signaling has been described in a large number of sporadic tumors (Giles et al. 2003).

Immunohistochemistry can be used to study β-catenin protein localization as a marker of Wnt pathway activation. When the pathway is not activated, β-catenin is localized at the cell membrane. After activation by an extracellular ligand or a genetic alteration, β-catenin is visible in the cytoplasm and/or the nucleus. In both benign and malignant ACTs, β-catenin delocalization can be observed. In the majority of ACCs, a diffuse delocalization is observed, consistent with an abnormal activation of the Wnt signaling pathway (Tissier et al. 2005). In a subset of these tumors, this can be explained by activating mutations of β-catenin.
In patients with FAP and ACC, biallelic inactivation of APC can activate the Wnt signaling pathway. By contrast, alterations of APC are not observed in sporadic ACC (Gaujoux et al. 2010, 2011). It remains to be demonstrated whether other components of the Wnt signaling pathway, such as AXIN, could take part in the activation of this pathway and contribute to the pathogenesis of ACC.

Abnormal retinoic acid signaling in ACCs

A recent study described the retinoic acid signaling of ACT transcriptome as a potentially relevant pathway (Szabo et al. 2010). Both retinoic acid production and action may be reduced in ACC. Retinoid X receptor alpha (RXRA) and aldehyde dehydrogenase 1a1 and 3 (ALDH1A1 and ALDH1A3), involved in oxidation and synthesis of retinoic acid, were found to be decreased in ACC in several microarray studies (Giordano et al. 2003, 2009, Velazquez-Fernandez et al. 2005, Laurell et al. 2009, de Reynies et al. 2009, Soon et al. 2009).

Retinoids are involved in several cancers, and are used in cancer therapy. 9-cis retinoic acid is a specific ligand for both the retinoid acid receptors and RXRs (Shimizu et al. 2009), and is able to inhibit proliferation of the ACC cell line H295 (Ferruzzi et al. 2005).

Malignancy status determination with fewer genes

The aim of transcriptome utilization is to reduce the number of genes studied, lower costs, and increase the reproducibility of results.

We recently took advantage of this ability, discriminating benign from malignant tumors and identifying molecular markers for ACC diagnosis (de Reynies et al. 2009). Indeed, the combined expression of two genes, large homolog 7 Drosophila (DLG7) and PTEN-induced putative kinase 1 (PINK1), was the best predictor of disease-free survival. The accuracy of DLG7–PINK1 was tested on an independent validation cohort of 94 non-metastatic tumors (adenomas and carcinomas). The molecular prediction appears to be at least as good as the Weiss score-based prediction. Prospective, multicenter validations are underway.

Subgroups of ACCs and their corresponding altered pathways

The transcriptome identifies two types of ACCs with different outcomes

Two recent studies showed that classifying ACCs according to their transcriptome resemblance identifies two distinct groups (Giordano et al. 2009, de Reynies et al. 2009). In both studies, overall survival differed significantly between the two groups (Fig. 3A and B). Tumors in the poor prognostic group showed more advanced disease. This raised the question as to whether these groups correspond to distinct types of ACC, or to distinct stages of similar tumors. The very distinct transcriptome profile, as well as the statistical independence of the transcriptome-based survival prediction from the tumor stage, argues in favor of the existence of distinct types of ACC.

Giordano et al. (2009) showed that carcinomas with a high degree of mitoses were more abundant in the poor-outcome group than in the good-outcome group.
Cell cycle regulators associated with ACC prognosis

In our series, 1850 genes were differentially expressed between the two groups of ACC. However, the genes driving this clustering, overexpressed in the poor-outcome group, were mainly involved in transcription and the cell cycle. The good-outcome group was enriched in genes involving cell metabolism and intracellular transport (Table 3).

TP53 mutations in ACCs

When compared to ACA, we observed that the p53 signaling pathway group of genes was up-regulated in ACC (de Reynies et al. 2009). We recently identified, by unsupervised clustering analysis, a subgroup of ACC which contained all ACC with an inactivating mutation of the tumor protein p53 (TP53) gene (Ragazzon et al. 2010). Our analysis showed that this alteration had a major influence on tumor biology. Indeed, global expression of p53-positive target genes was altered in this subgroup. Some of these target genes have been implicated in oncogenesis.

TP53 was first considered in the pathophysiology of ACC because of Li-Fraumeni syndrome. This syndrome is caused by germline mutations of the tumor suppressor gene p53 (TP53). This gene plays an important role in the control of cellular growth and division. Carriers of TP53 mutations can develop various tumors: breast cancer, brain tumors, acute leukemia, soft tissue sarcomas, bone sarcomas, and ACC (Hisada et al. 1998).

The p53 protein is important for cell cycle regulation, and causes cell death when DNA is damaged. Its gene, located at 17p13, is the most frequently mutated across all cancers (Hollstein et al. 1991, Caron de Fromentel & Soussi 1992). In the majority of cancer types, acquired mutations of this tumor suppressor gene have been identified (Caron de Fromentel & Soussi 1992).

In North America and Europe, 50–80% of children with sporadic ACC have a germline mutation of TP53 (Wagner et al. 1994, Varley et al. 1999). In Southern Brazil, pediatric ACCs are ten times more frequent than in other parts of the world. In almost all cases, an identical mutation in the tetramerization domain of the TP53 gene (R337H) has been found (Latronico et al. 2001, Ribeiro et al. 2001). The effects of this mutation may be pH-dependent during adrenal development (DiGiammarino et al. 2002).

Somatic mutations of TP53 are mostly located within exons 5–8, and are found in 25–35% of sporadic ACCs (Ohgaki et al. 1993, Reincke et al. 1994, Libe et al. 2007b). Interestingly, these mutations are usually observed in larger and more advanced tumors.

Moreover, a loss of heterozygosity at the 17p13.1 locus has been demonstrated in ~85% of ACCs, but in <30% of ACAs (Yano et al. 1989, Gicquel et al. 2001).

Wnt/b-catenin pathway activation occurs more often in the poor prognostic subgroup of ACCs

A majority of ACCs show a b-catenin nuclear delocalization in immunohistochemistry. However, in a subgroup of ACCs (approximately one quarter of cases), this delocalization is explained by activating mutations of catenin (cadherin-associated protein), beta 1 (CTNNB1) gene (Tissier et al. 2005,
Gaujoux et al. (2011). This suggests that other genetic alterations influence the β-catenin protein profile.

In our series, β-catenin nuclear delocalization and mutations were enriched in the poor-outcome group of ACCs, delineating a specific subgroup (Ragazzon et al. 2010).

Prognosis of ACC, determined by a few genes

We took advantage of this subclassification of ACC, using gene profiling to identify molecular markers for survival prognostication in ACC. The combined expression of budding uninhibited by benzimidazoles 1 homolog beta (BUB1B) and PINK1 was the best predictor of overall survival in ACC (de Reynies et al. 2009). Tumor classification based on IGF2 expression levels is less accurate than these molecular markers for both malignancy diagnosis and survival prediction. These molecular tools, derived from microarray studies, will be a significant aid to patient management. They are both technically simple and standardized, and could therefore be used in current practice. These tools would strongly complement the current clinical tools used for the pathological diagnosis of malignancy, especially in doubtful cases or in centers with limited expertise in adrenal tumors.

Conclusion

Since the first report of ACC transcriptome by Giordano et al. (2003) 7 years ago, this large-scale approach, now virtually pan genomic, has been the source of significant progress in the field of this rare but dramatic cancer. Some of the results produced from transcriptome analysis have underlined the importance of previous observations, such as IGF2 overexpression. But the power of this pan genomic approach, and its ability to identify clusters of dysregulated genes, has clearly contributed to the development of a new vision of the molecular genetics of ACTs. Importantly, the various studies summarized in this review have reported constant findings for their major observations, underlying the value of this approach to studying ACTs. Transcriptome analysis has clearly demonstrated its strength for ACT classification. It not only differentiates benign from malignant ACTs, but can also now reveal ACC subgroups. This molecular subclassification of ACC might be important for the diagnosis and prognostication of these tumors. It has already been used successfully to develop molecular markers for this purpose. It is also likely that an understanding of the genes and molecular alterations driving this classification will reveal new aspects of ACC pathogenesis. The identification of signaling pathways playing an important role in ACC development is also likely to result in new, targeted therapies. The IGF2 signaling pathway identified by molecular studies of ACC can be targeted by IGFR inhibition. Such an approach is already being used in clinical trials. Wnt/β-catenin signaling is another pathway identified by this approach that has already been inhibited in in vitro models of ACC (Doghman et al. 2008). Other targets of ACC could potentially be developed from the signaling pathways described in this review.

Declaration of interest

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

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