The Role of mTOR as Target for Treatment of Adrenal Tumors

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De rol van mTOR als doelwit voor de behandeling van bijnier tumoren

THESIS

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General introduction and aims of the thesis

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THE ADRENAL GLANDS

Adrenals are two symmetric endocrine glands (about 2x5x1 cm; weight approximately 4 g) with a pyramidal shape, placed on the top of the kidneys in the retroperitoneum. Each adrenal has two distinct structures: the external part is named adrenal cortex and the internal medulla, producing different hormones (Figure 1). The anatomy of these glands was firstly described by Bartholomeo Eustacius almost 450 years ago.[1]

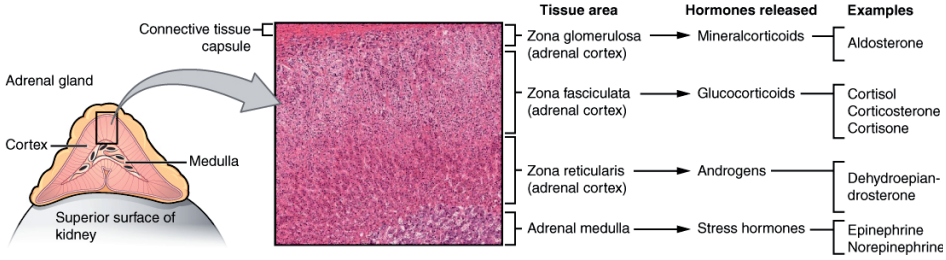

Figure 1. The anatomy of adrenal glands. Adapted from Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013.

Adrenal cortex

The adrenal cortex cells originate from mesoderm and in adult humans are organized in three zones or layers that, from the external to the internal, are named, zona glomerulosa (15%), zona fasciculata (75%) and zona reticularis (10%). In these zones, cells are organized in different histological structures that can be recognized microscopically. The adrenal cortex produces steroid hormones, which are derived from their common precursor cholesterol and have a common cyclopentano-perhydro-phenanthrene structure. The different adrenal cortex zones produce different steroids, depending on the differential expression of specific enzymes involved in steroid hormone synthesis. The *zona glomerulosa* is the site for production of mineralocorticoids, particularly aldosterone, that has a central role in the long-term regulation of blood pressure under the principal control of angiotensin II.[1] The *zona fasciculata* is the site for production of glucocorticoids, among which cortisol represents the most important in humans. Glucocorticoids are regulated by ACTH and they have an important role in the systemic stress response, cardiovascular function and regulation of metabolism.[1] The *zona reticularis* produces the adrenal androgens (DHEA; DHEA-S and androstenedione), which play a role particularly in premenopausal women, but rarely in some pathological conditions, their excess can have a clinical significance in men as well.[1]

Adrenal medulla

The cells forming the adrenal medulla are called chromaffin cells because they stain brown with chromium salts. These cells originate from the embryonic neural crest and can be considered as modified neurons that are mainly regulated directly by the sympathetic nervous system through preganglionic fibers originating in the thoracic spinal cord. Therefore, the adrenal medulla can be considered as a specialized sympathetic ganglion that releases its secretions directly into the blood. The adrenal medulla occupies the central portion of the adrenal gland and accounts for 10% of the total gland volume and produces catecholamine (epinephrine, norepinephrine and dopamine). In humans, catecholamines regulate many cardiovascular and metabolic processes.[1]

ADRENAL GLAND TUMORS

Primary adrenal tumors (AT) include benign and malignant adrenocortical tumors (ACT) and benign and malignant tumors of the adrenal medulla named pheochromocytomas (PCC).[2, 3] Both benign and malignant ATs can be hormonally inactive or they can be hormone-secreting, resulting in specific clinical syndromes including hyperaldosteronism or Conn's syndrome, hypercortisolism or Cushing's syndrome, virilizing or feminizing syndrome in case of ACTs and catecholamine excess syndrome in case of PCCs. The clinical symptoms and signs can be also related to mass effects that generally occur in malignant ATs because of their growth and/or metastases. However, ATs can also be clinically silent and discovered occasionally during diagnostic imaging procedure performed for indications other than an evaluation for adrenal disease. These adrenal masses (generally 1 cm or more in diameter) accidentally discovered are defined as *incidentalomas*. Adrenal masses are quite common with an overall frequency of about 6% (range, 1-32) and 4% (up to 10% in elderly patients) in radiological and in autopsy studies respectively.[4, 5] Most ATs are benign, hormonally inactive and clinically silent, and are often discovered incidentally.[4, 5] However, they can require long-term clinical follow-up.[6] On the other hand, malignant ATs are rare and aggressive with poor prognosis and scant treatment options.[7] The surgical removal of the tumor represents the treatment of choice for hormone-secreting tumors and for adrenal masses with a clinical and radiological suspicion of malignancy malignancy.[2, 8, 9] However, in malignant ATs, surgery allows a complete remission only in patients with a diagnosis during the early stages of disease.[3, 7, 10] In benign AT, medical treatment is currently restricted to the use of symptomatic drugs such as spironolactone and adrenal blocking agents in ACTs of the cortex and anti-adrenergic agents in PCCs. New treatment options are required for malignant ACTs and PCCs.

Adrenocortical carcinomas

Epidemiology

In contrast to the high prevalence of benign ATs, the malignant tumor of the adrenal cortex, named adrenocortical carcinoma (ACC), is rare.[11-13] The incidence of ACC is about 0.5-2 new cases/million/year, with an increased occurrence during the 5th-6th decades of life.[11-14] A second peak of increased incidence during childhood has also been described.[13, 15-18] Apart from some genetic factors that are discussed below, to date no risk factors have been identified that clearly predispose for the development of ACC.

Genetic aspects

Several genetic syndromes have been associated with the development of adrenal hyperplasia/neoplasia.[19] Most ACCs are sporadic and although adrenal malignancies have been described only in few cases with a proven genetic background, the study of these syndromes has supported the potential role of some molecular pathways in ACC pathogenesis.

The Beckwith–Wiedemann syndrome (BWS) is a genetic syndrome associated with ACC, together with different childhood tumors and a somatic overgrowth syndrome.[19, 20] BWS is caused by various epigenetic and/or genetic alterations that involve genes on chromosomal locus 11p15, which contains the genes *CDKN1C*, *IGF2* and *H19*, structurally organized in a cluster.[19, 20] In patients with BWS deregulation of imprinted genes on chromosomal locus 11p15 leads to a biallelic expression of *IGF2* and inactivation of *CDKN1C* and *H19*,.[20] *IGF2* encodes for the insulin-like growth factor 2, a growth factor mainly expressed during fetal life and involved in fetal growth that is thought to play a growth-promoting role in various malignancies. The transcriptional product of *H19* is a noncoding RNA acting as a transcriptional repressor of IGF2. *CDKN1C* encodes for the cyclin-dependent kinase inhibitor 1C (p57, Kip2), a strong inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation.[19] Although the estimated prevalence of BWS in patients with ACC is very low and restricted to the childhood,[19, 21] IGF2 has been reported to be over-expressed in about 70-90% of the sporadic ACCs, as compared with normal adrenals or benign ACTs.[22-31]

ACC develops in 3-10% of patients with Li-Fraumeni syndrome (LFS), an autosomal dominant disorder characterized by an increased susceptibility to early-onset development of several types of cancer, including breast cancer, soft tissue sarcomas, brain, and hematologic cancers.[19, 32, 33] About 70% of the patients with LFS harbor a germline mutation in the tumor suppressor gene *TP53* encoding for p53. P53 is a protein considered "the guardian of the genome" because it plays a central role in the activation of pathways involved in apoptosis, cell cycle arrest and DNA repair in response to genotoxic stress and oncogene activation, preventing genome mutation and preserving stability.[19, 33, 34]

A high frequency (50-80%) of germline *TP53* mutations has been described in children with ACC.[18, 35-37] An increased frequency of these mutations contributes to the increased incidence of childhood ACC in some regions such as the southern Brazil.[13, 15-18] Two recent non-Brazilian studies reported that germline *TP53* mutations are present in 3.9 and 5.8% of the respective adult ACC population.[38, 39] Therefore, the role of germline *TP53* mutations in the pathogenesis of adult ACC seems less important than in childhood ACC.[33] Somatic *TP53* mutations have been described in 15-70% of ACC samples,[40-46] supporting a potential role of p53 pathway in a subset of ACCs.

The Gardner's syndrome or familial colorectal polyposis (FAP) is an autosomal dominant form of polyposis characterized by the presence of multiple polyps in the colon with an increased risk for early onset colon cancer and to develop other tumors outside the colon including gastric carcinoma, periampullary carcinoma, astrocytoma, fibrosarcoma, small intestine carcinoid and papillary thyroid carcinoma.[47, 48] It has been recognized that a substantial proportion of patients develop bilateral adrenocortical nodular hyperplasia, which are characteristically non-functional and benign, but ACCs have also been described.[21, 49-51] FAP is caused by germline inactivating mutations of the *APC* gene, which encodes for a downstream regulator of the Wnt/β-catenin pathway, functioning as a classic tumor suppressor gene by antagonizing Wnt/β-catenin activation[52-55] (Nishisho et al.,1991). Canonical Wnt signalling relies on accumulation of the multifunctional β-catenin protein which translocates to the nucleus and regulates the transcription of target genes.[52] Mutations of *CTNNB1* (β-catenin gene) induce a constitutive of β-catenin target genes.[55] Although the estimated prevalence of FAP in patients with ACC is very low,[19] *CTNNB1* mutations have been frequently described (16-31%) in ACCs.[44, 51, 56-60] These evidences support a potential role of Wnt/β-catenin pathway in a subset of ACTs.

Other genetic syndromes potentially predisposing for ACC include: Lynch syndrome, Multiple Endocrine Neoplasia 1 and Neurofibromatosis 1.[19, 21, 61] Other syndromes such as Carney's complex and McCune–Albright, are predominantly related to benign adrenal pathology but no to ACC.[13, 19]

Clinical presentation and diagnosis

The clinical presentation of ACC mainly includes clinical symptoms related to hormone excess, nonspecific symptoms related to tumor growth and only rarely cachexia.[13, 14] Hypercortisolism is present in more than half of ACC patients and Cushing's syndrome is the most common clinical presentation of these tumors.[13, 14] Hyperandrogenism, associated or not with hypercortisolism, is also frequent (in about one-third of cases) and causes virilization syndrome particularly in women (acne, hirsutism, androgenetic effluvium, oligomenorrhea) and children (precocious pseudopuberty) with ACCs.[13, 14] Less frequently, ACC can be associated to hyperestrogenism, causing feminization syndrome

in men. Autonomous aldosterone secretion (causing hypertension and hypokalemia) is rare in ACC.[13, 14]

In 2005 the European Network for the Study of Adrenal Tumors (ENSAT) suggested a preoperative laboratory workup with evaluation of (precursor) hormone excess and urinary steroid metabolite profile in all patients with a suspicion of ACC (Table 1) (www.ensat.org/).[14] This extensive panel can be useful to support the adrenocortical origin, to suggest malignancy and to document hypercortisolism, although the cost effectiveness of this approach has not been proved yet.[62]

Table 1 preoperative laboratory workup in patients with ACC suggested by ENSAT

Hormonal work up	
Glucocorticoid excess (minimum 3 out of 4 tests)	• dexamethasone suppression test (1 mg, 23:00 h) • excretion of free urinary cortisol (24h urine) • basal cortisol (serum) • basal ACTH (plasma)
Sexual steroids and steroid precursors	• DHEA-S (serum) • 17-OH-progesterone (serum) • androstenedione (serum) • testosterone (serum) • 17-beta-estradiol (serum, only in men and postmenopausal women)
Mineralocorticoid excess	• potassium (serum) • aldosterone/renin ratio (only in patients with arterial hypertension and/or hypokalemia)
Exclusion of a phaeochromocytoma	• Exclusion of a phaeochromocytoma • Catecholamine or metanephrine excretion (24h urine) • meta- and normetanephrines (plasma)
Imaging	
	• CT or MRI of abdomen and CT thorax • Bone scintigraphy (when suspecting skeletal metastases) • FDG-PET (optional)
Staging during follow-up	
	• CT or MRI of abdomen and CT thorax every 2 - 3 months (depending on treatment)

The upper panel of the table shows the preoperative laboratory workup that ENSAT suggests to perform in all patients with a suspicion of ACC, the middle panel indicates the imaging procedure suggested by ENSAT in all patients with a suspicion of ACC at time of the diagnosis of the tumor and the lowest panel indicates the imaging procedure suggested by ENSAT during the follow-up of patients with ACCs.

At the time of diagnosis, unfortunately most ACC are large tumors and already at an advanced stage of disease.[11, 13] Therefore, an appropriate evaluation using computed tomography (CT) and/or magnetic resonance imaging (MRI) to define the tumor burden is required.[7, 13, 63] F-18/flourodeoxyglucose-positron emission tomography (FDG-PET) can help to detect suspect malignancy when an adrenal lesion is radiologically uncertain,[7, 62] while other diagnostic techniques such as bone scintigraphy are required only in

selected cases or are experimental such as [^{11}C]Metomidate PET or [^{123}I]Iodometomidate scan.[7, 13, 62, 64, 65]

On the basis of the ENSAT classification,[66] advanced ACC is defined by locoregional spread (stage III) or the presence of distant metastases (stage IV). At the time of diagnosis about 18-26% and 21-46% of ACC patients have stage III and stage IV disease, respectively.[11, 66-68]

Prognostic factors

ACC is one of the most aggressive solid tumors in humans, as evidenced by a 5-year survival rate below 15% at the metastatic stage.[11, 12] In the absence of local invasion and/or metastasis, the differential diagnosis between benign ACT [or adrenocortical adenoma (ACA)] and ACC is challenging and is based on the use of pathological scores evaluating the presence or absence of particular features.[13] The most widely used score is the Weiss's score (WS), which includes 9 parameters: mitosis, atypical mitosis, necrosis, venous invasion, sinusal invasion, capsular invasion, nuclear atypia, diffuse architecture, and clear cell.[69-71] ACTs having at least 3 of the 9 parameters indicated in the WS can more often behave as malignant tumors and they are therefore classified as ACC, although this distinction still has some limitations.[7, 13, 71] The status of tumor margin after resection[12] and the TNM according with the ENSAT classification[66] are well established prognostic factors. Other prognostic factors include the mitotic count (which is also included in the WS),[72] proliferative index (Ki67),[73] number of organs involved[72] and cortisol excess.[74] Recently, some molecular markers like IGF2, and β-catenin have also been suggested to have a prognostic role[75, 76] but these require a further validation.[7, 13]

Therapy

Surgery remains the only curative treatment in patients diagnosed at an early stage and should be performed in centers with consolidated experience (more than 10 ACCs/years).[7, 11-14] In patients with ACC, open surgery is the standard surgical approach for stage I-III. The role of laparoscopy, which can be considered as the standard surgical approach for benign adrenal masses and which can be performed safely by a skilled surgeon, also in case of small localized ACC, is still matter of debate.[7, 13] The aim of surgery in locally advanced ACC is to obtain a margin-free complete resection, which might require resection of parts of some other adjacent organs.[7] Adjuvant therapies can include radiotherapy and/or mitotane as shown in figure 2. These therapies aim to decrease the chance of recurrence, particularly in case in which surgery failed to obtain microscopic margin-free resection or in case of tumors with high risk of recurrence as defined by the presence of a stage III disease or a Ki67 higher than 10%.[7]

All therapies of unresectable or metastatic ACC must be considered palliative.[13] In the case of inoperable local infiltrating or metastatic ACC, surgical excision of the primary

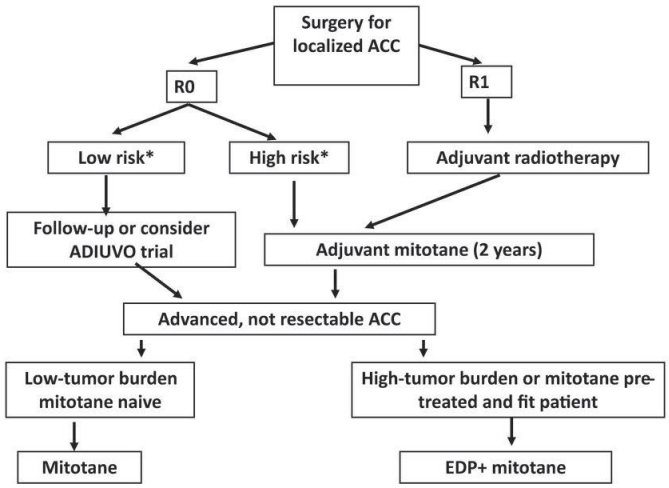

Figure 2. Algorithm of the management of adrenocortical carcinoma (ACC) according to stage, risk factors, and disease characteristics. *Low-risk ACC is defined stage I–II and Ki67 expression in ≤10% of neoplastic cells; high-risk ACC: stage III or Ki67 expression in >10% of neoplastic cells. Reproduced with permission from reference n 6.

tumor and/or metastasis should be considered in the case of an objective response after neoadjuvant chemotherapy, when a radical resection seems to be feasible.[7, 67] Additionally, surgery can be considered in patients with tumor recurrence after an initial resection, in case of patients with a time to first recurrence longer than 12 months, and when radical resection seems to be feasible.[7]

Mitotane, a derivate of the DDT pesticide, is currently the only drug approved in Europe and in the United States for the treatment of advanced ACC.[7] As monochemotherapy, mitotane induces an objective tumor response in 13-35% of patients.[7, 11] Several retrospective studies reported a higher objective response rate (up to 66%) and improved survival when the patients presented plasma mitotane level above 14 mg/L.[11, 14, 77] Response rates ranging between 14 and 55% were reported when mitotane was combined with cytotoxic chemotherapy in retrospective or phase II trials.[11, 14, 77] Recently the FIRMACT study, the first phase III trial ever performed in ACC compared the two polychemotherapy regimens believed to be the most active in ACC, mitotane plus etoposide, doxorubicin and cisplatin (M-EDP) versus mitotane plus streptozocin (M-S). M-EDP yielded a significantly higher response rate (23% vs 9%), but overall survival was not significantly different compared to M-S (14.8 months vs 12 months).[78] These results have been interpreted as a superiority of M-EDP, which is currently considered the first line regimen in ACC patients requiring cytotoxic therapy.[7] Unfortunately, no new treatment options have emerged in the last four decades underlining the urgent need for new therapeutic options.[11, 14, 77, 78]

Pheochromocytomas (PCCs)

Epidemiology

PCCs and paragangliomas (PGLs) are neuronal crest-derived neuroendocrine tumors arising from the chromaffin cells of the adrenal medulla or the extra-adrenal paraganglia.[3, 7, 79] The incidence of PCCs/PGLs is about 2-8 new cases/million/year.[3, 7, 80] Malignancy is defined by the presence of metastasis in non-chromaffin tissues and it is rare representing about 10-17% of all PCCs/PGLs, with an overall incidence below 1 new cases/million/year.[7, 80, 81]

Genetic aspects

During the last 20 years important progress has been made in discovering genetic alterations that can lead to development of PCCs and PGLs in the context of familial syndromes and in some sporadic cases.[82, 83] About 40% of the PCC/PGL patients harbor a germline mutation in one of the susceptibility genes identified, which include kinase receptor and signalling regulators [such as RET and neurofibromin 1 (NF1)], transcription factors [such as MYC-associated factor X (MAX)], energy metabolism components [such as succinate dehydrogenase (SDH) subunits SDHA, SDHB, SDHC, SDHD and cofactor SDHAF2], constituents of the cellular response to hypoxia [such as von Hippel–Lindau (VHL) and hypoxia-inducible factor 2A (HIF2A)], as well as endosomal signaling [such as transmembrane protein 127 (TMEM127)].[83]

An additional 25-30% of the remaining apparently sporadic cases harbor a somatic mutation in one of the above-mentioned genes.[82, 83]

Based on their transcriptional profiles, PCCs/PGLs have been classified in two different clusters: cluster 1 includes tumors with an increased expression of genes related to the hypoxic response, whereas cluster 2 includes tumors with an increased expression of genes related to kinase pathways.[83-85] PCCs/PGLs harboring mutations in *VHL*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2* and *HIF2A* belong to cluster 1, whereas tumors harboring mutations in *RET*, *NF1*, *TMEM127* and *MAX* belong to cluster 2.[83, 85]

Clinical presentation and diagnosis

In most patients, the clinical presentation of PCCs/PGLs is related to catecholamine excess which can cause acutely a variety of clinical symptoms (hypertension, tachycardia, pallor, headache, anxiety, hyperglycemia, weight loss, nausea, constipation, fever, flushing etc.). As such, this type of tumor is often referred to as the great mimic.[10, 80] The catecholamine hypersecretion causes in these patients an increased cardiovascular morbidity and mortality. Symptoms related to tumor growth are less common, but they can be the first clinical presentation of some tumors that are unable to secrete catecholamines.[80] In the case of patients affected by a familial syndrome, the presence

of other clinical manifestation associated with the syndrome or the periodic radiological screening can lead to the diagnosis of PCCs/PGLs. About 25% of all PCCs are discovered incidentally and about 5% of all incidentalomas are PCCs.[10, 86, 87]

When a PCCs/PGLs is clinically suspected the measurement of plasma free (nor) methanephrine (catecholamine metabolites) or urinary fractionated (nor)methanephrine is recommended.[80] In patients with a clear biochemical evidence of a PCCs/PGLs, localization of the tumor is performed with CT of the thorax, abdomen and pelvis with nonionic contrast as first-choice imaging modality, while MRI is preferred in case of head/neck paragangliomas PGLs.[80] In all patients with metastatic disease and in some patients with increased risk of metastatic disease functional imaging using ^{18}F-FDG PET/CT and ^{123}I-metaiodobenzylguanidine (MIBG) and/or somatostatin receptor imaging with ^{111}In-Pentetreotide and/or ^{18}F-DOPA can be required for a better definition of tumor burden, but also to guide treatment.[80]

Prognostic factors

The overall 5 years survival rate in malignant PCCs/PGLs ranges between 34-60%.[7, 10, 88] As above mentioned, most PCCs/PGLs are benign, but in absence of proven metastasis, it is difficult to predict whether a tumor behaves as benign or malignant. Some prognostic factors, such as the presence of a *SDHB* mutation, a tumor diameter larger than 5 cm and an extra-adrenal localization, can help to identify patients that require a closer follow-up.[10, 88-92] However, since metastasis can be discovered even a long time after the resection of the primary tumor a long term follow-up (at least 10 years) is required in all patients.[7, 10, 80, 81, 93]

Therapy

Different from the important progress made in discovering genetic alterations that can lead to development of PCCs and PGLs, only scant progress has been made in treating patients with malignant PCCs/PGLs. In all patients with resectable tumors, surgery is the first treatment approach.[7] The treatment with anti-adrenergic agents (i.e. α-adrenergic blockers) can help to manage symptoms and is recommended in all hormonally active PCCs/PGLs to reduce perioperative cardiovascular complications.[80] In patients with metastatic PCCs/PGLs, treatment is palliative and it aims to control excessive catecholamine secretion and tumor burden.[7] According with the recently published European Society for Medical Oncology (ESMO) clinical practice guidelines, in patients with unresectable/metastatic disease, when non progressive and with low tumor burden a wait and see approach and/or a loco-regional approach is feasible, while in patients with an apparently more aggressive disease treatment should be recommended.[7]

In progressive PCCs, treatment with meta-iodobenzylguanidine (^{131}I-MIBG) or certain types of systemic chemotherapy are used, but a low rate of response and frequent recurrences underline the need of new treatment approaches.[7, 94]

THE ROLE OF THE mTOR PATHWAY IN NORMAL AND TUMORAL ADRENAL CELLS

Introduction

The limited efficacy of conventional antineoplastic treatment in malignant ATs increases the need for novel and more effective treatment options. In recent years, progress in the understanding of the pathogenesis of tumors is encouraging and has initiated the development of so called "targeted drugs", compounds that specifically interfere with molecular mechanisms involved in tumor cell growth and/or tumor vascular supply. This category of drugs includes compounds interfering with growth factor receptors (including pro-angiogenesis factors) and their related signaling pathways. Alterations in expression of growth factors and their cognate receptors are considered to be involved in the pathogenesis of both ACCs and PCCs.[95-97] Moreover, tumor angiogenesis is essential for the growth and progression of solid tumors, including ATs.[95, 96, 98, 99] Therefore, compounds interfering with tumor angiogenesis and growth factor signaling pathways represent a potential novel treatment opportunity for the management of malignant ATs.

The mTOR pathway

The mammalian target of rapamycin (mTOR) is a protein kinase of the phosphoinositide 3 kinase (PI3Ks)/protein kinase B (PKB or AKT) signaling pathway, that forms multimolecular intracellular complexes and functions as a gatekeeper of metabolism, as well as cell growth. mTOR receives signals from sensors of cell stress, intracellular nutrients levels and several growth factors, including vascular endothelial growth factor (VEGF), insulin-like growth factors (IGFs), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF).[100-103] The complexes formed by mTOR are named mTORC1 and mTORC2, and are sensitive or insensitive to rapamycin respectively, the oldest compound among the mTOR inhibitors.[104, 105] The binding of many growth factors to their cognate receptor tyrosine kinases (RTKs), leads to the recruitment of PI3K to the membrane, where this enzyme converts phosphatidylinositol-bisphosphate (PIP2) to phosphatidylinositol-trisphosphate (PIP3), providing docking sites for signaling proteins, including 3-phosphoinositide-dependent protein kinase 1 (PDK1) and serine-threonine kinases AKT. These events are antagonized by PTEN (phosphatase and tensin-homologue) that limits the growth factors signaling by dephosphorylating PIP3. Figure 3 shows a simplified

scheme of growth factor induced activation of the mTOR pathway and the sites of action of compounds acting as mTOR inhibitors.

PDK1 is a serine-threonine kinase that phosphorylates and activates AKT, which elicits many downstream signaling events including the activation of mTORC1. AKT promotes mTORC1 activity mainly by phosphorylating tuberosus-sclerosis-complex (TSC1/2).[104, 106] This phosphorylation leads to the inactivation of TSC2 that is an indirect inhibitor of mTORC1 and results in the activation of this complex.[104] mTOR as part of mTORC1 complex can be also directly phosphorylated by AKT, forming phospho-mTOR (p-mTOR) that is often studied as marker of mTOR activation. However, whether this phosphorylation is crucial or not in the activation of mTORC1 complex is still matter of discussion.[104, 106] The activation of the mTORC1 complex leads to the phosphorylation and activation of downstream effectors of the pathway: the protein kinase p70 ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E binding proteins (4EBP1).[104, 105] S6K1 and 4E-BP1 are both regulators of mRNA translation and stimulate the synthesis of several oncogenic proteins such as c-Myc, hypoxia-inducible factor-1α (HIF-1α), VEGF, IGF-II and cyclin D.[101] mTORC2 regulates the cytoskeleton function and it has been proposed to be an important activator of the AKT function. Growth factors stimulate mTORC2, activity and some components of mTORC2 complex are phosphorylated as consequences of growth factor stimulation, but the responsible kinases are uncertain.[104, 105] Therefore, there are evidences that mTOR, as part of both complexes (mTORC1 and mTORC2), mediates the intracellular signaling induced by growth factor receptor activation (Figure 3). However, mTORC1 is downstream to AKT and mTORC2 is to be upstream to AKT.[101, 104]

It is noteworthy that mTOR is an intracellular mediator of IGFII and VEGF activity and, in turn, it is able to control the synthesis of these growth factors.[101] VEGF is one of the most important mediators of angiogenesis in solid tumors including adrenal tumors. Moreover, growth factors such as IGFs and VEGF are autocrine/paracrine regulators of tumoral cell growth in many different types of tumors, including ATs (Figure 4).[95, 97-99, 107]

Dysregulation of the mTOR pathway has been found in many human tumors, including neuroendocrine tumors.[101, 102, 108, 109] Therefore, the mTOR pathway is as an important target for antineoplastic therapies and it is conceivable that this pathway could also play a role in the pathogenesis of ATs. mTOR inhibitors may exert their antitumor effects indirectly, by inhibiting tumor angiogenesis, and directly by inhibiting cell growth and proliferation.[104] As such, a role for mTOR inhibitors in inhibiting ATs growth, particularly because of central role of mTOR pathway as mediator of several mitogenic and angiogenic factors, may be suggested.

A clinical role for an mTOR inhibitor as anticancer treatment was first demonstrated in a large phase III study that showed an improved overall survival in patients with renal cell carcinoma treated with temsirolimus (CCI-779), as compared with other treatments.[110]

Subsequently, many clinical trials have been conducted and others are still ongoing, to investigate the antineoplastic effects of mTOR inhibitors, alone or in combination with other compounds, in several kinds of solid tumors and hematological malignancies.[111] This has led to the approval of some of these compounds for the treatment of some type of cancer including particular categories of renal cancer, neuroendocrine tumors and breast cancer.[110-113] Most of the evaluated compounds such as sirolimus (rapamycin), temsirolimus, everolimus (RAD001) and deforolimus (AP23573), mainly target the mTORC1 complex (shown in Figure 3 as "traditional" mTOR inhibitors),.[101, 102, 106, 108, 111]

The activity of mTORC2 is not directly inhibited by the above listened compounds, but it has been reported that long-term treatment with rapamycin can inhibit the activity of mTORC2 by the sequestering of mTOR as part of the mTORC1 complex.[101, 105] However, new compounds, able to inhibit directly both mTORC complexes, such as AZD8055 and OSI-027 (reported in figure 3 as "new" mTOR inhibitors), are currently being evaluated for their efficacy in preclinical and clinical studies.[106, 111] The results of these studies should answer the question whether the use of compounds blocking both mTORC complexes could have an additional advantage over the use of traditional mTOR inhibitors in the treatment of tumors.

At the time the studies presented in this thesis were initiated, there were no preclinical or clinical studies evaluating the effects of mTOR inhibitor drugs in ACTs and only very limited data in PCCs.[109, 114, 115]

Therefore, the overall aim of this thesis is to explore the role of the mTOR pathway as a potential target for new treatment option in ATs.

Role of the mTOR pathway in the normal adrenal gland

There are only limited studies that addressed the mTOR pathway in normal adrenal gland. The expression of mTOR, p-mTOR, AKT and phospho-AKT (p-AKT) in the normal adrenal gland was initially evaluated only as a control for ATs.[109, 116] In 3 out of 3 normal adrenals no staining for p-mTOR and p-AKT, evaluated by immunohistochemistry, was reported in normal adrenal-medullas.[116] Fassnacht *et al.* evaluated the expression of total-AKT and p-AKT in 4 normal adrenal glands by immunohistochemistry.[109] These investigators described a notable staining for total-AKT and p-AKT in the reticularis layer, whereas a weaker expression of total-AKT and p-AKT was found in the zona fasciculata and zona glomerulosa and only a faint staining for total-AKT in the normal medulla. These preliminary observations already suggested a layer-specific pattern of activation of the PI3Ks/AKT/mTOR pathway in the normal adrenal gland, which appeared to be predominantly inactive in the normal adrenal medulla and active in the reticularis layer of the adrenal cortex. IGFs have been reported to stimulate the adrenal steroidogenesis,

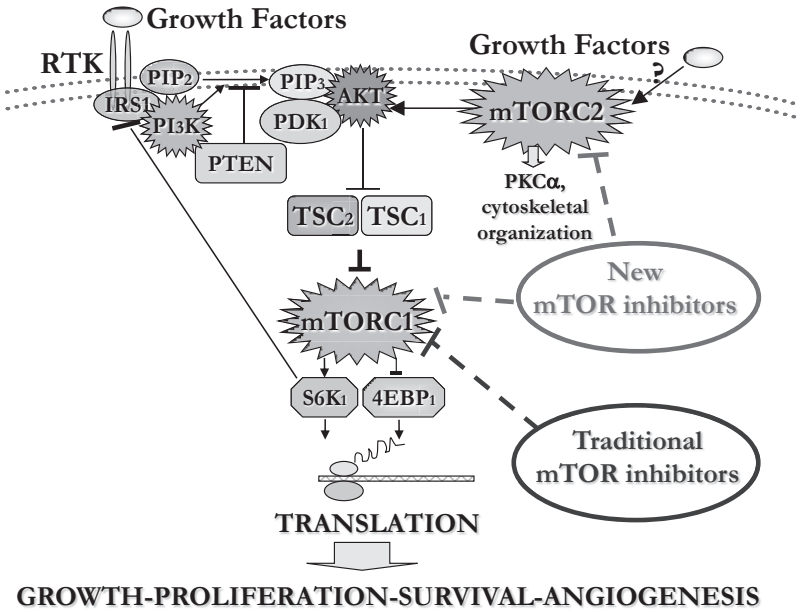

Figure 3. Simplified scheme of growth factors induced activation of mTOR pathway and mTOR inhibitors actions.

particularly the androgen production.[107] The activation of PI3K/AKT/mTOR-pathway in the reticularis of normal adrenals suggests a potential role of this pathway in the control of androgen production, probably as an intracellular mediator of the IGFs.

On the basis of the scarce existing information on the mTOR pathway in the adrenal gland, the first aim of this thesis is to evaluate the expression of the main components of the mTOR pathway in normal adrenals (chapter 3).

Potential role of the mTOR pathway in adrenal tumors

At the time the studies presented in this thesis were initiated, the role and function of mTOR and its pathway in adrenal tumors were not extensively clarified. Total-AKT is found to be overexpressed both in ACCs and in PCCs, compared to normal adrenals or adenomas.[109] The corresponding activated form, p-AKT, has also been reported to be overexpressed in PCCs.[109, 117] Equivocal data exist with respect to p-AKT expression in ACC.[25, 109] Indeed, some authors reported overexpression of p-AKT in ACC,[25] while others did not.[109] Mice with a heterozygous deletion of the PTEN gene are known to have an increased tendency to develop PCCs.[118] However, PTEN mutations appear to be rare in

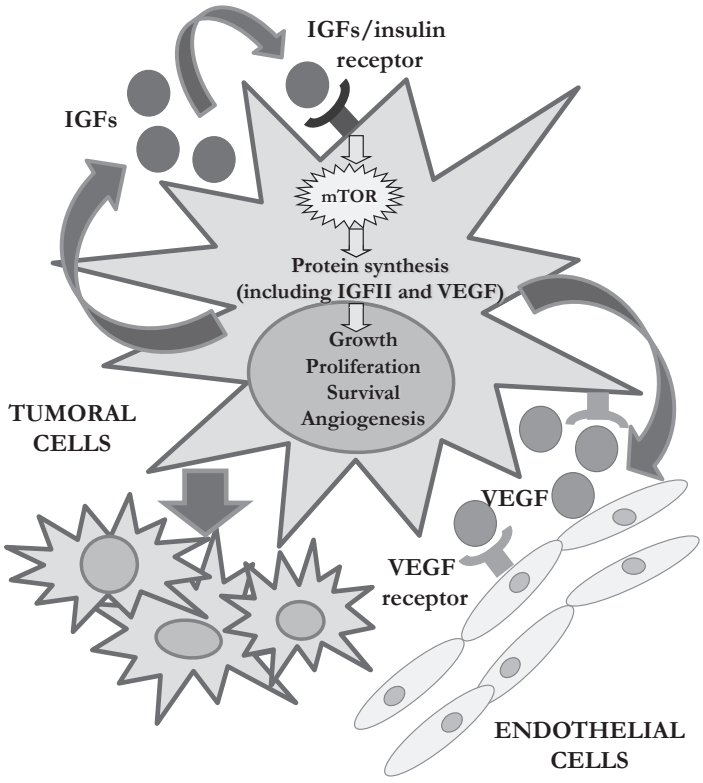

Figure 4. Potential role of the mTOR pathway in adrenal tumors

human PCCs,[119, 120] which has recently been confirmed by an integrated genetic analysis.[121] Conversely, other type of mutations such as *RET* and *NF1* described in PCCs/PGLs appear to be associated with an activation of the mTOR pathway in humans[83]

In H295 and SW13, two different human ACC cell lines[25, 122] and in PC12, a rat PCC cell line,[123] IGF-I stimulation has been correlated with increased AKT phosphorylation, suggesting that AKT acts as an intracellular mediator of IGF signaling in ATs. In PC12 cells, the effects of some growth factors on downstream components of mTOR pathway have also been evaluated. EGF and nerve growth factor (NGF) were reported to stimulate the phosphorylation of 4EBP1[124] and EGF was shown also to increase the phosphorylation of S6K1.[125] These effects were prevented by the administration of rapamycin.[125]

As indicated above, it has been shown that mTOR can stimulate the translation of IGF-II and VEGF. In turn, this stimulation can be induced by the same growth factors.[101] Therefore, mTOR may act as an intracellular mediator of growth factors in ATs as well, and could be an intracellular component of the pro-growth autocrine loops considered to be involved in the pathogenesis of ATs (particularly the IGFs), as shown in Figure 4.

To better clarify the role of the mTOR pathway in ATs, the second aim of this thesis is to describe the expression of the main components of the mTOR pathway in different types of ATs (chapters 2, 3 and 7), as well as to describe the potential relationship between these components and the main components of the IGF pathways in these tumors (chapters 2, 4 and 7).

The role mTOR as a drug target in the treatment of adrenal tumors

At the time the studies presented in this thesis were initiated there were no clinical or preclinical published data evaluating the effects of mTOR inhibitors in ACCs. However, compounds blocking IGF-I receptor activity were found to inhibit the proliferation of H295 cells.[25, 28] These effects were correlated with a reduction of p-AKT,[25] suggesting that AKT itself and/or mTOR could be a potential target for the treatment of ACCs. Inhibition of the IGF-I-receptor in preclinical models of human ACCs has been associated with a reduction of VEGF production.[25] According with the described role of mTOR in VEGF production,[101] it is conceivable that the inhibition of VEGF induced by the IGF-I receptor inhibitors is related to the inhibition of mTOR pathway. LY294002, an AKT inhibitor, has been shown to inhibit proliferation of PC12 PCC cells in a time- and dose-dependent manner.[117] In neuroblastoma cell lines (a tumor closely related to PCC), rapamycin inhibits cell proliferation by inducing cell cycle arrest.[126] Rapamycin, evaluated with a single dosage, significantly inhibits cell growth in the normal chromaffin rat cells, but not in PC12 cell line.[115] In a study evaluating the effects of glucacon-like-peptide-1 (GLP-1) in PC12 cells, used as model for neuronal cells, the authors show that GLP-1, through the activation of PI3K /AKT/mTOR pathway, was able to protect these cells from oxidative stress-induced cell apoptosis.[127] These protective effects were inhibited by the treatment with rapamycin. In an animal model for PCC, temsirolimus was found to inhibit tumor progression.[118] This effect could either be attributed to a direct effect of the mTOR inhibitor on tumoral cell proliferation or to the inhibitory effects of temsirolimus on vascular growth. Therefore, further preclinical studies should be performed to clarify whether these compounds might have a direct effect on PCC cells, whether these effects could depend on the presence of growth factors, such as IGFs, in the cell environment, and whether the combination treatment using mTOR inhibitors and other compounds, such as IGF-I receptor antagonists, could have stronger antineoplastic effects than the monotherapy. The promising effects of mTOR inhibitors in the treatment of neuroendocrine tumors[128, 129] have encouraged the use of mTOR inhibitors in the treatment of some patients with advanced malignant pheochromocytomas.[114] Unfortunately, in these 4 cases the treatment did not produce promising results. However, all these patients started treatment in a very late stage of disease, therefore, this early negative clinical experience

cannot exclude that the use of mTOR inhibitors in an earlier stage of disease may have more beneficial effects.

Overall, the efficacy of mTOR inhibitors in AT has not been extensively evaluated. In addition, the effects of this class of drugs in combination with other compounds, such as drugs targeting the IGF pathway or drugs frequently used in the treatment of this type of tumors (e.g. mitotane in ACC), warrants investigation.

Therefore, the third aim of this thesis is to evaluate the effects of mTOR inhibitors, alone or in combination with mitotane in ACC cell models (chapters 2, 3 and 5), and alone or in combination with drugs targeting the IGF pathway in ACC and PCCs cell models (chapters 2, 3 and 7).

Finally, since the results of the studies presented in chapters 2, 3, 4 and 5 of this thesis, as well as novel data from the literature, suggest that the role IGF in adult ACC may have been over-estimated, and because it became apparent that mTOR inhibitors alone may not fully fulfill the requirement of an effective treatment option for patients with advanced ACC, novel targets for ACC still need to be explored.

As such, the fourth aim of this thesis is to explore the presence of a large number of molecular events that can be targetable with mTOR inhibitors or other types of targeted drugs. This issue was investigated in a large series of advanced ACCs (chapter 6).

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The role of mTOR inhibitors in the inhibition of growth and cortisol secretion in human adrenocortical carcinoma cells

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ABSTRACT

Patients with adrenocortical carcinoma (ACC) need new treatment options. The aim of this study was to evaluate the effects of the mTOR-inhibitors sirolimus and temsirolimus on human-ACC cell growth and cortisol production.

In H295, HAC15 and SW13 cells we evaluated mTOR, IGF2 and IGF1-receptor expression; the effects of sirolimus and temsirolimus on cell growth; the effects of sirolimus on apoptosis, cellcycle and cortisol production. Moreover, the effects of sirolimus on basal- and IGF2-stimulated H295 cell-colony-growth and on basal- and IGF1-stimulated phospho-AKT, phospho-S6K1 and phospho-ERK in H295 and SW13 were studied. Finally, we evaluated the effects of combination treatment of sirolimus with an IGF2-neutralizing antibody.

We have found that H295 and HAC15 expressed IGF2 at a >1800-fold higher level than SW13. mTOR-inhibitors suppressed cell growth in a dose/time-dependent manner in all cell lines. SW13 were the most sensitive to these effects. Sirolimus inhibited H295 colony-surviving-fraction and size. These effects were not antagonized by IGF2, suggesting the involvement of other autocrine regulators of mTOR-pathways. In H295, sirolimus activated escape-pathways. The blocking of endogenously produced IGF2 increased the antiproliferative effects of sirolimus on H295. Cortisol production by H295 and HAC15 was inhibited by sirolimus.

The current study demonstrates that mTOR-inhibitors inhibit the proliferation and cortisol production in ACC cells. Different ACC cells have different sensitivity to the mTOR-inhibitors. mTOR could be a target for the treatment of human ACCs, but variable responses might be expected. In selected cases of ACC the combined targeting of mTOR and IGF2 could have greater effects than mTOR-inhibitors alone.

INTRODUCTION

Adrenocortical carcinomas (ACC) are uncommon malignancies with an incidence of 1-2 new cases/million/year.[1, 2] ACCs are highly aggressive tumors, associated with a 5-years survival ranging between 37 and 47%, for which novel treatment options are required.[1-3]

The insulin-like growth factor (IGF) system seems a major actor in the pathogenesis of ACC and is presently considered an attractive target for new treatments in these cancers.[4-6]

mTOR is a protein kinase of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway, functions as a gatekeeper of cell growth, metabolism and proliferation, receiving signals from sensors of cell stress, intracellular nutrients levels and several growth factors receptors including IGFs and vascular-endothelial-growth-factors receptors.[7-9] mTOR exists as part of two complexes: mTORC1 and mTORC2, respectively sensitive or insensitive to rapamycin (sirolimus).[10] The binding of growth factors to their receptor leads to the phosphorylation and activation of AKT, which elicits many downstream signaling events including the activation of mTOR as part of the mTORC1 complex. Activation of the mTORC1 complex leads to the phosphorylation and activation of downstream effectors of the pathway: the protein-70-ribosomal-protein-S6-kinase-1 (S6K1 (RPS6KB1)) and eukaryotic-translation-initiation-factor-4E-binding-proteins (4EBP1 (EIF4EBP1)).[10] Both S6K1 and 4EBP1 are regulators of mRNA translation and stimulate the synthesis of several proteins involved in cell proliferation.[10]

Alterations in the mTOR pathway have been found in many human tumours, regardless of deregulation of IGF system.[8, 9, 11] Therefore, the mTOR pathway is considered a target for antineoplastic therapy in several malignancies and it has very recently been proposed as target for ACC treatment.[12, 13] mTOR inhibitors may exert their antitumor effects directly by inhibiting cell growth and proliferation and indirectly, by inhibiting tumor angiogenesis.[10, 11]

Presently, many clinical trials are investigating the effects of compounds inhibiting mTORC1 activity (traditional mTOR inhibitors), such as sirolimus, temsirolimus, and everolimus, alone or in combination with other compounds, in several types of malignancy.[8, 9, 11, 14]

The role and function of mTOR and its pathway in ACC have not been clarified yet.[12] Recently, Doghman *et al.* showed that mTOR is activated in childhood ACCs and that everolimus is able to inhibit *in vitro* cell proliferation in ACC cell lines and growth of ACC xenografts in immunodeficient mice.[13]

The aims of this study were: 1) to evaluate the expression of mTOR, IGF2 and IGF-1receptor (IGF1R) in different human ACC cell lines, 2) to test the *in vitro* effects of the mTOR inhibitors sirolimus and temsirolimus on ACC cell lines in order to understand the mechanism of mTOR inhibitor-induced cell growth inhibition, 3) to explore the role

of the IGF2 autocrine loop in the effects of mTOR inhibitors, 4) to evaluate the effect of mTOR inhibitors on cortisol secretion.

MATERIALS AND METHODS

Study methodology

In this study we characterized the expression of the mTOR and IGF2 in three different human ACC cell lines: NCI-H295R (H295), HAC15 and SW13. In H295 and SW13 we also evaluated the expression of IGF1R. In all these cell lines we tested the dose- and time-dependent effects of sirolimus and temsirolimus on cell growth and the effects of sirolimus on induction of apoptosis and cell cycle. In H295, we tested the effects of sirolimus in the presence and absence of IGF2 stimulation on colony formation and determined the effect of blocking of endogenously produced IGF2 by an IGF2-specific neutralizing antibody on sirolimus-induced cell growth inhibition. In H295 and SW13 we explored the effects of sirolimus on basal- and IGF1 induced AKT, ERK1/2 and S6K1 phosphorylation. In the hormonally active ACC cells (H295 and HAC15), the effect of sirolimus on cortisol production was evaluated.

Cell lines and culture conditions

The human hormonally active ACC cell line H295, its clone HAC15, and the hormonally inactive ACC cell line SW13 were obtained from the American Type Culture Collection (Manassas, VA), from Dr. W. Rainey (as gift) and from ECACC (Salisbury, Wiltshire, UK), respectively.[15]

The cells were cultured in 75-cm^2 culture flasks at 37°C in a humidified incubator at 5% CO_2. For all cell lines, the culture medium consisted of DMEM/F12K medium, supplemented with 5% fetal calf serum (FCS), penicillin ($1x10^5$ U/liter), and l-glutamine (2 mmol/liter). Cells were harvested with trypsin (0.05%)-EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 95%. Media and supplements were obtained from Invitrogen (Breda, The Netherlands).

Drugs and reagents

mTOR inhibitors sirolimus and temsirolimus were purchased from LC Laboratories (Inc. Woburn, MA, USA). They were dissolved in dimethylsulfoxide (DMSO) as concentrated (10^{-3}M) stock solutions (stored at -20°C) and diluted in DMSO before use. IGF1 and IGF2 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and from Biosource (Tilburg, The Netherlands) respectively; both IGFs were diluted in 0.01M acetic acid solution as concentrated (10^{-5}M) stock solutions (stored at -20°C) and diluted in medium before use.

Anti-IGF2 neutralizing antibody (m610 human monoclonal antibody to IGF2) was kindly provided by Dr. Dimiter Dimitrov and Dr. Yang Feng.[16]

Quantitative RT-PCR

The expression of mTOR and IGF-II mRNA in human ACC cells was evaluated by quantitative RT-PCR.

From human ACC cell lines, total-RNA was isolated using a commercially available kit (High Pure RNA Tissue kit; Roche, Almere, The Netherlands). cDNA was synthesized using 500 ng of total-RNA in a Super Reverse Transcriptase (RT) buffer (HT Biotechnology Ltd., Cambrige, UK), together with 40 nmol of each deoxynucleotide-triphosphate, 15 ng oligo-dT primer, 20 U RNAse inhibitor, and 4 U AMV/Super RT (HT Biotechnology) in a final volume of 40 µl. This mixture was incubated for 1 hour at 40°C and thereafter 5 times diluted in bidest. A quantitative PCR was performed by TaqMan Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI-PRISM-7900 sequence Detection System (Perkin Elmer, Groningen, The Netherlands) for real-time amplifications, according to manufacturer's protocol. The assay was performed using 7,5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Alphen a/d Ryn, The Netherlands), primers and probes amount as reported in supplementary materials 1 and 5 µl cDNA template, in a total reaction volume of 12,5 µl. After an initial heating at 50°C for 2 min and 95°C for 10 min, samples were subjected to 40 cycles of denaturation at 95°C for 15 s and annealing for 1 min at 60°C. The primers and probes were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The sequence of the primers primers and probes used are reported in the supplementary material 1. Samples were normalized against the expression of the housekeeping gene hypoxanthine-phospho-ribosyl-transferase-1 (HPRT (HPRT1)). PCR efficiencies (E) were calculated for the primer-probe combinations used (supplementary material 1).[17] The relative expression of genes were calculated using the comparative threshold method, $2^{-\Delta C_t}$[18] after efficiency correction[19] of target and reference gene transcripts (HPRT).

Immunohistochemistry (IHC): AgarCyto cell-block

The expression of mTOR and IGF2 proteins in human ACC cell lines was evaluated by IHC using AgarCyto cell-blocks. H295, HAC15 and SW13 cell pellets were fixed in 4% formaldehyde solution in PBS, embedded in 2% agarose and afterwards in paraffin.[20] AgarCyto cell-blocks were cut in 5µm sections, deparaffinized and dehydrated. Antigen-retrieval was performed by microwave treatment in Tris–EDTA Buffer (pH 9.0). The slides were cooled for 1 hour at +4˚C and later incubated for 1 hour at room temperature (RT) with the anti-mTOR primary antibodies or over-night at +4˚C with the anti-IGF2 primary antibodies. The slides incubated with anti-IGF2-antibodies were subsequently washed and incubated for 30' at RT with Poly-Rabbit anti-Goat IgG. The slides were further

washed and incubated for 30 min at RT with Poly-AP-Goat anti-Mouse/Rabbit IgG. After washing, staining was visualized by 30 min incubation in new fuchsin solution. Slides were counterstained with hematoxylin and coverslipped. The antibody and the controls used are listened in supplementary material 2.

Immunocytochemistry : chamber slides

Cells were plated on poly-L-lysine-coated culture chamber slides (NUNC A/S, Roskilde, Denmark). After 48h, medium was removed and cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer, pH 6.9, for 40 min. at RT. After washing, the cells were treated for 3 min with 50% methanol and for 3 min with 100% methanol. After another washing, the cells were treated with a 3% H_2O_2-PBS solution for 15 min at RT in the dark, to quench endogenous peroxidase. After washing, the cells were incubated for 1 hour at RT with an IGFIR monoclonal antibody (supplementary material 2). Finally, the cells were incubated for 30 min at RT with HRP/anti-Rabbit/Mouse (Dako Detection System). Bound antibodies were visualized by incubation with freshly prepared DAB (Dako Detection System). Slides were counterstained with haematoxylin and cover slipped. For negative controls, the primary antibody was omitted.

Cell proliferation assay

Measurement of total DNA content. Cells were plated in 1 ml of medium in 24-well plates at the density necessary to obtain a 65-70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later, sirolimus or temsirolimus were added to wells in quadruplicate. The concentrations of compounds tested in H295 and HAC15 ranged between 10^{-9}M and 10^{-5}M. In the SW13, a maximal effect of compounds was observed already at 10^{-8}M. Therefore, we tested concentrations of compounds ranged between 10^{-12}M and 10^{-8}M. Controls were vehicle-treated. The cells inoculated for 6 and 9 days were refreshed every 3 days by adding fresh compounds. After 24 hours, 3, 6 and 9 days of treatment, the cells were harvested for DNA measurement, as a measure of cell number. Measurement of total DNA content was previously described in detail.[21]

Cell Proliferation Reagent WST-1 (WST). In H295 cells, the effect IGF2-specific neutralizing antibody on sirolimus-induced inhibition of cell proliferation was determined by WST-1 assay (Cell Proliferation Reagent WST-1 (Roche, UK)), according to the protocol provided by the manufacturer. Cells were plated in 100µl of medium+5%FCS (standard medium) in 96 well-plates (20.000 cells/well). After 24h, the medium was changed with medium+1%FCS and the following reagents were added: vehicle, sirolimus (10^{-9}M), anti-IGF2 ($4*10^{-8}$M), sirolimus+anti-IGF2. After 72h cell proliferation was measured by WST-1 assay. The experiment was repeated twice and each experiment was performed in quadruplicate.

DNA fragmentation assay

DNA fragmentation assay was used to determine the effects of the compounds on apoptosis. The cells were plated in 24-well plates and treated as above described for the cell proliferation assay. After 24 hours and 3 days of incubation, DNA fragmentation was determined using a commercially available ELISA kit (Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used. The same plates were also analyzed for the measurement of total DNA content. The amount of DNA-fragmentation (apoptosis) was corrected for the total DNA content in each well.

Cortisol secretion assay

In H295 and HAC15, we evaluated the effects of sirolimus on cortisol production. The cells were plated in 24-well plates and treated for 6 days as above described for the cell proliferation assay. We tested the effects of the sirolimus on cortisol production in concentrations corresponding to the EC_{50} on cell proliferation after 6 days of treatment. The culture supernatants from experiment performed in H295 and HAC15 cells were collected and stored at –20ºC until determination of the cortisol concentration.

The cortisol concentration was determined by a non-isotopic, automated chemiluminescence immunoassay system (Siemens Medical Solutions Diagnostics, Breda, The Netherlands). Cortisol levels were expressed as percentage of control and were corrected for the total DNA content in each well, thereby reflecting cortisol production per cell.

Cell cycle analysis

Cells were plated in 12-well plates at the density necessary to obtain a 65-70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later sirolimus was added to wells in triplicate. In each cell line the effects of sirolimus on cell cycle were tested in concentrations corresponding to the EC_{50} on cell proliferation after 6 days of treatment. In addition, the effects of the compounds at concentrations of 10^{-6}M in H295 and HAC15 and 10^{-8}M in SW13 were tested. Control groups were vehicle-treated. We evaluated the effects of the compounds on cell cycle after 24 hours of treatment in SW13, and in H295 and HAC15 cell lines after 72 hours of treatment according to the different growth rates of these cells. Following treatment, cells were harvested by gentle trypsinization, washed with ice-cold PBS and collected by centrifugation. Cells were resuspended in 200µl PBS and fixed in 70% ice-cold ethanol, followed by an overnight incubation at -20°C. After centrifugation, the cells were washed once with PBS and incubated for 30 min at 37°C in PBS containing 40µg/ml propidium iodide (Sigma Aldrich, Zwijndrecht, The Netherlands) and 10µg/ml of DNase-free RNase (Sigma Aldrich, Zwijndrecht, The Netherlands). For each tube, 20,000 cells were immediately measured on a FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) and analyzed using CellQuest Pro Software.

Colony forming assay

Cells were plated in poly-L-lysine (10 µg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) coated 12-well plates (2500 cells/well) and cultured in complete medium for three weeks.

Cells were allowed to attach for 24 hours before to be treated with the vehicle, sirolimus ($5*10^{-9}$M), IGF2 (10^{-8}M) or the combination of the two compounds. The experiment has been performed two times in triplicate. Cells were treated continuously and medium plus the compounds were refreshed every three or four days. After 3 weeks the formed colonies were fixed with 100% ethanol and stained with hematoxylin to allow calculation of the average colony forming efficiency. Colonies containing more than 50 cells were automatically counted with a Multi Image Light Cabinet from Alpha Innotech Corporation (Cell Biosciences, San Leandro).

Plating efficiency was defined as the mean number of colonies formed divided by the number of plated cells for control cultures expose to the vehicle express as percentage. The surviving fraction (SF) was calculated as (mean number of colonies)/(number of plated cells x plating efficiency).[22]

Protein Extraction

H295 and SW13 cells were plated in 3 ml of medium in 6-well plates at the density required to obtain 65-70% cell confluence at the end of the experiment. Seventy-two hours later cells were starved for 12 hours and then incubated for 1 hour with sirolimus or vehicle. The final concentrations of sirolimus tested were 10^{-6}M and $5x10^{-9}$M in H295 and 10^{-8}M and 10^{-10}M in SW13. Thirty minutes prior to collection of cells, IGF1 (10^{-8}M) was added to selected wells. Cells were washed with ice-cold PBS.Whole-cell lysates were prepared by adding 200µl of ice-cold RIPA lysis buffer (Pierce Biotechnology,Inc., Rockford, USA) with the addition of 1% Halt Phosphatase Inhibitor Cocktail (Pierce Biotechnology, Inc., Rockford, USA) to each well. After 1 minute of incubation on ice the cell lysates were transferred to the labelled tubes. After 15 minutes incubation on ice (mixing every 5 minutes), the samples were spun down at 13000 rpm at 4°C. The supernatants were stored at -80°C.

The total amount of proteins was calculated with dye-binding assay (Bio-Rad Protein Assay), using bovine serum albumin as standard curve and a spectrophotometer set to 595nm as reader.

Western Blotting

Total protein solution (30 µg) diluted in a water solution containing 20% SDS sample buffer were denatured (5 minutes in a bath at 95°C) and separated by electrophoresis on 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were first incubated for 2 hours with blocking buffer (0.1%Tween20-PBS/5% bovine

serum albumin for membranes for AKT mesurement and 0.1%Tween20 PBS/3% non-fat dry milk for the remaining membranes) and subsequently incubated overnight at 4°C with the primary antibody (supplementary material 3). After 1 hour of washing in 0.1% Tween20-PBS, the membranes were incubated for 1 hour with the secondary antibody at room temperature, followed by 1 hour wash in 0.1% Tween20-PBS. Starting from the incubation with the secondary antibodies the membranes were preserved in dark condition. Immunodetection was performed using the Odyssey infrared imaging system (LI-COR Biosciences, Cambridge, UK). The optical density of the appropriately sized bands was measured using the Odyssey molecular imaging software (LI-COR Biosciences). The relative expression of total-Akt, total-S6K, or total-ERK was calculated as a ratio to the expression of actin. The relative expression of phospho-Akt, phospho-S6K, or phospho-ERK was calculated as a ratio to total Akt, S6K, or ERK respectively.

Statistical analysis

All the experiments were carried out at least three times, with the exception of colony forming assay and the western blot that were performed twice. The repeated experiments gave comparable results. For the statistical analysis statistical software of SPSS (SPSS 15.0; SPSS Inc., Chicago,IL) and GraphPad Prism 5.0 (GraphPhad Software, San Diego, CA) were used.

We used non-parametric tests to evaluate the differences among groups (Mann-Whitney test and Kruskall-Wallis).

The comparative statistical evaluations among treatment groups were performed by ANOVA, followed by a multiple comparative test (Newman-Keuls or Dunnett's test).

RESULTS

Expression of mTOR IGFII and IGFIR in ACC cell lines

The IGF2 mRNA levels in H295 and HAC15 (H295=59.3±31; HAC15=50.6±9; ration over HPRT, mean±SD) cells were more than 1800-fold higher ($p<0.001$) than in SW13 in which IGF2 mRNA was just detectable (0.03±0.03; mean±SD), whereas the mRNA expression levels of mTOR were comparable between the cell lines (H295=0.12±0.05; HAC15=0.12±0.08; SW13=0.14±0.06). The higher expression levels of IGF2 in H295 and HAC15 compared to SW13 were also confirmed at protein level by the immunostaining (figure 1). In addition, the mTOR immunostaining showed a strong positivity in all cell lines (figure 1). A positive immuno-reactivity for IGF1R was shown in both H295 and SW13 cell lines (figure 2).

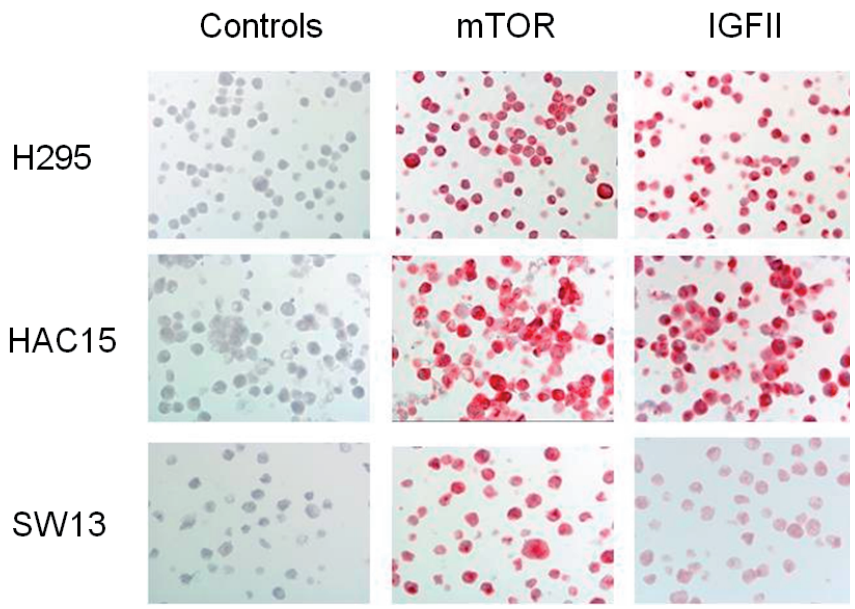

Figure 1. Immunocytochemical detection of mTOR (middle panel) and IGF2 (right panel) in human ACC cell lines. Left panel shows the absence of staining in the negative controls. Magnification, X100.

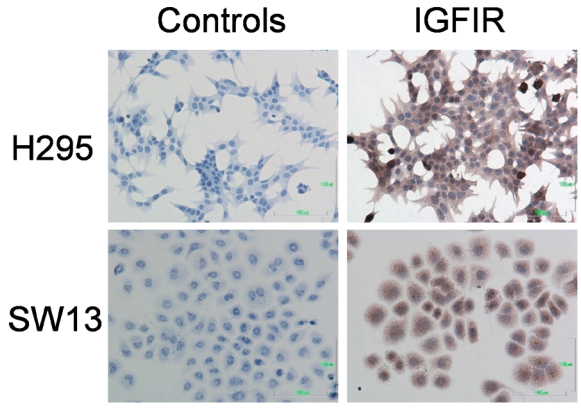

Figure 2. Immunocytochemical detection of IGF1R (right panel) in the human ACC cell lines H295 and SW13. Left panel shows the absence of staining in the negative controls. Magnification, X200.

Effects of mTOR inhibitors on cell growth and apoptosis in ACC cell lines

In ACC cell lines, sirolimus and temsirolimus significantly suppressed the cell growth in a dose and time-dependent manner (figure 3 shows the effects in H295 and SW13; effects in HAC15 are shown in supplementary figure 1). In H295, both compounds were able to significantly inhibit the cell growth with a comparable potency after 9 days of treatment (EC_{50}: $4.8x10^{-9}$M vs $1.9x10^{-8}$M). The effects of sirolimus ranged between 61.7% inhibition ($p<0.001$) at the maximal dose (10^{-5}M) and 16.7% ($p<0.01$) at the minimal dose tested (10^{-9}M). The effects of temsirolimus ranged between 57.1% inhibition ($p<0.001$) at the maximal dose (10^{-5}M) and 16% ($p<0.05$) at the minimal dose tested (10^{-9}M).

In HAC15, both compounds were able to significantly inhibit the cell growth with a comparable potency after 9 days of treatment (EC_{50}: $1.4x10^{-8}$M vs $4.3x10^{-7}$M). The effects

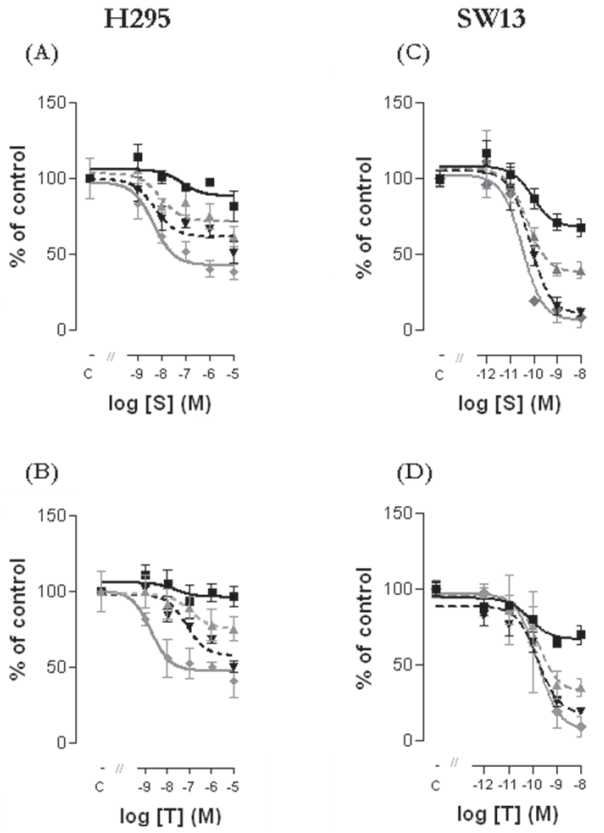

Figure 3. Dose-/time-dependent effect of sirolimus (S; panels A and C) and temsirolimus (T, panels B and D) treatment on cell proliferation, expressed as DNA content/well after 24 h (closed square), 3 days (closed triangle), 6 days (closed down triangle), and 9 days (closed diamond) treatment in H295 (left panels) and SW13 (right panels) cells. Data are expressed as the percentage of control and represent the meanGS.D. Control is set as 100%.

of sirolimus ranged between 79.9% inhibition ($p<0.001$) at the maximal dose (10^{-5}M) and 24.4% ($p<0.05$) at the minimal dose tested (10^{-9}M). The effects of temsirolimus were ranged between 81% inhibition ($p<0.001$) at the maximal dose (10^{-5}M) and 24.6% ($p<0.05$) at the minimal dose tested (10^{-9}M).

Nine days of treatment with sirolimus (figure 3C) and temsirolimus 10^{-10}M were already able to significantly inhibit the cell growth of SW13 cells. Sirolimus was significantly more potent than temsirolimus in terms of EC_{50} (EC_{50}: $3.3x10^{-11}$ M vs $1.7x10^{-10}$ M; $p=0.02$), but not with respect to the maximal and the minimal effective concentration of the two compounds. The effects of sirolimus ranged between 91.7% inhibition ($p<0.001$) at the maximal dose (10^{-8}M) and 49.3% ($p<0.05$) at the dose of 10^{-10}M. The effects of temsirolimus were ranged between 91.5% inhibition ($p<0.001$) at the maximal dose (10^{-8}M) and 34.9% ($p<0.05$) at the dose of 10^{-10}M.

In H295 sirolimus was able to significantly induce DNA fragmentation only at the highest dose used (10^{-5}M) (figure 4). Sirolimus 10^{-5}M was able to induce apoptosis also in HAC15. These effects were more pronounced after 24 hours than after 3 days of treatment. At the doses tested (10^{-8}M to 10^{-12}M), 24 hours and 3 days of treatment, sirolimus was not able to significantly induce apoptosis in SW13 (not shown).

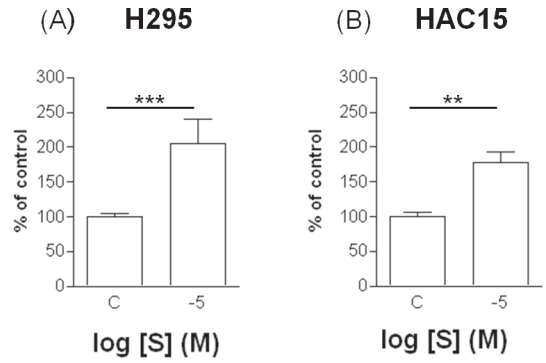

Figure 4. Twenty-four hour treatment with sirolimus at high dose (10^{-5} M) was able to induce cell apoptosis, as measured by the induction of DNA fragmentation, in H295 (A) and HAC15 (B) cells. Data are expressed as percentage of control and represent the mean±S.D. Control is set as 100%. **P<0.01;***P<0.001 vs control.

Effects of sirolimus on cortisol secretion in ACC cell lines

Sirolimus induced a significant inhibition of cortisol secretion in H295 and HAC15. These effects were still present after the correction for the estimated cell number in each well, suggesting a direct effect of sirolimus on hormonal secretion in ACC cells (figure 5). Cortisol secretion was inhibited by 21.7% in the in H295 sirolimus ($5x10^{-9}$M) treated cells ($P<0.01$) (figure 5A) and by 41.3% in sirolimus (10^{-8}M) treated HAC15 cells ($P<0.001$) (figure 5B).

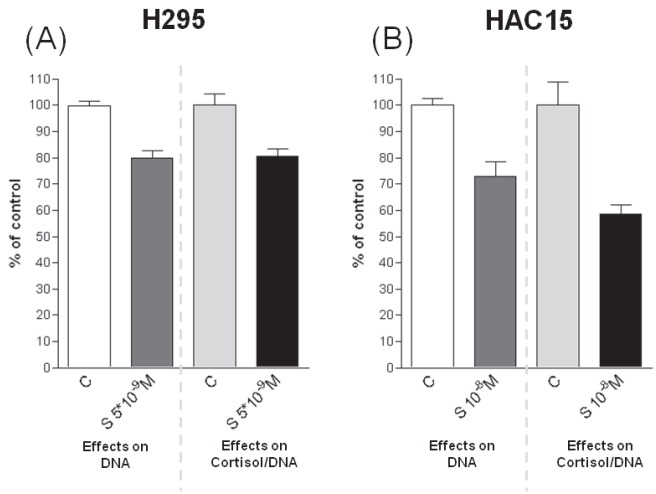

Figure 5. Six-day treatment with sirolimus inhibits cortisol production in ACC cells H295 (A) and HAC15 (B) independently of the inhibition of cell proliferation. The graphs showin parallel the effects of sirolimus on the cell proliferation (total DNA content) and on cortisol production for both cell types. The dose of sirolimus used ($5x10^{-9}$ M in H295 and 10^{-8} M in HAC15) corresponded at the EC50 of cell growth inhibition in each cell type. The cortisol levels measured were normalized for the total number of cells present in each well (Cortisol/total DNA content). Data are expressed as percentage of control and represent the mean±S.D. Control is set as 100%.

Effects of sirolimus on cell cycle progression and colony formation in ACC cell lines

To explore the mechanisms involved in mTOR inhibitor induced inhibition of cell proliferation we performed FACS analysis and colony forming assay. At FACS analysis sirolimus 10^{-6}M induced a significant G1-phase arrest in H295 and HAC15 (table 1). In SW13 we observed a significant G1-phase arrest by sirolimus at 10^{-8} and 10^{-10}M (table 1). This effect at the highest concentration of compound used was accompanied by a decrease in S-phase and G2-phase (table 1).

The plating efficiency for H295 in colony forming was 8.6%. Three weeks of treatment with sirolimus $5x10^{-9}$M, significantly inhibited the formation and growth of colonies as measured by a reduced surviving fraction (69.9% vs control; $p<0.001$) and average colony size (56.6%; $p<0.001$) (figure 6). IGF2 10^{-8}M was able to significantly increase the colony growth by increasing their size (62.7%; $p<0.001$) and the surviving fraction (29%; $p<0.05$). At the condition tested, the effects of sirolimus on surviving fraction and colony size were not reverted by the coadministration of IGF2. Similar results were also obtained when we repeated the experiment using IGFII $5x10^{-8}$ M (results not shown).

Table 1: Effects of sirolimus on cell cycle distribution in human adrenocortical cancer cell lines (H295, HAC15 and SW13 cells)

	Phase sub G0	Phase G0/1	Phase S	Phase G2/M
	(*mean±SD*)	(*mean±SD*)	(*mean±SD*)	(*mean±SD*)
H295				
Control	2.9±0.6	55.8±5.1	12.4±2.8	27.8±3.7
Sirolimus 10^{-6}M[a]	2.8±0.7	59.4±3.8[*]	11.3±2.9	26.4±3.8
Sirolimus $5x10^{-9}$ M[a]	3.9±1.8	55.9±4	11.6±1.4	28.6±4.2
HAC15				
Control	7.8±2.9	60.7±1.5	15.7±0.9	15.8±3.4
Sirolimus 10^{-6} M[a]	6.8±1.5	63.8±2.3[*]	14.2±1.6	15.3±2
Sirolimus 10^{-8} M[a]	8±2.2	61.6±1.9	15.6±1.3	14.8±1.2
SW13				
Control	1.8±0.3	56.7±5	15.9±0.9	25.7±5.2
Sirolimus 10^{-8} M[b]	1.9±0.6	72.9±5.7[**]	11.2±2.4[**]	14±3.6[**]
Sirolimus 10^{-10} M[b]	1.8±0.3	67±6.2[**]	14.4±1.7	16.8±4.7[**]

SD: standard deviation; [a]cell cycle distribution measured after 72 hours of incubation; [b]cell cycle distribution measured after 24 hours of incubation; [*]p<0.05 vs control; [**] p<0.001 vs control.

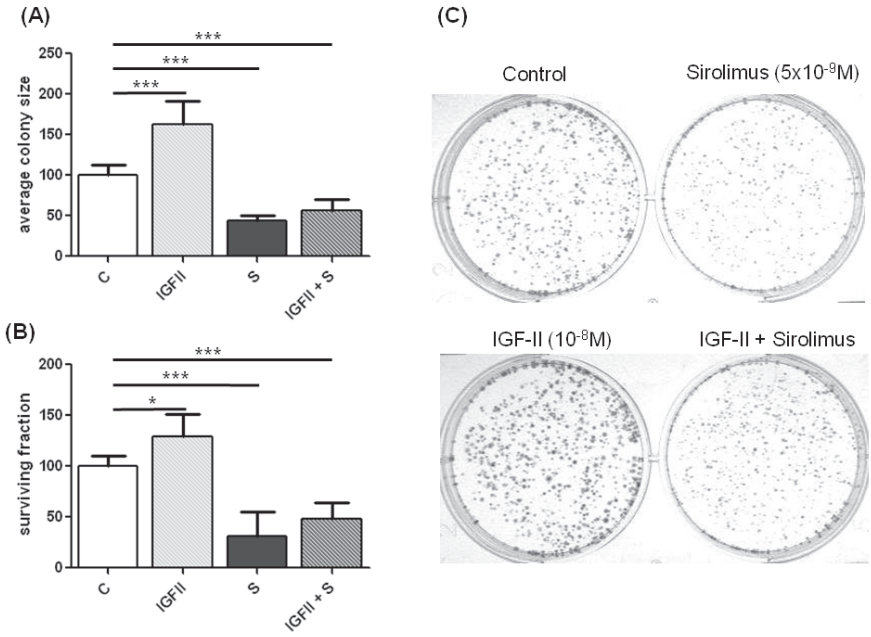

Figure 6. Effects of 3-week treatment with IGF2 (10^{-8} M) and/or sirolimus ($5x10^{-9}$ M) on colony formation and growth of the human ACC cell line H295. Left panel: IGF2 stimulates H295 cell proliferation by increasing the average size of colonies (A) as well as the surviving fraction (B). Both these effects are efficiently antagonized by the coadministration of sirolimus. Data are expressed as percentage of control and represent the mean±S.D. Control is set as 100%. The right panel (C) shows a representative photograph of the wells containing treated and untreated cells as used to perform colony-forming experiments.***P<0.001 vs control.

Effects of sirolimus on the IGF activated intracellular pathways in ACC cells

To further understand the mechanisms responsible for the effects of the mTOR inhibitor in ACC cell lines, we studied the effects of sirolimus and/or IGF1 on some key intracellular components of the IGF pathway in H295 and SW13 (Figure 7). Thirty minutes IGF1 (10^{-8}M) stimulation significantly increased the phosphorylation of S6K1 and AKT in both cell lines. IGF1 stimulation increased the phosphorylation of ERK1/2 in H295. H295 cells treated with sirolimus 10^{-6}M and $5x10^{-9}$M had significant lower phospho/total-S6K1 than control and IGF1-stimulated cells, as expected by a successful inhibition of the mTORC1 complex activity. Sirolimus 10^{-8} and 10^{-10}M reduces phospho/total-S6K1 also

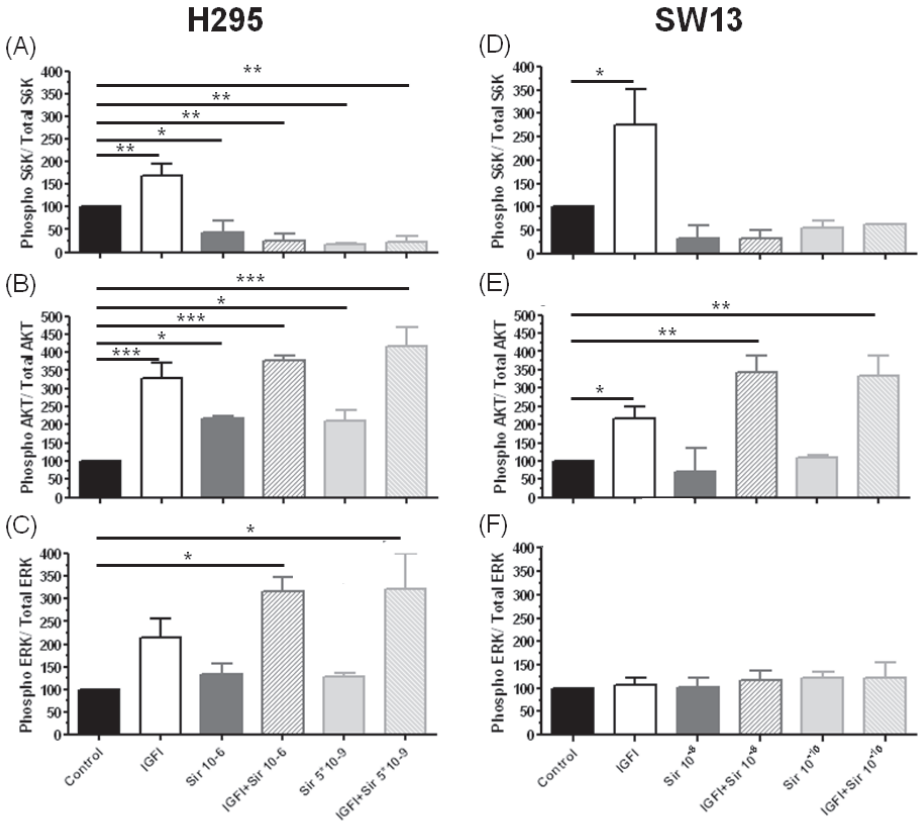

Figure 7. Western blotting results. In H295 ACC cell line (left panel): effects of sirolimus (10^{-6} and $5x10^{-9}$ M) with/without IGF1 (10^{-8} M) administration on phospho-S6K/total S6K (A), phospho-ERK/total ERK (B), and phospho-AKT/total AKT (C). In SW13 ACC cell line (right panel): effects of sirolimus (10^{-8} and $5x10^{-10}$ M) with/without IGF1 (10^{-8} M) administration on phospho-S6K/total S6K (D), phospho-ERK/total ERK (E), and phospho-AKT/total AKT (F). In the two ACC cell lines, the administration of sirolimus±IGF1 induced different effects on IGF-activated intercellular pathways (explanation in the text). Protein activation is measured as the ratio of phosphoprotein band density/total protein band density. Values are expressed as percentage of the control, mean±S.D.*P<0.05; **P<0.01; ***P<0.001 vs control.

in SW13, but this reduction resulted statistically significant only when compared to the IGF1-stimulated cells and not when compared to the control (12 hours starved cells). In both cell lines the sirolimus induced inhibition of S6K1 phosphorylation was not reverted by the IGF1 stimulation. In H295 the treatment with sirolimus was associated with an increased AKT-phosphorylation and this AKT stimulation was enhanced by the IGF1 stimulation. In SW13 the treatment with sirolimus alone did not increase the AKT-phosphorylation. However, an increased phospho/total-AKT was observed in all IGF1 stimulated cells despite the sirolimus treatment. In SW13 the ERK1/2 phosphorylation was not affected by the IGF1 and/or sirolimus treatment.

Effect of IGFII neutralization on sirolimus-induced H295 cell growth inhibition

To better address the potential influence of the IGF2 autocrine loop on ACC cell sensitivity to the mTOR inhibitors, we tested effects of sirolimus (10^{-9}M) on cell growth in presence or absence of anti-IGF2 neutralizing antibodies ($4x10^{-8}$M). This concentration of anti-IGF2 was previously shown to completely block IGF2 (10^{-8}M) –induced cell proliferation.[16] FCS medium (1%) was used to minimize the presence of exogenous growth factors. In these conditions a 72h treatment with sirolimus combined with anti-IGF2Abs was able to almost totally block H295 cell proliferation (90% inhibition compared to controls). Sirolimus or anti-IGF2 antibody alone induced an inhibition in H295 cell proliferation of 64% and 42%, respectively (figure 8).

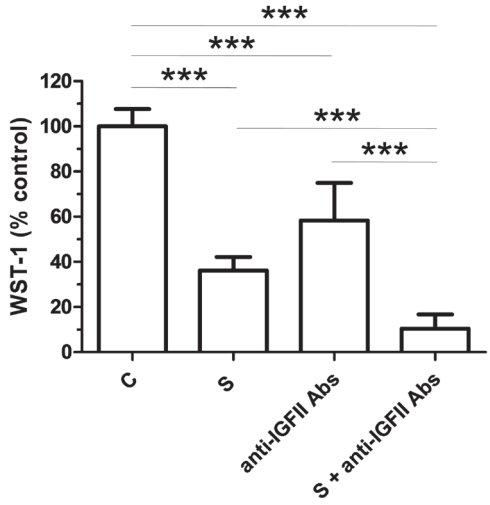

Figure 8. Effect of 72 h combination treatment with anti-IGF2 neutralizing antibodies (anti-IGF2 Abs) at a concentration of $4x10^{-8}$ M and sirolimus (S, 10^{-9} M) oncell proliferation (WST-1) of H295 cells. Data are expressed as the percentage of control and represent the mean±S.D. Control is set as 100%. ***P<0.001.

DISCUSSION

In the present study, we describe the expression of mTOR, IGF2 and IGF1R and the *in vitro* anti-proliferative and anti-secretive effects of the mTOR inhibitors in the currently available human ACC cell lines. All ACC cell lines expressed comparable mRNA and protein mTOR levels. Both H295 and SW13 showed a significant IGF1R protein expression. Conversely, the expression levels of IGF2 were considerably higher in H295 and its clone HAC15 than those in SW13. These results show that mTOR is expressed in human ACC cell lines and its expression appears to be unrelated to IGF1R expression or IGF2 overexpression.

The mTOR inhibitors caused a significant inhibition of cell growth *in vitro* and sirolimus induced a significant reduction of hormonal production in the hormonally active cells, independent of the effect on cell growth. Sirolimus appeared to be more potent than temsirolimus in inhibiting cell proliferation in SW13. Temsirolimus acts as direct inhibitor of mTOR, but *in vivo* temsirolimus is also converted in sirolimus.[23] After having proven that temsirolimus inhibits the *in vitro* cell-growth in ACC cell lines also in a direct way, we continued the experiments using sirolimus only. Cell cycle arrest appeared the predominant mechanism responsible for the observed antiproliferative effects of sirolimus, as already reported in other cancer cell lines.[24]

In H295 and SW13, IGF1 stimulation has been correlated with increased AKT-phosphorylation.[6, 25] In this study we describe an IGF1 induced AKT and S6K1 phosphorylation in both ACC cell lines confirming the role of the AKT/mTOR pathway as intracellular mediator of the IGF signalling in ACCs. Moreover, we prove that long term exposure to IGF2promotes colony growth in H295 and these effects are antagonized by mTOR inhibitors. It has been reported that everolimus can produce anti-vascular effects in *in vivo* model ACCs.[13] Therefore, mTOR plays a role as intracellular mediator of the autocrine/paracrine loops considered to be involved in the pathogenesis of ACCs.[12, 26]

In the present manuscript we found that the different ACC cell lines display a differential sensitivity to the anti-growth effects of the mTOR inhibitors. The antiproliferative effects of the drugs were observed at concentrations of sirolimus and temsirolimus that can be reached in vivo in humans.[23] Moreover, cell growth inhibition at these concentrations was considerably higher in SW13 than in H295 (and its clone HAC15). Many factors can contribute to this difference. SW13 cells differ from H295 because they harbour TP53 mutation,[27] they are less differentiated, they do not over-produce IGF2 and steroids and they present a higher growth rate. Cells with TP53 mutation have been suggested to be more sensitive to mTOR inhibitors.[14, 28] The absence of the IGF2 overproduction can contribute to the higher sensitivity of SW13 cells, compared to other two cell lines. The overstimulation of the growth-factor-receptors can determine the over-activation of the mTOR pathway upstream to mTOR (i.e. increased phosphorylation of AKT), or can

over-activate other pro-growth pathways such us the Ras/Raf/MEK/ERK pathway (i.e. increased phosphorylation of ERK1/2), determining resistance or escape to the effects of traditional mTOR inhibitors.[14, 29-31] We performed all our experiments using the same culture medium in all cell lines. In such a setting, the endogenus production of growth factor by the cells may determine differential sensitivity to mTOR inhibitors. The proliferation of H295 is stimulated by an autocrine/paracrine IGF2/IGF1-receptor-loop.[32] The over-activation of this loop can negatively influence the sensitivity of H295 to the mTOR inhibitors. This hypothesis is supported by the results obtained in WB, the experiments using anti-IGF2 neutralizing Abs and the colony-forming experiments. Using WB we demonstrated that the effects of sirolimus on the IGF activated intracellular pathways are different in H295 and SW13 cells. At the condition tested, IGF1 induced the activation of the AKT/mTOR pathway in both cell lines and ERK activation only in H295. Sirolimus suppressed the mTORC1 activity in both cell lines. However, in H295, but not in SW13, the inhibition of mTORC1 activity was associated with a significant increased phosphorylation of AKT, supporting an over-activation of the mTOR pathway upstream to mTORC1 in H295, likely representing an escape pathway. This activation could result by the IGF2 endogenous production that persistently stimulates the IGF1-receptor (autocrine IGF loop) even in starved H295 cells. The effects of sirolimus on AKT were even enhanced by IGF1 administration which also induced ERK stimulation in the sirolimus treated H295, despite the fact that both basal and IGF1 S6K phosphorylation was fully blocked by sirolimus. These results show that in H295 cells treated with sirolimus, IGF can stimulate two pathways potentially associated with traditional mTOR inhibitors treatment escape: AKT and ERK pathways.[14, 28] To further provide evidence that in H295 the endogenous overproduction of IGFII has a negative interference with the effects of mTOR inhibitors, we explored the effects of sirolimus alone or in presence of anti-IGF2 neutralizing antibodies (at concentration predicted to effectively neutralize the endogenous IGF2production). These experiments demonstrated for the first time that IGF2 neutralization increases the anti-proliferative effects of mTOR inhibitors in an ACC model. This raises the question whether the cotreatment of traditional mTOR inhibitors and IGF1 receptor antagonists should be considered for patients with ACCs, known to have a strong IGF autocrine loop.

In colony forming assay, H295 cells were more sensitive to sirolimus than observed with the DNA measurement. Possible mechanisms that could explain this higher sensitivity include: the disruption of the growth factor paracrine/autocrine loops; the selection of "aggressive clones" and the mTORC2 complex inhibition. In colony forming experiments the cell density is very low leading to the disruption of growth factor paracrine/autocrine loops and reducing the growth factor induced activation of escape pathways. However, in the colony experiments IGF2 (used at a dose comparable to the concentration reached in the medium of H295 in DNA-measurement experiments) stim-

ulated cell proliferation, but did not revert the effects of sirolimus, suggesting that IGF2 is not the only autocrine/paracrine regulator of the mTOR pathway activity in H295. By disrupting the autocrine/paracrine loops we forced the cells to grow in a less favourable condition and this could lead to the selection of "more aggressive clones". H295 cells showed a low plating efficacy suggesting that only a small percentage of cells are able to adapt and grow under these conditions. These cells could be less dependent by the autocrine/paracrine loops, less sensitive to the IGF2 and consequentially less exposed to the growth factor-induced activation of the escape pathways. This hypothesis could also contribute to explain the observed incapability of the IGF2 to revert the sirolimus induced inhibition on H295 colony growth. Three weeks of continuous treatment of H295 cells with sirolimus induced a significant reduction of the cell surviving fraction compared to controls. These results can suggest that long time treatment with mTOR inhibitor does not only block the cell growth, but also induces cell death. Traditional mTOR inhibitors as sirolimus and temsirolimus have the mTORC1 complex as target. The presence of activated mTORC2 can stimulate the AKT activation representing a potential mechanism of escape to the effects of traditional mTOR inhibitors for cancer cells. However, it has been suggested that long term treatment with traditional mTOR inhibitors can also indirectly inhibit the TORC2 complex by sequestering mTOR as part of the TORC1 complex.[7, 33] This double block is considered one of the potential mechanisms of mTOR inhibitor induced tumor cell death.[14] Moreover, mTORC2, as well as mTORC1, is activated by growth factors. Therefore, in colony experiments the disruption of the autocrine/paracrine loops may also contribute to the mTORC2 inhibition.[34, 35] This raises the question whether the use of drugs simultaneously blocking mTORC1 and mTORC2 or mTORC1 and PI3K could have a place in the treatment of selected patients with ACCs.

For the first time we show an anti-secretive effect (inhibition of cortisol production) of mTOR inhibitors in ACC cell lines. The mechanisms responsible for this effect still need to be clarified. mTOR inhibitors are already used in the clinical setting and no signs or symptoms of hypo-adrenalism have been described.[36] Therefore, it is probable that mTOR inhibitors are not able to suppress the physiological adrenal steroid production. The IGFs are able to stimulate adrenal steroid production. It is thus possible that mTOR could play a role as intracellular mediator of the effects of IGFs and that mTOR inhibition could antagonize this pro-secretive effect of IGFs in ACC cells.

Conclusions, translational aspects and future directions

The results of the current study suggest that ACCs may be considered for treatment with traditional mTOR inhibitors. The effects of these compounds *in vitro* at concentration potentially reachable *in vivo* are predominantly cytostatic, although it is shown that long time treatment with traditional mTOR inhibitors (in conditions disrupting the autocrine loops) can lead to cell death. Additional clinical benefit in patients with hypersecretive

ACCs could be an inhibitory effect of these compounds on cortisol secretion. However, several factors such as cell type, cell differentiation, the presence of an autocrine growth factor loop, as well as the cell environment could largely influence the sensitivity of ACCs to these drugs. These differences point out the importance to investigate the presence of biomarkers predictive of potential clinical benefit and to eventually proceed in the clinical investigation of these compounds only in selected patients with higher chance to respond to this treatment. This study also suggest to investigate the role of TP53 mutations, cell differentiation, proliferative index, the presence of activated autocrine loops as potential marker predictive of mTOR inhibitor effects in ACCs and the activation of AKT and ERK during the treatment as potential markers of escape. Lastly, it is suspected that in some cases of ACCs, combined treatment with mTOR inhibitors and other compounds should be considered to overcome possible mechanisms of resistance to mTOR inhibitors used as mono-therapy.

Particularly the effects of treatment targeting the IGF2 autocrine loop, in combination with mTOR inhibitors, warrants further investigation in ACC.

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SUPPLEMENTARY MATERIAL

Supplementary material 1: Primers probes and relative amount in PCR mixtures.

			Amount (nmol/l) added in the total reaction volume (12,5 µl) used for each sample	E factor for primers-probe mixture used
IGF-II				1,98
	forward	5'-CCAAGTCCGAGAGGGACGT-3'	300	
	reverse	5'-TTGGAAGAACTTGCCCACG-3'	300	
	probe	5'-FAM-ACCGTGCTTCCGGACAACTTCCC-TAMRA-3'	200	
mTOR				1,91
	forward	5'-TGCTGCGTGTCTTCATGCAT-3'	300	
	reverse	5'-GGATTGCAGCCAGTAACTTGATAG-3'	300	
	probe	5'-FAM-ACAGCCCAGGCCGCATTGTC-TAMRA-3'	100	
HPRT				1,98
	forward	5'-CACTGGCAAAACAATGCAGACT-3'	500	
	reverse	5'-GTCTGGCTTATATCCAACACTTCGT-3'	500	
	probe	5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'	100	

Supplementary material 2: Antibodies used for IHC. Protein detected use type and source purcha

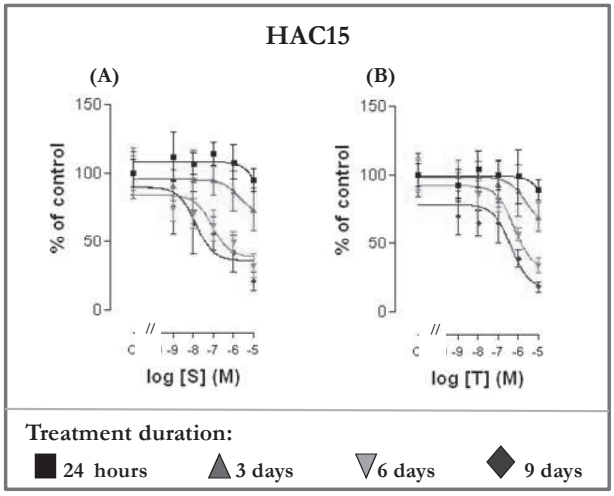
Protein detected	use	type and source	purchase by	dilution used	Positive controls
anti-mTOR	primary antibodies	mono-Rabbit	Cell Signaling Technology	1:25	breast cancer
anti-IGF-II	primary antibodies	poly-Goat	Santa Cruz Biotechnology	1:500	human ACC
anti-IGFIR	primary antibodies	mono-Mouse	Novus Biologicals	1:500	human pancreas
anti-Goat IgG	secondary antibodies	poly-Rabbit	DakoCytomation	1:200	
anti-Mouse/Rabbit-IgG	secondary antibodies	poly-AP-Goat	PowerVision+ (ImmunoVision Technologies)	as provided by the manufacturer	

Negative controls included omission of the primary antibodies and the incubation with secondary antibodies (PolyAPGoat anti Mouse/Rabbit IgG for mTOR and PolyA

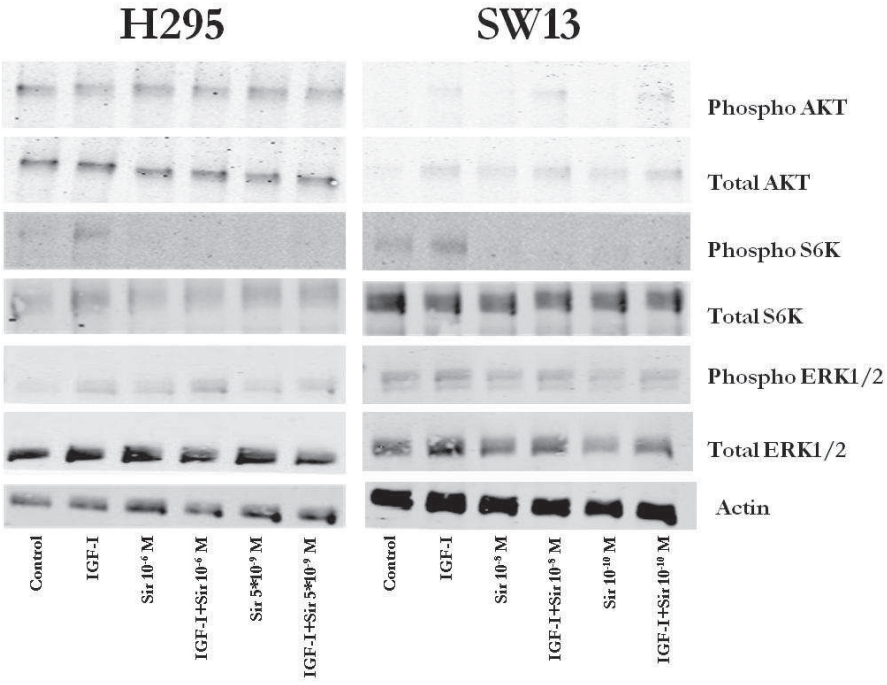
Supplementary material 3: Antibodies used for WB.

Protein detected	Molecular weight	use	type and source	purchase by	dilution used
anti-phospho-S6K(Thr389)	70 kDa	primary antibodies	mono-Rabbit	Cell Signaling Technology	1:1000
anti-total-S6K	70 kDa	primary antibodies	mono-Mouse	Santa Cruz Biotechnology	1:1000
anti-phospho-AKT(Ser473)	60 kDa	primary antibodies	poly-Rabbit	Cell Signaling Technology	1:1000
anti-total-AKT	60 kDa	primary antibodies	poly-Rabbit	Cell Signaling Technology	1:1000
anti-phospho-ERK1/2 (Tyr 204)	42,44 kDa	primary antibodies	mono-Mouse	Santa Cruz Biotechnology	1:200
anti-total- ERK	42 kDa	primary antibodies	mono-Mouse	Santa Cruz Biotechnology	1:500
anti-actin	42 kDa	primary antibodies	mono-Mouse	Sigma-Aldrich	1:1000

Precision Plus Protein Standard from BioRad Laboratories, Inc, was used as protein marker.



Supplementary figure 1. Sirolimus (A) and temsirolimus (T) significantly suppressed the cell growth in a dose and timedependent manner in HAC15 cells).



Supplementary figure 2. Exemplary figure of the blotting gels corresponding to the results presented in figure 7

Characterization of the mTOR pathway in human normal adrenal and adrenocortical tumors

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ABSTRACT

mTOR-pathway has been recently suggested as a new potential target for therapy in adrenocortical carcinomas (ACCs).

The aim of the current study is to describe the expression of the mTOR-pathway in normal (NAs) and pathological adrenals and to explore whether there are correlation between the expression of these proteins and the *in vitro* response to sirolimus. At this purpose, the MTOR, S6K1 (RPS6KB1), and 4EBP1 (EIF4EBP1) mRNA expression was evaluated in 10 NAs, 10 hyperplasia (AHs), 17 adenomas (ACAs) and 17 ACCs by qPCR whereas total(t)/phospho(p)-mTOR, t/p-S6K and t/p-4EBP1 protein expression was assessed in three NAs, three AHs, six ACAs and 20 ACCs by immunohistochemistry. The effects of sirolimus on cell survival and/or cortisol secretion in 12 human primary cultures of adrenocortical tumors (ATs) were also evaluated.

In the NAs and AHs a layer-specific expression of evaluated proteins was observed. S6K1 mRNA levels were lower in ACCs compared with NAs, AHs and ACAs ($p<0.01$). A subset of ATs presented a moderate-high staining of the evaluated proteins. Median t-S6K1 protein expression in ACCs was lower than ACAs ($p<0.01$). Moderate to high staining of p-S6K1 and/or p-4EBP1 was observed in most ATs. A subset of ACCs not having moderate to high staining had higher Weiss than others ($p<0.029$). In primary AT cultures sirolumus significantly reduced cell survival or cortisol secretion only in sporadic cases.

In conclusion these data suggest the presence of an activated mTOR-pathway in a subset of ATs and a possible response to sirolimus only in certain ACC cases.

INTRODUCTION

Adrenocortical cancer (ACC) is a rare and aggressive cancer with a 5-year survival at the metastatic stage below 15%.[1-3] The insulin-like growth factor (IGF) system has been considered a major actor in the pathogenesis of ACCs and an attractive target for new treatments in patients affected by this malignancy.[4-6] Another important factor may be mTOR, which is a protein kinase of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway, playing a pivotal role in cell growth, metabolism and proliferation.[7] Activation of the mTOR pathway leads to the phosphorylation and activation of downstream effectors such as the protein-70-ribosomal-protein-S6-kinase-1 (S6K1)(RPS6KB1) and eukaryotic-translation-initiation-factor-4E-binding-proteins (4EBP1)(EIF4EBP1).[7] S6K1 and 4EBP1 are both regulators of mRNA translation and stimulate the synthesis of several proteins involved in cell proliferation and survival.[7] Alterations in the mTOR pathway have been found in many human tumours.[8-10] Therefore, the mTOR pathway is considered a target for antineoplastic therapy in several malignancies and it has recently been proposed as target for ACC treatment.[11-13]

MATERIALS AND METHODS

Subjects

A total of 65 adrenal samples (26 ACC, 19 adrenocortical adenomas [ACAs], ten adrenal hyperplasia [HAs] and ten normal adrenals [NAs]) were selected from the tissue bank (from 1992 and July 1, 2010) of the Erasmus Medical Centre, Rotterdam (The Netherlands). HAs were obtained from adult patients undergoing bilateral adrenal removal for Cushing's syndrome. All tissues were frozen within 60 minutes after surgical removal. NAs were obtained from adult donors or adult patients undergoing normal adrenal removal during surgery for kidney cancer.

An additional 12 adrenal samples (seven ACC, five ACA) were collected during surgery and they were processed immediately to obtain primary adrenal tumor cell cultures.

This study was approved by the Medical Ethics Committee of the Erasmus Medical Center.

The Weiss score, assessed by an expert pathologist in adrenal disease (RRdK), was used to make the distinction between adenomas and carcinomas.[14]

The following clinical parameters were recorded in all patients: date of diagnosis, age, gender, ENSAT stage,[15] Weiss score, mitotic count (as defined by the presence number of mitoses equal or higher than five in 50 high-power fields), hormonal status, and type of hormonal secretion (cortisol and/or androgens and/or estrogens and/or mineralocorticoids).[3]

Total RNA isolation and quantitative RT-PCR (qRT-PCR)

From snap frozen adrenal tissues, total-RNA was isolated using a commercially available kit (High Pure RNA Tissue kit; Roche, Almere, The Netherlands).

Total RNA from the human ACC cell line NCI-H295R was used as a positive control.

The cDNA synthesis from total-RNA and quantitative PCR were performed as described previously.[11]

The mRNA expression of *MTOR, 4EBP1, S6K1* and of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT (HPRT1) were evaluated by quantitative RT-PCR in human adrenocortical tissue samples depending on the availability of frozen tissues.

The primers and probes were purchased from Sigma-Aldrich. The sequence of the primers and probes used are reported in the Supplementary table 1, see section on supplementary data given at the end of this article. Samples were normalized against the expression of HPRT. PCR efficiencies (E) were calculated for the primer-probe combinations used (Supplementary table 1).[16] The relative expression of genes was calculated using the comparative threshold method, $2^{-\Delta Ct}$,[17] after efficiency correction[18] of target and reference gene transcripts (*HPRT*).

Immunohistochemistry (IHC)

The expression of total/phospho-mTOR, total/phospho-4EBP1 and total/phospho-S6K1 in adrenal samples were evaluated.

Paraffin-embedded tissue specimens were cut in 5 µm sections, deparaffinized and dehydrated. Antigen-retrieval was performed by microwave treatment in Tris–EDTA Buffer (pH 9.0). The slides were cooled for 1 hour at +4˚C and incubated for 1 hour at room temperature (RT) with the primary antibodies. The primary monoclonal antibodies (MABs) to detect mTOR, phospho-mTOR, 4EBP1, phospho-4EBP1 and phospho-S6K1 were purchased from Cell Signaling Technology (Inc.-3 Trask Lane-Danvers, MA 01923, USA, dilution: 1:25, 1:50, 1:1200; 1:25 and 1:100 respectively). The primary MAB to detect S6K1 was purchased from Santa Cruz Biotechnology, Inc. (dilution 1:25). The slides were washed and incubated for 30 min at RT with secondary antibodies (Poly-AP-Goat anti-Mouse/Rabbit IgG PowerVision+; ImmunoVision Technologies, Duiven, the Netherlands) at the concentration provided by the manufacturer. After washing, staining was visualized by incubation for 30 min with new fuchsin solution.[19] Only phospho-S6K1 staining was performed and visualized with a Dako Detection System, following a different protocol described previously.[11] All slides were counterstained with hematoxylin and coverslipped. Positive controls included cases of breast cancer with previously proven positivity at immunohistochemistry (IHC) for the protein evaluated. Negative controls included omission of the primary antibody and the incubation with secondary antibodies.

The staining was evaluated independently by two investigators and any discrepancy was resolved by a consensus review. In tumor specimens (ACAs and ACCs) the results were interpreted in a semiquantitative manner by using an intensity-proportion scoring system as described previously.[20] The score was calculated by the sum of the intensity score and the proportion of the stained cells; this provided a score between 0 and 6. The proportion score was as follows: 0 = no positivity (or less than 10%); +1 = less than 1/3 tumor cell positivity; +2 = 1/3 to 2/3 tumor cell positivity; and +3 = more than 2/3 tumor cell positivity. The intensity score was as follows: +1 = weak staining; +2 = intermediate staining; +3 = strong staining. The score 0 was considered as negative; 2-3 as low; 4-5 as intermediate and 6 as high. Finally, adrenocortical tumors (ATs) were dichotomously grouped as having intermediate to high expression of the evaluated protein and phosphoproteins (IHC score equal-higher than 4) or not (IHC score lower than 4).

In AHs and in NAs, we used only the intensity score as indicated above.

To evaluate the correlation between the in vitro response to sirolimus (rapamycin) and the protein expression of the main components of the mTOR pathway, the expression of total/phospho-mTOR, total/phospho-4EBP1 and total/phospho-S6K1 were evaluated in 12 additional AT samples used to perform AT primary cell cultures. In this additional series, the staining was performed and visualized with a Dako Detection System, following a different protocol described previously (De Martino, van Koetsveld et al. 2012). In this system, dilution of total/phospho-mTOR, total/phospho-4EBP1 and total/phospho-S6K1 antibodies were: 1:50, 1:100, 1:2400; 1:50; 1:50 and 1:100 respectively.

Drugs and reagents

The mTOR inhibitor sirolimus was purchased from LC Laboratories Inc. (Woburn, MA, USA), dissolved in dimethylsulfoxide (DMSO) as concentrated (10^{-3}M) stock solution (stored at -20°C) and diluted in 40% DMSO before use. IGF1 was purchased from Sigma-Aldrich, diluted in 0.01M acetic acid as concentrated ($5x10^{-6}$M) stock solution (stored at -20°C) and diluted in culture medium before use.

Cell lines and culture conditions

The human ACC cell lines H295 and SW13 were obtained from the American Type Culture Collection (Manassas, VA, USA) and from ECACC (Salisbury, Wiltshire, UK), respectively. Cells were cultured, as described previously in detail.[11]

Immunocytochemistry: chamber slides

To evaluate the expression of total/phospho-4EBP1 and total/phospho-S6K1 in ACC cell lines, H295 and SW13 cells were plated on poly-L-lysine-coated culture chamber slides (NUNC A/S, Roskilde, Denmark), for 48h in full medium. Subsequently, medium was refreshed and cells were incubated for 16h in a complete medium or starved in serum-

free medium containing 0.1% human serum albumin (medium-HSA), according with the different treatment group assigned. Finally, media were refreshed again and cells were incubated for 30 min in complete medium or medium containing HSA with or without IGF1 (10^{-8}M), according with the different treatment group assigned (Figure 1). Immunocytochemistry in chamber slides was performed, as described previously in detail.[11] The

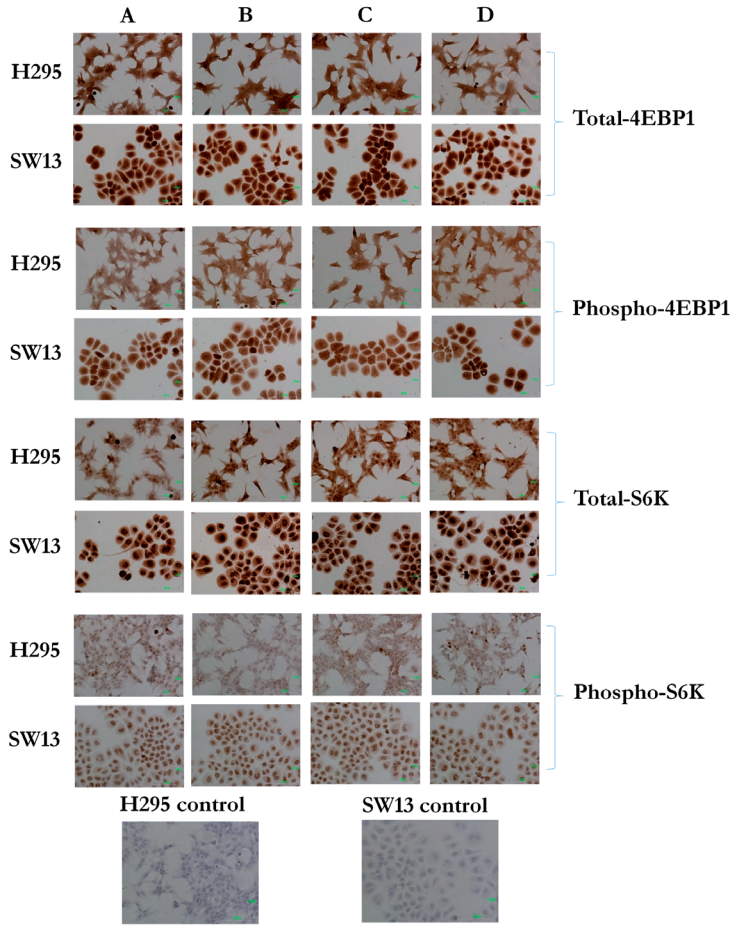

Figure 1. Immunocytochemical detection of total 4EBP1, phospho-4EBP1, total S6K1, and phospho-S6K1 in two human adrenocortical carcinoma cell lines H295 and SW13 plated in different medium conditions (details in the text). Panel A medium conditions include a complete continuous medium. Panel B medium conditions include 48 h in a complete medium followed by16 h in a serum-free medium containing 0.1% human serum albumin (medium-HSA), thus an additional 30 min in medium-HSA. Panel C medium conditions include 48 h in a complete medium followed by 16 h in medium-HSA and thus 30 min in a complete medium. Panel D medium conditions include 48 h in a complete medium followed by 16 h in medium-HSA and thus 30 min in medium-HSA with IGF1 (10^{-8} M). The two pictures at the bottom show the absence of staining in the negative controls. Magnification, x200.

dilution of total/phospho-mTOR, total/phospho-4EBP1 and total/phospho-S6K1 antibodies were: 1:2400; 1:50; 1:50 and 1:100 respectively.

Primary cell culture of human adrenocortical tumors

Immediately after surgery, the adrenal specimens were minced into small pieces and dissociated with collagenase type I (2 mg/ml; Sigma-Aldrich). Cell viability, after Ficoll density gradient separation, was determined by trypan blue exclusion and always exceeded 90%. Cells were cultured in DMEM/F12K medium (Invitrogen), supplemented with 5% FCS, penicillin ($1x10^5$ U/l; Bristol-Meyers Squibb, Woerden, The Netherlands) and L-glutamine (2 mmol/l; GIBCO). Cells were plated at a density of 100.000 cells per well in 24-well plates (Corning Costar, Schiphol, The Netherlands). After 3-4 days of incubation at 37°C in a humidified incubator containing 5% CO_2, the medium was changed and incubations without or with sirolimus were performed for 7 days in quadruplicate. Controls were treated with the vehicle. The medium and test substances were washed every 3 days. On day 7, media were collected, cells were washed twice with saline, followed by lysis for DNA analysis as described previously in detail (Hofland, et al. 1990). Owing to a limitation in the cell yield obtained per tissue, and the absence of cortisol secretion in some cases, not all experiments could be carried out in each individual case.

Cortisol secretion assay

In cortisol-secreting adrenal tumor primary cultures, we evaluated the effects of sirolimus on cortisol production. The culture supernatants from primary culture experiments, performed as above described, were collected and stored at -20°C until determination of the cortisol concentration.

The cortisol concentration was determined by a non-isotopic, automated chemiluminescence immunoassay system (Siemens Medical Solutions Diagnostics, Breda, The Netherlands). Cortisol levels were expressed as percentage of control and were corrected for the total DNA content in each well, thereby reflecting cortisol production per cell.

Statistical analysis

Statistical analysis was performed using the statistical software of SPSS (SPSS 15.0; SPSS Inc.). Quantitative data were expressed using means and standard deviations (SD) and medians and range. Qualitative data are expressed as percentage. Mann-Whitney U test and Kruskall-Wallis test were used to compare two or more groups. The comparative statistical evaluations among treatment groups in primary culture experiments were performed by ANOVA, followed by a multiple comparative test (Newman-Keuls). Spearman's rank correlation coefficient was used to test correlation.

RESULTS

Study population

This study included samples from 65 adrenal patients. Only two of the included ACC patients were children (9.5 and 4.2 years old respectively).

To describe the mTOR pathway, the mRNA expression levels of *MTOR, 4EBP1, and S6K1*, were evaluated by qRT-PCR in 54 human adrenocortical tissue samples (17 ACC, 17 ACAs, ten AHs and ten NAs) and the protein expression levels of mTOR, phospho-mTOR, 4EBP1, phospho-4EBP1 total-S6K1 and phospho-S6K1 were evaluated by IHC in 32 human adrenocortical tissue (20 ACCs, six ACAs, three AHs, and three NAs). For 20 cases (11 ACC including the two children, and three ACA, three AH, and three NA patients we had adequate material to perform the analysis of the mTOR pathway by both qRT-PCR and IHC.

mRNA expression of mTOR pathway components in human adrenal samples

The mRNA expression of *MTOR, 4EBP1*, and *S6K1*were evaluated by quantitative RT-PCR in 54 adrenocortical tissue samples. In the samples, no statistically significant differences between the expression levels of *MTOR* (ACCs: 0.16±0.29; ACAs: 0.35±0.21; AHs: 0.29±0.07; NAs: 0.27±0.19, *median±SD*) and *4EBP1* (ACCs: 0.36±0.60; ACAs: 0.41±0.64; AHs: 0.36±0.42; NAs: 0.33±0.08) were found, while the mRNA expression levels of *S6K1* were significantly lower in ACCs than in other groups (ACCs: 0.10±0.08 *vs* ACAs: 0.20±0.11; AHs: 0.29±0.07; NAs: 0.23±0.08; p<0.01) (figure 2A, B and C). In the group of ACCs, a significant correlation was found between the mRNA levels of *MTOR* and ***4EBP1*** (p=0.043), (p=0.003); *S6K1* and *4EBP1* (p=0.011), but no relationships were observed

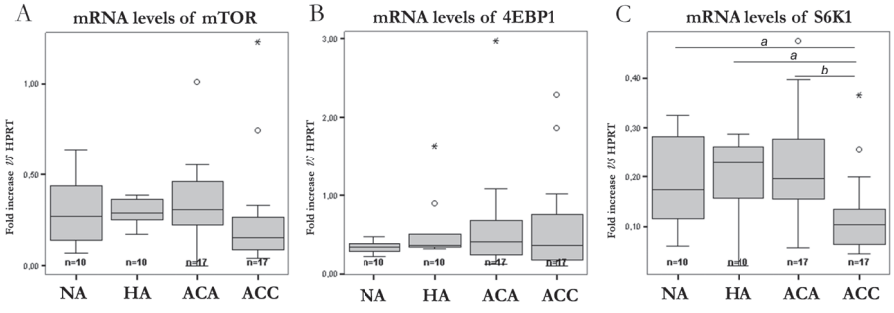

Figure 2. Box plot representation of the relative (A) mTOR, (B) 4EBP1, and (C) S6K1 mRNA expression observed in normal and pathological human adrenal samples. A significantly lower (P<0.01) relative mRNA expression of S6K1 was observed in adrenocortical carcinomas (ACCs) compared with all the other groups (C). ([a]P<0.01; bP<0.001; °outliers; *extreme outliers).

with the Weiss score, mitotic index and TNM. The correlations were also present when whole series of samples was considered.

Protein expression of mTOR pathway components in human normal adrenal and adrenal hyperplasia.

All the evaluated components of the mTOR pathway were expressed in cortex of the evaluated NA and AH specimens. However, a layer-specific expression of the evaluated proteins was observed. The intensity of staining for the majority of the evaluated proteins was moderate to strong in glomerulosa and reticularis layers, weak to moderate in fasciculata and faint to weak in NA medulla, with the exception of phospho-S6K1 which showed moderate to strong staining in normal medulla.

Representative pictures of the staining observed in NAs are reported in Figure 3.

Protein expression of mTOR pathway components in human adrenocortical tumors

In 26 ATs (20 ACCs and six ACAs), the protein expression of the total and phospho-mTOR, total and phospho-4EBP1, total and phospho-S6K1 were evaluated by IHC. Table 1 summarizes the results of the IHC and the main clinical features of the evaluated patients. Figure 4 shows 2 exemplary cases of immunostaining in ATs (a case of ACC [left panel] and a case of ACA [right panel]).

All ACCs, except one case (19/20; 95%), and all ACAs evaluated showed a positive staining for total-mTOR. This expression was intermediate to high in 12 ACCs (60%) and in all ACA samples. The staining of phospho-mTOR was present in a lower number of cases (5/20 ACCs and 3/6 ACAs) and only in some of these it was intermediate to high (two ACCs and two ACAs). A positive staining for total 4EBP1 was observed in all tumor samples evaluated with the exception of one ACC. This staining was intermediate to high in 15 ACCs (75%) and in all ACAs evaluated. The staining for phospho-4EBP1 was positive in 19 ACCs (95%) and in all ACAs. This staining was intermediate to high in 12 ACCs (60%) and in all ACAs. A positive total S6K1 staining was observed in 15 (75%) ACCs and all ACAs. This staining was intermediate to high in five ACCs (25%) and four ACAs (67%). The staining for phospho-S6K1 was positive in eight (40%) ACCs and four ACAs (67%). This staining was intermediate to high in six ACCs (30%) and three ACAs (50%) respectively. The overall total-S6K1 score observed in ACAs was higher than ACCs ($p=0.009$) (Figure 5 A).

Considering together the staining score of phospho-S6K1 and phospho-4EBP1, an intermediate to high staining of at least one of the two components of the mTOR pathway was found in 16 ACCs (80%) and in all ACAs. An intermediate to high staining of both components was described in two ACCs (10%) and in three ACAs (50%). Interestingly, all ATs (six ACAs and 20 ACCs), which did not present any intermediate to high staining of

Table 1. Immunocytochemical detection of mTOR-pathway components in human adrenocortical tumors.

Patient number	Tumor Type	Sex	Weiss	Hormonal secretion	mTOR protein expression		Phospho mTOR protein expression		4EBP1 protein expression		Phospho-4EBP1 protein expression		S6K protein expression		Phospho S6K protein expression		phospho-4EBP1 and/or phospho S6K
					Score	Considerable expression	Score	Considerable expression	Score	Considerable expression	Score	Considerable expression	Score	Considerable expression	Score	Considerable expression	Considerable expression
1	ACC	F	3	C	4	Yes	2	No	4	Yes	4	Yes	0	No	0	No	Yes
2	ACC	F	5	C and A	3	No	0	No	4	Yes	3	No	0	No	4	Yes	Yes
3	ACC	F	9	A	0	No	0	No	4	Yes	4	Yes	2	No	4	Yes	Yes
4	ACC	M	6	C, A and E	5	Yes	0	No	4	Yes	4	Yes	4	Yes	0	No	Yes
5	ACC	F	6	A	6	Yes	0	No	2	No	4	Yes	2	No	0	No	Yes
6	ACC	F	7	C and A	5	Yes	0	No	4	Yes	3	No	0	No	2	No	No
7	ACC	M	8	none	6	Yes	4	Yes	6	Yes	5	Yes	3	No	0	No	Yes
8	ACC	F	4	A	2	No	0	No	3	No	4	Yes	3	No	2	No	Yes
9	ACC	F	7	none	5	Yes	0	No	4	Yes	3	No	4	Yes	0	No	No
10	ACC	F	3	none	4	Yes	0	No	4	Yes	2	No	0	No	4	Yes	Yes
11	ACC	F	7	C and A	3	No	0	No	4	Yes	2	No	3	No	4	Yes	Yes
12	ACC	F	5	A	6	Yes	2	No	4	Yes	5	Yes	4	Yes	2	No	Yes
13	ACC	F	7	none	3	No	0	No	5	Yes	4	Yes	2	No	4	Yes	Yes
14	ACC	M	8	C and M	3	No	0	No	4	Yes	0	No	3	No	0	No	No
15	ACC	F	8	none	2	No	0	No	5	Yes	3	No	3	No	0	No	No
16	ACC	M	4	A	5	Yes	0	No	0	No	2	No	0	No	4	Yes	Yes
17	ACC	F	6	none	2	No	0	No	2	No	5	Yes	3	No	2	No	Yes
18	ACC	F	4	none	6	Yes	2	No	2	No	4	Yes	6	Yes	0	No	Yes
19	ACC	F	5	A	6	Yes	0	No	5	Yes	4	Yes	3	No	0	No	Yes
20	ACC	F	4	none	5	Yes	4	Yes	5	Yes	6	Yes	5	Yes	3	No	Yes
			Median 6; Range 3-9		***Median 4,5; Range 0-6***	***Frequency 12/20 (60%)***	***Median 0; Range 0-4***	***Frequency 2/20 (10%)***	***Median 4; Range 0-6***	***Frequency 15/20 (75%)***	***Median 4; Range 0-6***	***Frequency 12/20 (60%)***	***Median 3; Range 0-6***	***Frequency 5/20 (25%)***	***Median 2; Range 0-4***	***Frequency 6/20 (30%)***	***Frequency 16/20 (80%)***
21	ACA	F	1	none	5	Yes	0	No	5	Yes	4	Yes	5	Yes	3	No	Yes
22	ACA	M	0	C	5	Yes	0	No	5	Yes	5	Yes	4	No	0	No	Yes
23	ACA	F	0	none	5	Yes	4	Yes	5	Yes	4	Yes	5	Yes	4	Yes	Yes
24	ACA	F	0	C	4	Yes	4	Yes	4	Yes	4	Yes	3	No	4	Yes	Yes
25	ACA	F	0	None	5	Yes	0	No	5	Yes	5	Yes	5	Yes	5	Yes	Yes
26	ACA	M	2	none	5	Yes	2	No	4	Yes	5	Yes	5	Yes	0	No	Yes
			Median 0; Range 0-2		***Median 5; Range 4-5***	***Frequency 6/6 (100%)***	***Median 2; Range 0-4***	***Frequency 2/6 (33%)***	***Median 5; Range 4-5***	***Frequency 6/6 (100%)***	***Median 4,5; Range 4-5***	***Frequency 6/6 (100%)***	***Median 5; Range 3-5***	***Frequency 4/6 (67%)***	***Median 3,5; Range 0-5***	***Frequency 3/6 (50%)***	***Frequency 6/6 (100%)***

ACC: adrenocortical carcinoma; ACA: adrenocortical adenoma; C: cortisol; A: androgens; E: estrogens; M: mineralocorticoids.

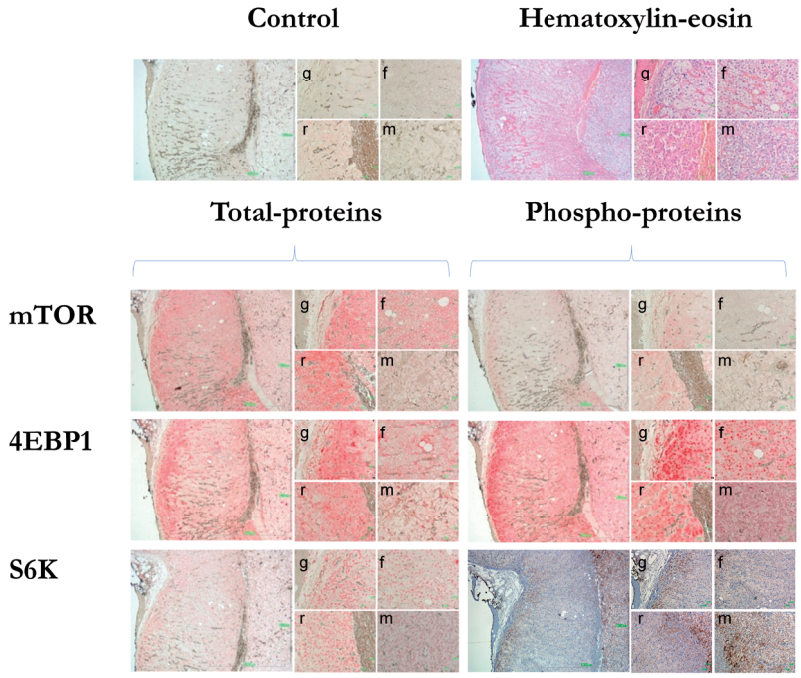

Figure 3. Exemplary pictures of the immunocytochemical detection of total mTOR, phospho-mTOR, total 4EBP1, phospho-4EBP1, total S6K1, and phospho-S6K1 in normal adrenals. Large pictures represent all the normal adrenal ayers (magnification, x40) and smaller pictures represent the higher magnification (100x) of each adrenal layers (from the outer to the inner: glomerulosa (g); fasciculata (f); reticularis (r); and medulla (m)).

the two above-mentioned phosphoproteins had significantly higher Weiss scores than others (p=0.009) (Figure 5 B). This difference was still present considering only ACCs (p=0.029) (Figure 5 C).

In ACC group a higher total-S6K1 (5±2 *vs* 2±2; p=0.04) and phospho-4EBP1 (5±1 *vs* 3±2; p=0.04) protein expression was observed in tumors having a mitotic count lower than 5.

Several positive correlations between the staining score of different components of the mTOR pathway in adrenocortical tumors were observed (Supplementary table 2 see section on supplementary data given at the end of this article), potentially suggesting common regulators.

In the 14 ATs, for which adequate material was available to perform both IHC and qRT-PCR, none of the evaluated components showed a significant correlation between mRNA expression levels and protein expression levels.

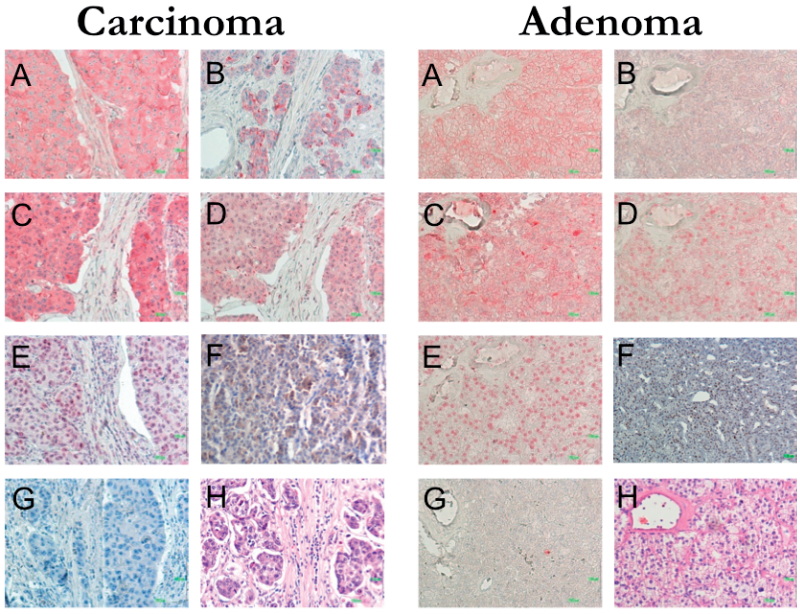

Figure 4. Immunocytochemical detection of total mTOR (A), phospho-mTOR (B), total 4EBP1 (C), phospho-4EBP1 (D), total S6K1 (E), and phospho-S6K1 (F) in a case of human adrenocortical carcinoma (ACC (left panel)) and a case of human adrenocortical adenoma (ACA (right panel)). Picture 'G' shows the absence of staining in the negative controls, and picture 'H' the HE staining in both panels. Magnification, X200.

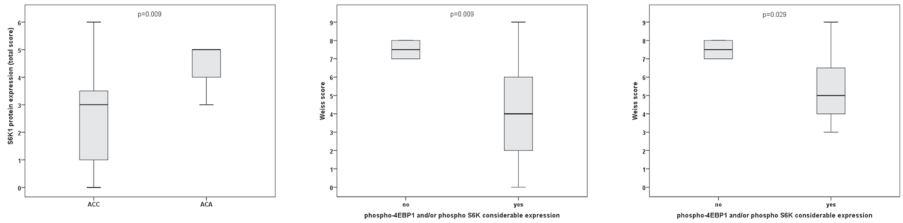

Figure 5. (A) Box plot representation of total S6K1 protein expression observed in malignant (ACCs) and benign (ACAs) adrenocortical tumors. The expression of S6K1 (total score; explanation in the text) in ACCs was significantly lower than that in ACAs. (B) Box plot representation of the Weiss score values in adrenocortical tumors (including six ACAs and 20 ACCs) divided into two groups according to the presence (yes) or absence (no) of an intermediate to high IHC score for phospho-4EBP1 and/or phospho-S6K1 (explanation in the text). (C) Box plot representation of the Weiss score values in the malignant subgroup of adrenocortical cancer (ACC) divided into two groups according to the presence (yes) or absence (no) of an intermediate to high IHC score for phospho-4EBP1 and/or phospho-S6K1. Tumors not having an intermediate to high IHC score for phospho-4EBP1 and/or phospho-S6K1 had a significantly higherWeiss score than other tumors (B); this difference was still present considering only ACCs (C).

ACCs associated with a hormonal production did not present any significant difference in the mRNA and protein expression levels of the evaluated components when compared with the non-secreting ACCs.

Protein expression of mTOR pathway components and in vitro response to sirolimus in preclinical models of human ATs

In order to explore whether the protein expression of the main components of the mTOR pathway are predictive for the effects of mTOR inhibitors in *in vitro* models of human ATs, we performed experiments in human ACC cell lines and in primary cell cultures of ACCs and ACAs.

Human ACC cell lines. To define whether total/phospho-4EBP1 and total/phospho-S6K1 protein expression are correlated with the *in vitro* response of ACC cell lines to mTOR inhibitors, immunocytochemistry was performed in H295 and SW13. We had previously described the effects of sirolimus in these cell lines[11] and demonstrated that SW13 are more sensitive to sirolimus treatment than H295 cell line. All the evaluated proteins were well expressed in both cell lines, either in basal or in serum/IGF1-stimulated conditions (Figure 1). No major differences were observed in total/phospho-4EBP1 and total/phospho-S6K1 protein expression between the cell lines.

Primary human ACC and ACA cell cultures. To determine whether the protein expression of all evaluated components of the mTOR pathway were correlated with the *in vitro* response of primary human AT cell cultures to mTOR inhibitors, immunocytochemistry was performed in tissue of seven ACC and five ACA from which primary cultures were performed. In these cell cultures, the response to sirolimus (10^{-10} and 10^{-6}M) was defined based on the presence or absence of a significant inhibition in cell number (as evaluated by DNA measurement) and/or cortisol secretion (in the cortisol-secreting tumors). Supplementary table 3, see section on supplementary data given at the end of this article shows the results of the effect of sirolimus in primary cultures. Only one ACC primary culture showed a significant cell number reduction after sirolimus treatment (figure 6 A). In a different case which was a cortisol-secreting ACC, sirolimus treatment significantly inhibited cortisol secretion (figure 6 C), but it did not affect cell number (figure 6 B). There were no clear differences in the expression of the evaluated proteins between the two responder cases and the others. These results suggest that only a small subset of ACC show a response to mTOR inhibitors and that there is not a clear correlation between response to sirolimus and protein expression of the main components of the mTOR pathway.

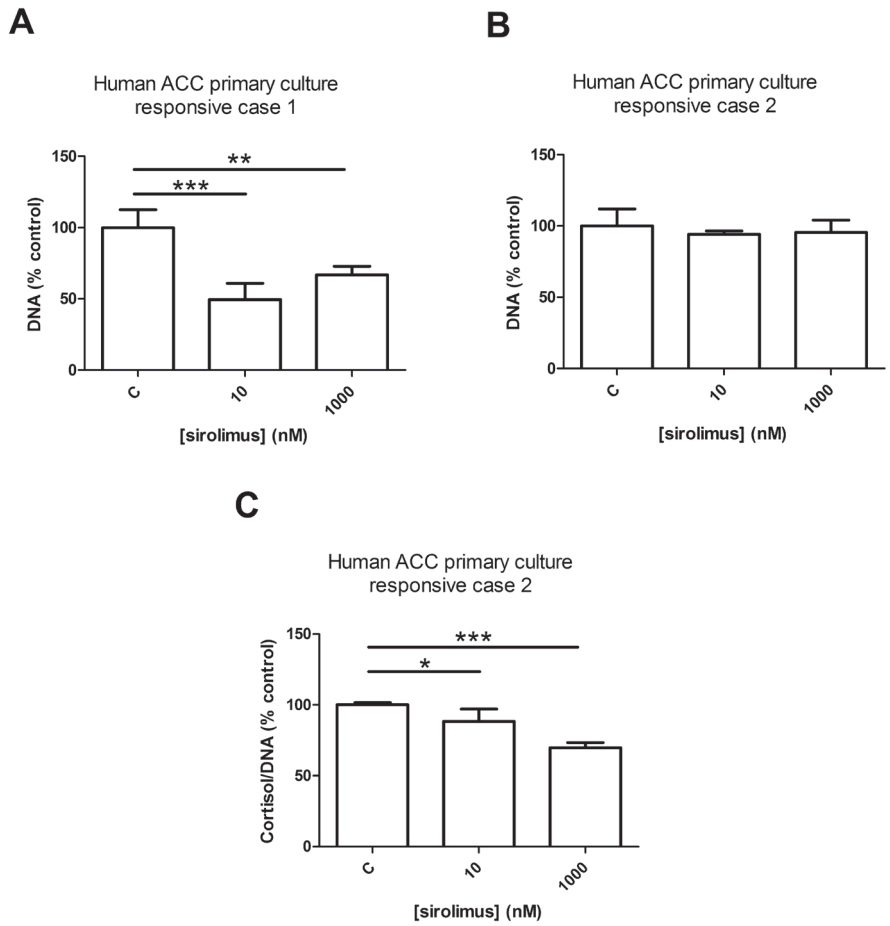

Figure 6. Effects of sirolimus in primary cultures of adrenocortical cancer: two responder cases. (A) A case with a significant reduction in cell survival as measured by the total DNA content. (B and C) A case with a significant reduction in cortisol secretion normalized for the total DNA content (C). Values are expressed as the percentage of the control and are described as mean±S.D. of four repeated measurements. *P<0.05; **P<0.01;***P<0.001.

DISCUSSION

This study demonstrates a layer-specific protein expression pattern of the major components of the mTOR pathway in normal adrenals and suggests the presence of an activated mTOR pathway in a subset of adrenal tumors.

Generally, malignant adrenal tumors (ACCs) showed variable protein expression of the evaluated components of the mTOR pathway and lower *S6K1* mRNA and protein levels than adrenal benign tumors, suggesting a possible deregulation of the mTOR pathway

in ACCs. However, it is difficult to establish whether these downregulations could be related to an abnormal mTOR pathway activity. Phospho-S6K1 and/or phospho-4EBP1 have been considered as potential markers of mTOR pathway activation in human cancers.[21, 22] Although a higher percentage of benign tumors presented an intermediate to high staining of these phospho-proteins compared to the ACCs, the total scores of phospho-S6K1 or phospho-4EBP1 were not significantly higher in ACAs compared with ACCs. Interestingly, tumors not having an intermediate to high staining of these phosphoproteins had a significantly higher Weiss score than tumors presenting an intermediate to high staining of at least one phosphoprotein. These data suggest that the mTOR pathway activity was downregulated in a subgroup of tumors with a more aggressive pathological phenotype (although not in all cases with aggressive pathological phenotype), thus indicating that a subset of less differentiated ACCs could be less dependent on activation of the mTOR pathway.

The different expression of the evaluated proteins in the different layers of the adrenal cortex suggests a specific role of the mTOR pathway in particular adrenal functions. For example the stronger expression of several components of the mTOR pathway in the reticularis could suggest a role of this pathway in the androgen production and the stronger expression of these components in the glomerulosa may be related to angiotensin II induced activation of the mTOR pathway.[23] We did not perform a real comparison between the staining observed in NAs and that observed in ATs because the presence of different layers in NAs, expressing the evaluated proteins at different intensities, made it difficult to attribute a semiquantitative IHC score as done for tumors. However, if we consider as reference, for example, the most representative layer of the adrenal cortex, which is the fasciculata, some tumors over-express and others have lower expression of the evaluated proteins. This observation reinforces the suggested heterogeneity of adrenocortical tumors, particularly in ACCs, in the expression of the mTOR pathway.

Several positive correlations between the mRNA levels of the different evaluated components of the mTOR pathway were observed. Similarly, correlations were also observed among the different protein expression levels, suggesting that different components of the mTOR pathway could have common regulators of transcription and/or protein translation. Conversely, we did not find correlations between the expression observed at mRNA levels and the expression observed at protein levels. This absence of correlation between protein and mRNA expression could suggest different mechanisms of regulation in protein and mRNA expressions, such as the presence of post-transcriptional regulators or could simply be explained by the small sample size.

The PI3-K/AKT/mTOR pathway is an intracellular pathway that mediates the effects of many growth factors including the IGFs.[12, 24] Preclinical studies demonstrated that mTOR inhibitors inhibit *in vitro* cell proliferation in ACC cell lines[11] and used at high dose; they also inhibit growth of ACC xenografts in immunodeficient mice.[13] Moreover, mTOR path-

way has been found to be activated in childhood ACCs.[13] However, genomic abnormality associated with the potential PI3-K/AKT/mTOR pathway activation such as the loss of the tumor suppressor gene *PTEN* or *PI3KCA* mutations are not common events in ACCs.[25, 26]

Nakamura *et al.,* evaluating the presence of potential surrogate markers of targeted drugs in ACCs by IHC, failed to demonstrate a significant overexpression of phospho-S6K1 and phospho-4EBP1 in 41 ACCs as compared with 54 ACAs and 5 NAs. Conversely their results suggest a trend to a down-regulation of expression of these proteins in ACCs. This study demonstrates the expression of these proteins for each individual tumor and the percentage of positive/negative cases as established considering only intermediate to high staining. Adopting this approach we could define different subsets of ACC patients: 10% with an intermediate to high staining of both phospho-S6K1 and phospho-4EBP1; 30 or 60% expressing respectively phospho-S6K1 or phospho-4EBP1 and 80% expressing at least one of these proteins. Based on these results it can be hypothesized that a subset of patients with ACCs could potentially be candidate for treatment with mTOR inhibitors. However, it must be considered that tissue biomarkers capable of defining the sensitivity of patients to the mTOR inhibitors have not been yet been validated in the clinical setting. To explore the role of the main components of the mTOR pathway as predictor of response in *in vitro* models of ATs, we performed experiments in human ACC cell lines and in primary cell cultures of human ACCs and ACAs. Our previous study had demonstrated that sirolimus inhibits cell proliferation in H295 and SW13 human ACC cell lines although with a different potency, SW13 cell lines being more sensitive than H295 cell lines.[11] These two cell lines express total/phospho-4EBP1 and total/phospho-S6K1 at a comparable level, suggesting that the expression of these proteins is not useful to predict the different sensitivity to sirolimus observed in these cell lines. We further investigated the role of mTOR, phospho-mTOR, 4EBP1, phospho-4EBP1 total-S6K1 and phospho-S6K1 as predictor of response to sirolumus treatment in human ACC and ACA cell primary cultures. Also in this context protein expression was found to be unable to predict a differential sensitivity to sirolimus. In primary cultures experiments sirolimus significantly inhibited total DNA content only in one case of ACC with sarcomatoid features.[27] These results suggest that only a small subset of ACCs might respond to treatment with mTOR inhibitors. Additionally, they might indicate that ACCs with this particular phenotype could be more sensitive to this treatment. While both ACC cell lines showed a significant response to sirolimus in terms of DNA,[11] only one of the seven evaluated ACCs showed a response in this type of experiments. In this regard, it should be considered that primary cultures are in fact unable to proliferate, and therefore, DNA analysis in this context represents cell survival rather than a cell proliferation. For this reason the effects of sirolimus on total DNA content in primary cultures could be underestimated.

IGF2 is known to be overexpressed in ACC compared to ACA or non-pathological adrenals and this explains why the IGF pathway has been historically considered an attractive target for ACCs. The absence of an overactivation of the PI3-K/AKT/mTOR pathway raises the question whether there is dissociation between the known over expression of the IGF2 in ACC and the activation of the classical IGF stimulated intracellular pathway. The variable expression of the evaluated components of the mTOR pathway suggests that mTOR inhibitors must be used with caution in unselected patients with ACC. Few patients with advanced ACCs who received everolimus, as salvage treatment, did not receive any major benefit.[28] Few ACC patients have been treated in registered clinical trials.[29-31] These studies failed to prove the hoped efficacy of these compounds, but they suffer of limitations. As mitotane can interfere with the metabolism of other drugs including mTOR inhibitors, it became necessary to determine mitotane levels in these patients. However, these early clinical experiences suggest that a subset of patients could benefit (more than 6 month tumor stabilization) from combined treatment including mTOR inhibitors and IGF1R antagonists.[30, 31] In a previous study, we demonstrated that in H295 ACC cells, IGFs can activate mechanisms of escape from mTOR inhibitors which could be responsible for a reduced sensitivity to the treatment with these drugs.[11] The blocking of endogenously produced IGF2 increased the antiproliferative effects of sirolimus.[11] These preclinical studies support the rational to combine IGF1R antagonist and mTOR inhibitor treatment in a subset of patients with ACCs.

In conclusion, despite the well-known IGF2 overexpression and the potential role of the mTOR pathway in ACCs, presently there is no evidence that clearly supports a key role of this pathway in the pathogenesis of ACCs. However, this study suggests that a subset of ACCs have an activated mTOR pathway. Presently, there is not a strong proven rationale for the use of the mTOR inhibitors alone in ACCs. Therefore, further studies are warranted to investigate the potential role of mTOR inhibitors, alone or in association with other drugs, in ACC patients. The association between the Weiss score and the expression of the mTOR pathway components should be confirmed in larger series. Moreover, the role of the expression of the mTOR pathway components as prognostic parameters and as predictive biomarker for treatment with mTOR inhibitors in ACC warrants further investigation.

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SUPPLEMENTARY MATERIAL

Supplementary table 1. Primers/probes used, relative amount in PCR mixtures and relative efficiency-factor (E-factor).

			Amount (nmol/l) added in the total reaction volume (12,5 µl) used for each sample	E factor for primers-probe mixture used
mTOR				1,91
	forward	5'-TGCTGCGTGTCTTCATGCAT-3'	300	
	reverse	5'-GGATTGCAGCCAGTAACTTGATAG-3'	300	
	probe	5'-FAM-ACAGCCCAGGCCGCATTGTC-TAMRA-3'	100	
4EBP1				1,94
	forward	5'-GGCGGCACGCTCTTCA-3'	300	
	reverse	5'-TCAGGAATTTCCGGTCATAGATG-3'	300	
	probe	5'- FAM-ACCACCCCGGGAGGTACCAGGA-TAMRA-3'	100	
S6K1				1,86
	forward	5'-TGGAAGACACTGCCTGCTTTT-3'	300	
	reverse	5'-TGATCCCCTTTTGATGTAAATGC-3'	300	
	probe	5'- FAM-CTTGGCAGAAATCTCCATGGCTTTGG-TAMRA-3'	200	
HPRT				1,98
	forward	5'-CACTGGCAAAACAATGCAGACT-3'	500	
	reverse	5'-GTCTGGCTTATATCCAACACTTCGT-3'	500	
	probe	5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'	100	

Supplementary table 2. Correlation among the investigated protein expression according to the Spearman's method.

Adrenocortical tumors

		Weiss	Total-mTOR protein	phospho-mTOR protein	Total-4EBP1 protein	phospho-4EBP1 protein	Total-S6K1 protein	phospho-S6K1 protein
Weiss	Corelation coefficient	1,0000	-0,0100	-0,2290	-0,1000	-0,2850	-0,2880	-0,3140
	P value		*0,9630*	*0,2600*	*0,6270*	*0,1580*	*0,1540*	*0,1180*
Total-mTOR protein	Corelation coefficient		1,0000	0,3660	0,1360	0,3430	,393*	-0,3510
	P value			*0,0660*	*0,5060*	*0,0860*	***0,0470***	*0,0790*
Phospho-mTOR protein	Corelation coefficient			1,0000	0,1940	,482*	0,3760	-0,0670
	P value				*0,3410*	***0,0130***	*0,0580*	*0,7460*
Total-4EBP1 protein	Corelation coefficient				1,0000	0,3130	0,3040	0,0420
	P value					*0,1190*	*0,1320*	*0,8370*
Phospho-4EBP1 protein	Corelation coefficient					1,0000	,534**	-0,1240
	P value						***0,0050***	*0,5470*
Total-S6K1 protein	Corelation coefficient						1,0000	-0,1780
	P value							0,3840
Phospho-S6K1 protein	Corelation coefficient							1,0000
	P value							

* Significant correlation 0,05 (bilateral).

** Significant correlation 0,01 (bilateral).

Adrenocortical cancers

		Weiss	Total-mTOR protein	phospho-mTOR protein	Total-4EBP1 protein	phospho-4EBP1 protein	Total-S6K1 protein	phospho-S6K1 protein
Weiss	Corelation coefficient	1,0000	0,1364	-0,0825	0,3089	-0,0543	0,2418	-0,2374
	P value		*0,5665*	*0,7296*	*0,1851*	*0,8201*	*0,3044*	*0,3136*
Total-mTOR protein	Corelation coefficient		1,0000	,488*	0,0363	0,2697	0,2840	-0,4267
	P value			***0,0290***	*0,8792*	*0,2501*	*0,2249*	*0,0606*
Phospho-mTOR protein	Corelation coefficient			1,0000	0,2606	,617**	0,3923	-0,2394
	P value				*0,2672*	***0,0038***	*0,0871*	*0,3094*
Total-4EBP1 protein	Corelation coefficient				1,0000	0,1880	0,1286	-0,0911
	P value					*0,4274*	*0,5890*	*0,7025*
Phospho-4EBP1 protein	Corelation coefficient					1,0000	0,4349	-0,1860
	P value						*0,0553*	*0,4323*
Total-S6K1 protein	Corelation coefficient						1,0000	-0,4324
	P value							0,0569
Phospho-S6K1 protein	Corelation coefficient							1,0000
	P value							

* Significant correlation 0,05 (bilateral).

** Significant correlation 0,01 (bilateral).

Supplementary table 3. Effects of siroluimus on primary cultures of adrenocortical tumors.

Patient ID	Tumor Type	Effects of sirolimus on total DNA content			Effects of sirolimus on total cortisol secretion adjested for DNA content			Responders
		Control	10 nM	1000M	Control	10 nM	1000M	
A	ACC	100±7.4	99,8±3.6	96±9.6	-	-	-	no
B	ACC	100±9.4	55.6±13.4***	66.7±7.2**	-	-	-	yes
C	ACC	100±12	99.9±9.7	110.5±14.7	-	-	-	no
D	ACC	100±4.3	ND	94.3±10.8	-	-	-	no
E	ACC	100±13.4	116.2±18	113.4±19.4	100±16.4	91.4±16.7	94.5±13.8	no
F	ACC	100±11.9	94.1±2.5	95.4±9.1	100±1.3	88.4±8.2*	69.8±3.8***	yes
G	ACC	100±20.8	98.6±19.2	96.9±19.6	100±17.4	86.3±10.4	74±23	no
H	ACA	100±3.6	75.7±37.2	69.6±3.5	-	-	-	no
I	ACA	100±6	90.1±14.4	98.2±7.6	-	-	-	no
J	ACA	100±70.7	76.6±14.4	98.6±33.3	-	-	-	no
K	ACA	100±7.2	97.7±8.8	86.7±8	-	-	-	no
L	ACA	100±4.4	84.9±34.4	94±1	100±6.8	108.2±17.8	96±17.1	no

*p<0,05; **p<0,01; ***p<0,001

Effects of the dual IGF1-/Insulin-Receptor inhibitor OSI-906 and mTOR inhibitors in an *in vitro* model of human adrenocortical carcinoma

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4

ABSTRACT

The IGF and mTOR-pathways are considered as potential targets for therapy in patients with adrenocortical carcinoma (ACC).

This study aims to describe the IGF pathway in ACC and to explore the *in vitro* response to the combined treatment with the dual IGF1-/Insulin-Receptor inhibitor OSI-906, (linsitinib) and mTOR inhibitors (sirolimus and everolimus) in an *in vitro* model of ACC. At this purpose, the protein expression level of IGF2, IGF1-Receptor [IGF1R] and IGF2R was evaluated by immunohistochemistry in 17 human ACC samples and the mRNA expression level of *IGF1*, *IGF2*, *IGF1R*, Insulin-Receptor[*IR]A*, *IRB*, *IGF2R*, IGF-Binding-Proteins [*IGF-BP*] 1, 2, 3 and 6 was evaluated by qRT-PCR in 12 samples. In H295 and HAC15 ACC cell lines the combined effects of OSI-906 and sirolimus or everolimus on cell survival were evaluated.

A high protein expression of IGF2, IGF1R and IGF2R was observed in 14 (82%), 11 (65%) and 17 (100%) of the samples, respectively. A high relative expression of *IGF2* mRNA was found in the majority of samples. The mRNA levels of the *IRA* were higher than that of *IRB* and *IGF1R* in the majority of samples (75%). OSI-906 inhibits cell growth in the H295 and HAC15 cell lines and, combined with sirolimus or everolimus, OSI-906 showed a significant additive inhibitory effect.

In conclusion, in addition to IGF2 and IGF1R, ACC express IGF2R, IRA and several IGF-binding proteins, suggesting that the interplay between the different components of the IGF pathway in ACC could be more complex than previously considered. The addition of mTOR inhibitors to OSI-906 may have stronger antiproliferative effects than OSI-906 alone.

INTRODUCTION

Malignant tumors of the adrenal cortex, defined as adrenocortical carcinoma (ACC), are rare but aggressive cancers for which new treatment options are required.[1-3] Although most ACC are sporadic, rarely ACC develop in the context of certain genetic syndromes such as the Beckwith–Wiedemann syndrome (BWS), Li-Fraumeni syndrome and familial colorectal polyposis. The study of these syndromes has supported the potential role of some molecular pathways in ACC pathogenesis.[4] Particularly the BWS is a genetic syndrome associated with childhood ACC, other childhood tumors and a somatic overgrowth syndrome in which deregulation of imprinted genes on chromosomal locus 11p15 leads to a biallelic expression of *IGF2*.[4, 5] Although the estimated prevalence of BWS in patients with ACC is very low and restricted to childhood,[4, 6] IGF2 has been reported to be over-expressed in about 70-90% of sporadic ACCs as compared to normal adrenals or benign adrenocortical tumors.[7-16] Therefore, the insulin-like growth factor (IGF) system is considered as a promising target for new medical treatment options in ACC.[10, 13, 17] The IGF system participates in the regulation of growth, lifespan and metabolism and includes circulating ligands, exerting their effects as endocrine and/or paracrine factors [insulin, IGF1 and IGF2 (IGFs)]; binding proteins (IGF-BP1-6 that modulate the bioavailability of IGFs) and multiple receptors.[18] Among the receptors, the IGF1 receptor (IGF1R) and the insulin receptor isoforms A and B (IRA and IRB) are tyrosine-kinase receptors. The mannose 6-phosphate/insulin-like growth factor2 receptor (IGF2R) is a scavenger receptor involved in the internalization and degradation of IGF2. In adult humans, insulin predominantly exerts metabolic effects through the activation of IRB, whereas IGFs, particularly IGF1, mainly exerts growth-stimulating effects through the activation of IGF1R receptors. IRA is predominantly expressed during fetal development when it is an important mediator of pro-growth effects of insulin and IGFs. IRA expression in malignant tumor tissue has been suggested to be involved in cancer development.[18] Currently, the efficacy of several IGF1R and IGF1R/IR inhibitors is evaluated in clinical trials, alone or in combination with other agents for the treatment of several malignant disorders.[19, 20] mTOR is a protein kinase of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signalling pathway and plays a pivotal role in cell growth, metabolism and proliferation, by mediating the effects of various growth factors, including the IGFs.[21] The mTOR pathway is considered a target for antineoplastic therapy in several malignancies and it has recently been proposed as a target for ACC treatment.[22-24]

This study aims at describing the IGF pathway in ACC and to explore the *in vitro* response to the combined treatment with a dual IGF1-R/INS-R inhibitor (OSI-906) and the mTOR inhibitor (sirolimus) in an *in vitro* model of ACC using ACC cell lines and primary human ACC cell cultures.

MATERIALS AND METHODS

Subjects

Seventeen ACC and 6 normal adrenal tissue samples (NA) samples were used for this study. Fresh tissue was snap frozen within 60 minutes after surgical removal. NA samples were collected for *in vitro* studies from adrenalectomy (NA) due to renal cell carcinoma. This study was approved by the Medical Ethics Committee of the Erasmus MC and all patients gave written informed consent.

The following clinical parameters were recorded in all patients: date of diagnosis, age, gender, ENSAT stage,[25] Weiss score (assessed by an expert pathologist in adrenal disease [RRdK]),[26] mitotic count (as defined by the presence number of mitoses equal or higher than 5 in 50 high-power fields), hormonal status and type of hormonal secretion (cortisol and/or androgens and/or estrogens and/or mineralocorticoids).[27]

Total RNA isolation and quantitative RT-PCR (qRT-PCR)

From snap frozen adrenal tissues (available for 12 ACC cases and 6 NA cases), total RNA was isolated using a commercially available kit (High Pure RNA Tissue kit; Roche, Almere, The Netherlands).

Total RNA from the human ACC cell line NCI-H295R (H295) was used as a positive control.

The cDNA synthesis from total RNA and quantitative PCR were performed as previously described.[22] mRNA expression of IGF1, IGF2, IGF1-Receptor [IGF1-R], Insulin-Receptor[IR] A, IRB, IGF2R, IGF-Binding-Proteins [BP] 1, 2, 3 and 6 and of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was evaluated by RT-qPCR in human ACC tissue samples, depending on the availability of frozen tissues.

The primers and probes were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and they were previously reported (Supplementary material table 1). Samples were normalized to the expression of HPRT. PCR efficiencies (E) were calculated for the primer-probe combinations used (Supplementary material table 1).[28] The relative expression of genes was calculated using the comparative threshold method, 2-ΔCt,[29] after efficiency correction[30] of target and reference gene transcripts (HPRT).

Immunohistochemistry (IHC)

The expression of IGF2, IGF1-R and IGF2R in adrenal samples was evaluated. Paraffin embedded tissue specimens were cut in 5 µm sections, deparaffinized and dehydrated. Antigen-retrieval was performed by microwave treatment in Tris–EDTA Buffer (pH 9.0). The slides were cooled for 1 hour at +4˚C and incubated for 1 hour at room temperature (RT) with the primary monoclonal antibodies and incubated overnight at +4˚C with the primary polyclonal antibodies. The primary monoclonal antibodies to detect IGF1-R

were purchased from Novus Biologicals (NB110-87052; dilution: 1:500) and the primary polyclonal antibodies to detect IGF2 and IGF2R were purchased from R&D Systems (AF-292-NA; dilution: 1:500) and Santa Cruz Biotech (SC-25462; dilution: 1:50) respectively. The slides were washed and incubated for 30 min at RT with secondary antibodies (Poly-AP-Goat anti-Mouse/Rabbit IgG PowerVision+; ImmunoVision Technologies) at the concentration provided by the manufacturer. After washing, staining was visualized by a 30 min incubation in new fuchsin solution. Only IGF1-R staining was performed and visualized with a Dako Detection System, following a different protocol previously described.[22] All slides were counterstained with hematoxylin and coverslipped. Positive controls included cases of adrenocortical cancer and normal human pancreas with previously proven positivity at IHC for the protein evaluated. Negative controls included omission of the primary antibody and the incubation with secondary antibodies.

The staining was evaluated independently by two investigators and any discrepancy was resolved by a consensus review. The results were interpreted in a semiquantitative manner by using an intensity-proportion scoring system previously described.[31] The score was calculated by the sum of the intensity score and the proportion of the stained cells; this provided a score between 0 and 6. The proportion score was as follows: 0 = no positivity (or less than 10%); +1 = less than 1/3 tumor cell positivity; +2 = 1/3 to 2/3 tumor cell positivity; and +3 = more than 2/3 tumor cell positivity. The intensity score was as follows: +1 = weak staining; +2 = intermediate staining; +3 = strong staining. The score 0 was regarded as negative; 2-3 as low; 4-5 as intermediate and 6 as high. Finally adrenocortical tumors were dichotomously grouped as having intermediate to high expression of the evaluated protein and phospho-proteins (IHC score equal-higher than 4) or not (IHC score lower than 4).

Drugs and reagents

The dual IGF1-R/IR inhibitor OSI-906 and the mTOR inhibitors sirolimus and everolimus were purchased from LC Laboratories (Inc. Woburn, MA, USA) and prepared as a 10^{-3}M stock solution in dimethylsulfoxide (DMSO). Compounds were stored at -20°C and further diluted in 40% DMSO before the use. Final DMSO concentration, also added as vehicle to controls, was 0.4%.

Cell lines and culture conditions

The human ACC cell lines H295 and HAC15 were obtained from the American Type Culture Collection (Manassas, VA) and from Dr. W. Rainey (as gift), respectively.[32] The cells were cultured as previously described in detail.[22]

Measurement of total DNA content assay

Measurement of total DNA content per well was used to determine the effects of the compounds on cell proliferation. Cells were plated in 1 ml of medium in 24-well plates at the density required to obtain a 65-70% cell confluence in the control groups at the end of the experiment. The experiments were performed using medium containing high (5% FCS) or low serum (1% FCS). Twenty-four hours later compounds were added to wells in quadruplicate, medium was refreshed at day 3 and fresh compounds were added again. After 3 or 6 days of treatment with the selected compounds, cells were harvested for DNA measurement, as a measure of cell number. All controls were vehicle treated. Measurement of total DNA content was previously described in detail.[33]

Apoptosis assay

Apoptosis has been studied using two methods: "DNA fragmentation assay" and "MuseTM Annexin V & Dead Cell Kit".

DNA fragmentation assay. The cells were plated in 24-well plates and treated as above described for the cell proliferation assay. After 24 hours compounds were or vehicle were added and after 3 days of incubation, DNA fragmentation was determined using a commercially available ELISA kit (Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used. The same plates were also analyzed for the measurement of total DNA content. The amount of DNA-fragmentation (apoptosis) was corrected for the total DNA content in each well.

Muse™ Annexin V & Dead Cell Kit (Millipore, Germany). Cells were plated in 12-well plates at the density necessary to obtain a 65-70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later sirolimus was added to wells in duplicate. Control groups were vehicle-treated. After seventy-two hours of treatment, cells were harvested by gentle trypsinization and processed for staining according to the protocol provided by the supplier of the assay. The experiments were repeated twice.

Cell cycle assay

The effects of compounds on cell cycle progression were evaluated using the "MuseTM Cell Cycle Assay" (Millipore, Germany). Cells were plated in.12-well plates at the density necessary to obtain a 65-70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later sirolimus was added to wells in duplicate. Control groups were vehicle-treated. After seventy-two hours of treatment, cells were harvested by gentle trypsinization and processed for fixation and staining according to the protocol provided by the supplier of the assay. The experiments were repeated twice.

Statistical analysis

All the experiments were carried out at least three times, with the exception of the apoptosis assays and cell cycle assay that were performed twice. The repeated experiments gave comparable results. For the statistical analysis statistical software of SPSS (SPSS 15.0; SPSS Inc., Chicago,IL) and GraphPad Prism 5.0 (GraphPhad Software, San Diego, CA) was used. The Spearman's rank coefficient (rho) was used to test correlation.

We used non-parametric tests to evaluate the differences among groups (Mann-Whitney test and Kruskall-Wallis). The comparative statistical evaluations among treatment groups were performed by ANOVA, followed by a multiple comparison test (Newman-Keuls).

RESULTS

Study population

This study included samples from seventeen patients with ACC (main clinical characteristics reported in table 1). Only two of the included ACC patients were children in which the presence of a genetic cause was not known (case 6 and 8; 9.5 and 4.2 years old respectively).

To describe the IGF pathway, the protein expression levels of IGF2, IGF1-R and IGF2R were evaluated by IHC in the ACC samples. In twelve of these samples, the mRNA expression levels of *IGF1*, *IGF2*, *IGF1-R*, *IRA*, *IRB*, *IGF2R*, *IGF-BP* 1, 2, 3 and 6 were evaluated by RT-qPCR.

Table 1: IGF2, IGF1R and IGF2R protein expression in 17 adrenocortical cancer samples

Patient number	Sex	Weiss	Hormonal secretion	IGFII protein expression		IGFIR protein expression		IGFIIR protein expression	
				Score	Considerable expression	Score	Considerable expression	Score	Considerable expression
1	F	3	C	4	yes	5	yes	4	yes
2	F	5	C and A	6	yes	4	yes	5	yes
3	F	9	A	6	yes	3	no	4	yes
4	M	6	C and A	3	no	6	yes	5	yes
5	F	6	A	5	yes	5	yes	5	yes
6	F	7	C and A	6	yes	6	yes	5	yes
7	M	8	none	4	yes	5	yes	6	yes
8	F	4	A	6	yes	3	no	5	yes
9	F	7	none	4	yes	3	no	5	yes
10	F	3	none	3	no	4	yes	5	yes
11	F	7	C and A	6	yes	4	yes	5	yes
12	F	5	A	5	yes	6	yes	6	yes
13	F	7	none	4	yes	3	no	4	yes
14	F	8	none	6	yes	3	no	5	yes
15	M	4	A	6	yes	4	yes	6	yes
16	F	6	none	6	yes	2	no	5	yes
17	M	6	C	3	no	5	yes	6	yes
		Median 6; Range 3-9		***Median 5; Range 3-6***	***Frequency 14/17 (82%)***	***Median 4; Range 2-6***	***Frequency 11/17 (65%)***	***Median 5; Range 4-5***	***Frequency 17/17 (100%)***

mRNA expression of the components of the IGF pathway in human ACC and NA samples

The mRNA expression of several components of the IGF pathway was evaluated by RT-qPCR in 12 ACC samples and in 6 NA samples. As shown in Figure 1, the expression levels of most of these IGF pathway components is quite variable in the different samples evaluated, although a high relative expression of *IGF2* was found in the majority of samples observed (mean 66,8±106,4; median levels 24.82; range 0.01-289.68). As com-

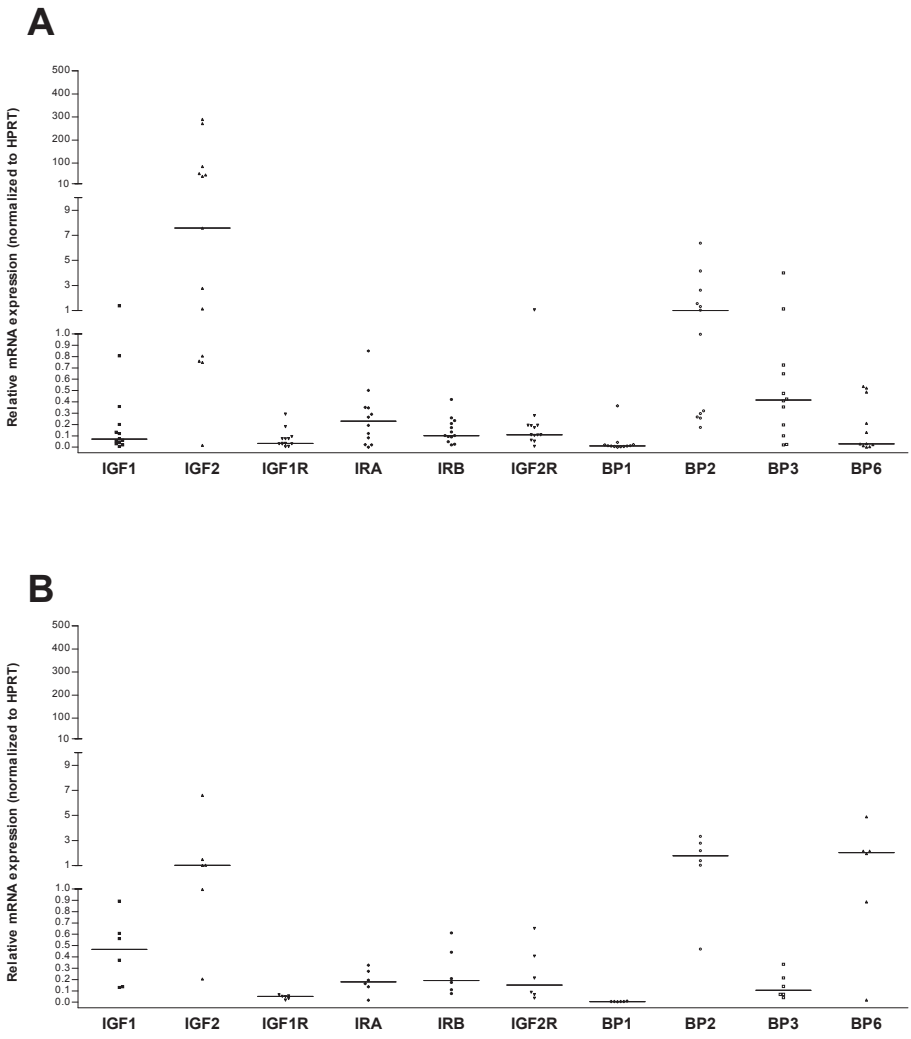

Figure 1. mRNA expression levels of the main components of the IGFs and mTOR pathways (expressed as relative mRNA expression as normalized to the house-keeping gene HPRT) in a series of 17 human ACC samples (panel A) and in a series of 6 normal adrenals (panel B).

pared with other receptors evaluated, the receptor expressed at highest levels within tumors was *IRA* in 7 of 12 samples (58.3%); *IGF2R* in 3 (25%); *IGF1R* in one (8.3%) and IRB in the remaining one (8.3%). Considering only the tyrosine-kinase receptors, *IRA* was the receptor expressed at highest levels in the majority of samples (83%). Mean levels of *IRA* were significantly higher than mean levels of *IGF1R* (0.25±0.26 *vs* 0.07±0.09; $p<0.05$). In all the evaluated samples, excepted for three cases, *IRA/IRB* ratio was higher than 1 (2.19±1.59). As compared with other IGFBPs evaluated, the *IGFBP* expressed at highest levels within tumors was *IGFBP2* in 7 of 12 samples (58.3%); *IGFBP3* in 4 (33.3%) and *IGFBP6* in only one case (8.3%). Mean levels of *IGFBP2* were significantly higher than the mean level of *IGFBP1* and *IGFBP6* (1.16±1.9 *vs* 0.04±0.1; $p<0.01$ and *vs* 0.18±0.07; $p<0.05$, respectively). A negative correlation was found between *IGF2* and *IGFBP6* (rho: -0.8; $p<0.003$), whereas a positive correlation was found between *IGF1R* and *IGF2R* (rho: 0.7; $p<0.009$); I*GF1R* and *IRB* (rho: 0.8; $p<0.003$); *IGF1R* and *IGFBP1* (rho: 0.8; $p<0.001$) and *IRA* and *IGFBP2* (rho: 0.8; $p<0.003$). No relationship was observed between the mRNA levels of the IGF components and any clinical parameters evaluated including hormone production, Weiss score, mitotic index and TNM.

Mean levels of IGF1 and IGFBP6 were significantly lower in ACC as compared with NA (0.3±0.4 vs 0.5±0.3; $p<0.05$ and 0.2±0.2 vs 2.0±1.6 $p<0.01$, respectively). Mean levels of IGF2 were considerably higher in ACC as compared with NA (66.8±103.6 vs 1.9±2.3; $p<0.05$), but this difference did not reach statistical significance, probably as a consequence of the small sample size and the high variation of IGF2 levels within the tumor samples. Comparing the expression of the evaluated components of the IGF pathway in the 12 ACC evaluated to the median value of each component in the NA, we observed an over expression of: IRA in 7 cases (58%); IRB in 4 (33,3%); IGF1 in 2 (17%); IGF2 in 9 (75%); IGF1R in 5 (41.6%); IGF2R in 5 (41.6%); IGFBP1 in 10 (83.3%); IGFBP2 in 3 (25%); IGFBP3 in 9 (75%) and IGFBP6 in none. In addition, 9 ACC samples showed an IRA-IRB ratio higher than the median value observed in normal adrenals.

Protein expression of the components of the IGF pathway in human ACC samples

The protein expression of IGF2, IGF1R and IGF2R was evaluated by IHC in 17 human ACC. Table 1 summarizes the results of the IHC and the main clinical features of the evaluated patients. An intermediate to high staining for IGF2 (82%; median score 5; range 3-6) and IGF1R (65%; median score 4; range 2-6) was observed in most tumor tissues and for IGF2R (median score 5; range 4-6) in all ACC. No correlations were observed between the expression of the different proteins that were evaluated and between these proteins and the main clinical-pathological characteristics of the corresponding patients. No correlations were observed between the protein and mRNA expression of IGF2, IGF1R and IGF2R although a trend to positive correlation was found between IGF2 protein and mRNA expression. Figure 2 shows an exemplary case of immunostaining in ACC.

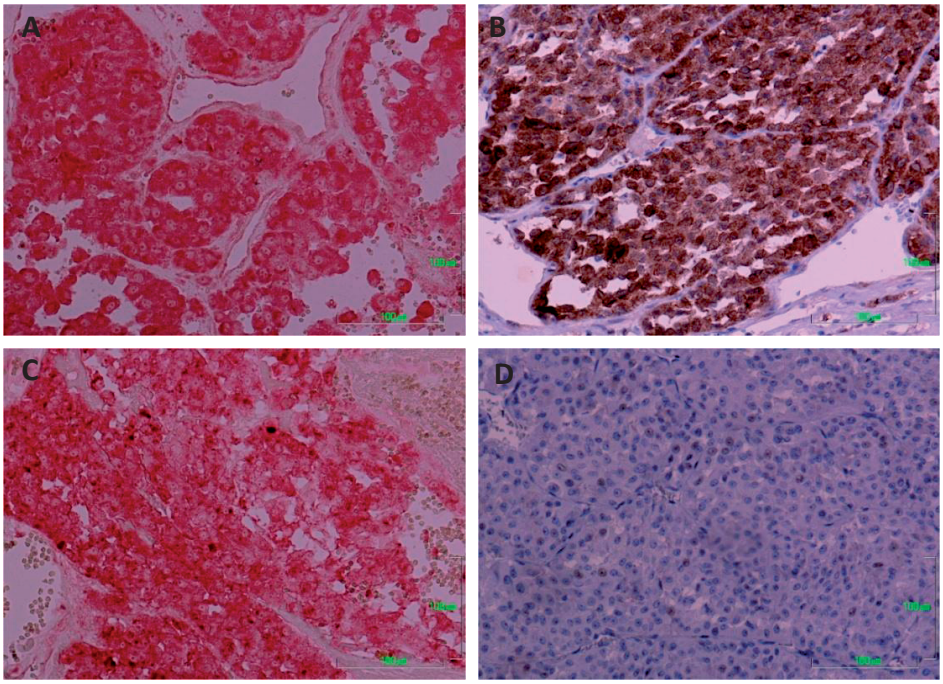

Figure 2. Immunohistochemical detection of IGF2 (A), IGF1R (B) and IGF2R (C) in a case of human adrenocortical carcinoma. Pictures "D" shows the absence of staining in the negative control for panel B. Magnification, X200.

Effects of dual IGF1-R/INS-R inhibitor in human adrenocortical cell lines

In both H295 and HAC15 cell lines OSI-906 inhibited cell proliferation in a dose- and time-dependent manner (figure 3A and 3D). OSI-906 was slightly, but significantly, more potent in inhibiting cell proliferation in HAC15 compared to H295. HAC15 cells were slightly more sensitive to OSI906 than H295 cells. After 6 days of treatment in full medium the IC_{50} of OSI-906 in H295 was $1.5x10^{-7}$M and in HAC15 $2.9x10^{-8}$M ($p<0.01$). The maximal inhibition observed In H295 and HAC15 was 90% and 95%, respectively (not statistically significant; p=0.3). In both H295 and HAC15 cells the potency of OSI-906 and the maximal inhibition observed were similar in cell cultured in medium with high serum compared with cells cultured in medium with low serum (figure 3B and 3E). At the condition tested, OSI-906 induced DNA-fragmentation in a dose-dependent manner in both H295 and HAC15 (figure 3C and 3F).

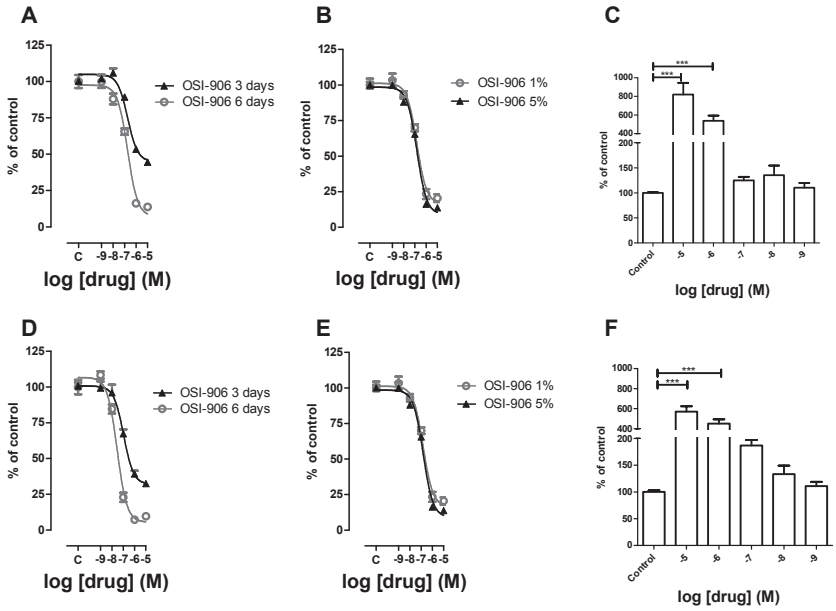

Figure 3. Dose and time-dependent effect of OSI-906 treatment on H295 (A-B) and HAC15 (D-E) cell proliferation, expressed as DNA content/well after 3 days and 6 days (panels A and D) and after 6 days in medium with high or low serum (panels B and E). Dose-dependent effects of 3-day treatment with OSI-906 on apoptosis of H295 (C) and HAC15 (F) cells, expressed as DNA fragmentation (normalized to the DNA content of each well). Data are expressed as the percentage of control and represent the mean± SEM. Control is set as 100%. *** $p<0.001$.

Effects of the dual IGF1R/INS-R inhibitor OSI-906 in combination with mTOR inhibitors on human ACC cells

Sirolumus and everolimus inhibited cell proliferation in H295 and HAC15 cells in a dose-dependent manner in both experimental conditions tested (high *vs* low serum concentration medium) data not shown. Sirolimus was slightly, but not significantly, more potent than everolimus. The potency of both compounds was similar in medium containing either high or low serum concentration. Selected doses of sirolimus or everolimus combined with OSI-906 $5x10^{-8}$M had statistically significant additive effect on cell proliferation (figure 4). Particularly both concentrations used of sirolimus and everolimus showed additivity with OSI-906 in inhibiting H295 and HAC cell proliferation when tested in medium containing low serum concentration (figure 4B, 4D, 4F and 4H). Only the highest concentrations used of mTOR inhibitors (10^{-6}M) showed some additivity with OSI-906 in inhibiting H295 and HAC cell proliferation when tested in medium containing high serum concentration (figure 4A, 4C, 4E and 4G).

At the condition tested only the highest concentrations used of sirolimus (10^{-6}M) showed significant additivity with OSI-906 in increasing annexin V, used as measure of

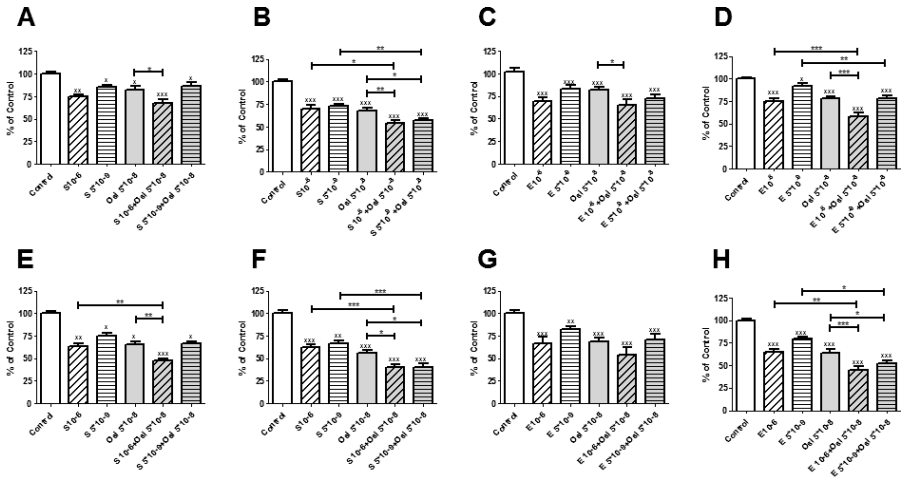

Figure 4. Effect of OSI-906 (Osi), alone or in combination with the mTOR inhibitors sirolimus (S) or everolimus (E), on H295 (A-D) and HAC15 (E- H) cell proliferation. Results are expressed as DNA content/well. Two different conditions have been tested: medium with high (panels: A, C, E and G) or low serum (panels: B, D, F and H). Data are expressed as the percentage of control and represent the mean± SEM. Control is set as 100%. *p<0.05; **p<0.01; *** p<0.001; ^{x} p<0.05 *vs* control; ^{xx} p<0.01 vs control; ^{xxx} p<0.001 *vs* control.

apoptosis, in H295 (figure 5A). Everolimus did not show a statistically significant additivity in increasing annexin V in H295 (figure 5B).

OSI-906 (10^{-7}M) alone or in combination with sirolimus 10^{-6}M or $5x10^{-9}$M significantly increased the proportion of cells in G_0/G_1 phase of the cell cycle (p<0.05, p<0.05 and p<0.01 respectively). OSI-906 in combination with sirolimus 10^{-6}M or $5x10^{-9}$M significantly reduced the proportion of G_2/M (p<0.05) (figure 5C). Additionally the combined treatment with OSI-906 and sirolimus showed a trend to have additive effects in inducing G1-cell cycle block. Statistically significant additive effects in increasing the proportion of cells in G_0/G_1 phase (p<0.05) and reducing the proportion of cells in G_2/M phase (p<0.05) were observed when combining OSI-906 10^{-7}M and sirolimus $5x10^{-9}$M as compared with sirolimus alone (figure 5C).

OSI-906 (10^{-7}M) alone or in combination with everolimus 10^{-6}M or $5x10^{-9}$M significantly increased the proportion of cells in G_0/G_1 phase of the cell cycle (p<0.01). OSI-906 alone or in combination with everolimus 10^{-6}M or $5x10^{-9}$M significantly reduced the proportion of cells in G_2/M phase (p<0.01) (figure 5D). Additionally, the combined treatment with OSI-906 and everolimus showed significant additive effects in inducing G1-cell cycle block. Particularly, when combining OSI-906 10^{-7}M and everolimus 10^{-6}M or $5x10^{-9}$M, statistically significant additive effects in increasing the proportion of cells in G_0/G_1 phase (p<0.05 and p<0.01, respectively) and in reducing the proportion of G_2/M (p<0.05) as compared with everolimus alone were observed (figure 5D).

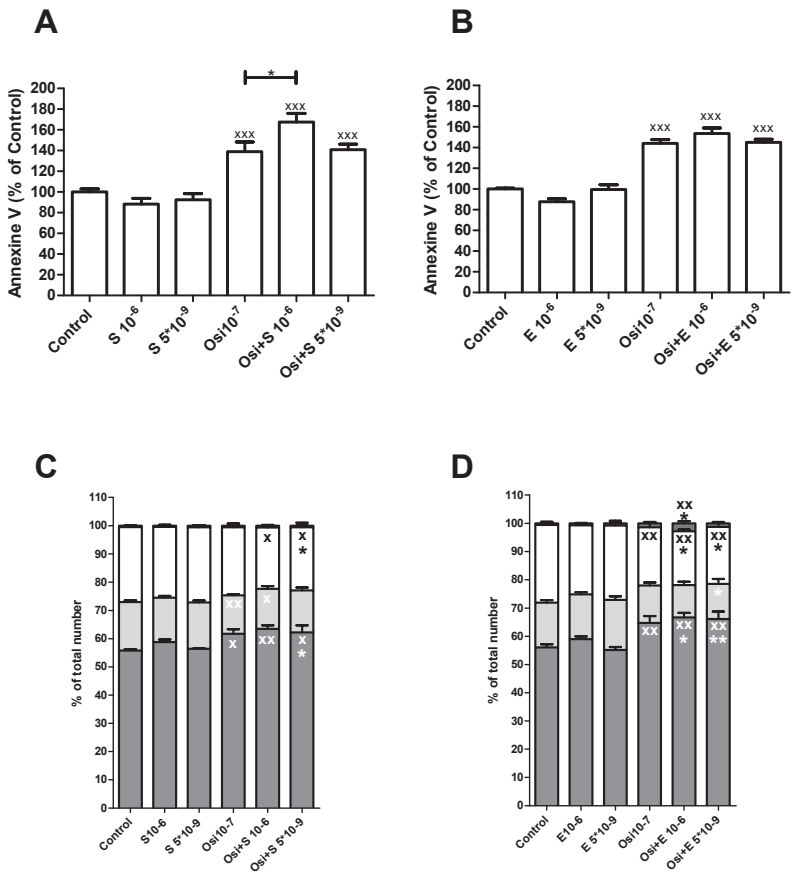

Figure 5. Panels A and B: Effect of OSI-906 (Osi), alone or in combination with the mTOR inhibitors sirolimus [(S); panel A] or everolimus [(E); panel B], on Annexin V as a measure of induction of apoptosis in H295 cell line. Data are expressed as the percentage of control and represent the mean± SEM. Control is set as 100%. *p<0.05 vs Osi alone; xxx p<0.001 vs control. Panels C and D: Effect of OSI-906 (Osi), alone or in combination with the mTOR inhibitors sirolimus (panel C) or everolimus (panel D), on cell cycle in H295 cell line. Data are expressed as mean± SEM. *p<0.05 and **p<0.01 *vs* S (panel C) or E (panel D) alone; ^{x} p<0.05 and ^{xx} p<0.01 *vs* control.

DISCUSSION

The results of this study show that the majority of ACC express IGF2, IGF1R and IGF2R mRNA and protein and demonstrate IRA mRNA expression in these tumors, suggesting that factors such as IGF2R and IRA, not well described before, could interact with IGF2, potentially modulating the role of IGF2 in adrenocortical tumorigenesis. Mean levels of IGF1 and IGFBP6 were significantly lower in ACC as compared with NA. Additionally, this study demonstrates that treatment of human ACC cells with OSI-906, a dual IGF1R/

IR inhibitor, reduces cell proliferation and that combined treatment with OSI-906 and mTOR inhibitors can have additive antiproliferative effects.

A high mRNA and protein expression of IGF2 is found in most evaluated samples, in agreement with the already well known IGF2 overexpression in 70-90% of ACC.[7-16] IGF1R protein expression was demonstrated in all the evaluated ACC samples and an intermediate to high staining was observed in more than 50% of cases. These data are in agreement with previous studies describing IGF1R expression in most ACC.[10, 13, 34] The protein expression of IR in most ACCs has been previously described as well,[34] however, to the best of our knowledge, the differential expression of IRA and IRB isoforms of the IR in ACCs, has never been explored. Unfortunately, to the best of our knowledge, there are currently no antibodies available to distinguish between the IR isoforms. We could therefore only evaluate IR isoform expression at mRNA level. While IRB is considered as the main mediator of metabolic effects of insulin and IGFs in adult tissue, IRA is an isoform of the IR, predominantly expressed during fetal development and is considered as an important mediator of growth-promoting effects of insulin and IGFs.[18] IRA has a higher affinity for IGF2 compared with the IGF1R and its expression in malignant tumor tissue has been suggested to be involved in cancer development.[18] To our knowledge this is the first study demonstrating the presence of IRA in ACCs and showing that in these cancers, IRA is often expressed at higher level compared with IGF1R and IRB. The expression of IGF2 and the IGF1R has suggested a potential role of an IGF2-IGF1R autocrine loop in adrenocortical tumorigenesis.[35] The current study suggests a role of IRA as potential additional mediator of the IGF2 effects in ACC. However, in addition to the tyrosine kinase receptors involved in the IGF pathway, also IGF2R and IGFBPs could play a role in modulating the IGF effects. The IGF2R serves a function in the degradation of IGF2, intracellular trafficking of lysosomal enzymes and activation of transforming growth factor beta. Down-regulation of IGF2R has been found in some type of cancers and it has been suggested that IGF2R could play a role as a tumor suppressor gene in some malignancies.[36, 37] Loss of heterozygosis at the locus of IGF2R gene has been reported to be a frequent event in ACC, supporting a potential role of IGF2R as a tumor suppressor gene also in ACC development.[38] However, a low protein expression of IGF2R in ACC has never been described. Conversely, the current study demonstrates the presence of a high IGF2R protein expression in most ACCs, suggesting that a high level of IGF2R protein might counteract the growth-stimulating effects of IGF2 in adrenocortical tumorigenesis. In line with previously published data, in the current study a variable expression of IGFBPs was found in ACCs.[9, 39] Several correlations between the different components of the IGF pathway were found suggesting the existence of common mechanisms of regulation. However no correlations with clinical-pathological parameters were found. This lack of correlation might be related to the small sample size and to the complexity of the IGF pathway. Among the IGFBPs evaluated, the IGFBP expressed

at higher levels was IGFBP2, whereas IGFBP6 was expressed at lowest level. Additionally the expression of IGFBP6 was significantly lower in ACC than in normal adrenals. Therefore, whether high IGFBP2 and/or low IGFBP6 could play a role in the regulation of IGF pathway in adrenocortical tumorigenesis deserves further investigation.

The IGF pathway is considered as one of the most promising targets for a novel medical treatment modality in patients with ACC.[10, 13, 23, 40] In preclinical models of ACC, two types of drugs targeting the IGF1R, i.e. NVP-AEW541, a selective IGF1R kinase inhibitor and IMC-12, an IGF1R antibody, have been reported to have antiproliferative effects,[10, 13] thus encouraging the development of clinical trials in ACC patients using drugs targeting the IGF pathway. The current study confirms that OSI-906 (linsitinib), an IGF1R/IR inhibitor, inhibits the proliferation of the human ACC cell lines H295 and HAC15 *in vitro* already at a concentration lower than the concentrations reached *in vivo* in humans (about $5x10^{-6}$M). OSI-906 has been recently tested in ACC patients in a phase III clinical trial (NCT00924989). The results of this study have been presented at ASCO meeting in 2014[41] showing that a small subgroup of patients seems to benefit from treatment with this drug, although improvements in overall or progression-free survival were not observed. These apparently controversial results between preclinical and clinical studies, could be explained in several ways. First, it might indicate that our preclinical models are not representative enough for the population of patients with ACC, because these tumors are heterogeneous. The role of the IGF pathway as a potential target for treatment in ACC might have been overestimated, as suggested by the fact that up-to-date *in vivo* experiments demonstrated that isolated IGF2 overexpression has no oncogenic potential.[42] Since disappointing results emerged in clinical trials adopting different types of drugs targeting the IGF pathway in different types of malignancies, despite apparently promising preclinical data,[43] it could be hypothesized that current strategies adopted to target this pathway might still be inadequate. Indeed, biomarkers that can predict tumor response to IGF-targeting drugs, that might drive the selection of patient candidates to these drugs, have not been identified yet. Additionally, the complexity of the system could have been underestimated (such as the expression of potential regulators of the IGF pathway, as IGF2R in ACC) and the existence of interfering factors may not have been characterized yet. For example, in case of ACC patients the potential pharmacokinetic interactions between mitotane and drugs acting on the IGF pathway should be better investigated. Mitotane is strong a inducer of CYP3A4 and was shown to decrease bioavailability of sunitinib in patients with ACC.[44] Finally, targeting only the IGF pathway might not be sufficient to suppress cell growth because other pathways, that in part also interact with the IGF pathway (e.g. the mTOR pathway) are still activated. As such, before to finally declare a "game over"[42] for the role of IGF2 in adrenocortical tumorigenesis and as potential target for novel treatment in ACC patients, it could probably be useful to return to the bench and try to better explore the IGF pathway in its

whole complexity. In line with this, the results of the current study point out that ACC express components of the IGF pathway, such as IRA and IGF2R, that have not been considered before.

In a previous study from our group it was demonstrated that mTOR inhibitors inhibit cell proliferation in H295 and SW13 human ACC cell lines, but in H295, probably as consequence of the IGF2 overexpression, this treatment could activate two potential pathways of escape to treatment with traditional mTOR inhibitors, i.e. the AKT and ERK pathways.[45] These data provide the rational for experiments combining mTOR inhibitors and drugs targeting the IGF pathway in ACC. In the current study the effects of OSI-906 in combination with mTOR inhibitors were evaluated and the results of these experiments demonstrated that these compounds can have additive antiproliferative effects in some of the tested conditions. Particularly additive antiproliferative effects were more pronounced when the experiments were performed using medium with low serum, suggesting that cell environment and the presence of growth factors different from IGF2 could influence the effects of these combination of compounds. These results are in line with a recently published phase I study demonstrating that a subgroup (about 40%) of ACC patients treated with cixutumumab (IGF1R inhibitor) and temsirolimus experienced a long term disease stabilization.[46] These results suggest that treatment strategies combining mTOR inhibitors and OSI-906 warrant further investigation, although considering the heterogeneous expression of the main components of the IGF pathway in the different ACC samples, the apparently modest antiproliferative effects observed at a low concentration of these compounds as well as the potential limits of the used human cell lines as model of human ACC, caution is recommended before moving from the bench to the bedside.

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SUPPLEMENTARY MATERIAL

Supplementary table 1

Oligo Name	Forward	Reverse	Probe
IGF1R	CCAAAACTGAAGCCGAGAAG	GGGTCGGTGATGTTGTAGGT	AAGCAGGAACACCACGGCCG
IGF2R	ACCGACCCCTCCACGC	CCTCCAAGGCCACCTTCAG	AGCAGTACGACCTCTCCAGTCTGGCAAA
IGF1	TTGTGATTTCTTGAAGGTGAAGATG	CGTGGCAGAGCTGGTGAAG	TACCTGGCGCTGTGCCTGCTCA
IGF2	CCAAGTCCGAGAGGGACGT	TTGGAAGAACTTGGCCACG	ACCGTGCTTCCGGACAACTTCCC
IRA	CGTTTGAGGATTACCTGCACAA	GCCAAGGGACCTGCGTTT	TGGTTTTCGTCCCCAGGCCATC
IRB	CCCAGAAAAACCTCTTCAGGC	GGACCTGCGTTTCCGAGA	CTGGTGCCGAGGACCCTAGGCC
mTOR	TGCTGCGTGTCTTCATGCAT	GGATTGCAGCCAGTAACTTGATAG	ACAGCCCAGGCCGCATTGTC
p70S6K	TGGAAGACACTGCCTGCTTTT	TGATCCCCTTTTGATGTAAATGC	CTTGGCAGAAATCTCCATGGCTTTGG
4EBP1	GGCGGCACGCTCTTCA	TCAGGAATTTCCGGTCATAGATG	ACCACCCCGGGAGGTACCAGGA
HPRT	TGCTTTCCTTGGTCAGGCAGTAT	AAATCCAACAAAGTCTGGCTTATATC	CAAGCTTGCGACCTTGACCATCTTTGGA

Combined *in vitro* effects of sirolimus and mitotane in the inhibition of growth in human adrenocortical carcinoma cells

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ABSTRACT

Adrenocortical cancer (ACC) is a rare cancer with poor prognosis and scant treatment options. Mitotane alone, or in combination with cytotoxic chemotherapy, represents the current referral treatment for patients with unresectable ACC. Recent studies have shown that mTOR inhibitors suppress growth of ACC cells. The current study aimed at evaluating the effects of mitotane in combination with the mTOR inhibitor sirolimus.

In human H295 and SW13 ACC cells the effects of a 6-day treatment with increasing doses of mitotane in the presence or absence of selected doses of sirolimus has been tested on cell proliferation, as measured by the total DNA content. The tested doses of mitotane ranged between 10^{-7} and 10^{-5}M in both cell lines, whereas those of sirolimus were $5x10^{-9}$ and 10^{-6}M in H295 and $5x10^{-11}$M and 10^{-10} M in SW13.

In H295, mitotane significantly inhibited cell proliferation at all concentrations tested, with an IC_{50} of $4.5x10^{-6}$M and a maximal inhibition of 87% as compared with vehicle-treated controls ($p<0.001$). In SW13, mitotane significantly inhibited cell proliferation at concentrations higher than $2.5x10^{-6}$M, with an IC_{50} of $1.6x10^{-5}$M and a maximal inhibition of 81% as compared with vehicle-treated controls ($p<0.001$). In both H295 and SW13 sirolimus significantly inhibited cell proliferation at both concentrations tested and when combined with mitotane, it showed significant additive effects. This additivity was observed only with low mitotane doses (between 10^{-7} and $5x10^{-6}$M). Using mitotane doses higher than $5x10^{-6}$M the cell proliferation inhibition was already nearly maximal and no significant additive effects could be observed.

The current study demonstrates that sirolimus has additive antiproliferative effects when combined with low doses of mitotane. These doses correspond to concentrations lower than the therapeutic range of mitotane. If this effect can also be achieved *in vivo*, our data suggest that the addition of sirolimus to mitotane might be useful in ACC patients when the therapeutic range of mitotane range is not reached.

INTRODUCTION

ACC is a rare but aggressive solid cancer with and incidence of about 1-2 new cases/million/year and a 5-year survival rate of below 15% at the metastatic stage.[1, 2] Surgery remains the only curative treatment in patients diagnosed at an early stage.[1-3]

Mitotane, is currently the only drug approved in Europe and in the United States for the treatment of advanced ACC.[4] As monochemotherapy, a response rate of mitotane between 13 and 35% has been reported.[1, 4] However, in patients achieving plasma mitotane level above 14 mg/L a higher response rate (up to 66%) and an improved survival have been reported.[1, 3, 4] In combination with chemotherapy response rates of mitotane range between 14 and 55%.,[1, 3, 4, 5] No novel treatment option has emerged in the last four decades,[1, 3-5] underling the urgent need of new therapeutic options for patients affected by this malignancy.

The mTOR pathway is considered a target for antineoplastic therapy in several malignancies and it has recently been proposed as target for ACC treatment.[6-8] In preclinical models of ACC, mTOR inhibitors such as sirolimus, temsirolimus and everolimus inhibit cell proliferation in a dose- and time-dependent manner.[6-8] However, the human ACC cell line SW13 was found to be more sensitive to mTOR inhibition than the H295 cell line, which is considered as the most representative model of ACC.[9] These preclinical data, together with the expected heterogeneity of ACC, suggest that caution is required before using this class of drugs in unselected ACC patients[9, 10] and caution is also supported by preliminary clinical experience with the use of everolimus in some ACC patients with a late stage of disease.[11] Unfortunately, due to the absence of a clear predictor of the effectiveness of mTOR inhibitors in this malignancy, it is difficult to define selection criteria for patients, who are candidates for this class of drugs.[12] Therefore, combination of mTOR inhibitors with different drugs, potentially active in ACC, could be a more prudent clinical approach than the use of these inhibitors as monotherapy in unselected ACC patients. Among the possible combination treatment options, there is a rationale to use mTOR inhibitors in association with drugs targeting the IGF pathway.[9] This combination treatment has recently been evaluated in an early-phase clinical trial suggesting a potential clinical effect in a subset of ACC patients that experienced prolonged disease stabilizations.[13] To our knowledge, there are no studies evaluating the effects of mTOR inhibitors combined with mitotane in ACC. Therefore, this study aimed at evaluating the effects of mitotane in combination with sirolimus in two ACC cell lines the H295 and the SW13.

MATERIALS AND METHODS

Cell lines and culture conditions

Two human ACC cell lines the NCI-H295R (H295) and the SW13, were obtained from the American Type Culture Collection (Manassas, VA) and from ECACC (Salisbury, Wiltshire, UK), respectively.[14]

The cells were cultured in 75-cm^2 culture flasks at 37°C in a humidified incubator at 5% CO_2. For all cell lines, the culture medium consisted of DMEM/F12K medium, supplemented with 5% fetal calf serum (FCS), penicillin ($1x10^5$ U/liter), and l-glutamine (2 mmol/liter). Cells were harvested with trypsin (0.05%)-EDTA (0.53 mM) solution and resuspended in culture medium. Cell viability always exceeded 95%. Media and supplements were obtained from Invitrogen (Breda, The Netherlands).

Drugs and reagents

Mitotane was purchased from Sigma-Aldrich and dissolved in ethanol as a concentrated (10^{-2}M) stock solution (stored at -20°C) and diluted in ethanol prior to use. The mTOR inhibitor sirolimus (rapamycin) was purchased from LC Laboratories (Inc. Woburn, MA, USA) and dissolved in dimethylsulfoxide (DMSO) as a concentrated (10^{-3}M) stock solution (stored at -20°C) and diluted in 40% DMSO prior to use.

Measurement of total DNA content.

Cells were plated in 1 ml of culture medium in 24-well plates at the density required to obtain a 65-70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later mitotane and/or sirolimus were added to the wells in triplicate, medium and compounds were refreshed after three days of treatment and controls were vehicle-treated. The tested doses of mitotane ranged between 10^{-7} and 10^{-5}M in both cell lines, sirolimus was tested at concentrations of $5x10^{-9}$ and 10^{-6}M in H295 and $5x10^{-11}$M and 10^{-10}M in SW13. The different doses of sirolimus used were selected on the bases of the previously reported dose-response curves of sirolimus in the used cell lines.[9] After 6 days of treatment, the cells were harvested for DNA measurement, as a measure of cell number. Measurement of total DNA content was previously described in detail.[15]

Statistical analysis

All the experiments were carried out at least three times. The repeated experiments gave comparable results. For the statistical analysis statistical software of GraphPad Prism (GraphPhad Software, San Diego, CA) was used. The comparative statistical evaluations among treatment groups were performed by ANOVA, followed by a multiple comparative test (Newman-Keuls). The half maximal inhibitory concentration (IC_{50}) was calculated

assuming that log(inhibitor) vs. response curves follow a symmetrical sigmoidal shape with a standard slope of of -1.0. Results are expressed as means±standard-errors.

RESULTS

Effects of Mitotane plus sirolimus on cell growth in ACC cell lines

In both H295 and SW13, mitotane significantly inhibits cell proliferation in a dose-dependent manner as shown in Figure 1. In H295 the antiproliferative effects of mitotane were observed already at the lowest concentration tested (10^{-7}M); these effects ranged between 10% ($p<0.05$) and 87% ($p<0.001$), as compared with vehicle-treated control. In SW13 antiproliferative effects of mitotane were observed at concentrations between $2.5x10^{-6}$M and 10^{-5}M. These effects ranged between 30% ($p<0.01$) and 81% ($p<0.001$) as compared with vehicle-treated controls. Mitotane was slightly, but significantly more potent in H295 (IC_{50} $4.5x10^{-6}$M) than in SW13 ($IC_{50}1.6x10^{-5}$M) ($p<0.01$).

In both H295 and SW13, the selected concentrations of sirolimus significantly inhibited cell proliferation. In H295, sirolimus at concentrations of $5x10^{-9}$M and 10^{-6}M reduced cell proliferation by 24% ($p<0.001$) and 45% ($p<0.001$), as compared with vehicle-treated controls, respectively. In SW13 cells, sirolimus at concentrations of $5x10^{-11}$M and 10^{-10}M, reduced cell proliferation by 57% ($p<0.001$) and 70% ($p<0.001$) as compared with vehicle-treated controls, respectively.

When mitotane was used at low concentrations (between 10-7 and 5x10-6M), sirolimus had additive effects, when compared with mitotane alone. In H295, this additivity was 9-15% and 14-30% when using 5x10-9M or 10-6M of sirolimus, respectively, and the maximal additive effects were observed at the lowest mitotane concentration tested (Figure 1 A). In SW13, the additivity was 19-58% and 25-66% when using 5x10-11M or 10-10M of sirolimus, respectively, and the maximal additive effects were observed at the lowest mitotane concentration tested (Figure 1 B). Of note, mitotane alone used at concentration of 10^{-7} and 10^{-6}M, was unable to inhibit cell proliferation in this cell line, while an inhibition of cell proliferation was observed when these low concentrations of mitotane where combined with sirolimus alone, even at very low concentrations. The addition of sirolimus to mitotane did not significantly interfere with the dose-response curves of mitotane in the tested cell lines (Figure 2). In H295, as compared with sirolimus alone, mitotane at a concentration higher than 10-7M had significant additive effects when combined with both the concentrations of sirolimus tested ($p<0.01$ or $p<0.001$; Figure 1A). In SW13, as compared with sirolimus alone, mitotane had significant additive effects only when the highest concentration of mitotane (10-5M) was combined with the lowest concentration of sirolimus (5x10-11M) ($p<0.05$; Figure 1B).

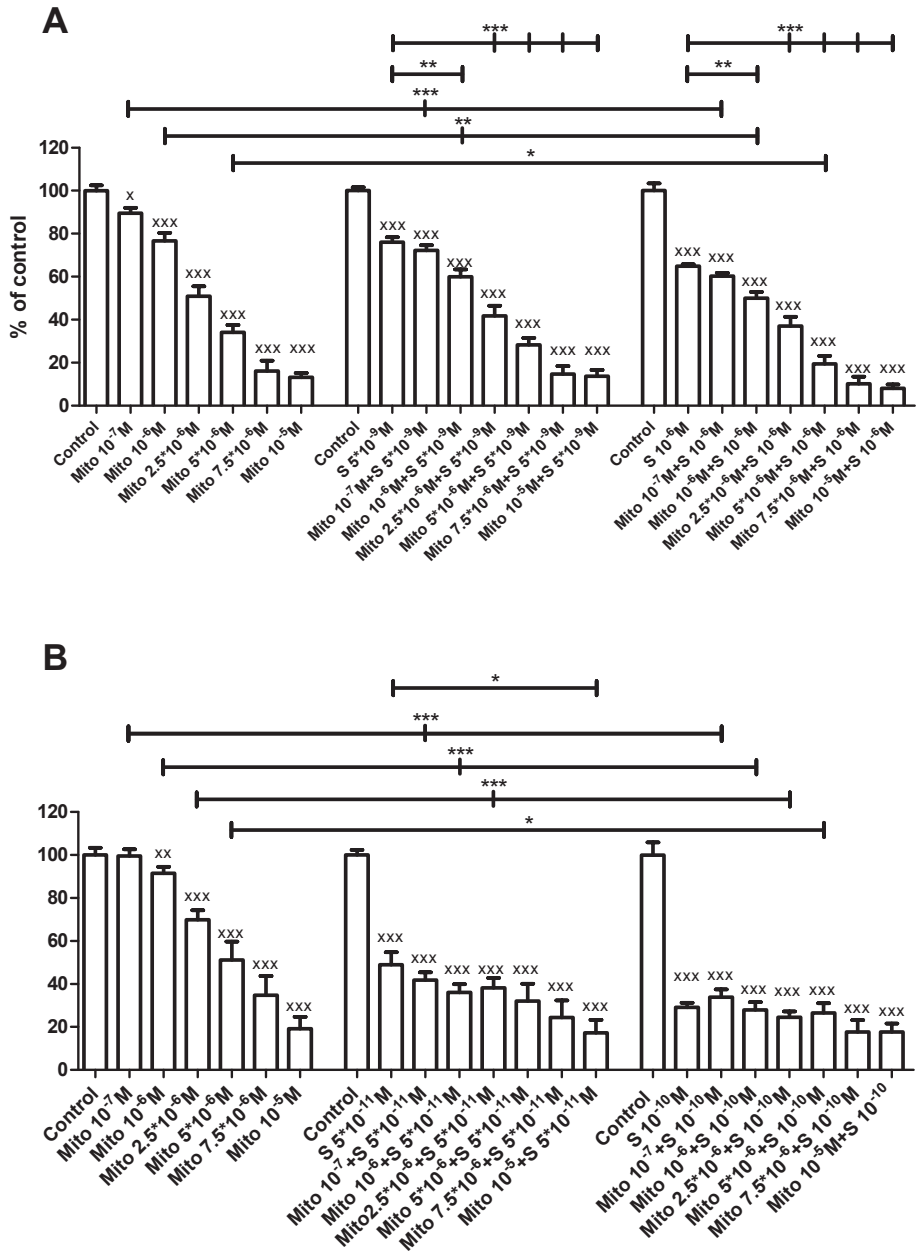

Figure 1. Combined effects of a 6-day treatment with increasing concentrations of mitotane (Mito) and selected concentrations of sirolimus (S) in two human ACC cell lines: H295 (panel A) and SW13 (panel B). At some of the combinations tested significant additive inhibitory effects on cell growth are observed in both cell lines. *p< 0.05, ** p<0.01 and ***p< 0.001; ^{x}p< 0.05, ^{xx}p< 0.01 and ^{xxx}p< 0.001 *vs* control.

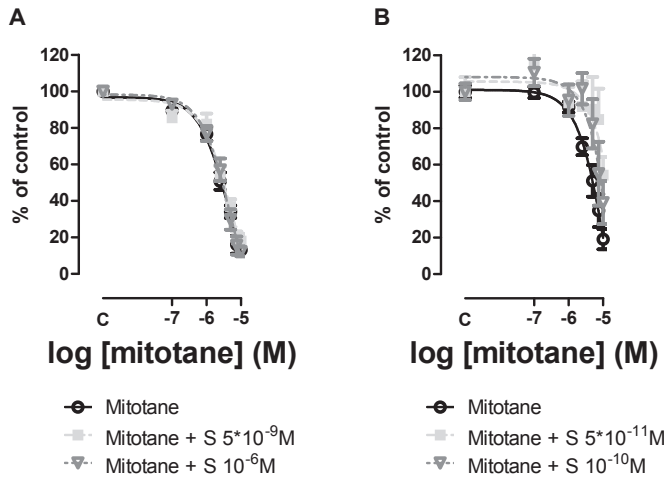

Figure 2. Dose-response curve of 6-days treatment with increasing mitotane M) concentrations, alone or in combination with two selected concentrations of sirolimus (S), in two ACC cell lines: H295 (panel A) and SW13. The addition of sirolimus to mitotane did not significantly change the dose response curve of mitotane. Data are expressed as the percentage of control and represent the mean±SD. Control is set as 100% and in the curve of mitotane alone the control is represented by vehicletreated cells, whereas in the curves of mitotane combined with sirolimus the control is represented by treated cells with sirolimus alone.

DISCUSSION

The current study demonstrates that in human ACC cell lines, sirolimus has additive antiproliferative effects when combined with low mitotane doses.

Mitotane is considered as a referral drug in the treatment of patients with advanced ACC, but unfortunately there are patients who do not respond and/or tolerate the drug, raising the requirement of new treatment options.[1, 4]

The monitoring of mitotane plasma levels during treatment is very important, because a response rate up to 66% has been reported in patients achieving mitotane plasma level above 14 mg/L, but a higher rate of adverse effects is reported when the plasma mitotane level exceeds 20 mg/L, making the therapeutic window of this drug very narrow. A rapid achievement and long-term maintenance of these therapeutic range (14-20 mg/L) has been suggested as predictor of mitotane response in patients with ACC.[1, 4, 16, 17] However, this clinical goal is sometimes difficult to be reached because mitotane has a complex pharmacokinetical profile that causes large variation in individual drug availability and in some patients the onset of adverse events can preclude a fast drug escalation or the use of a full dose of the drug. Treatment strategies combining mitotane with other drugs could increase the response rate of patients, as compared with monotherapy, for several reasons. The combination could have additive antiprolif-

erative effects potentially increasing the *in vivo* antineoplastic effects and/or potentially reducing the concentration of each drug required to obtain a desired antineoplastic effect and consequentially decreasing the risks of adverse events. On the other hand, in absence of appropriate predictors of clinical response that can help to select patients for the most appropriate treatment, the combination of two drugs, both potentially effective, but acting with different mechanisms, could increase the chance of patients to get a clinical benefit from at least at one of the two treatments. Additionally, in particular in the case of mitotane, the combined treatment with other active drugs could reduce the risk of tumor progression during the treatment phases in which mitotane plasma level is still below the therapeutic range.

Recently, mTOR inhibitors have been suggested as a new potential treatment for ACC. Preclinical data suggest sirolimus, temsirolimus and everolimus can inhibit ACC cell proliferation.[6-8] However, preliminary experience with the use of everolimus as salvage treatment in few ACC patients did not show promising results,[11] whereas combined treatment of an IGF1R antibody with temsirolimus in a phase I study including ACC patients, showed more promising results.[13] Therefore, combination treatment with mTOR inhibitors and other drugs might have higher effects than mTOR inhibitors alone. To our knowledge there are no in vitro studies evaluating the effects of mitotane in combination with mTOR inhibitors.

In the current study all the concentrations of mitotane used (from 10^{-7} to 10^{-5}M) were lower than the concentrations (4.3-6.3x10^{-5}M) corresponding to the mitotane plasma level at the therapeutic range.[18] The addition of sirolimus to these low concentrations of mitotane showed higher antiproliferative effects than mitotane alone suggesting that combined treatment might have additive effects to the antineoplastic action of mitotane, permitting to reduce the dose required to obtain desired clinical effects. This additivity was higher when the concentration of mitotane used were lower, suggesting that combined treatment might be particularly useful during the phases of treatment in which mitotane plasma level are below the therapeutic range, such as during the initial dose titration and/or for those subjects in which the therapeutic range of mitotane is hardly maintained due to bad tolerance or other reasons.

mTOR inhibitors are antineoplastic compounds largely developed for clinical use, with an acceptable safety profile.[19-22] Among the mTOR inhibitors, sirolimus can be considered as a referral compound since it is the first drug that has been discovered in this category, and because other mTOR inhibitors have been developed starting from it. Additionally, the *in vitro* effects of sirolimus in H295 and SW13 ACC cell lines cultured in similar experimental conditions had already been demonstrated, showing that H295 were significantly less sensitive than SW13 to sirolimus.[9] Based on the results of this previous report, in the current study two different concentrations of sirolimus were selected for each cell line. With the exception of 10^{-6}M sirolimus used in H295 cells,

all the tested concentrations where within the range of blood drug levels reached in humans during sirolimus treatment (maximal concentration about 10^{-7}M).[23, 24] In both cell lines, the addition of mitotane to sirolimus showed some significant additivity as compared with sirolimus alone, but only at some of the experimental conditions tested. Particularly, in the H295 cell model that is more sensitive to mitotane but less sensitive to sirolimus than the SW13 cell model, the additivity was more pronounced and observed at all concentration of mitotane tested with the exception of the lowest one. Conversely, in SW13 cells that were very sensitive to sirolimus, the addition of mitotane showed a significant additive inhibitory effect as compared to sirolimus alone, only when the highest mitotane concentration was combined with a very low sirolimus concentration. This results might be related to the potent inhibitory effects of sirolimus alone in SW13 cells, that induced already a near maximal inhibition of cell proliferation. While H295 cells are well accepted as a good model of ACC, a large debate is still open about the appropriateness of SW13 cells as a model for this type of cancer.[25] Taking in account this and the other potential limitations of ACC cell lines as preclinical model of ACC, the results of the current study might suggest that among ACC patients it could be possible to find subgroups of patients with a high sensitivity to sirolimus, in which the use of combined treatment could induce an important antineoplastic effect even in absence of mitotane effect, such as in mitotane resistant patients. If this condition would be confirmed *in vivo*, it could represent an additional reason to obtain an increased tumor respose rate by using combined treatment strategies rather than monotherapy, regardless the presence or absence of additivity. Future studies should also examine the antiproliferative and antisecretory effects of mitotane combined with sirolimus on primary cultures of human functional and non-functional ACC.

Among the potential limitations of the current study there is the lack of possibility to explore pharmacokinetic interaction between drugs. In the *in vivo* setting, mitotane is a well known inducer of microsomal liver enzyme cytochrome P450 (CYP3A4/5).[26] The induction of this enzyme can reduce the circulating concentrations of drugs metabolized by it, including mTOR inhibitors.[27] This pharmacokinetic interaction should be kept in mind when translating the *in vitro* results to the potential *in vivo* applications, and attention should be used in monitoring the blood concentrations of drugs to reach the desired therapeutic levels.

In conclusion, this study suggests a potential advantage of combining mitotane with sirolimus, but these data are still preliminary. Clearly, additional studies, including animal models, are mandatory before moving from the bench to the bedside.

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Molecular Screening for a Personalized Treatment Approach in Advanced Adrenocortical Cancer

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ABSTRACT

Context: adrenocortical cancer (ACC) is a rare cancer with poor prognosis and scant treatment options. In ACC, no personalized approach has emerged but no extensive molecular screening has been performed to date.

Objective: to evaluate the presence of a large number of potentially targetable molecular events in a large cohort of advanced ACC.

Design, setting, and participants: we used hotspot gene sequencing (Ion Torrent, 40 patients) and comparative genomic hybridization (CGH, 28 patients; a subset of the entire cohort) in adult stage III-IV ACC samples, to screen for mutations and copy number abnormalities of potential interest for therapeutic use in 46 and 130 genes respectively.

Results: At least one copy number alteration or mutation was found in 19 patients (47.5%). The most frequent mutations were detected on *TP53, ATM* and *CTNNB1* (6/40 [15%], 5/40 [12.5%] and 4/40 [10%] respectively). The most frequent copy number alterations identified were: amplification of the *CDK4* oncogene (5/28; 17.9%) and deletion of the *CDKN2A* (4/28; 14.3%) and *CDKN2B* (3/28; 10.7%) tumor suppressor genes. Amplifications of *FGFR1, FGF9* or *FRS2* were discovered in 3 subjects (10.7%). Associated alterations were: deletions of *CDKN2A, CDKN2B* with *ATM* mutations and *TP53* mutations with *CTNNB1* mutations.

Conclusions: No simple targetable molecular event emerged. Drugs targeting the cell cycle could be the most relevant new therapeutic approach for patients with advanced ACC. Inhibitors of the fibroblast growth factor receptor pathway could also be a therapeutic option in a subset of patients, while other targeted therapies should be considered on a case-by-case basis.

INTRODUCTION

Adrenocortical cancer (ACC) is one of the most aggressive solid tumors in humans, as evidenced by a 5-year survival rate of below 15% at the metastatic stage.[1, 2] Its incidence is about 1-2 new cases/million/year, with an increased occurrence during childhood and the fourth to fifth decades of life. Surgery remains the only curative treatment in patients diagnosed at an early stage while treatment options for patients with advanced ACC are still scant.[1-4]

A personalized approach to cancer treatment is based on the use of drugs able to target specific molecular alterations playing pivotal roles in oncogenesis which are targetable in a given patient. The study of ACC-associated syndromes has suggested that the IGF2 signaling pathway (Beckwith-Wiedemann syndrome), p53 (Li-Fraumeni syndrome) or Wnt/β-catenin signaling (Familial Adenomatous Polyposis) are currently the most attractive targets for ACC.[2] Among these targets, only IGF1 receptor antagonists are currently under investigation in ACC. The results of phase I studies evaluating the effects of IGF1 receptor antagonists have already been published, demonstrating that drugs targeting the IGF1receptor can induce tumor stabilization in a subset of patients, but objective tumor response are rare.[5] Regarding p53 or Wnt/β-catenin signaling, drugs targeting these pathways are still in early development phases. Preliminary studies using antiangiogenics or epithelial growth factor (EGFR)-targeted drugs in patients with ACC have yielded disappointing results.[2, 6-11] However, both the lack of screening for the relevant targeted events and the full demonstration of the relevance of such targets in ACC may explain these results.

In sporadic ACC, several studies have used gene expression microarrays or comparative genomic hybridization (CGH), mainly to establish criteria aimed at differentiating benign from malignant tumors or to identify prognostic markers.[12, 13] Few studies have evaluated the presence of putative biomarkers for new targeted agents in ACC. Indeed, small numbers of molecular alterations (up to five per series) were screened in 8-35 ACC patients among whom only 11 exhibited indisputable criteria of malignancy, as evidenced by the presence of extraadrenal disease.[6, 14-16]

We hypothesize that testing a large number of potentially targetable molecular events in patients with indisputably malignant ACC could accelerate the drug development process in this rare and aggressive solid tumor. To achieve this goal, we collected a large number of primary or locally recurrent well-characterized malignant ACCs. We used hotspot gene sequencing and comparative genomic hybridization (CGH) to evaluate the presence of mutations and copy number abnormalities in, respectively, 46 and 130 genes of potential interest.

MATERIALS AND METHODS

Patient population and sample acquisition

Samples from different ACC patients were collected in four centers of the French COMETE network (Institut National du Cancer): the "Institut Gustave Roussy", the "Centre Hospitalier Régional et Universitaire-Tumorothèque-CRRC de Lille"; the "Hospices Civils de Lyon"; the "Hôpital Cochin, Paris". Samples were snap frozen and stored in tumor biobanks according to national ethics recommendations and local procedures. The forty samples selected for the present study and processed for DNA extraction had to meet the following inclusion criteria: ACC diagnosis confirmed by a local expert pathologist,[17] indisputable malignancy (stage III-IV) based on the ENSAT staging definition,[18] the presence of more than 50% of tumor cells based on histological examination, age older than 17 years. The following clinical parameters were recorded: date of diagnosis, age, gender, Weiss score,[17] mitotic count (available in 39 patients and calculated in 50 or 10 high-power fields [HPF] for 29 or 10 patients, respectively), hormonal status based on hormonal measurements, tumor node metastasis stage and previous systemic treatments. Informed consent was obtained from each patient.

DNA isolation

Genomic DNA was extracted from several 10-20 µm sections of each tumor specimen after digestion with proteinase K (3 hours), using the DNeasy Tissue Kit (QIAGEN), according to the manufacturer's protocol. DNA concentrations were assessed with the Qubit fluorometer (Invitrogen).

Mutational analysis

DNA sequencing was performed with the Ion Torrent technique (PGM-Sequencer Life Technologies). Ten nanograms of each sample were amplified with multiplex PCR based on the Cancer-Panel primers pool followed by library preparation according to recommendations of the Ion AmpliSeq Library Kit 2.0 protocol (Life Technologies), using 17 cycles for multiplex PCR and adding Ion Xpress barcode adapters during the ligation step to allow for subsequent pooling of the samples. The list of sequences covered by multiplex PCR (.bed file) is available on www.ampliseq.com. Each individual library was quantified using the Qubit fluorometer (Invitrogen) and controlled using a Bioanalyzer (Agilent Technologies). Libraries were diluted to obtain a final dilution of 3ng/µL for each library, and 8 or 16 libraries were pooled together for amplification on spheres using the Ion OneTouch 200 Template Kit version 2 (Life Technologies). Spheres obtained by 8 different libraries were loaded onto an Ion 316 chip and spheres obtained by the remaining 32 libraries were loaded onto an Ion 318 chip for sequencing using the Ion PGM 200 Sequencing Kit for 520 flows.

Using 190 primer pairs, this approach allowed the simultaneous study of hotspot regions of 46 critical oncogenes or tumor suppressor genes of potential interest to predict drug sensitivity (reported in supplementary table 1). All reported somatic genetic variants were compared with the relative GRCh37 (h19) reference sequences using Torrent Suite version 2.2 software (variantCaller v2.2.3-31149; Life Technologies) and annotated using Alamut version 2.2 software (Interactive Biosoftware). The variants with a read frequency higher than 10%, none synonymous with and not known as common polymorphisms, were retained as interesting variants (mutations), and were confirmed by Sanger direct sequencing, as previously described.[19] Moreover AKT1 (exon 4), PI3KCA (exons 5-10-21) and CTNNB1 (exon 3) were sequenced in all samples to complete the information obtained with the Ampliseq Cancer Panel.

Oligonucleotide CGH microarrays.

For microarray hybridizations, 400 ng of DNA from each DNA sample was digested and sample integrity was measured using an Agilent bioanalyzer. The test DNA samples were labeled with Cy5 fluorescent dye and the reference DNA samples were labeled with Cy3 fluorescent dye using the Genomic DNA Enzymatic Labeling Kit (Agilent Technologies), following the manufacturer's protocol. Cy3-labeled and Cy5-labelled DNAs were hybridized to the SurePrint G3 Human CGH Microarray 4x180K (Agilent Technologies), prior to washing and scanning with Agilent Scanner G2565CA.

Oligonucleotide CGH microarray analysis.

Oligonucleotide CGH array processing was performed as detailed in the manufacturer's protocol (version 7.1, December 2011; http://www.agilent.com). Data were extracted from scanned images using the Feature Extraction software (version 10.7.3,1, Agilent Technologies), along with protocol CGH_107_Sep09. Acquired signals were normalized according to their dye and local GC percentage content using in-house scripts under the R statistical environment (http://cran.r-project.org). The resulting log2(ratio) values were segmented using the circular binary segmentation algorithm[20] implementation from the DNAcopy package for R. Aberration status calling was automatically performed for each profile according to its internal noise (absolute variation of log2(ratio) values across consecutive probes on the genome). All genomic coordinates were established on the University of California Santa Cruz build hg19 *Homo Sapiens* genome.[21] The analysis focused on 130 genes of potential interest to predict drug sensitivity, including the 46 genes studied by sequencing and additional genes involved in the IGF pathway as reported in supplementary table 1. We described all copy number alterations above zero as gains and all the alterations below zero as losses. However, only copy number gains with log2 ratio values higher than 1 were considered as amplified, and copy number losses with a log2 ratio value lower than 1 were considered as deletions.

Statistical analysis.

Descriptive parameters were calculated using statistical software (SPSS v 15.0). Quantitative data were expressed using means and standard deviations (SD) and medians and ranges. Qualitative data were expressed using percentages.

RESULTS

Study population

The study population included 40 adult patients. The main clinical characteristics of these patients are reported in table 1. Malignancy was ascertained by the stage: 10 and 30 patients had stage III or IV, disease respectively. All samples were collected from primary tumors (34 samples) or local recurrences (6 samples). Each sample corresponded to a different patient. In two patients with stage IV disease, a complete Weiss score could not be assigned because of insufficient available material.

Hotspot gene sequencing

Using the Cancer-Panel primers pool for Ion Torrent sequencing, all 40 samples were informative for the analysis of sequence variants of the evaluated hotspot regions. The mean number of mapped reads per tumor was 167680±122768 (mean ± SD); base coverage depth per tumor was 835X ±598; mean read on-target was 90%±6 (median 93%; 61-95%); the average coverage at 100x was 93.4±2% (median 93%; 89-99%) and the median of 8 variants (range 5-16) was detected. Variants were reported per tumor. The interesting variants retained were all confirmed by Sanger sequencing.

More than one quarter (14/40, 35%) of the samples exhibited at least one mutation, as defined above (details reported in supplementary table 2). Single mutations in the *TP53* gene were found in six samples (15%). All of these mutations are predicted to be associated with a disruption of p53 function (www-p53.iarc.fr). Single mutations in the *ATM* gene were found in five samples (12.5%). Single mutations of *CTNNB1* were found in four samples (10%), all located in exon 3.

In individual cases (1/40; 2.5%), single mutations in the genes coding for *ERBB4, FLT3, STK11, SMO,* and *GNAS* were found (supplementary table 2). A *GNAS*-activating mutation was found in a patient presenting with an isolated Cushing's syndrome (stage III ACC, WS 6) and no clinical evidence of fibrous dysplasia. A *STK11* mutation was found in a patient presenting with an isolated non-secreting ACC (stage III, WS 6).

DNA copy number changes

Twenty-eight of the 40 evaluated samples (a subset of the entire cohort) generated informative profiles by CGH. The average profile of the 28 samples is reported in fig-

Table 1. Patient clinical characteristics

Parameters	Class	Number	Frequency (%)
Gender		40	
	Female	29	72,5
	Male	11	27,5
Age: median 55 (19-77) years; mean 52±16			
	19-29 years	3	7,5
	30-39	9	22,5
	40-49	4	10
	50-59	8	20
	60-69	12	30
	≥70	4	10
Weiss score : median 7 (4-9) ; mean 6,9±1,4			
	4	2	5
	5	4	10
	6	10	25
	7	10	25
	8	4	10
	9	8	20
	Unknown	2	5
Mitotic index			
	≤5	3	7,7
	>5 but <10	9	23,1
	≥10	27	69,2
	Unknown	1	2,5
Hormonal secretion			
	Present	26	65
	Cortisol	*13*	*32,5*
	Cortisol and androgens	*9*	*22,5*
	Androgens	*3*	*7,5*
	Androgens and estrogens	*1*	*2,5*
	Absent	14	35
Stage	III	10	25
	IV	30	75
Origin of sampling	Primary tumors	34	85
	Recurrence	6	15
Previous systemic treatment			
	yes	14	35
	Mitotane	*9*	*22,5*
	Mitotane and Chemotherapy	*5*	*12,5*
	no	26	65

ure 1. Most samples exhibited gains of oncogenes or loss of tumor suppressor genes, as reported in supplementary table 3.

Twelve of these profiles (42.9%) contained at least one deletion or amplification in the expression of the 130 evaluated genes. Among these genes, the recurrent retained abnormality was the amplification of the *CDK4* oncogene (Chr12q14) observed in 5 of

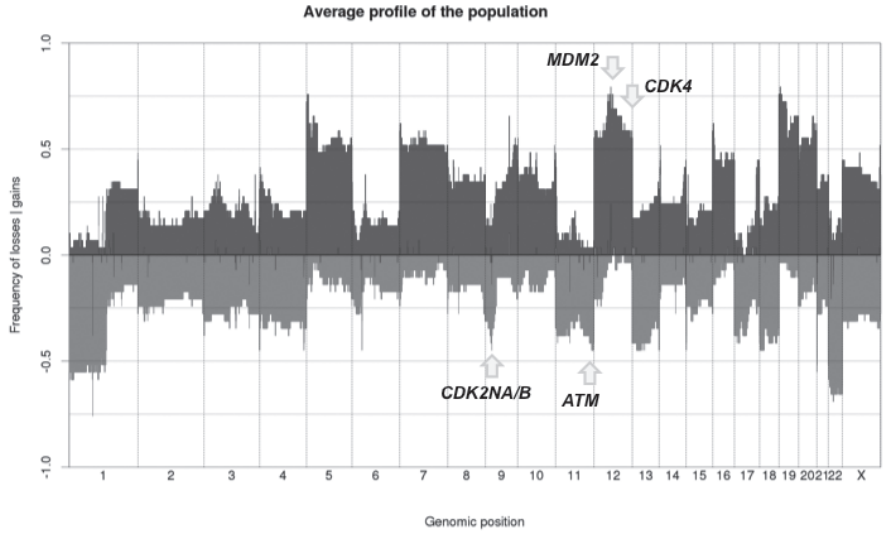

Figure 1. Frequency of chromosome gains and losses in 28 adult advanced ACC samples as detected by comparative genomic hybridization. The X-axis shows each numbered chromosome region, starting from chromosome 1 and ending with chromosome X with short-arm regions (p) preceding the long-arm regions (q). The Y-axis shows the frequency of samples exhibiting a gain (blue and upper part of the graph) or a loss (red and lower part) of each hybridized chromosome region. The arrows indicate the chromosome regions containing *CDK4, CDKN2A/B, MDM2*, and *ATM*.

the 28 evaluated samples (17.9%); the deletion of the *CDKN2A* tumor suppressor gene (Chr9p21) observed in four other samples (14.3%) and the deletion of the tumor suppressor gene *CDKN2B* (Chr9p21) observed in 3 samples. All these 3 patients with deletion of *CDKN2B* were also deleted for *CDKN2A* (figure 2 and supplementary table 3). Overall 32.1% of the samples exhibited an amplification/deletion (figure 2 and 3) of one of these genes. Amplification of three different components of the fibroblast growth factor (FGF) pathway (FGFR1; FGF9 and FRS2) was found in 3 different samples (3/28; 10.7%) (figure 2).

Integrated results of hotspot gene sequencing and DNA copy number variation

Taken together, the results of DNA copy number variation and hotspot gene sequencing data (figure 2), showed that almost half of the evaluated samples exhibited at least one molecular event (19/40; 47.5%).

Some alterations were recurrently associated, such as the *ATM* mutation and loss of *CDKN2A* observed in 3 of the 5 samples harboring *ATM* mutations. Three of the four patients with a *CTNNB1* mutation were also carrying a *TP53* mutation.

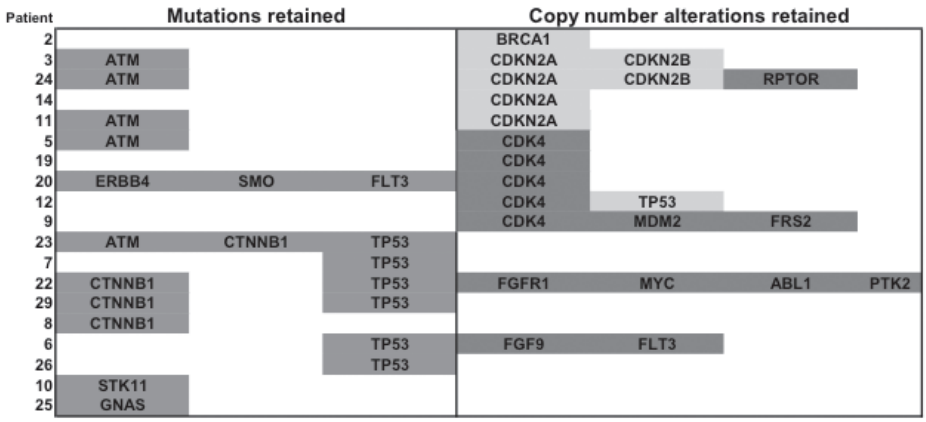

Figure 2. Molecular abnormalities detected by CGH and hot spot gene sequencing (Ion Torrent; Life Technologies)) in a cohort of 40 adult ACC samples. The left panel shows the mutated genes in pink (according to the definition described in the text). The right panel shows the copy number variations detected by CGH in the screened genes. Amplifications are in red and deletions (according to the definition described in the text) in green.

DISCUSSION

To the best of our knowledge, this is the first study to screen a large series of indisputably malignant ACC for the presence of a large number (more than 40) of structural DNA changes that could help to select new targeted drugs. The strengths of this study include a selective use of malignant ACC samples based on documented local or distant invasion and the use of an innovative sequencing system, the Ion Torrent which allows one to generate results concerning a large set of genes in a time span compatible with clinical needs. None of the previous studies evaluated more than 5 potential targets by DNA sequencing and none of them were able to integrate the results of DNA sequencing with the study of DNA copy number alterations in this type of malignancy. Thirty-five percent of the samples exhibited at least one mutation and 42.9% had at least one deletion or amplification in the evaluated genes. Overall, almost half of the samples (47.5%) had at least one molecular abnormality. The present study provides evidence that drugs targeting the cell cycle represent the most relevant potential new therapeutic strategy for patients with advanced ACC. Inhibitors of the FGFR pathway could be a potential target for treatment in a subset of ACC patients, whereas treatment with other targeted therapies could be considered exclusively on a case-by-case basis. The rarity of the molecular alterations usually used in personalized oncology, suggests that no short-term results can be expected from the use of new available licensed agents in the majority of ACC patients.

Based on DNA sequencing of 46 potentially actionable oncogenes or tumor suppressor genes, we identified alterations including mutations in *ERBB4* and *FLT3* genes (figure 2). No mutations were identified on *EGFR*, *BRAF*, *KIT, PIK3CA, RET* or *PDGFR-A*. Previously, two studies evaluated the results of *EGFR* (exons 18-21) sequencing in ACC and reported a mutation frequency of respectively 0 (0/30) and 11% (4/35) in the cases.[6, 14] A previous study conducted by our group found no mutation in *EGFR* (exon 18,19 and 21), *BRAF, PIK3CA* and *JAK2* in 18 ACCs, which is in line with the present results.[15] Because some target gene regions were not well explored by Ampliseq Cancer-Panel primers, we further validated our results by direct sequencing and we confirmed the absence of *AKT1* (exon 4) mutations and *PI3KCA* mutations (exons 5, 10 and 21) in all of our samples (data not shown).

Using CGH, different components of the FGFR pathway were found to be amplified in three different samples. A growing number of studies indicate that inhibition of the FGFR pathway may be an effective therapeutic option against cancer and several drugs targeting the FGFR pathway are under clinical development (phase I-III).[22] A microarray gene expression analysis performed in 11 ACC samples evidenced frequent up-regulation of FGFR1 and 4 in one study.[23] A recent study suggested that FGFR-4 overexpression and gene amplification have a prognostic value in ACC.[24] In the present study FGFR1-4 were not frequently amplified, but gains of FGFR 1-4 were frequently observed (22 of 28 [78.6%] samples exhibited gains in at least one of these receptors; supplementary table 3). Conversely, the study of copy number variations suggests that *HER2* (ERBB2) amplification is not present in ACC, in accordance with previous reports.[25-27] In addition, many other putative biomarkers of targeted treatments such as *PTEN*, *AKT*, *ALK*, *c-MET/HGFR*, *EGFR*, *PDGFR*, and *ESR1* did not exhibit amplification/deletion, which completes the negative results of the mutation screening approach.

CTNNB1 and *TP53* mutations are genetic alterations well known to play a role in the pathogenesis of ACC.[2] Based on DNA sequencing, we identified *CTNNB1* mutations in four samples (10%) and TP53 mutations in 6 samples (15%). Interestingly, both *CTNNB1* and *TP53* mutations (figure 2) were found in three samples. Moreover, *CTNNB1* gains were observed in all abnormal (mutated or deleted) *TP53* samples (supplementary table 3), while they were rare (9%) in wild-type *TP53* samples. This observation, which is very intriguing from the pathogenic point of view, and hitherto never clearly described in ACC, suggests an association between the status of *TP53* and *CTNN1B* genes. *CTNNB1* mutations are early events in adrenocortical oncogenesis.[16, 28] Previously published studies reported *CTNNB1* mutations in 20-30% of the evaluated samples.[16, 28] The frequency of *CTNNB1* mutations in our population was in the low range of previously published reports. Because some rare *CTNNB1* exon 3 mutations previously described in ACC were outside the hotspot regions investigated in our study, we performed direct sequencing of exon 3 of the *CTNNB1* gene in all ACC samples. This technique allowed us to detect

CTNNB1 mutations in two additional patients. This led to a whole *CTNNB1* mutation rate of 15%, which is still in the low range of previously published reports.

TP53 mutations were suggested to be late events in adult adrenocortical oncogenesis.[29] Previously published studies reported *TP53* mutations in 10-70% of the evaluated samples.[30] The frequency of *TP53* mutation in our population was also in the low range of previously published reports. The difference in the frequency of *CTNNB1* and *TP53* in our study compared to previously published reports could be due to the low sample size and the heterogeneity of the ACC patient population. Interestingly, based on CGH, one sample exhibited a *TP53* deletion and another *MDM2* amplification (figure 2 and supplementary table 3). The *MDM2* gene has been reported to be overexpressed in ACC[13] and is a potential target for treatment (www.clinicaltrials.gov). Regarding the IGF pathway, no major alteration was found with the methods used in our (study supplementary table 3).

DNA-damage response and G1 cell cycle progression are new pathways whose exploration could be interesting in ACC patients. By sequencing, we demonstrated for the first time the presence of *ATM* mutations/interesting variants in five ACC patients. *ATM* plays a role in cell response to DNA damage and genome stability. Mutations in this gene are associated with ataxia telangiectasia, a disorder associated with high frequency of cancer.[31] All the detected *ATM* variants are considered of interest because they have been described as potentially involved in malignancy.[32-34] The frequencies of these *ATM* variants in our series is higher than that expected in the general population (relative database dbSNP [build 137]; supplementary table 2). A recent study demonstrated ATM gene copy number is reduced in ACC compared with adrenal adenomas.[35] We detected alterations of several key components of the cell cycle by CGH analysis: *CDK4* amplification and *CDKN2A* and *CDKN2B* deletion. *CDK4* encodes for a cyclin-dependent kinase that plays a crucial role in G1-S phase cell cycle progression. CDK4 has already been suggested to be overexpressed in ACC as compared to normal adrenals.[36] By alternative splicing, *CDKN2A* can encode for two different gene products: the tumor suppressor protein p16 (a CDK4 inhibitor) and ARF (a stabilizer of p53) (figure 3).[37] *CDKN2B* encodes for p15, another CDK4/6 inhibitor. Loss of nuclear immunostaining for p16 has been reported in three out of seven ACCs.[38] The integration of data obtained by sequencing and CGH allowed us to discover that three of the five *ATM* mutated samples also had a *CDKN2A* deletion and one had *CDK4* amplification suggesting a functional synergism between DNA damage checkpoints and G1 cell cycle progression pathways in the pathogenesis of ACC (figure 2 and 3).

Finally, by sequencing, we detected two mutations described as part of well-characterized genetic disorders and hitherto never reported in adult ACC: mutations in *GNAS* and *STK11* genes. Activating mutations in the *GNAS* gene have been described in sporadic ACTH-independent macronodular adrenal hyperplasia (AIMAH) and rarely in benign adrenal tumors. These mutations can cause the McCune Albright (MAS) syn-

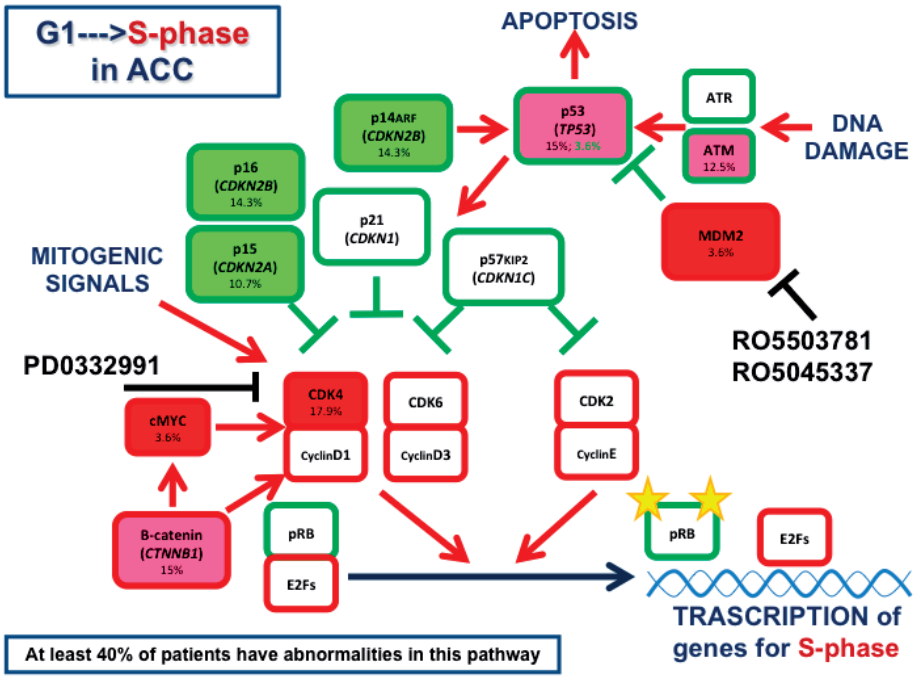

Figure 3. Schematic representation of the G1 cell cycle progression pathway. Using CGH (28 samples) and hot spot gene sequencing (Ion Torrent;Life Technologies; 40 samples) in a large cohort of malignant adrenocortical cancer samples, we detected several abnormalities in this pathway. Oncogenes are represented in red frames; tumor suppressor genes are represented in green frames. The abnormalities detected are indicated as follows: amplified genes are represented with a red filling, deleted genes are represented with a green filling, and mutated genes are represented with a pink filling. Red arrows signify stimulation and green and black lines signify inhibition. Some cell processes are represented in blue (eg,apoptosis). The percentage of samples that exhibit an abnormality in a given gene is in the lower part of some boxes (in black). The percentage in green in the lower part of the TP53 box refers to a case of TP53 deletion. p14ARF is encoded by the same gene as p16. Two drugs (in black) potentially interfering with this pathway and currently being used in phase I clinical trials (www.clinicaltrials.gov).

drome which is associated with AIMAH.[39] A single case of ACC with a somatic mutation of *GNAS* was recently reported in a child unaffected by MAS. Mutations in the *STK11* tumor suppressor gene are associated with Peutz-Jegher's Syndrome (PJS). A single case of ACC was recently reported in a child with PJS.[40] We did not find signs or symptoms respectively suggestive of MAS or PJS in either of these patients nor in their family members. We suspect that both of these mutations could play a role in ACC oncogenesis in the affected patients.

Our study suffers from several limitations: 35% of the patients had received previous medical treatment and we were unable to discriminate between germline and somatic mutations. In addition, we did not explore mRNA and protein expression and protein

function. Finally, we used a sequencing system that detects many, but not all, mutations in the evaluated genes. However, we decided to focus on the selected genomic events that are already widely used in oncology as predictors of drug responses.

CONCLUSIONS

In conclusion, we identified 47.5% mutations or CGH alterations in a large series of ACC patients. To our knowledge, this is the first time that *ATM, STK11* and *GNAS* mutations have been reported in adult ACC patients. No relevant molecular alteration suggests the likelihood of a simple molecular-driven targeted approach in ACC patients in the short term. However, our study predicts a potential future role for new compounds targeting DNA-damage responses, G1 cell cycle progression and the FGFR pathway.

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SUPPLEMENTARY MATERIAL

Supplementary table 1

Gens eveluated by Ion Torrent (hotspot gene sequencing) and Comparative Genomic Hybridization [CGH] (copy number alterations)

Symb	Name	Chr	Cytoband	Ion Torrent	CGH
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	9	9q34.1	yes	yes
AKT1	v-akt murine thymoma viral oncogene homolog 1	14	14q32.32	yes	yes
AKT2	v-akt murine thymoma viral oncogene homolog 2	19	19q13.1-q13.2	no	yes
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	1	1q44	no	yes
ALK	anaplastic lymphoma receptor tyrosine kinase	2	2p23	yes	yes
APC	adenomatous polyposis coli	5	5q21-q22	yes	yes
ATM	ataxia telangiectasia mutated	11	11q22-q23	yes	yes
ATR	ataxia telangiectasia and Rad3 related	3	3q23	no	yes
BRAF	v-raf murine sarcoma viral oncogene homolog B1	7	7q34	yes	yes
BRCA1	breast cancer 1, early onset	17	17q21	no	yes
BRCA2	breast cancer 2, early onset	13	13q12.3	no	yes
BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B	15	15q15	no	yes
CCNA1	cyclin A1	13	13q12.3-q13	no	yes
CCNA2	cyclin A2	4	4q27	no	yes
CCNB1	cyclin B1	5	5q12	no	yes
CCNB2	cyclin B2	15	15q22.2	no	yes
CCNB3	cyclin B3	23	Xp11	no	yes
CCND1	cyclin D1	11	11q13	no	yes
CCND2	cyclin D2	12	12p13	no	yes
CCND3	cyclin D3	6	6p21	no	yes
CCNE1	cyclin E1	19	19q12	no	yes
CCNH	cyclin H	5	5q13.3-q14	no	yes
CDC6	cell division cycle 6	17	17q21.3	no	yes
CDC7	cell division cycle 7	1	1p22	no	yes
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	16	16q22.1	yes	yes
CDK1	cyclin-dependent kinase 1	10	10q21.1	no	yes
CDK2	cyclin-dependent kinase 2	12	12q13	no	yes
CDK4	cyclin-dependent kinase 4	12	12q14	no	yes
CDK6	cyclin-dependent kinase 6	7	7q21-q22	no	yes
CDK7	cyclin-dependent kinase 7	5	5q12.1	no	yes
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	6	6p21.2	no	yes
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	12	12p13.1-p12	no	yes
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	11	11p15.5	no	yes
CDKN2A	cyclin-dependent kinase inhibitor 2A	9	9p21	yes	yes
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	9	9p21	no	yes
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	1	1p32	no	yes
CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	19	19p13	no	yes
CHEK1	checkpoint kinase 1	11	11q24.2	no	yes
CHEK2	checkpoint kinase 2	22	22q12.1	no	yes
CSF1R	colony stimulating factor 1 receptor	5	5q32	yes	yes
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	3	3p21	yes	yes
E2F1	E2F transcription factor 1	20	20q11.2	no	yes
EGFR	epidermal growth factor receptor	7	7p12	yes	yes
EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	8	8p12	no	yes
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (	17	17q12	yes	yes
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	2	2q33.3-q34	yes	yes
ESR1	estrogen receptor 1	6	6q25.1	no	yes
FBXW7	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase	4	4q31.3	yes	yes
FGF4	fibroblast growth factor 4	11	11q13.3	no	yes
FGF9	fibroblast growth factor 9 (glia-activating factor)	13	13q11-q12	no	yes
FGFR1	fibroblast growth factor receptor 1	8	8p12	yes	yes
FGFR2	fibroblast growth factor receptor 2	10	10q26	yes	yes
FGFR3	fibroblast growth factor receptor 3	4	4p16.3	yes	yes
FGFR4	fibroblast growth factor receptor 4	5	5q35.1-qter	no	yes
FLT3	fms-related tyrosine kinase 3	13	13q12	yes	yes
FRS2	fibroblast growth factor receptor substrate 2	12	12q15	no	yes
GNAS	GNAS complex locus	20	20q13.3	yes	yes
GSK3B	glycogen synthase kinase 3 beta	3	3q13.3	no	yes
HDAC9	histone deacetylase 9	7	7p21.1	no	yes
HNF1A	HNF1 homeobox A	12	12q24.2	yes	yes
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	11	11p15.5	yes	yes
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	14	14q32.33	no	yes
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	2	2q33.3	yes	yes
IGF1	insulin-like growth factor 1 (somatomedin C)	12	12q23.2	no	yes
IGF1R	insulin-like growth factor 1 receptor	15	15q26.3	no	yes
IGF2	insulin-like growth factor 2 (somatomedin A)	11	11p15.5	no	yes
IGF2R	insulin-like growth factor 2 receptor	6	6q26	no	yes
IGFBP1	insulin-like growth factor binding protein 1	7	7p13-p12	no	yes
IGFBP2	insulin-like growth factor binding protein 2, 36kDa	2	2q33-q34	no	yes
IGFBP3	insulin-like growth factor binding protein 3	7	7p13-p12	no	yes
IGFBP4	insulin-like growth factor binding protein 4	17	17q12-q21.1	no	yes
IGFBP5	insulin-like growth factor binding protein 5	2	2q33-q36	no	yes
IGFBP6	insulin-like growth factor binding protein 6	12	12q13	no	yes
INS	insulin	11	11p15.5	no	yes
INSR	insulin receptor	19	19p13.3-p13.2	no	yes
IRS1	insulin receptor substrate 1	2	2q36	no	yes
IRS2	insulin receptor substrate 2	13	13q34	no	yes
IRS4	insulin receptor substrate 4	23	Xq22.3	no	yes
JAK2	Janus kinase 2	9	9p24	yes	yes
JAK3	Janus kinase 3	19	19p13.1	yes	yes
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)	4	4q11-q12	yes	yes
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	4	4q11-q12	yes	yes
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	12	12p12.1	yes	yes
MAP3K1	mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase	5	5q11.2	no	yes
MDM2	Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)	12	12q14.3-q15	no	yes
MDM4	Mdm4 p53 binding protein homolog (mouse)	1	1q32	no	yes
MET	met proto-oncogene (hepatocyte growth factor receptor)	7	7q31	yes	yes
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	3	3p21.3	yes	yes
MPL	myeloproliferative leukemia virus oncogene	1	1p34	yes	yes
MTOR	mechanistic target of rapamycin (serine/threonine kinase)	1	1p36.2	no	yes
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	8	8q24.21	no	yes
NOTCH1	notch 1	9	9q34.3	yes	yes
NOTCH4	notch 4	6	6p21.3	no	yes
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	5	5q35.1	yes	yes
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	1	1p13.2	yes	yes
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	11	11q13-q14	no	yes
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	4	4q12	yes	yes
PDPK1	3-phosphoinositide dependent protein kinase-1	16	16p13.3	no	yes
PGR	progesterone receptor	11	11q22-q23	no	yes
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	3	3q26.3	yes	yes
PIK3CB	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta	3	3q22.3	no	yes
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	5	5q13.1	no	yes
PLK1	polo-like kinase 1	16	16p12.2	no	yes
PSMA2	proteasome (prosome, macropain) subunit, alpha type, 2	7	7p13	no	yes
PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3	14	14q23	no	yes
PSMA4	proteasome (prosome, macropain) subunit, alpha type, 4	15	15q25.1	no	yes
PSMA6	proteasome (prosome, macropain) subunit, alpha type, 6	14	14q13	no	yes
PSMB5	proteasome (prosome, macropain) subunit, beta type, 5	14	14q11.2	no	yes
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	6	6p21.3	no	yes
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	6	6p21.3	no	yes
PSMD5	proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	9	9q33.2	no	yes
PSME3	proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	17	17q21	no	yes
PTEN	phosphatase and tensin homolog	10	10q23.3	yes	yes
PTK2	protein tyrosine kinase 2	8	8q24.3	no	yes
PTPN11	protein tyrosine phosphatase, non-receptor type 11	12	12q24	yes	yes
RB1	retinoblastoma 1	13	13q14.2	yes	yes
RET	ret proto-oncogene	10	10q11.2	yes	yes
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	17	17q23.1	no	yes
RPTOR	regulatory associated protein of MTOR, complex 1	17	17q25.3	no	yes
SMAD4	SMAD family member 4	18	18q21.1	yes	yes
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	22	22q11	yes	yes
SMO	smoothened, frizzled family receptor	7	7q32.3	yes	yes
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	20	20q12-q13	yes	yes
STK11	serine/threonine kinase 11	19	19p13.3	yes	yes
TOP2A	topoisomerase (DNA) II alpha 170kDa	17	17q21-q22	no	yes
TP53	tumor protein p53	17	17p13.1	yes	yes
TSC1	tuberous sclerosis 1	9	9q34	no	yes
TSC2	tuberous sclerosis 2	16	16p13.3	no	yes
VEGFA	vascular endothelial growth factor A	6	6p12	no	yes
VHL	von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase	3	3p25.3	yes	yes

Supplementary table 2: Mutations detected by Ion Torrent

Number of observation	Chr	Position	*Gene*	Target ID	Type	Ref	Variant	HotSpot(1)	Reference SNP(2)	Protein	Type of SNP		NM
3	chr11	108138003	***ATM***	AMPL82011	SNP	T	C	COSM21826	rs1800056	p.Phe858Leu	Substitution	Missense	NM_000051.3(ATM):c.2572T>C
1	chr11	108119823	***ATM***	AMPL82666	SNP	T	C	COSM21825	rs56128736	p.Val410Ala	Substitution	Missense	NM_000051.3(ATM):c.1229T>C
1	chr11	108170506	***ATM***	AMPL83653	SNP	A	C	---	rs1800059	p.Ser1691Arg	Substitution	Missense	NM_000051.3(ATM):c.5071A>C
1	chr3	41266082	***CTNNB1***	AMPL423052	SNP	C	T	---	nr	p.Gln27*	Substitution	Nonsense	NM_001904.3(CTNNB1):c.79C>T
1	chr3	41266085	***CTNNB1***	AMPL423052	SNP	C	T	---	nr	p.Gln28*	Substitution	Nonsense	NM_001904.3(CTNNB1):c.82C>T
1	chr3	41266136	***CTNNB1***	AMPL423052	SNP	T	C	COSM5663;COSM5685	rs121913407	p.Ser45Pro	Substitution	Missense	NM_001904.3(CTNNB1):c.133T>C
1	chr3	41266137	***CTNNB1***	AMPL423052	SNP	C	T	COSM5689;COSM5667;COSM5692	nr	p.Ser45Pro	Substitution	Missense	NM_001904.3(CTNNB1):c.134C>T
1	chr2	212576820	***ERBB4***	AMPL33659	SNP	G	T	---	nr	p.Thr360Asn	Substitution	Missense	NM_005235.2(ERBB4):c.1079C>A
1	chr13	28592611	***FLT3***	AMPL87967	SNP	C	A	---	nr	p.Arg845Met	Substitution	Missense	NM_004119.2(FLT3):c.2534G>T
1	chr20	57484421	***GNAS***	AMPL104671	SNP	G	A	COSM27895	rs121913495	p.Arg201His	Substitution	Missense	NM_000516.4(GNAS):c.602G>A
1	chr7	128851596	***SMO***	AMPL492615	SNP	C	T	---	nr	p.Pro641Ser	Substitution	Missense	NM_005631.4(SMO):c.1921C>T
1	chr19	1223125	***STK11***	AMPL490549	SNP	C	G	COSM21360	rs59912467	p.Phe354Leu	Substitution	Missense	NM_000455.4(STK11):c.1062C>G
1	chr17	7574018	***TP53***	AMPL110679	SNP	G	A	---	nr	p.Arg337Cys	Substitution	Missense	NM_000546.4(TP53):c.1009C>T
1	chr17	7574003	***TP53***	AMPL110679	SNP	G	A	COSM11073	nr	p.Arg342*	Substitution	Nonsense	NM_000546.4(TP53):c.1024C>T
1	chr17	7577106	***TP53***	AMPL117792	SNP	G	T	COSM10939	nr	p.Pro278Thr	Substitution	Missense	NM_000546.4(TP53):c.832C>A
1	chr17	7578212	***TP53***	AMPL226789	SNP	G	A	COSM10654		p.Arg213*	Substitution	Nonsense	NM_000546.4(TP53):c.637C>T
1	chr17	7578190	***TP53***	AMPL226789	SNP	T	C	COSM10758	nr	p.Tyr220Cys	Substitution	Missense	NM_000546.4(TP53):c.659A>G
1	chr17	7577527	***TP53***	AMPL94170	SNP	G	T	---	nr	p.Leu252Ile	Substitution	Missense	NM_000546.4(TP53):c.754C>A

SNP: Single nucleotide polymorphism (variant); NM: NCBI gene identifiers (http://www.ncbi.nlm.nih.gov/gene)
(1)http://www.sanger.ac.uk/genetics/CGP/cosmic/
(2)http://www.ncbi.nlm.nih.gov/projects/SNP/

Supplementary table 3: CGH results in 28 ACC samples

Patiets Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
ABL1	0,24	-0,36	0,00	0,31	0,00	0,62	0,10	0,00	0,49	0,50	0,00	0,10	0,00	0,41	0,00	0,08	-0,07	0,00	0,08	0,00	-0,25	1,07	0,42	0,33	-0,12	0,68	0,00	-0,26
AKT1	0,00	0,28	0,31	0,05	0,00	0,00	0,36	0,00	0,00	0,11	-0,08	0,00	0,00	0,00	0,00	0,06	0,08	0,00	0,00	0,00	-0,25	0,00	0,17	0,18	0,00	0,07	0,00	-0,29
AKT2	0,18	0,26	0,61	0,00	0,63	0,63	0,65	0,19	0,00	0,13	0,50	0,50	0,00	0,00	0,00	0,29	-0,58	0,00	0,08	0,00	0,35	0,58	0,00	-0,47	-0,73	0,00	0,24	0,34
AKT3	0,00	-0,41	0,00	0,33	0,00	0,35	0,41	0,00	0,00	0,18	0,00	0,00	0,42	0,41	0,00	0,00	0,06	0,00	0,00	0,00	-0,24	0,00	0,44	0,62	0,00	0,00	0,00	-0,38
ALK	0,00	-0,41	0,00	0,33	0,00	0,00	0,00	-0,17	0,00	0,20	0,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,67	-0,26	0,00	-0,48	0,41	0,00	0,29	0,06	-0,37
APC	0,54	0,31	0,00	0,00	0,67	0,44	0,61	0,12	0,00	0,47	0,00	0,44	0,00	0,28	0,00	0,13	0,00	0,75	0,48	0,00	0,74	0,41	0,00	-0,48	-0,73	-0,44	0,42	0,30
ATM	-0,21	-0,44	0,00	0,00	0,00	0,00	-0,12	-0,19	0,00	0,15	0,00	0,00	0,00	0,00	0,00	0,00	-0,44	0,00	0,00	-0,67	-0,13	0,00	0,00	-0,48	0,00	-0,45	0,00	-0,40
ATR	0,00	-0,42	-0,38	-0,41	0,00	0,00	0,56	0,06	0,00	0,30	0,00	0,00	0,00	0,12	0,00	0,00	-0,50	0,00	0,00	0,00	0,00	0,15	0,13	0,00	-0,14	0,32	0,00	0,00
BRAF	-0,20	0,00	0,00	0,00	0,67	0,53	0,64	0,15	0,00	0,36	0,00	0,78	0,00	0,00	0,00	0,15	0,12	0,00	0,47	0,00	0,41	0,40	0,12	0,00	0,00	0,31	0,40	0,23
BRCA1	0,00	-1,00	0,00	-0,40	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,00	-0,57	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,51	0,23	-0,06	-0,45	0,00	0,00
BRCA2	0,00	-0,43	0,00	-0,40	0,00	0,17	-0,46	-0,18	0,00	0,31	-0,34	0,47	0,00	0,00	0,00	0,00	-0,62	0,00	0,46	-0,67	0,00	0,14	0,00	-0,42	0,00	-0,41	0,00	-0,92
BUB1B	0,00	0,00	0,00	0,00	0,00	0,18	0,00	-0,18	0,80	-0,15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,47	-0,67	-0,39	0,69	-0,46	0,00	0,00	-0,46	0,00	-0,37
CCNA1	0,00	-0,43	0,00	-0,40	0,00	0,17	-0,46	-0,18	0,00	0,31	-0,34	0,47	-0,64	0,00	0,00	0,00	-0,62	0,00	0,46	-0,67	0,00	0,14	-0,47	-0,42	0,00	-0,41	0,00	-0,92
CCNA2	-0,17	0,31	-0,37	-0,14	0,64	-0,23	-0,09	0,00	0,00	0,00	0,00	0,00	-0,66	0,00	0,00	0,00	-0,38	0,00	0,48	0,00	0,00	0,16	-0,17	0,34	0,00	-0,46	0,00	0,00
CCNB1	0,54	0,31	0,00	0,00	0,67	0,44	0,61	0,12	0,00	-0,15	0,00	0,44	0,00	0,00	0,00	0,13	0,00	0,75	0,48	0,00	0,74	0,41	0,00	-0,48	0,00	-0,21	0,18	0,30
CCNB2	0,00	0,00	0,00	0,00	0,00	0,18	0,00	-0,18	0,54	-0,15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,47	-0,67	-0,39	0,69	-0,46	0,00	0,00	-0,46	0,00	-0,37
CCNB3	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
CCND1	-0,17	-0,41	0,00	0,00	0,00	0,00	0,05	-0,09	0,00	0,23	0,00	0,00	0,07	0,00	0,00	0,07	-0,44	0,00	0,00	-0,67	0,00	0,23	0,00	-0,46	-0,09	0,56	0,00	-0,25
CCND2	0,14	0,32	0,34	-0,38	0,71	0,40	-0,07	0,21	0,75	-0,18	0,36	0,00	0,00	-0,58	0,00	0,23	0,00	0,74	0,52	0,00	0,42	0,89	-0,48	-0,39	0,00	0,38	0,69	0,00
CCND3	0,43	-0,40	-0,37	0,00	0,00	0,00	0,05	0,15	0,00	0,74	0,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,24	0,97	0,00	0,00	0,00	0,37	0,23
CCNE1	0,10	0,26	0,61	0,00	0,63	0,63	0,65	0,19	0,00	0,00	0,49	0,41	0,00	0,00	0,00	0,29	0,00	0,00	0,48	0,00	0,35	0,58	0,00	-0,48	-0,18	0,00	0,40	0,34
CCNH	0,54	0,31	0,00	0,00	0,67	0,44	0,61	0,12	0,00	-0,15	0,00	0,44	0,00	0,00	0,00	0,13	0,00	0,75	0,48	0,00	0,74	0,41	0,00	-0,48	-0,73	-0,44	0,00	0,30
CDC6	0,00	-1,00	0,00	-0,40	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,00	-0,57	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,51	-0,51	-0,06	-0,45	0,00	0,00
CDC7	-0,19	-0,42	-0,38	0,15	0,00	0,00	0,00	-0,19	0,00	-0,14	-0,33	0,00	-0,64	-0,62	0,00	0,00	0,08	0,00	0,00	-0,71	-0,27	0,00	-0,48	-0,48	-0,73	0,00	0,00	-0,39
CDH1	0,16	0,00	0,00	0,00	0,63	-0,17	0,00	0,15	0,00	0,00	0,00	0,44	0,00	0,00	0,00	0,25	0,00	0,68	0,47	0,00	0,43	0,64	0,00	0,23	0,00	0,30	0,00	0,29
CDK1	0,00	0,32	0,30	0,28	0,08	0,18	0,33	0,00	-0,72	-0,16	0,00	0,44	0,00	0,00	0,00	0,23	0,11	0,00	0,48	0,00	0,00	0,25	-0,49	0,00	0,00	0,00	0,00	0,00
CDK2	0,28	0,29	0,34	0,00	0,66	0,44	0,44	0,14	0,37	0,07	0,41	0,00	0,00	0,25	0,00	0,23	0,10	0,70	0,44	0,00	0,42	0,68	0,21	0,00	-0,09	0,32	0,57	0,00
CDK4	0,28	0,29	0,34	0,00	1,53	0,68	0,44	0,87	2,31	1,01	0,73	1,39	0,00	0,25	0,00	0,22	0,10	0,71	1,14	0,00	0,42	0,68	0,22	0,00	0,00	0,32	0,71	0,00
CDK6	-0,20	0,31	0,00	0,00	0,67	0,53	0,64	0,16	0,00	0,34	0,00	0,79	0,00	0,00	0,00	0,13	0,15	0,00	0,48	0,00	0,41	0,28	0,12	0,00	0,00	0,32	0,42	0,23
CDK7	0,54	0,31	0,00	0,00	0,67	0,44	0,61	0,12	0,00	-0,15	0,00	0,44	0,00	0,00	0,00	0,13	0,00	0,75	0,48	0,00	0,74	0,41	0,00	-0,48	0,00	-0,21	0,18	0,30
CDKN1A	0,00	-0,40	-0,37	-0,39	0,00	0,00	0,05	0,15	0,00	1,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,69	0,00	0,00	0,43	0,00	0,00	0,00	0,37	0,00
CDKN1B	0,16	0,32	0,34	-0,39	0,66	0,38	-0,05	0,16	0,50	-0,11	0,42	0,00	0,00	-0,58	0,00	0,23	0,00	0,70	0,48	0,00	0,43	0,89	-0,91	-0,51	0,00	0,33	0,72	0,00
CDKN1C	-0,20	-0,42	0,00	0,13	0,00	-0,08	0,00	0,00	0,95	-0,17	0,00	0,00	0,00	0,35	0,00	0,06	-0,45	0,00	0,14	-0,39	0,00	0,00	0,00	-0,47	-0,07	0,50	0,00	-0,40
CDKN2A	0,33	-0,42	-2,23	0,00	0,00	-0,53	0,19	-0,40	0,00	0,00	-1,59	0,00	0,00	-1,92	0,00	0,00	0,00	0,00	0,00	-0,60	-0,25	0,00	-0,45	-2,91	-0,73	0,63	0,00	-0,38
CDKN2B	0,33	-0,42	-2,23	0,00	0,00	-0,53	0,19	-0,40	0,00	0,00	0,00	0,00	0,00	-1,92	0,00	0,00	0,00	0,00	0,00	-0,60	-0,25	0,00	-0,45	-2,91	-0,73	0,63	0,00	-0,38
CDKN2C	-0,19	-0,42	-0,38	0,37	0,00	0,00	0,00	-0,19	0,00	-0,20	-0,31	0,00	-0,65	-0,62	0,00	0,00	-0,42	0,00	0,00	-0,69	-0,23	0,00	-0,48	-0,59	-0,73	0,00	0,00	-0,38
CDKN2D	0,10	0,26	0,61	0,00	0,59	0,60	0,65	0,07	0,15	0,15	0,51	0,63	0,09	0,00	0,00	0,29	0,34	0,00	0,49	0,00	0,35	0,58	0,00	0,12	-0,16	0,00	0,17	0,34
CHEK1	-0,21	-0,38	0,00	0,00	0,00	0,00	-0,12	-0,17	0,00	0,07	-0,09	0,00	0,00	0,00	0,00	0,00	-0,44	0,00	0,00	-0,67	-0,13	0,00	0,00	-0,43	0,00	-0,45	0,00	-0,39
CHEK2	-0,19	-0,40	-0,38	-0,40	0,00	-0,18	0,00	-0,38	0,00	0,21	-0,28	0,00	-0,59	-0,58	0,00	0,00	-0,47	0,00	0,00	-0,64	-0,21	0,22	-0,85	-0,09	-0,08	-0,45	-0,56	-0,35
CSF1R	0,54	0,32	0,00	0,00	0,67	0,45	0,61	0,13	0,00	-0,18	0,00	0,44	0,00	0,25	0,00	0,13	0,00	0,75	0,48	0,00	0,74	0,41	0,00	-0,45	-0,73	-0,44	0,42	0,32
CTNNB1	0,00	-0,42	-0,38	-0,42	0,00	0,68	0,64	-0,10	0,00	0,21	0,00	0,44	0,00	0,60	0,00	0,00	-0,50	0,00	0,00	-0,68	0,00	0,29	0,33	0,00	-0,13	0,32	0,00	0,00
E2F1	0,71	-0,40	0,00	0,31	0,67	0,19	0,72	0,17	0,00	0,48	0,00	0,45	0,00	-0,59	0,00	0,22	0,00	0,71	0,00	-0,28	0,74	0,69	-0,07	0,00	-0,76	0,62	0,54	0,54
EGFR	-0,20	0,81	0,00	0,00	0,67	0,37	0,64	0,16	0,00	0,30	0,00	0,79	0,00	0,00	0,00	0,00	0,11	0,00	0,48	0,00	0,41	0,27	0,12	0,00	0,00	0,32	0,42	0,23
EIF4EBP1	-0,18	-0,40	0,18	0,00	0,00	0,50	0,00	-0,18	0,00	0,54	0,07	0,43	0,00	0,00	0,00	0,00	0,17	0,00	0,00	0,00	0,75	1,17	-0,96	0,00	0,00	-0,41	0,00	0,30
ERBB2	0,00	-1,00	0,00	-0,40	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,00	-0,57	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,51	-0,51	-0,06	-0,45	0,00	0,00
ERBB4	0,00	-0,43	0,00	0,33	0,00	0,00	0,00	0,00	0,00	-0,14	0,27	0,00	0,00	0,42	0,00	0,00	0,00	0,00	0,00	-0,54	-0,26	0,00	0,22	0,00	0,00	0,31	0,00	-0,39
ESR1	0,18	-0,42	-0,37	0,00	0,00	-0,24	0,00	0,15	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,47	0,00	0,00	0,00	0,71	0,00
FBXW7	-0,17	0,31	-0,37	-0,14	0,64	-0,23	-0,09	0,00	0,00	0,00	0,00	0,00	-0,66	0,00	0,00	0,00	-0,38	0,00	0,48	0,00	0,00	0,16	0,15	0,34	0,00	-0,46	0,00	0,00
FGF4	-0,17	-0,41	0,00	0,00	0,00	0,00	0,05	-0,09	0,00	0,23	0,00	0,00	0,07	0,00	0,00	0,07	-0,44	0,00	0,00	-0,67	0,00	0,23	0,00	-0,46	-0,09	0,56	0,00	-0,25
FGF9	0,00	-0,40	0,00	-0,40	0,00	1,59	-0,46	-0,16	0,00	0,28	-0,30	0,47	0,00	0,00	0,00	0,00	-0,62	0,00	0,46	-0,67	0,00	0,14	-0,47	-0,42	0,00	-0,41	0,00	-0,87
FGFR1	-0,18	-0,40	0,18	0,00	0,00	0,50	0,00	-0,18	0,00	0,54	0,07	0,43	0,00	0,00	0,00	0,00	0,17	0,00	0,00	0,00	0,75	1,17	-0,96	0,00	0,00	-0,41	0,00	0,30
FGFR2	0,00	0,00	0,30	0,32	0,10	0,00	0,33	0,00	0,00	-0,17	0,00	0,44	0,00	0,00	0,00	0,23	-0,58	0,00	0,47	0,00	0,00	0,36	0,49	0,00	0,00	0,00	0,00	0,00
FGFR3	-0,13	-0,37	-0,44	0,00	0,69	0,00	-0,09	0,19	0,39	0,00	0,00	0,00	0,00	0,00	0,04	0,28	0,18	0,73	0,50	0,00	0,00	0,82	-0,15	-0,16	-0,08	0,00	0,00	0,00
FGFR4	0,54	0,32	0,00	0,00	0,66	0,45	0,50	0,16	0,82	-0,18	0,00	0,43	0,00	0,25	0,00	0,13	0,00	0,75	0,00	0,00	0,74	0,27	0,00	-0,45	-0,09	0,25	0,61	0,32
FLT3	0,00	-0,43	0,00	-0,40	0,00	1,32	-0,46	-0,18	0,00	0,28	-0,30	0,47	0,00	0,00	0,00	0,00	-0,62	0,00	0,46	-0,67	0,00	0,14	-0,47	-0,42	0,00	-0,41	0,00	-0,87
FRS2	0,28	0,31	0,34	0,00	0,67	0,45	0,44	0,16	1,70	0,00	0,41	0,00	0,00	0,25	0,00	0,22	0,10	0,71	0,48	0,00	0,42	0,68	0,22	0,00	0,00	0,32	0,71	0,00
GNAS	0,71	-0,39	-0,06	0,30	0,67	0,12	0,72	0,17	0,00	0,21	0,00	0,45	0,00	0,00	0,00	0,22	0,00	0,71	0,51	-0,28	0,74	0,69	-0,07	0,00	-0,71	0,62	0,73	0,54
GSK3B	0,00	-0,42	-0,38	-0,41	0,00	0,00	0,57	0,07	0,00	0,30	0,00	0,00	0,00	-0,42	0,00	0,00	-0,49	0,00	0,00	0,00	0,00	0,15	0,13	0,00	-0,14	0,32	0,00	0,00
HDAC9	-0,20	0,33	0,00	0,00	0,67	0,37	0,42	0,16	0,00	0,00	0,00	0,79	0,00	0,00	0,00	0,14	0,00	0,00	0,48	0,00	0,41	0,27	0,15	0,00	0,00	0,32	0,42	0,23
HNF1A	0,22	0,00	0,34	0,00	0,65	0,00	0,44	0,16	0,00	0,07	0,40	0,00	0,00	0,25	0,00	0,25	0,00	0,71	0,46	0,00	0,42	0,68	0,22	0,00	-0,08	0,32	0,51	0,00
HRAS	-0,20	-0,32	0,09	0,13	0,00	-0,08	0,07	0,00	0,95	-0,17	0,00	0,00	0,00	0,00	0,12	0,21	-0,45	0,00	0,14	-0,39	0,11	0,09	0,00	-0,47	-0,07	0,50	0,00	-0,19
HSP90AA1	0,00	0,28	0,31	0,05	0,00	0,00	0,36	0,00	0,00	0,28	0,06	0,00	0,00	0,00	0,00	0,06	0,08	0,00	0,00	0,00	-0,25	0,00	0,17	0,18	0,00	0,07	0,00	-0,29
IDH1	0,00	-0,43	0,00	0,33	0,00	0,00	0,00	0,00	0,00	-0,14	0,27	0,00	0,00	0,42	0,00	0,00	0,00	0,00	0,00	-0,54	-0,26	0,00	0,22	0,00	0,00	0,31	0,00	-0,39
IGF1	0,28	0,00	0,34	0,00	0,67	0,00	0,44	0,16	0,00	0,00	0,41	0,00	0,00	0,25	0,00	0,22	0,10	0,71	0,48	0,00	0,42	0,68	0,22	0,00	0,00	0,32	0,71	0,00
IGF1R	0,00	-0,38	0,00	0,00	0,00	0,17	0,00	-0,18	0,11	-0,18	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,47	-0,61	-0,39	0,69	0,20	0,00	0,00	-0,45	0,00	-0,38
IGF2	-0,20	-0,42	0,00	0,13	0,00	-0,08	0,07	0,00	0,95	-0,17	0,00	0,00	0,00	0,00	0,00	0,06	-0,45	0,00	0,14	-0,39	0,00	0,09	0,00	-0,47	-0,07	0,50	0,00	-0,19
IGF2R	0,18	-0,42	-0,37	0,00	0,00	-0,24	0,00	0,15	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,47	0,00	0,00	0,00	0,71	0,00
IGFBP1	-0,20	0,81	0,00	0,00	0,67	0,37	0,64	0,16	0,00	0,00	0,00	0,79	0,00	0,00	0,00	0,13	0,11	0,00	0,48	0,00	0,41	0,27	0,12	0,00	0,00	0,32	0,42	0,23
IGFBP2	0,00	-0,40	0,00	0,33	0,00	0,00	0,00	0,00	0,00	-0,14	0,27	0,00	0,00	0,42	0,00	0,00	0,00	0,00	0,00	-0,54	-0,26	0,00	0,22	0,00	0,00	0,31	0,00	-0,34
IGFBP3	-0,20	0,81	0,00	0,00	0,67	0,37	0,64	0,16	0,00	0,00	0,00	0,79	0,00	0,00	0,00	0,13	0,11	0,00	0,48	0,00	0,41	0,27	0,12	0,00	0,00	0,32	0,42	0,23
IGFBP4	0,00	-1,00	0,00	-0,40	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,00	-0,57	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,51	0,23	-0,06	-0,45	0,00	0,00
IGFBP5	0,00	-0,40	0,00	0,33	0,00	0,00	0,00	0,00	0,00	-0,14	0,27	0,00	0,00	0,42	0,00	0,00	0,00	0,00	0,00	-0,54	-0,26	0,00	0,22	0,00	0,00	0,31	0,00	-0,34
IGFBP6	0,28	0,29	0,34	0,00	0,66	0,44	0,44	0,14	0,79	0,07	0,41	0,00	0,00	0,25	0,00	0,23	0,10	0,70	0,44	0,00	0,42	0,68	0,21	0,00	-0,09	0,32	0,57	0,00
INS	-0,20	-0,42	0,00	0,13	0,00	-0,08	0,07	0,00	0,95	-0,17	0,00	0,00	0,00	0,00	0,00	0,06	-0,45	0,00	0,14	-0,39	0,00	0,09	0,00	-0,47	-0,07	0,50	0,00	-0,19
INSR	0,10	0,26	0,61	0,00	0,59	0,35	0,65	0,07	0,15	0,15	0,51	0,63	0,09	0,00	0,00	0,29	0,34	0,00	0,49	0,00	0,35	0,58	0,00	0,12	-0,16	0,65	0,17	0,34
IRS1	0,00	-0,40	0,00	0,33	0,00	0,00	0,00	0,00	0,00	-0,14	0,27	0,00	0,00	0,42	0,00	0,00	0,00	0,00	0,00	-0,54	-0,26	0,00	0,22	0,00	0,00	0,31	0,00	-0,38
IRS2	0,00	-0,37	0,00	-0,40	0,00	0,15	-0,50	-0,18	-0,69	0,29	-0,34	0,47	0,00	0,00	0,00	0,00	0,19	0,00	0,46	-0,67	0,00	0,14	0,46	0,38	0,00	-0,41	0,00	-0,94
IRS4	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
JAK2	0,33	-0,42	0,00	0,00	0,00	-0,53	0,19	0,00	0,46	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,00	0,00	0,00	-0,60	-0,25	0,00	-0,45	-0,48	0,00	0,63	0,00	-0,38
JAK3	0,10	0,26	0,61	0,00	0,59	0,60	0,65	0,07	0,15	0,15	0,51	0,41	0,09	0,00	0,00	0,29	0,34	0,00	0,49	0,00	0,35	0,58	0,00	0,12	-0,16	0,00	0,17	0,34
KDR	-0,17	-0,42	-0,37	-0,14	0,64	0,00	-0,09	0,00	0,00	0,00	0,00	0,00	-0,66	0,00	0,00	0,00	-0,38	0,00	0,48	0,00	0,00	0,66	-0,17	0,34	0,00	-0,46	0,00	0,00
KIT	-0,17	-0,42	-0,37	-0,14	0,64	0,00	-0,09	0,00	0,00	0,00	0,00	0,00	-0,66	0,00	0,00	0,00	-0,38	0,00	0,48	0,00	0,00	0,66	-0,17	0,34	0,00	-0,46	0,00	0,00
KRAS	0,16	0,32	0,34	-0,39	0,66	0,38	-0,05	0,16	-0,71	-0,11	0,42	0,00	0,00	-0,64	0,00	0,23	0,00	0,70	0,48	0,00	0,42	0,68	0,63	0,00	0,00	0,33	0,72	0,00
MAP3K1	0,54	0,31	0,00	0,00	0,67	0,44	0,61	0,12	0,00	-0,15	0,00	0,44	0,00	0,00	0,00	0,13	0,00	0,75	0,48	0,00	0,74	0,41	0,00	0,00	0,00	-0,18	0,71	0,30
MDM2	0,28	0,31	0,34	0,00	0,67	0,45	0,44	0,16	2,54	0,00	0,41	0,00	0,00	0,25	0,00	0,22	0,10	0,71	0,48	0,00	0,42	0,68	0,22	0,00	0,00	0,32	0,71	0,00
MDM4	0,00	-0,41	0,00	0,32	0,00	0,35	0,41	0,00	0,00	0,24	0,00	0,00	0,42	0,41	0,00	0,00	0,06	0,00	0,00	0,00	-0,24	0,00	0,67	0,62	0,00	0,00	0,00	-0,38
MET	-0,20	-0,05	0,00	0,00	0,67	0,53	0,64	0,15	0,00	0,32	0,00	0,78	0,00	0,00	0,00	0,10	0,14	0,00	0,47	0,00	0,41	0,28	0,12	0,00	0,00	0,31	0,40	0,23
MLH1	0,00	-0,42	-0,38	-0,42	0,00	0,68	0,64	-0,10	0,45	0,21	0,00	0,44	0,00	0,60	0,00	0,00	-0,50	0,00	0,00	-0,68	0,00	0,29	0,33	0,00	-0,13	0,32	0,00	0,00
MPL	-0,20	-0,42	-0,38	0,14	0,00	0,00	0,00	-0,15	0,00	-0,10	-0,12	0,00	-0,56	-0,62	0,00	0,00	-0,42	0,00	0,00	-0,69	-0,23	0,00	-0,48	-0,50	-0,73	0,00	0,00	-0,31
MTOR	-0,20	-0,42	-0,38	-0,40	0,00	0,00	0,00	-0,15	0,00	-0,10	0,00	0,00	-0,56	-0,62	0,00	0,06	-0,38	0,00	0,00	-0,63	-0,23	0,00	-0,48	-0,50	-0,73	0,00	-0,08	-0,31
MYC	-0,18	-0,42	0,18	0,00	0,00	0,94	0,00	-0,18	0,00	0,24	0,07	0,44	0,00	0,00	0,00	0,00	0,17	0,00	0,00	0,00	0,75	1,17	0,00	0,00	0,00	-0,37	0,00	0,29
NOTCH1	0,24	-0,36	0,00	0,00	-0,11	0,62	0,10	0,00	0,17	0,31	-0,11	0,00	0,00	0,00	0,05	0,08	0,00	0,00	0,08	0,00	-0,22	0,68	0,42	0,33	-0,12	0,68	0,00	-0,26
NOTCH4	0,00	-0,40	-0,33	-0,39	0,00	0,00	0,00	0,15	0,00	0,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,22	0,00	0,00	0,00	0,37	0,00
NPM1	0,54	0,32	0,00	0,00	0,66	0,45	0,61	0,13	0,82	-0,18	0,00	0,43	0,00	0,25	0,00	0,13	0,00	0,75	0,00	0,00	0,74	0,27	0,00	-0,45	0,00	-0,44	0,61	0,32
NRAS	-0,19	-0,42	-0,37	0,00	0,00	0,00	0,00	-0,18	0,00	-0,14	-0,32	0,00	-0,61	-0,57	0,00	0,00	0,07	0,00	0,00	-0,67	-0,25	0,00	-0,48	-0,43	-0,73	0,00	0,00	-0,36
PAK1	-0,17	-0,41	0,00	0,00	0,00	0,00	0,00	-0,15	0,00	0,23	0,00	0,00	0,00	0,00	0,00	0,00	-0,44	0,00	0,00	-0,67	0,00	0,00	0,00	-0,48	-0,09	0,56	0,00	-0,34
PDGFRA	-0,17	-0,42	-0,37	-0,14	0,64	0,00	-0,09	0,00	0,00	0,00	0,00	0,00	-0,66	0,00	0,00	0,00	-0,38	0,00	0,48	0,00	0,00	0,66	-0,17	0,34	0,00	-0,46	0,00	0,00
PDPK1	0,16	-0,38	0,00	0,11	0,63	0,36	0,00	0,21	0,00	0,28	0,07	0,60	0,12	0,00	0,07	0,25	0,00	0,68	0,49	0,00	0,29	0,64	-0,09	-0,12	-0,14	0,43	0,00	0,29
PGR	-0,21	-0,44	0,00	0,00	0,00	0,00	-0,12	-0,19	0,00	0,15	0,00	0,00	0,00	0,00	0,00	0,00	-0,44	0,00	0,00	-0,67	0,00	0,00	0,00	-0,48	0,00	-0,45	0,00	-0,40
PIK3CA	0,00	-0,42	-0,38	-0,41	0,00	0,00	0,56	0,06	0,00	0,13	0,00	0,00	0,00	-0,63	0,00	0,00	-0,53	0,00	0,00	0,00	0,00	0,00	-0,10	0,00	-0,15	0,32	0,00	0,00
PIK3CB	0,00	-0,42	-0,38	-0,41	0,00	0,00	0,57	0,06	0,00	0,30	0,00	0,00	0,00	0,82	0,00	0,00	-0,50	0,00	0,00	0,00	0,00	0,15	0,13	0,00	-0,14	0,32	0,00	0,00
PIK3R1	0,54	0,31	0,00	0,00	0,67	0,44	0,61	0,12	0,00	-0,15	0,00	0,44	0,00	0,00	0,00	0,13	0,00	0,75	0,48	0,00	0,74	0,41	0,00	-0,48	0,00	-0,21	0,43	0,30
PLK1	0,16	-0,38	0,00	0,00	0,63	0,36	0,00	0,15	0,00	0,18	0,00	0,44	0,00	0,00	0,00	0,25	0,00	0,68	0,47	0,00	0,00	0,64	-0,16	-0,12	0,00	0,30	0,00	0,29
PSMA2	-0,20	0,81	0,00	0,00	0,67	0,37	0,64	0,16	0,00	0,00	0,00	0,79	0,00	0,00	0,00	0,13	0,12	0,00	0,48	0,00	0,41	0,27	0,12	0,00	0,00	0,32	0,42	0,23
PSMA3	0,00	0,31	0,34	0,00	0,00	0,00	0,36	-0,16	0,00	0,16	0,00	0,00	0,00	0,00	0,00	0,00	0,22	0,00	0,00	0,00	-0,25	0,00	0,10	0,32	0,00	0,00	0,00	-0,38
PSMA4	0,00	0,00	0,00	0,00	0,00	0,18	0,00	-0,18	0,78	-0,18	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,47	-0,67	-0,39	0,69	0,20	0,00	0,00	-0,45	0,00	-0,38
PSMA6	0,00	0,31	0,34	0,00	0,00	0,00	0,36	-0,16	0,00	0,16	0,00	0,00	0,00	0,00	0,00	0,00	0,22	0,00	0,00	0,00	-0,25	0,00	0,10	0,32	0,00	0,00	0,00	-0,38
PSMB5	0,00	0,31	0,46	0,00	0,00	0,41	0,36	-0,16	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,36	0,00	0,00	0,00	-0,25	0,00	0,00	0,32	0,00	0,00	0,00	-0,38
PSMB8	0,00	-0,40	-0,37	-0,39	0,00	0,00	0,05	0,15	0,00	0,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,69	0,00	0,00	0,43	0,00	0,00	0,00	0,37	0,00
PSMB9	0,00	-0,40	-0,37	-0,39	0,00	0,00	0,05	0,15	0,00	0,25	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-0,69	0,00	0,00	0,43	0,00	0,00	0,00	0,37	0,00
PSMD5	0,16	-0,39	0,00	0,31	0,00	0,53	0,08	0,00	0,41	0,39	0,00	0,00	0,00	0,41	0,00	0,00	0,00	0,00	0,00	0,00	-0,25	0,71	0,11	0,33	0,00	0,63	0,00	-0,37
PSME3	0,00	-1,00	0,00	-0,40	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,00	-0,57	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,51	0,23	-0,06	-0,45	0,00	0,00
PTEN	0,00	0,00	0,30	0,57	0,10	0,19	0,33	0,00	-0,69	-0,17	0,00	0,44	0,00	0,00	0,00	0,23	-0,58	0,00	0,47	0,00	0,00	0,25	-0,49	0,00	0,00	0,00	0,00	0,00
PTK2	-0,18	-0,40	0,18	0,00	0,00	-0,19	0,00	-0,09	-0,65	0,40	-0,10	0,44	0,00	0,00	0,00	0,00	0,12	0,00	0,00	0,00	0,75	1,17	0,00	0,00	-0,11	-0,37	0,00	0,29
PTPN11	0,22	0,00	0,34	0,00	0,65	0,00	0,44	0,16	0,00	0,07	0,40	0,00	0,00	0,25	0,00	0,25	0,00	0,71	0,46	0,00	0,42	0,68	0,22	0,00	-0,08	0,32	0,57	0,00
RB1	0,00	-0,43	0,00	-0,40	0,00	0,18	-0,46	-0,18	0,00	0,29	-0,34	0,47	-0,63	0,00	0,00	0,00	0,00	0,00	0,46	-0,67	0,00	0,14	-0,47	-0,42	0,00	-0,41	0,00	-0,87
RET	0,00	0,32	0,30	0,32	0,08	0,31	0,33	0,00	-0,75	-0,16	0,17	0,44	0,00	0,00	0,00	0,23	0,11	0,00	0,46	0,00	0,00	0,25	0,00	0,00	0,00	0,00	0,00	0,00
RPS6KB1	0,00	-0,42	0,00	0,00	0,00	0,00	0,00	0,00	0,38	0,37	0,00	0,00	-0,64	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,51	0,23	0,00	0,29	0,00	0,00
RPTOR	0,15	-0,36	0,00	0,00	0,00	0,32	0,61	0,08	0,40	0,37	0,00	0,09	-0,41	0,00	0,05	0,09	-0,47	0,00	0,10	-0,69	0,00	0,00	-0,51	1,22	-0,13	0,39	0,00	0,07
SMAD4	-0,42	-0,41	-0,39	0,00	0,00	0,23	0,00	-0,18	-0,69	0,15	0,14	0,00	0,00	-0,54	0,00	0,00	0,07	0,00	0,00	-0,66	-0,59	0,18	-0,43	0,37	0,00	-0,42	0,00	-0,92
SMARCB1	-0,11	-0,40	-0,38	-0,37	0,00	-0,18	0,00	-0,30	0,00	0,21	-0,28	0,00	-0,48	0,25	0,00	0,07	-0,47	0,00	0,00	-0,64	-0,21	0,00	-0,50	-0,09	-0,11	-0,38	-0,56	-0,29
SMO	-0,20	0,00	0,00	0,00	0,67	0,53	0,64	0,15	0,00	0,36	0,00	0,78	0,00	0,00	0,00	0,15	0,12	0,00	0,47	0,00	0,41	0,40	0,12	0,00	0,00	0,31	0,40	0,23
SRC	0,71	-0,39	0,00	0,30	0,67	0,19	0,72	0,17	0,00	0,48	0,00	0,45	0,00	-0,59	0,00	0,00	0,00	0,71	0,00	-0,28	0,74	0,69	-0,07	0,00	-0,76	0,62	0,54	0,54
STK11	0,10	0,40	0,61	0,00	0,59	0,35	0,65	0,07	0,15	0,15	0,51	0,52	0,09	0,00	0,00	0,29	0,34	0,00	0,49	0,00	0,00	0,58	0,00	0,12	-0,16	0,15	0,17	0,34
TOP2A	0,00	-1,00	0,00	-0,40	0,00	0,00	0,00	0,00	-0,67	0,00	0,00	0,00	-0,57	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,51	-0,51	-0,06	-0,45	0,00	0,00
TP53	-0,20	-0,41	0,00	-0,40	0,00	0,00	0,00	-0,14	-0,53	0,34	0,00	-1,58	0,00	0,00	0,00	0,00	-0,47	0,00	0,00	-0,69	0,00	0,00	-0,50	0,25	-0,12	-0,42	0,00	0,00
TSC1	0,24	-0,36	0,00	0,31	0,00	0,62	0,10	0,00	0,49	0,50	0,00	0,10	0,00	0,00	0,05	0,08	-0,07	0,00	0,08	0,00	-0,22	1,07	0,42	0,33	-0,12	0,68	0,00	-0,26
TSC2	0,16	-0,38	0,00	0,11	0,63	0,36	0,00	0,21	0,00	0,28	0,07	0,60	0,12	0,00	0,07	0,25	0,00	0,68	0,49	0,00	0,29	0,64	-0,09	-0,12	-0,14	0,43	0,00	0,29
VEGFA	0,17	-0,40	-0,37	0,00	0,00	0,00	0,05	0,15	0,00	0,56	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,97	0,00	0,00	0,00	0,37	0,00
VHL	0,00	-0,42	-0,38	-0,42	0,00	0,68	0,64	-0,10	0,00	0,28	0,00	0,44	0,00	0,00	0,00	0,00	-0,50	0,00	0,00	-0,68	0,00	0,29	-0,45	0,00	-0,13	0,32	0,00	0,00

Expression of IGF/mTOR pathway components in human pheochromocytomas and *in vitro* inhibition of pheochromocytoma cell growth by mTOR inhibitors and the dual IGF1/Insulin-Receptor antagonist OSI-906

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ABSTRACT

Context. Dysregulation of the IGF and mTOR pathways have been suggested to be involved in the pathogenesis of pheochromocytomas (PCC). mTOR inhibitors, as sirolimus and everolimus, as well as IGF1-Receptor [IGF1-R] antagonists, such as OSI-906 could be a novel treatment option for malignant PCC.

Objective. To evaluate the expression of the main components of the IGF/mTOR pathway in human PCC and to investigate the effects of sirolimus, everolimus and OSI-906 (IGF1-R/Insulin receptor [IR] blocker), alone and in combination, in a rat PCC cell model.

Design and Methods. mRNA expression of IGF1, IGF2, IGF1-R, IR subtypes A and B, IGF2R, IGF-Binding-Proteins 1, 2, 3 and 6, mTOR, 4EBP1 and S6K1 was evaluated in 24 human PCC by quantitative-PCR. In PC12 cells, the effect of mTOR inhibitors and OSI-906 on cell growth and apoptosis were tested by measurement of total DNA-content and DNA-fragmentation, respectively.

Results. All investigated components were expressed in human PCC. A high expression of IGF2 mRNA and an increased IRA/IRB ratio was found. mTOR inhibitors and OSI-906 were able to suppress PC12 proliferation in a dose and time-dependent manner. After a 6 days, maximal inhibitory effects of sirolimus, everolimus and OSI-906 on PC12 cell proliferation were 52%, 43%, and 69% respectively. OSI-906 strongly stimulated cell apoptosis. Combined treatment of sirolimus with OSI-906 had additive antiproliferative effects.

Conclusion. The results of the current study suggest the use of OSI-906, alone or in combination with mTOR inhibitors, as a new treatment option in progressive PCCs patients.

INTRODUCTION

Pheochromocytomas (PCCs) and paragangliomas PGLs are rare neuronal crest- derived neuroendocrine tumors arising from the adrenal medulla or the extra-adrenal paraganglia.[1, 2] During the last 20 years important progress has been made in discovering genetic alterations that can lead to development of PCCs and PGLs in the context of familial syndromes and in some sporadic cases.[1, 2] Malignant PCCs are rare and can be defined only based on the presence of metastatic disease.[1] Conversely, scant progress has been made in treating patients with malignant PCCs. In progressive PCCs, treatment with meta-iodobenzylguanidine (^{131}I-MIBG) or certain types of systemic chemotherapy are used, but a low rate of response and frequent recurrences underline the need of new treatment approaches.[1, 3]

The mammalian target of rapamycin (mTOR) is a protein kinase of the PI3Ks/AKT signaling pathway that mediates the pro-growth effects of several growth factors including insulin and insulin-like growth factors (IGFs).[4, 5] Dysregulation of the mTOR pathway has been found in many human tumors, including neuroendocrine tumors (NETs).[4-6] Recently, everolimus, an mTOR inhibitor has been approved for the treatment of pancreatic NETs.[6] PCCs and pancreatic NETs share some common pathogenic molecular events (NF1 and VHL mutations), therefore mTOR inhibitors have been suggested as a potential new treatment modality for progressive metastatic PCCs.[7, 8]

The insulin/IGF system plays a pivotal role in the regulation of growth, lifespan and metabolism. This system is composed of circulating ligands, i.e. insulin IGF1 and IGF2 (IGFs); circulating and tissue binding proteins (BP1-6 that modulate the bioavailability of IGFs) and multiple receptors.[9] The circulating ligands can exert their effects as endocrine and/or paracrine factors. Among the receptors, the IGF1 receptor (IGF1R) and the insulin receptor isoforms A and B (IR-A and IR-B) are tyrosine-kinase receptors. The IGF2 receptor (IGF2R) is a scavenger receptor involved in the internalization and degradation of IGF2. In adult humans, insulin predominantly exerts metabolic effects through the activation of IRB and IGFs. Particularly IGF1, mainly exerts pro-growth effects through the activation of IGF1R receptors. IGF2 and IRA are predominantly expressed during fetal development, while their expression in cancer tissues could contribute to cancer growth. Currently, the efficacy of several IGF1R and IGF1R/IR antagonists is evaluated in clinical trials alone or in combination with other agents for the treatment of several malignant disorders.[10, 11] IGF1R, IR and IGF2 are expressed in PCCs and IGF1R seems to be overexpressed in PCCs compared with normal adrenals.[12-14] Therefore, insulin and IGFs could be part of autocrine/paracrine loops and play a role in the pathogenesis of PCCs/PGL. To the best of our knowledge the expression of IRA in human PCCs and the effects of an IGF1R/IR antagonist alone or in combination with mTOR inhibitors in preclinical models of PCCs/PGL have not been described to date.

The aim of this study was to describe the mRNA expression of the main components of the IGF/mTOR pathways in human PCC and to evaluate the *in vitro* effects of mTOR inhibitors (sirolimus end everolimus) and the IGF1R/IR antagonist OSI-906 on cell proliferation and apoptosis in the PC12 rat pheochromocytoma cell line.

MATERIALS AND METHODS

Subject

Twenty-four human PCCs (including one paraganglioma) samples were collected at the Erasmus Medical Center, Rotterdam (The Netherlands), between 2001 and 2009.

The following clinical parameters were recorded: date of diagnosis, age, gender, presence of hormonal hyper-secretion, genetic cause, tumor size, origin of samples, local invasion (vaso-, capsule- and periadrenal fat-invasion), presence of metastatic disease (local lymph nodes, distant metastases) at sampling time, and systemic antineoplastic treatment or radiotherapy before tumor collection.

According to the current World Health Organization (WHO) classification, malignant PCCs were defined by the presence of metastases to a site where pheochromocytoma/paraganglionic tissue is not normally present. Genetic tests for genes predisposing to a genetic syndrome associated with PCCs were performed only in patients with clinical suspicion of genetic syndromes, according with local clinical practice at time of clinical diagnosis. Clinical data were obtained from medical records. This study was approved by the Medical Ethics Committee of the Erasmus Medical Center.

Total RNA isolation

From snap frozen PCC tissues, total-RNA was isolated using a commercially available kit (High Pure RNA Tissue kit; Roche, Almere, The Netherlands). The cDNA synthesis from total-RNA and quantitative PCR were performed as previously described.[15]

Quantitative RT-PCR

In all selected samples we evaluated the mRNA expression of: IGF1, IGF2, IGF1-Receptor [IGF-R], Insulin-Receptor[IR]A, IRB, IGF2R, IGF-Binding-Proteins [BP] 1, 2, 3 and 6, mTOR, 4EBP1, S6K1 and of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) by quantitative RT-PCR.

The primers and probes were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The sequence of the primers and probes used are reported in the supplemental material-table 1. Samples were normalized to the expression of HPRT as previously reported.[15]

Drugs and reagents

Sirolimus, everolimus and OSI-906 were purchased by LC Laboratories (Inc. Woburn, MA, USA) and prepared as a 10^{-3}M stock solution in dimethylsulfoxide (DMSO). Compounds were stored at -20°C and further diluted in 40% DMSO before the use. Final DMSO concentration, also added as vehicle to controls, was 0.4%.

Cell lines and culture conditions

The rat pheochromocytomas cell line PC12 cell line were an obtained from from Clontech and grown as specified by the manufacturer.

The cells were cultured in 75-cm^2 culture flasks at 37°C in a humidified incubator at 5% CO_2. The culture medium consisted of DMEM medium, supplemented with 10% horse serum, 5% fetal calf serum (FCS), penicillin ($1x10^5$ U/liter), and streptomycin (100 mg/L). Cells were harvested with trypsin (0.05%)-EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 95%. Media and supplements were obtained from Invitrogen (Breda, The Netherlands).

Measurement of total DNA content assay

Measurement of total DNA content assay was used to determine the effects of the compounds on cell proliferation. Cells were plated in 1 ml of medium in 24-well plates at the density necessary to obtain a 65-70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later compounds were added to wells in quadruplicate. The concentrations of sirolimus and everolimus tested in PC12 ranged between 10^{-12}M and 10^{-6}M. The concentrations of OSI-906 tested in PC12 ranged between 10^{-9}M and 10^{-5}M. In combination experiments the concentration of compounds tested were selected according on 6 days-cell proliferation experiments. For a 6 day incubation experiment, medium was refreshed at day 3 with fresh compounds added. After 3 and 6 days of treatment, the cells were harvested for DNA measurement, as a measure of cell number. Measurement of total DNA content was previously described in detail.[16]

DNA fragmentation assay

DNA fragmentation assay was used to determine the effects of the compounds on apoptosis. The cells were plated in 24-well plates and treated as above described for the cell proliferation assay. After 24 hours and 3 days of incubation, DNA fragmentation was determined using a commercially available ELISA kit (Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used. The same plates were also analyzed for the measurement of total DNA content. The amount of DNA-fragmentation (apoptosis) was corrected for the total DNA content in each well.

Statistical analysis

For the statistical analysis statistical software of SPSS (SPSS 15.0; SPSS Inc., Chicago,IL) and GrafPad Prism 3.0 (GraphPhad Software, San Diego, CA) was used.

Non-parametric tests were used to evaluate the differences among groups (Mann-Whitney test and Kruskall-Wallis test for comparison among two or more groups, respectively). The Spearman's rank coefficient was used to test correlation.

The comparative statistical evaluations among treatment groups were performed by ANOVA, followed by a multiple comparative test (Newman-Keuls).

RESULTS

Study population

The IGF and mTOR pathway components were examined in tumor tissue obtained from 24 adult PCC patients. The main clinical characteristics of these patients are reported in table 1. Three patients with PCC had metastatic disease, 20 had benign PCC and 1 had a benign paraganglioma. Among benign PCC only two had some unfavourable features (local invasion) at pathology. Four patients had multiple endocrine neoplasia type II (MEN2A), 2 patients had Von Hippel Lindau disease (VHL) and 2 patients had neurofibromatosis type 1 (NF1). Following the cluster definition as described by Dahia P.M.L. *et al*.[17] and based on the available genetic information, our study population could be divided in three groups: sporadic/genetic-unknown-background; cluster 1 (including the 2 VHL patients) and cluster 2 (including the 4 patients with MEN2A and 2 with NF1) (table 1).

mRNA expression of IGF and mTOR pathway components in human pheochromocytoma tissue

Figure 1 shows the mRNA levels of IGF1, IGF2, IGF1-Receptor [IGF1-R], Insulin-Receptor[IR] A, IRB, IGF2R, IGF-Binding-Proteins [BP] 1, 2, 3 and 6, mTOR, 4EBP1 and p70S6K1 (S6K1) as assessed by quantitative RT-PCR in the full study population. The examined IGF and mTOR components were expressed in all examined PCC tissue samples, but in particular a high expression of IGF2 mRNA was found. Indeed, mean IGF2 expression levels, normalized to HPRT, were about 80 to 30000 fold higher than the mean expression levels of any of the other evaluated components, normalized to HPRT. The majority of samples presented higher mRNA levels of IRA than IRB (0,109±0.016 vs 0.047±0,019; p=0.0097). Indeed IRA/IRB ratio was equal or higher than 1 in all samples but three. Among these last three samples both VHL samples were included. Median mRNA levels of IRA were similar to IGF1R (0.078±0,010; p=0.15), but significantly higher than IGF2R (0.035±0,005; p<0.0001). Overall, positive correlations were found between the mRNA levels of components of the IGF pathway with those of mTOR pathway components (table 2). In particular, the mRNA

Table 1. Main clinical characteristics of evaluated patients

Patient	Age at diagnosis (years)	Gender	Proven genetic syndrome	Hormonal secretion	Primary tumor localization	Local invasion	Lymph node metastasis	Distant metastasis	Tumor longer diameter (cm)	Privius systemic antineoplastic treatments or radiotherapy	Sample origin
1	17	m	Von Hippel Lindau	Yes	Right adrenal	No	No	No	5	No	Primary
2	39	m	Von Hippel Lindau	Yes	Bilateral	No	No	No	7	No	Primary
3	37	f	Neurofibromatosis	Yes	Right adrenal	No	No	No	8	No	Primary
4	53	f	Neurofibromatosis	Yes	Right adrenal	No	No	No	7	No	Primary
5	38	f	MEN IIA	No	Left adrenal	No	No	No	8	No	Primary
6	26	m	MEN IIA	Yes	Bilateral	No	No	No	2,1	No	Primary
7	21	m	MEN IIA	Yes	Left adrenal	Yes	No	No	1,9	No	Primary
8	61	f	MEN IIA	Yes	Bilateral	Yes	No	No	9	No	Primary
9	30	m	No	Yes	Right adrenal	No	No	No	6,5	No	Primary
10	66	f	No	Yes	Right adrenal	No	No	No	2,2	No	Primary
11	41	f	No	Yes	Right adrenal	No	No	No	5,6	No	Primary
12	63	f	No	Yes	Left adrenal	No	No	No	6	No	Primary
13	39	m	No	Yes	Right adrenal	No	No	No	6,5	No	Primary
14	47	m	No	Yes	Right adrenal	No	No	No	4	No	Primary
15	76	f	No	Yes	Right adrenal	No	No	No	5	No	Primary
16	32	f	No	Yes	Right adrenal	Yes	No	No	5	No	Primary
17	30	f	No	Yes	Left adrenal	No	No	No	7	No	Primary
18	43	m	No	Yes	Left adrenal	No	No	No	3	No	Primary
19	63	m	No	Yes	Right adrenal	No	No	No	4	No	Primary
20	73	f	No	Yes	Left adrenal	No	No	No	3,5	No	Primary
21	43	f	No	Yes	Left adrenal	Yes	Yes	No	1,3	No	Primary
22	75	m	No	Yes	Left adrenal	Yes	Yes	No	11	No	Primary
23	42	m	No	Yes	Right adrenal	Yes	Yes	No	8,5	No	Primary
24	56	f	No	Yes	Paraganglioma	No	No	No	6	No	Primary

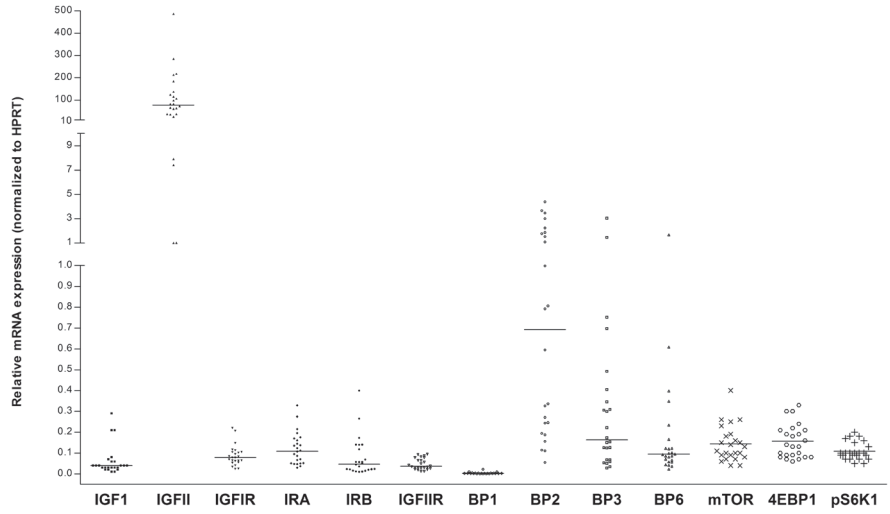

Figure 1. mRNA levels expression of the main component of the IGFs and mTOR pathways in a cohort of 24 human pheochromocytomas (expressed as relative mRNA expression as normalized to the house-keeping gene HPRT).

Table 2. Main correlation observed between the components of the IGF and mTOR pathways

	Corelation with	Coefficient	P value
mTOR	S6K1	,803**	*,000*
	IGF-IR	,451*	*,027*
	IR-A	,746**	*,000*
	IR-B	,643**	*,001*
	IGF-IIR	,850**	*,000*
	BP-1	,475*	*,019*
	BP-3	,464*	*,022*
4EBP1	IGF-I	,423*	*,039*
	IGF-II	,703**	*,000*
	IR-A	,621**	*,001*
	IR-B	,674**	*,000*
	BP-1	,522**	*,009*
	BP-3	,620**	*,001*
S6K1	mTOR	,803**	*,000*
	IGF-IR	,556**	*,005*
	IR-A	,725**	*,000*
	IR-B	,598**	*,002*
	IGF-IIR	,764**	*,000*

level of all evaluated components of the mTOR pathway strongly correlated with IRA and IRB mRNA expression. No correlation was observed between the mRNA levels of the evaluated components of the IGF or mTOR pathway and clinical features including the age of diagnosis and tumor diameter. In addition, no statistical significant differences were observed in IGF- and mTOR component mRNA expression between benign and malignant tumors and between PCCs associated with genetic syndromes, nor between samples belonging to cluster 1 or 2 and the apparently sporadic ones.

However, samples of the same cluster (1 or 2) showed similarities in terms of increased expression or reduced expression of some components of the IGF/mTOR pathways (figure 2). Of note, both VHL samples (cluster 1) and the PGL studied had very high IGF2, BP3 levels and IRA/IGF1R ratio, while low levels of these parameters were observed in most MEN2A and NF1 samples (cluster 2). Conversely, cluster 1 samples had low IRA/IRB ratio and low IGF1R levels, while often high levels of these parameters were observed in cluster 2. Anyway this observation are very speculative because the genetic test had been performed only in few cases.

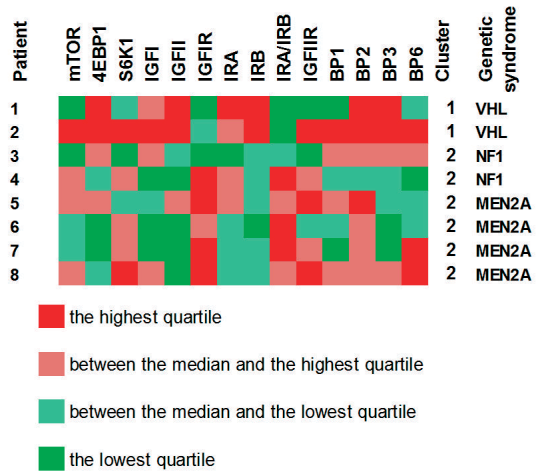

Figure 2. Graphic representation of the relative mRNA expression of the main component of the IGFs and mTOR pathways in a subgroup of 8 human pheochromocytoma with proven genetic syndrome as compared with the expression levels of the same components in the whole population of human pheochromocytoma evaluated (24 cases). The different colors represent different quartiles of expression levels.

Effects of sirolimus and everolimus in rat pheochromocytomas PC12 cell line

In the PC12 cell line sirolimus and everolimus significantly suppressed the cell growth in a dose and time-dependent manner (figure 3). After 6 days of treatment sirolimus was slightly, but significantly, more potent than everolimus in terms of EC_{50} (EC_{50}: $2x10^{-9}$ M vs $1.5x10^{-8}$ M; $p<0.0001$) and with respect to the maximal effect observed at the high-

est dose used (p=0.007). The effects of sirolimus after 6 days ranged between 52.4% inhibition (p<0.001) at the maximal dose (10^{-6}M) and 14% (p<0.05) at the dose of 10^{-12}M. The effects of everolimus after 6 days ranged between 43% inhibition (p<0.001) at the maximal dose (10^{-6}M) and 29.6% (p<0.05) at the dose of 10^{-8}M.

Three- days treatment with both compounds induced apoptosis in PC12 cells, only at the highest dose used (10^{-6}M; data not shown).

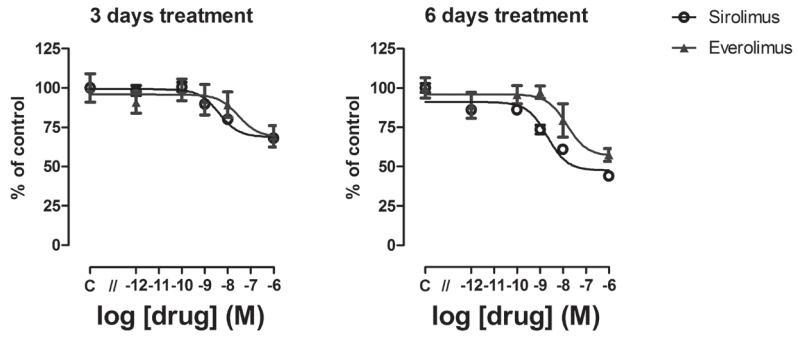

Figure 3: Dose/time-dependent effect of sirolimus (O) and everolimus (▲) treatment on PC12 cell proliferation, espresse as DNA content/well after 3 days (upper panel) and 6 days (lower panel). Data are expressed as the percentage of control and represent the mean± DS. Control is set as 100%.

Effects of OSI906 alone or in combination with sirolimus or everolimus in rat pheochromocytomas PC12 cell line

To evaluate the in vitro effects of IGF1R/IR antagonist in PCC, we tested the effects of 3 and 6 days treatment with OSI-906 on DNA content (representing cell number) and of 3 days on DNA fragmentation in PC12 cells.

OSI-906 significantly suppressed cell growth in a dose and time-dependent manner (figure 4A). Maximal inhibition by 98.6% (P<0.001) was observed at a dose of 10^{-5}M; EC_{50} was $2.7x10^{-7}$M.

OSI-906 significantly increased the apoptosis in a dose-dependent manner (figure 4B). These effects ranged between 33% stimulation (p<0.01) at the dose of 10^{-7}M and 1249% (p<0.001) at the dose of 10^{-5}M.

The anti-proliferative effects of 6 days combined treatment with OSI-906 ($2.5x10^{-7}$M and 10^{-7}M; approximately corresponding to the EC_{50} and EC_{25}) and sirolimus (10^{-8}M and $5x10^{-9}$M, approximately corresponding to the EC_{50} and EC_{25}) or everolimus (10^{-6}M and 10^{-8}M, approximately corresponding to the maximal concentration tested and the EC_{50}) were evaluated. Combination of both mTOR inhibitor with the OSI-906 showed statistically significant additive effects (figure 4C-F).

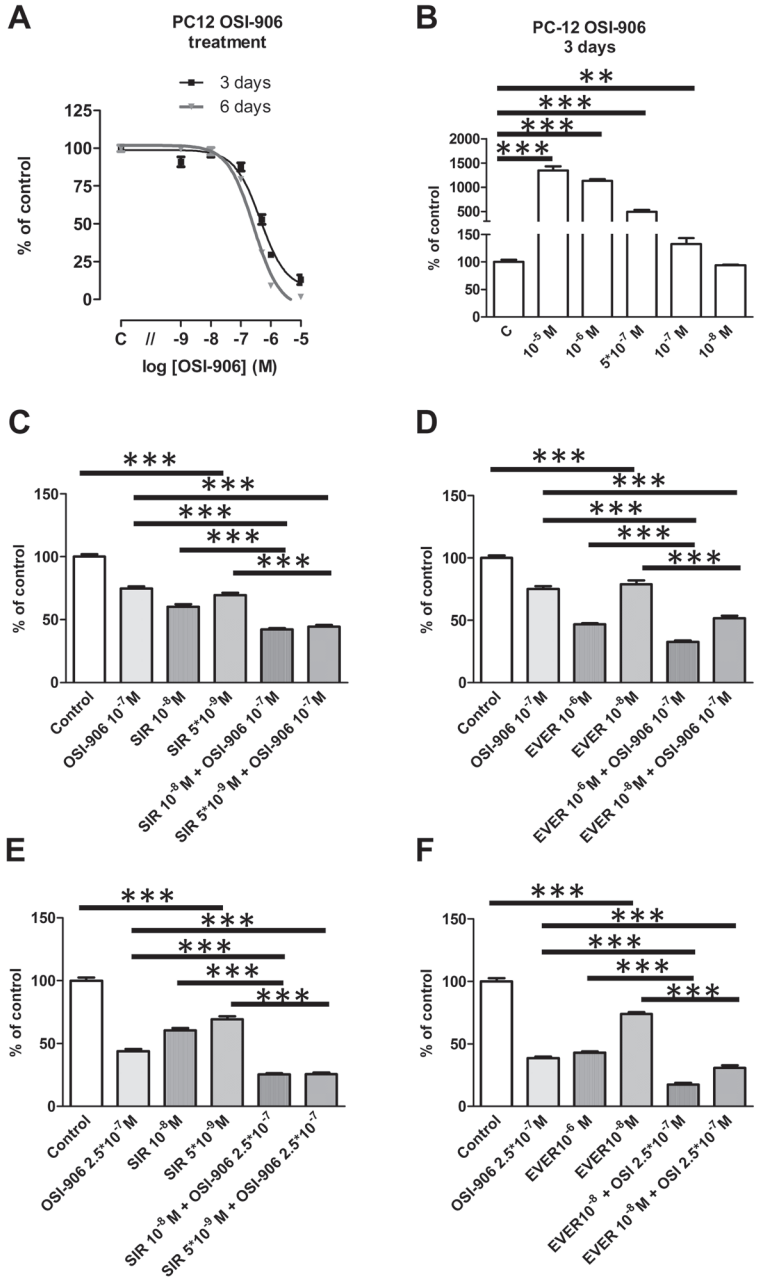

Figure 4: Effects of OSI-906 alone or in combination with mTOR inhibitors on PC12 cells. Panel A: dose/time-dependent effect of OSI-906 treatment on PC12 cell proliferation, expressed as DNA content/well. Panel B: dose-dependent effect of OSI-906 treatment on PC12 cell apoptosis, normalized versus the DNA content of each well. Panel C-F: additive effects of combined treatment OSI-906 and sirolimus or everolimus on PC12 cell proliferation. Data are expressed as the percentage of control and represent the mean±.S. D.. Control is set as 100%. **p<0,01; *** p<0,001.

DISCUSSION

In the present study we examined the interrelationship between IGF and mTOR pathways in PCC tissues, as well as the antiproliferative *in vitro* effects of mTOR inhibitors, an IGF1R/IRA inhibitor, and their combination in a PCC cell line model. This study demonstrates the presence of IGF/mTOR pathway components and a high IRA/IRB mRNA ratio in the majority of human PCCs, suggesting the existence of an IGF2-IRA autocrine/paracrine loop in human PCCs, in addition to the already suggested IGF2-IGF1R loop.[12-14] Drugs inhibiting mTOR or IGF1R/IRA suppress cell proliferation in the rat PCC cell line PC12. OSI-906, an IGF1R/IRA inhibitor, strongly induces cell apoptosis and combining OSI-906 with mTOR inhibitors shows additive antiproliferative effects.

The mTOR pathway is considered as a potential target for treatment of PCC patients with progressive disease.[7, 8] The mTOR, 4EBP1 and S6K1 mRNA levels were not significantly different in 2 VHL patients (cluster 1) as compared with the group including the MEN2A and NF1 (cluster 2) patients or in malignant versus benign PCCs. However, this could depend on the small study population. Several positive correlations between the mRNA levels of the different components of the IGF and mTOR pathways were observed. Particularly, both kinases of the mTOR pathway, mTOR and S6K1, showed a significant positive correlation with all the IGF receptors evaluated, suggesting that the expression of these genes share common mechanisms of regulation. Conversely 4EBP1, the negative regulator of the mTOR pathway activation is positively correlated with both IGF1 and IGF2, also suggesting that the expression of these genes share common mechanisms of regulation and that the increased expression of 4EBP1, in parallel with the increased expression of IGFs, could be a mechanism to balance the activation of mTOR pathway induced by IGFs. IRA is an isoform of the IR with higher affinity for IGF2 than IGF1R. IRA expression in malignant tumor tissue has been suggested to be involved in cancer development.[9] To our knowledge this is the first study demonstrating the presence of comparable levels of IRA and IGF1R mRNA and an increased IRA/IRB ratio in PCCs, suggesting a potential role of IGF2-IRA autocrine loop in PCC proliferation. In the samples for which genetic testing was available we could identify some similarities among samples belonging to the same cluster. Both VHL mutated PCC (cluster 1) presented a down-regulation of IGF1R and an up-regulation of BP3, while an inverse profile was observed in MEN2A and NF1 mutated samples (cluster 2), in agreement with previous reports.[18] IGF/insulin pathway could thus play a role in both cluster 1 and 2 PCC proliferation, although through potentially different mechanisms. In MEN2A and NF1 mutated samples (cluster 2) a high level of IGF1R and IRA/IRB ratio was observed in the majority of PCC (figure 2), whereas in VHL mutated samples (cluster 1) in particular high levels of IGF1, IGF2, IRA and IRA/IGF1R ratio were found.

In an animal model for pheochromocytoma, temsirolimus, an mTOR inhibitor, was able to inhibit tumor progression.[19] Few studies have evaluated the effects of mTOR inhibitors in PCC cell lines.[7] Rapamycin, evaluated as a single dosage (10^{-8}M), has been found to significantly inhibit cell growth in normal chromaffin rat cells, but not in the PC12 cell line.[20] Short term treatment with rapamycin or everolimus were found to have modest inhibitory effects on cell viability in MTT cells, a more recently established mouse PCC cell line.[21, 22] The present study confirmed that short term treatment (up to 3 days) with rapamycin or everolimus has only modest antiproliferative effects in the PC12 cell line, but it also showed that longer treatment (up to 6 days) results in a stronger inhibition of PC12 growth, already at a doses that can be achieved in vivo in humans (maximal concentration about 10^{-7}M).[23, 24] However, the observed anti-proliferative effects did not exceed 50% cell growth inhibition and were not associated to a significant induction of cell apoptosis. It was previously reported that S6K1 silencing induces apoptosis in PC12[25] whereas in the present study mTOR inhibitors induced significant apoptosis in PC12 only at high dose (10^{-6}M). Another study demonstrated by western-blot that high doses of rapamycin (10^{-6}M) were required to strongly inhibit pS6 phosphorylation (target of S6K1) in MTT PCC cells.[21] These results suggest that the inhibition of S6K1 can induce cell apoptosis in PCC cells, but the concentration of rapamycin required to stimulate cell apoptosis is probably too high. These results are consistent with the early clinical experience with the use of everolimus in PCC patients suggesting that everolimus can induce disease stabilization in some patients but it does not induce objective tumor response.[26, 27]

Similar to human PCC, PC12 cells express both IGF1R and IR and IGF2.[28] IGF1, IGF2 and insulin can stimulate PC12 proliferation.[28] Therefore, there is evidence of a potential autocrine/paracrine loop of IGFs in these cells. The pro-growth and anti-apoptotic effects of IGF1 have also been demonstrated in MPC cells, a more recently established mouse PCC cell line.[29] In both MPC and PC12 cells, IGF1 activates both AKT and ERK pathways.[29] *Fernández et al.* demonstrated that liver-IGF1-deficient mice (LID) were less predisposed to develop PCC than control mice when injected with MPC cells, but the administration of IGF1 to LID mice could accelerate PCC growth. Interestingly, the authors demonstrated an increased IR expression in PCC developing in LID mice as compared with controls,[29] suggesting a possible role of IR as well in PCC growth. This observation is in line with our hypothesis of a role of IRA in human PCCs. NVP-AEW541, an IGF1R antagonist with higher affinity for IGF1R than IR,[30] was shown to possess significant *in vitro* and *in vivo* antiproliferative activity in neuroblastoma cancer cell lines,[31] a tumor generally considered to be related with PCC. OSI-906 is a selective and orally bioavailable dual IGF-1R/IR kinase inhibitor which is currently evaluated alone or in combination with other compounds in several types of malignancy.[32] The present study demonstrates that OSI-906, used at a dose reached in human plasma as measured

after administration *in vivo*,[33] strongly inhibits cell proliferation and strongly stimulates cell apoptosis in PC12 cells, suggesting that OSI-906 could be a potential new treatment option for PCC patients with metastatic disease. Conversely, a recent study showed that high doses of NVP-AEW541 were required to inhibit cell viability in MPC and MTT PCC cell lines.[22] These different results could depend on the different cell lines and/or on the compound used. PC12 cells are generated by a spontaneously developed PCC in rats, while both MPC and MTT are derived from tumors in heterozygous neurofibromin (NF1) knockout mice.[29, 34-36] NF1 defective tumors could have an activation of AKT and ERK pathways independent of the growth factor receptor stimulation responsible for a reduced sensitivity to the IGF1R blocker. Alternatively, NVP-AEW541, considered to be a selective IGF1R blocker, could be less effective than OSI-906 in inhibiting PCC cell proliferation because it does not disrupt the IR induced cell proliferation.

In several experimental models, including neuroblastoma models, it has been demonstrated that combining mTOR inhibitors with IGF1R antagonists may have additive effects[37] and clinical trials combining compounds that belong to these two categories of drugs are ongoing.[38] To our knowledge there are no clinical studies or case reports evaluating the effects of IGF1R antagonist alone or in combination with mTOR inhibitors in PCC patients. In patients with PCCs there is a suggested rationale to use mTOR inhibitors. The present study showed potent in vitro antiproliferative effects of OSI-906 in PC12. If the data from PC12 cells can be extrapolated to human PCC, the combination of these two drugs could be an attractive strategy to treat these patients. Further studies are required to confirm this data in animal models of PCC. Moreover, in such a preclinical setting it would also be interesting to evaluate whether the expression levels of the different components of the mTOR and IGF pathways can influence the sensitivity of PCC to these treatments.

Conclusions

In PCCs, IGF- and mTOR pathways are simultaneously activated. In particular IGF2 is highly expressed and the subtype A of the IR is expressed at a comparable level of IGF1R and could play an important role in the autocrine/paracrine loop of IGFs in these tumors. mTOR inhibitors inhibit PC12 PCC cell line proliferation but do not stimulate cell apoptosis. Targeting of the IGF pathway with OSI-906 inhibits PC12 PCC cell line proliferation and strongly stimulates cell apoptosis. OSI-906 and mTOR inhibitors have additive antiproliferative effects on PCC cells. The current study suggests that the use of OSI-906 alone or in combination with mTOR inhibitors may be a potential future treatment option in progressive PCCs patients.

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SUPPLEMENTAL MATERIAL

Supplemental material table 1

Oligo Name	Forward	Reverse	Probe
IGF1R	CCAAAACTGAAGCCGAGAAG	GGGTCGGTGATGTTGTAGGT	AAGCAGGAACACCACGGCCG
IGF2R	ACCGACCCCTCCACGC	CCTCCAAGGCCACCTTCAG	AGCAGTACGACCTCTCCAGTCTGGCAAA
IGF1	TTGTGATTTCTTGAAGGTGAAGATG	CGTGGCAGAGCTGGTGAAG	TACCTGGCGCTGTGCCTGCTCA
IGF2	CCAAGTCCGAGAGGGACGT	TTGGAAGAACTTGGCCACG	ACCGTGCTTCCGGACAACTTCCC
IRA	CGTTTGAGGATTACCTGCACAA	GCCAAGGGACCTGCGTTT	TGGTTTTCGTCCCCAGGCCATC
IRB	CCCAGAAAAACCTCTTCAGGC	GGACCTGCGTTTCCGAGA	CTGGTGCCGAGGACCCTAGGCC
mTOR	TGCTGCGTGTCTTCATGCAT	GGATTGCAGCCAGTAACTTGATAG	ACAGCCCAGGCCGCATTGTC
p70S6K	TGGAAGACACTGCCTGCTTTT	TGATCCCCTTTTGATGTAAATGC	CTTGGCAGAAATCTCCATGGCTTTGG
4EBP1	GGCGGCACGCTCTTCA	TCAGGAATTTCCGGTCATAGATG	ACCACCCCGGGAGGTACCAGGA
HPRT	TGCTTTCCTTGGTCAGGCAGTAT	AAATCCAACAAAGTCTGGCTTATATC	CAAGCTTGCGACCTTGACCATCTTTGGA

General discussion and future perspectives

8

The main purpose of this thesis was to explore the role of the mammalian target of rapamycin (mTOR) pathway as a potential target for a novel treatment option in patients with adrenal tumors (AT).

While benign tumors of the adrenal cortex (adrenal adenomas, ACA) and medulla (pheochromocytomas, PCC) are generally cured by surgery, both adrenocortical carcinoma (ACC) and malignant PCC are rare tumors with scant treatment options. Therefore, new treatment options for these malignancies are required.[1-3]

The limited efficacy of conventional antineoplastic treatment in malignant ATs increases the need for novel effective treatment options. During the past 15 years, progress in understanding the pathogenesis of tumors has encouraged the development of so-called "targeted drugs", which are compounds that specifically interfere with molecular mechanisms involved in tumor cell growth and/or tumor vascular supply, leading to major advances in oncology.[4, 5]

Included in this category of drugs there are compounds interfering with growth factor receptors and their related signaling pathways. Alterations of growth factors and their cognate receptors are considered to be involved in the pathogenesis of both ACCs and PCCs.[6-10] Therefore, compounds interfering with tumor angiogenesis and growth factor signaling pathways represent a potential novel treatment options for the management of patients with malignant ATs. The mTOR pathway, being involved in both these processes, could represent a potential target for treatment of these malignancies (*chapter 1*).[2, 3]

Most research described in this thesis is focused on adrenocortical tumors (ACT) (*chapters 2, 3, 4, 5 and 6*), while the research described in one chapter (*chapters 7*) has focused on PCCs. For this reason, the following discussion is more extensively dedicated to the research described ACTs and to a lesser extent to PCCs.

THE mTOR PATHWAY IN NORMAL ADRENALS

The expression of the main components of the mTOR pathway in the normal adrenal gland has not been addressed before (*chapter 1*). This represented the starting point to understand whether this pathway could play a role in the normal physiology of the adrenal gland and whether alterations within the mTOR pathway (activity) could play a role in the pathogenesis of ATs. In *chapter 3* of the current thesis the mRNA and protein expression of the main components of the mTOR pathway in normal adrenals is described. A layer-specific protein expression pattern of the major components of the mTOR pathway was found, suggesting a specific role of the mTOR pathway in particular adrenal functions. For example, the stronger expression of several components [i.e

total-mTOR, total- /phospho- eukaryotic translation initiation factor 4E binding proteins (4EBP1) and total- /phospho- protein kinase p70 ribosomal protein S6 kinase 1 (S6K1)] of the mTOR pathway in the zona reticularis could suggest a role of this pathway in androgen production and the stronger expression of these components in the zona glomerulosa may be related to angiotensin II induced activation of the mTOR pathway.[11] Further studies are required to clarify the specific role of the mTOR pathway in regulating steroid production. In this respect it is interesting to note that in *chapter 2* we show an anti-secretive effect (e.g. inhibition of cortisol production) of mTOR inhibitors (drugs inhibiting the mTOR pathway) in ACC cell lines, whereas the use of mTOR inhibitors in the clinical setting appears not to result in signs or symptoms of hypo-adrenalism.[12]

EXPRESSION OF THE MAIN COMPONENTS OF THE mTOR PATHWAY IN ACTs

The expression of the main components of the mTOR pathway in ACTs has not extensively been investigated before (*chapter 1*). The study of the expression of these components in benign (ACA) and malignant (ACC) ACTs is an important step for several reasons. First, the demonstration of an abnormal expression of these components in ACTs may support a potential role of the mTOR pathway in the pathogenesis of ACTs, increasing the interest of this pathway as a new potential target for treatment. In *chapter 3* of the current thesis, the mRNA and protein expression of the main components of the mTOR pathway was evaluated, showing that ACC present a highly variable protein expression of these components and a lower protein S6K1 mRNA expression than normal adrenals. These data suggest that up-regulation of the mTOR pathway is not ubiquitously observed in ACC. Concomitantly, *Doghman, M. et al* reported that mTOR signaling is active in childhood ACTs.[13] There is increasing body of evidence supporting that adult ACC and childhood ACTs are different entities.[14, 15] Therefore, when comparing the results of the current thesis (*chapter 3*) with the study of *Doghman, M. et al,* it is important to keep in mind that most of our samples were adult ACC while all the samples used by *Doghman, M. et al* were childhood ACTs.[13] The results of the current thesis are in line with the results reported by *Nakamura, M. et al*, who did not find a significant over-expression of phospho-S6K1 and phospho-4EBP1 in ACCs (including both adult and childhood ACTs) as compared with ACAs and normal adrenals.[16] The studies of *chapter 3 also* focused on the description of the heterogeneity of the protein expression of the main components of the mTOR pathway in ACCs tumors. Based on these data, the mTOR pathway should not be expected to be widely involved in the pathogenesis of in ACCs, but might be involved in a subset of them.

The description of the expression of the main components of the mTOR pathway in ACTs was also important to address the question whether a differential expression in

different subgroups of ACTs could have a prognostic value. In the *chapter 3* of the current thesis, it is shown that ACCs present with a lower S6K1 mRNA and protein level compared with ACAs, and ACCs which do not have an intermediate to high staining of phospho-S6K1 or phospho- 4EBP1 have a significantly higher Weiss score than others, suggesting that a subset of less differentiated ACCs could have an inactivation of the mTOR pathway. These data suggest that the down-regulation of the mTOR pathway in ACTs warrants further investigation as a potential prognostic factor.

In the era of personalized medicine the description of the main components of the mTOR pathway in ACTs is an important step to explore in ACCs, as their presence can be considered as potential markers for treatment with mTOR inhibitors (drugs acting on this pathway). In *chapter 3* the protein expression of individual ACCs is described, demonstrating that 80% of carcinomas have an intermediate to high expression of phospho-S6K1 and/or phospho-4EBP1, which are considered as potential molecular biomarkers of mTOR pathway activation in human cancers.[17, 18] In addition, in *chapter 6* of the current thesis, we demonstrate that the presence of genomic alterations, currently considered as potential predictor of response to mTOR inhibitors, are not common events in stage III-IV ACCs and these results have been very recently confirmed also by integrated genomic characterization in ACC samples of patients not selected on the basis of staging.[19] Keeping in mind that molecular biomarkers capable to predict the clinical response to mTOR inhibitors have not been clearly identified yet, the data of this thesis suggest that a subset of patients have molecular evidence of mTOR pathway activation, but further studies are required to explore whether these molecular events could predict an increased sensitivity to mTOR inhibitors.

EFFECTS OF mTOR INHIBITORS IN ACTs

The effects of mTOR inhibitors in ACTs models have not been evaluated previously (*chapter 1*). The testing of mTOR inhibitors in preclinical models of ACTs is a mandatory step to explore whether these compounds could represent a novel treatment opportunity for the management of ACCs. In *chapter 2* the effects of two mTOR inhibitors, sirolimus and temsirolimus, on human ACC cancer cell lines (H295, their clone HAC15 and SW13) were studied. It was demonstrated that mTOR inhibitors inhibit the proliferation and cortisol production in ACC cells, but SW13 were significantly more sensitive than other cells to these compounds. In line with these results, *Doghman, M. et al* reported that everolimus, another mTOR inhibitor, inhibited ACC cell line proliferation and, used at high dose, inhibited H295 xenograft growth in mice.[13] The results of the current thesis demonstrate that the antiproliferative effects of sirolimus at concentrations potentially reachable *in vivo* are predominantly cytostatic (*chapter 2*). Additionally, sirolimus was found to

significantly reduce cell survival and cortisol secretion only in selected ACT primary cultures (*chapter 3*).[20] These data suggest that based on preclinical studies evaluating the effects of mTOR inhibitors in ACT models, a subset of patients with ACCs might be more sensitive than others to this treatment. Therefore, further studies are warranted to find potential biomarkers predictive of response to treatment with mTOR inhibitors in ACTs. In an attempt to answer to this requirement, in the experiments described in *chapter 3* the protein expression of the main components of the mTOR pathway was investigated in relation to the *in vitro* effects of mTOR inhibitors in ACT primary cultures. However, the expression of none of the evaluated proteins correlated with the *in vitro* response to these drugs (*chapter 3*). This absence of a correlation could be due to the low number of primary cultures used. Only specifically designed clinical trials can appropriately evaluate for biomarkers predictive of response to treatments, but this type of clinical trials is extremely difficult to perform in such a rare cancer as ACC. Therefore, progress in this direction can only be awaited from the results of clinical trials in other more common types of cancer. Once a clear predictive biomarker will be identified in other cancers, its value in ACC should be explored.

RELATIONSHIP BETWEEN THE mTOR AND THE IGF PATHWAYS IN ACTs

The relationship between the mTOR and the IGF pathways in ACTs has not been specifically addressed before (*chapter 1*). The mTOR pathway is an intracellular pathway that mediates the effects of many growth factors, including the IGFs.[21, 22] Therefore, the study of this relationship is important to understand whether the mTOR pathway could be involved in the mediation of the pathogenic effects of IGFs in ACC, whether a differential expression of the main components of the IGF pathway could influence the *in vitro* sensitivity to mTOR inhibitors and whether there is a rational to combine drugs targeting the IGF and the mTOR pathways.

The relationship between the mTOR and the IGF pathways in the H295 and SW13 ACC cell lines is addressed in *chapters 2* and *3,* demonstrating that both ACC cell lines have a similar protein expression of IGF1R and the main components of the mTOR pathway, but both mRNA and protein expression of IGF2 were considerably higher in H295 compared with SW13. Next it was evaluated whether in both cell lines the effects of IGF stimulation on the main components of the mTOR pathway is similar or not. In *chapter 2* it was demonstrated that IGF1 significantly stimulated AKT and S6K1 phosphorylation in both H295 and SW13, demonstrating that the mTOR pathway acts as an intracellular mediator of IGFs in both human ACC cell lines (a schematic representation of the pathway is shown in figure 1). Therefore, the mTOR pathway could be involved in the mediation of the pathogenic effects of IGFs in ACC cell lines. However, the effects of the mTOR

inhibitor sirolimus on the IGF-activated intracellular pathways were different between H295 and SW13 cells. At the experimental condition tested, IGF1 induced the activation of the AKT/mTOR pathway in both cell lines, but ERK activation only in H295. Sirolimus efficiently suppressed the mTORC1 activity in both cell lines. However, in H295, but not in SW13 cells, the inhibition of mTORC1 activity was associated with the activation of AKT, likely representing an escape pathway. This activation was further enhanced by

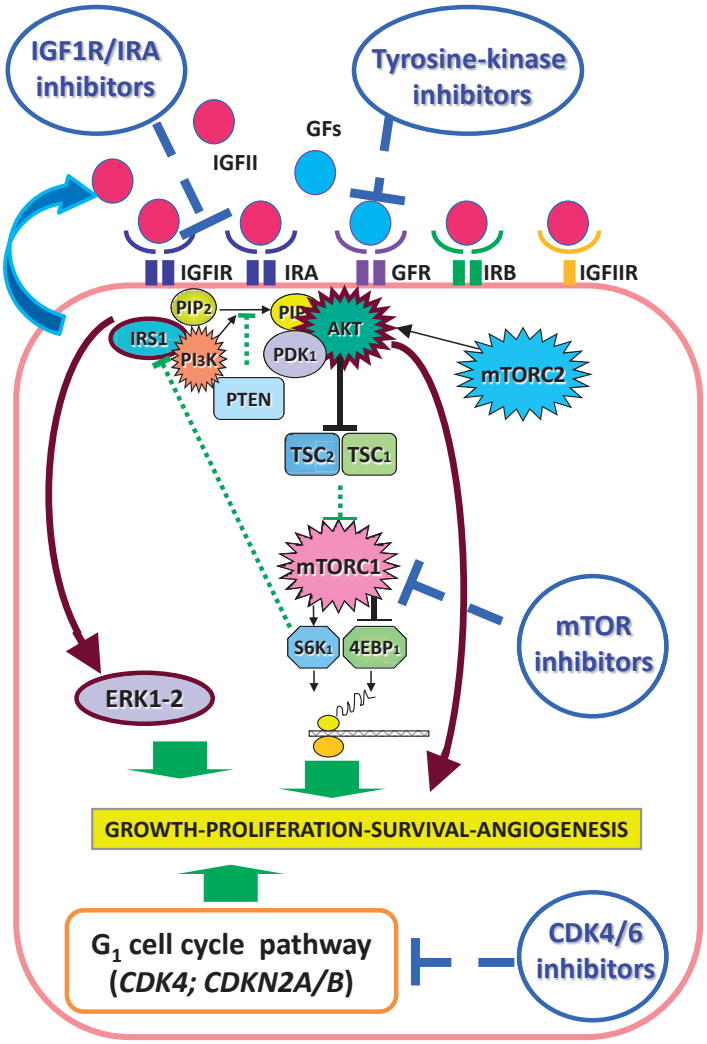

Figure 1. Schematic representation of the potential molecular pathways representing potential targets for treatment in patients with ACC, based on the results presented in the current thesis. GFs: growth factors; GFR: growth factor receptor. Brown lines shows two potential escape pathways to the treatment with mTOR inhibitors: AKT and ERK activation.

IGF1 administration which also induced ERK stimulation in the sirolimus treated H295 cells. These data suggest the presence in H295 of two potential pathways of escape to treatment with traditional mTOR inhibitors: the AKT and ERK pathways (see figure 1 for the potential escape pathways).[21, 23] The activation of these escape pathways could be related, at least partially, to the IGF2 overexpression in H295, which is not found in the SW13 cell model. Therefore, it could be speculated that high IGF2 expression could negatively influence the *in vitro* sensitivity of ACC cell lines to mTOR inhibitors, supporting the rationale to combine mTOR inhibitors and drugs specifically targeting the IGF pathway in ACC (*chapter 4*).

IGF2 overexpression is very common in ACC (about 80%),[10] whereas in the studies described in *chapter 3* only a subset of ACC samples strongly expressed the evaluated components of the mTOR pathway, particularly the phospho-proteins. In the studies included in this thesis, a subgroup of 16 ACC samples was characterized for both the protein expression of the main components of the mTOR pathway (*chapter 3*) and the protein expression of IGF2, IGF1R and IGF2R (*chapter 4*). Within this subgroup of ACC samples we were not able to find correlations between these proteins. Therefore, the results of the current thesis show that the expression of the main components of the mTOR and the IGF pathways are not strongly related, arising the question whether in ACC there is a dissociation between the expression of IGF2 and the activation of the classical IGF stimulated intracellular pathways. Actually, the results of *chapter 3* raises the doubt that the role of IGF2 in the pathogenesis of adult ACC may have been overestimated, in agreement with some other recent speculations.[24] However, it should also be considered that the complexity of the IGF system may have been underestimated. The study reported in *chapter 4* points out that ACC express components of the IGF pathway as well, such as IRA and IGF2R, which were not considered before. As such, before to finally declare a "game over"[24] for the role of IGF2 in adrenocortical tumorigenesis and as a potential target for novel treatment in ACC patients, it could be probably useful to return to the bench and try to better explore the IGF pathway in ACC in its whole complexity.

EFFECTS OF mTOR INHIBITORS IN COMBINATION WITH OTHER DRUGS IN ACTs

The data derived from the use of the mTOR inhibitors alone in the preclinical studies (*chapter 2 and 3*), together with the expected heterogeneity of ACC (*chapter 3 and 6*), suggested that caution is required before using this class of drugs in unselected ACC patients. Such caution was also suggested by preliminary clinical experience with the use of everolimus in some ACC patients with a late stage of disease.[25] Unfortunately, due to the current absence of molecular biomarkers capable to predict the response to mTOR inhibitors in ACTs (*chapters 3 and 6*), it is difficult to define selection criteria for patients that are candidate for

treatment with this class of drugs. Therefore, combination of mTOR inhibitors with other drugs, potentially active in ACC, could be a more prudent clinical approach than the use of these inhibitors as monotherapy in unselected ACC patients.

During the development of this thesis the IGF pathway was considered the most attractive target for new treatment in ACC (*chapter 1*).[26, 27] Additionally, the early results of this thesis (*chapters 1* and *2*) suggested a rationale to combine mTOR inhibitors with drugs targeting the IGF pathway. Therefore, in the studies described in *chapter 4*, the effects of mTOR inhibitors were explored in combination with OSI-906. OSI-906 (linsitinib) is an IGF1-R/Insulin receptor [IR] blocker that during the development of the current thesis has been tested in a phase III trial in ACC patients (NCT00924989). The results of this study have been presented at ASCO meeting in 2014[28] and show that a small subgroup of patients seems to benefit from treatment with this drug, but the hoped improvements in overall or progression-free survival were not observed. This observation again illustrates that ACC is a very heterogenous disease. However, whether combining drugs that target the IGF-system with other compounds such as mTOR inhibitors could be more effective requires further investigation. In *chapter 4* it was reported that in selected conditions combined treatment with mTOR inhibitors and OSI-906 have additive growth inhibitory effects on ACC cells, supporting a potential role for treatment strategies combining mTOR inhibitors and drugs targeting the IGF pathway in ACC. These results are in line with a recently published phase I study demonstrating that a subgroup (about 40%) of ACC patients treated with cixutumumab (IGF1R inhibitor) and temsirolimus (mTOR inhibitor) experienced long term disease stabilization.[29]

Another attractive candidate for new combination treatment strategies in ACC is mitotane, since this drug is currently considered as a referral drug in the treatment of patients with advanced ACC. However, unfortunately the majority of studies suggest that about two-thirds of patients do not respond and/or do not tolerate this drug.[30, 31] Therefore, in *chapter 5* of this thesis the effects of mTOR inhibitors in combination with mitotane were evaluated, demonstrating that in human ACC cell lines the addition of sirolimus to low concentrations of mitotane had stronger antiproliferative effects than mitotane alone. If these results can be translated to humans, they suggest that the addition of sirolimus might add to the antitumor action of mitotane, reducing the mitotane dose required to obtain a desired clinical effect with potentially less side effects. This additive effects were higher when the concentration of mitotane used was lower, suggesting that combined treatment might be particularly useful during the phases of treatment in which mitotane plasma levels are below the therapeutic range, such as during the initial dose titration and/or for those subjects in which the therapeutic range of mitotane is hardly maintained due to bad tolerance or other reasons. Although these data are still preliminary, they suggest a potential advantage of combining mitotane with sirolimus. Therefore, this combination treatment clearly warrants further investigation.

NEW POTENTIAL TARGETS FOR ACC IN ADDITION TO THE IGF AND mTOR PATHWAYS

During the development of the current thesis, most of the early clinical experience with targeted drugs, including drugs targeting the IGF pathway, failed to demonstrate the hoped effects in patients with ACC,[3, 24, 28] thereby arising the questions whether molecular events, potentially targetable with currently developed targeted drugs, are present in at least a subset of ACC patients. This question is addressed in *chapter 6*. Using hotspot gene sequencing and comparative genomic hybridization the presence of a large number of mutations and copy number abnormalities of potential interest for therapeutic aims were evaluated in a large group of adult stage III-IV ACC samples. At least one copy number alteration or mutation was found in about half of the patients. The most frequently detected mutations were on *TP53, ATM* and *CTNNB1* (15%, 12.5% and 10% respectively). The most frequently identified copy number alterations were: amplification of the *CDK4* oncogene (17.9%) and deletion of the *CDKN2A* (14.3%) and *CDKN2B* (10.7%) tumor suppressor genes. No relevant alteration in the evaluated components of the mTOR and IGF pathways were found with these techniques. No simple targetable molecular event emerged. Based on genomic alterations, the cell cycle appeared to be the most relevant new potential therapeutic target for patients with advanced ACC (figure1). Recently, a small-molecule CDK4/6 inhibitor (i.e. palbociclib or PD-0332991 and LEE011) has rekindled interest in the concept of blocking cell cycle progression to stop cancer cell growth.[32, 33] Currently these compounds are under the phase I-III of clinical development and preliminary results suggest a safety profile in humans and promising anti-tumor activity in different cancer types (http://clinicaltrials.gov). Further studies to explore the effects of these compounds in preclinical models of ACCs are warranted.

Overall these data underline that, despite the fact that during the last 10 years many progresses have been made in describing the molecular alteration in ACTs, the translation of these progress from bench to the bedside with the aim to improve the treatment of patients with ACC has not been easy, so far.

EXPRESSION OF THE MAIN COMPONENTS OF THE mTOR PATHWAY AND THE RELATIONSHIP BETWEEN THE mTOR AND THE IGF PATHWAYS IN PHEOCHROMOCYTOMAS (PCCs)

Dysregulation of the mTOR pathway have been suggested to be present at least in a subgroup of human PCCs.[9, 34] However, relationships between the mTOR and the IGF pathways has been poorly investigated. In *chapter 7* of the current thesis the mRNA expression of the main components of the mTOR and IGF pathways in a series of hu-

man PCCs is explored. All the evaluated components of the mTOR and IGF pathways are expressed in human PCC samples, with a high expression of IGF2 mRNA and a high IRA/IRB mRNA ratio in the majority of cases. These data suggest the existence of a potential IGF2-IRA autocrine/paracrine loop in human PCCs. This loop could play a role in the pathogenesis of these tumors representing a potential target for new treatment options in patients with malignant PCCs. Additionally, several correlations between the mRNA levels of the different components of mTOR and IGF pathways were found. In particular, the mRNA level of all evaluated components of the mTOR pathway strongly correlated with IRA and IRB mRNA expression, suggesting that the expression of these genes could share common mechanisms of regulation.

EFFECTS OF mTOR INHIBITORS AND OSI-906 IN PCCs

The effects of mTOR inhibitors in PCC models had hardly been investigated before the project of this thesis started (*chapter 1*). In *chapter 7* the effects of two mTOR inhibitors, sirolimus and everolimus, in PC12, a rat PCC cell line, are described, demonstrating that both compounds significantly suppress cell proliferation in a dose- and time-dependent-manner. However, in this experimental model the observed anti-proliferative effects did not exceed 50% cell growth inhibition and were not associated with a significant induction of cell apoptosis. These results are consistent with the early clinical experiences with the use of everolimus in PCC patients, suggesting that everolimus can induce disease stabilization in some patients, but to date no tumor objective response has been reported by using this drug in patients with this malignancy[-]

Additionally, during the development of this thesis some other studies explored the effects of mTOR inhibitors (particularly NVP-BEZ235 a dual PI3K/mTORC1/2 inhibitor and AZD8055, a dual mTORC1/2 inhibitor), alone or in combination with other drugs (particularly lovastatin, an ERK inhibitor), in preclinical PCC models adopting different PCC cell lines and methodologies as compared with the current thesis.[37, 38] Preclinical and early clinical studies support a potential role of sunitinib, a tyrosine kinase inhibitor that targets several receptors [platelet-derived growth factor (PDGF-Rs) and vascular endothelial growth factor receptors (VEGFRs)], in the treatment of PCC, giving the rationale for a phase II clinical trial in patients with progressive malignant PCC and paraganglioma (NCT01371201) that is currently ongoing.[9, 39-41] Considering these data further investigations are also required to explore the feasibility and the potential effects of treatment strategies combining drugs targeting the mTOR inhibitor pathway with tyrosine kinase receptor inhibitors. All together these preclinical experiments suggest that in PCCs treatment strategies combining mTOR inhibitors with other drugs warrant further investigation.

As above mentioned, in *chapter 7* of the current thesis the existence of an IGF2-IRA autocrine/paracrine loop in human PCCs is hypothesized for the first time. In PCC models the effects of drugs targeting simultaneously the IGF1-R and IR, such as OSI-906, have never been investigated before. Therefore, the effects of OSI-906 alone or in combination with sirolimus, were investigated in PC12 cells, demonstrating that OSI-906 significantly increased the apoptosis and suppressed cell growth in a dose and time-dependent-manner *(chapter 7)*. These antiproliferative effects were further increased by combining OSI-906 with sirolimus or everolimus, demonstrating a clear additivity in the cell growth inhibitory effects of these two classes of compounds. The next step will be to examine the antiproliferative and antisecretory effects of these drugs, alone and in combination, in primary cultures of human malignant PCCs. If the data from PC12 cells can be extrapolated to at least a subgroup of human PCCs, the combination of these two drugs could be an attractive strategy to treat patients with progressive PCCs. Therefore, future studies are warranted to define whether treatment with OSI-906 alone or in combination with mTOR inhibitors might be a potential future treatment option for patients with progressive PCCs.

CONCLUSION AND FUTURE DIRECTIONS

The results of the current thesis support a role for the mTOR pathway as a potential target for treatment of patients with ACCs, as well as for patients with malignant PCCs. However, in both types of tumors, treatment strategies combining mTOR inhibitors with other drugs might be more effective than the use of mTOR inhibitors alone. Additionally, considering the potential heterogeneity of these malignancies, treatment strategies based on the selection of patients with a potentially higher chance to respond to mTOR inhibitors based on their tumor characteristics, might be more effective than the use of mTOR inhibitors in unselected patients. Unfortunately, molecular biomarkers capable to predict a clinical response to mTOR inhibitors have not been clearly identified yet. Therefore, further preclinical and clinical investigation are required to find new molecular biomarkers useful to predict tumor response to both conventional and novel treatments for patients with ATs and to address the role of mTOR inhibitors, alone or in combination with other drugs, in selected subgroups of patients with these tumors. All these data could help to move into the direction of a more personalized approach to the treatment of malignant ATs, and hopefully this approach could lead to advantage in the clinical management of these rare but aggressive diseases.

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Summary of the thesis

9

SUMMARY

Adrenal tumors (AT) include benign and malignant cortical tumors named adrenocotical adenomas (ACA) and adrenocortical carcinomas (ACC), respectively and benign and malignant pheochromocytomas (PCC). The malignant ATs are aggressive tumors with a poor prognosis. The surgical removal of the tumor represents the treatment of choice for both benign and malignant hormone-secreting tumors, for all tumors suspected to be a PCC, as well as for all non hormone-secreting adrenal masses with clinical and radiological suspicion of malignancy. However, in malignant ATs, surgery allows a complete remission only in a minority of cases with a diagnosis in early stage of the disease. The limited efficacy of conventional antineoplastic treatments in malignant ATs increases the need for novel and more effective treatment options.

The mammalian target of rapamycin (mTOR) pathway is one of the most important intracellular mediators of growth factor receptors, including insulin-like growth factors (IGFs), which have been suggested to play a role in AT pathogenesis. Dysregulation of the mTOR pathway has been found in many human tumors. Therefore, the mTOR pathway is a target for antineoplastic therapy in several malignancies.

At time the studies presented in this thesis were initiated, the role and functions of mTOR pathway in the normal and pathological adrenal gland and the effects of mTOR inhibitors as novel treatment opportunity for the management of malignant ATs was not clarified.

The main aim of the present thesis was to explore the role of mTOR pathway as a potential target for new treatment options in patients with ATs.

Chapter 1 provides an overview of the pathophysiology and current treatment options for ATs, as well as an overview of the mTOR pathway. It also describes the background of the mTOR pathway as a potential novel target for treatment of patients with ATs.

Chapter 2 describes the effects of the mTOR-inhibitors sirolimus and temsirolimus on growth and cortisol production in cell line models of human ACC (H295, HAC15 and SW13 cell lines). This study demonstrated that mTOR-inhibitors inhibit the proliferation in a dose- and time-dependent manner in all three ACC cell lines. The antiproliferative effects appeared to be predominantly related to the inhibition of cell cycle. Moreover, sirolimus inhibits cortisol production in human secreting ACC cells. However, the different ACC cell lines were found to have a differential sensitivity to the mTOR-inhibitors, suggesting that mTOR could be a target for the treatment of human ACCs, but with the

expectation of variable responses. This study also suggested that the overexpression of IGF2 might be a reason for a reduced sensitivity to mTOR inhibitors and it was demonstrated that the blocking of endogenously produced IGF2 increased the antiproliferative effects of sirolimus on H295, a cell line that overexpresses IGF2. Therefore, this study suggested that in ACC, the combined targeting of mTOR and IGF2 may have stronger effects than treatment with mTOR-inhibitors alone.

Chapter 3 describes the mRNA, protein and phospho-protein expression of the main components of the mTOR pathway: mTOR, S6K1 and 4EBP1 in normal adrenal, adrenal hyperplasia and in benign and malignant adrenocortical tumors. This study demonstrated a layer specific expression of the evaluated components in the normal adrenal gland, as well as in adrenal hyperplasia, which is suggestive for a specific role of the mTOR pathway in particular adrenal functions, such as the regulation of androgens or aldosteron production. Although the evaluated proteins of the mTOR pathway were found to be expressed in most ACTs, this study underlined the heterogeneous expression of the main components of the mTOR pathway in ACCs, and suggested that some more aggressive tumors could have a down-regulation of the mTOR pathway. Therefore, this study pointed out the importance to find biomarkers that are predictive for a response to treatment with mTOR inhibitors in ACTs. Such biomarkers might drive the selection of patients candidate to this type of treatment. In our attempt, to answer to this requirement, in this study the protein expression of the main components of the mTOR pathway was also investigated in relation to the *in vitro* effects of mTOR inhibitors in ACT primary cultures. Unfortunately, the expression of none of the evaluated proteins correlated with the *in vitro* response to these drugs, although the number of primary cultures that were tested was low. As such, these preclinical data did not help to suggest a potential molecular biomarker capable of predicting a response to mTOR inhibitors in ACCs. Therefore, it is suggested that caution should be taken before using mTOR inhibitors as monotherapy in unselected ACC patients.

The data derived from the use of the mTOR inhibitors alone in preclinical studies, together with the expected heterogeneity of ACCs and a lack of molecular biomarker capable to predict the response to mTOR inhibitors, suggested that combination of mTOR inhibitors with other drugs, potentially active in ACC, could be a more prudent clinical approach than the use of these inhibitors as monotherapy in unselected ACC patients. Since IGF was considered as one of the most attractive targets for a new treatment in patients with ACC, in **chapter 4** the combination of drugs targeting the mTOR pathway and the IGF pathway was explored. This study showed that most ACC express IGF2, IGF1R and IGF2R proteins and we demonstrated IRA mRNA expression in these tumors. These data point out that ACCs express some components of the IGF pathway, such as

IRA and IGF2R, which were not considered before. As such, before to finally declare a "game over" for the role of IGF2 in adrenocortical tumorigenesis and as a potential target for novel treatment in ACC patients, it may probably be useful to return to the bench and try to better explore the IGF pathway in ACCs in its whole complexity. Additionally, we demonstrated that in human ACC cells, treatment with OSI-906, an IGF1R/IR antagonist, inhibits cell proliferation and that combined treatment with OSI-906 and mTOR inhibitors has additive antiproliferative effects.

Since mitotane is currently considered as a referral drug in the treatment of patients with advanced ACC (although unfortunately not all patients respond and/or tolerate this drug), this drug represents another attractive candidate for new combined treatment strategies in ACC. Therefore, in **chapter 5** the combination of drugs targeting the mTOR pathway and mitotane was explored. These studies demonstrated that sirolimus has additive antiproliferative effects when combined with low doses of mitotane. These doses corresponded to concentrations lower than the therapeutic range of mitotane, suggesting that the addition of sirolimus to mitotane might be useful in ACC patients when the therapeutic range of mitotane is not reached.

In addition to the mTOR and IGF pathways, **chapter 6** describes, in a large series of ACC samples, the presence of molecular events (detected by hotspot gene sequencing and comparative genomic hybridization), potentially targetable with currently developed targeted drugs. This study demonstrated that the presence of genomic alterations of the main components of the mTOR pathway are not common events in advanced ACCs. At least one copy number alteration or mutation was found in about half of the patients. The most frequent mutations were detected in *TP53, ATM* and *CTNNB1*. The most frequent copy number alterations identified were: amplification of the *CDK4* oncogene and deletion of the *CDKN2A* and *CDKN2B* tumor suppressor genes. Therefore, no simple targetable molecular event emerged and based on genomic alterations, the cell cycle appeared to be the most relevant new potential therapeutic target for patients with advanced ACC.

Chapter 7 describes the expression of the main components of the mTOR pathway and the relationship between the mTOR and the IGF pathways in PCCs, as well as the effects of mTOR inhibitors, alone or in combination with OSI-906, in a rat PCC cell line (PC12). This study demonstrated that all the investigated components of the mTOR and the IGF pathways were expressed in human PCC. A high expression of IGF2 mRNA and an increased IRA/IRB ratio was found. mTOR inhibitors and OSI-906 were able to suppress PC12 proliferation in a dose- and time-dependent manner. OSI-906 strongly stimulated cell apoptosis. Combined treatment of sirolimus with OSI-906 had additive antiprolifera-

tive effects. Therefore, this study suggested that OSI-906, alone or in combination with mTOR inhibitors, could represent a new treatment option in progressive PCC patients.

In **chapter 8**, the general discussion, the results of these studies are integrated and further discussed. Special emphasis is given to those issues which address the role of the mTOR pathway as a potential target for the treatment of patients with ACCs or malignant PCCs. A need to move into the direction of a more personalized approach for the treatment of patients with malignant ATs is emphasized.

Samenvatting

10

Bijnier tumoren omvatten benigne (goedaardige)- en maligne (kwaadaardige) tumoren uitgaande van de bijnierschors (bijnierschors adenomen en bijnierschors carcinomen), alsmede benigne- en maligne mergtumoren, de feochromocytomen. Maligne bijnierschors tumoren zijn zeer agressief en patiënten met deze tumoren hebben een slechte prognose. Chirurgische verwijdering van de tumor is de voorkeursbehandeling voor zowel debenigne- als maligne hormoonproducerende tumoren, voor alle als feochromocytoom verdachte tumoren, alsmede voor niet-hormoon producerende ruimte-innemende processen in de bijnier, die klinisch en radiologisch verdacht worden van maligniteit. Bij maligne bijnier tumoren resulteert chirurgie echter slechts in een klein deel van de patiënten met een vroege diagnose van de ziekte in complete genezing. Deze beperkte effectiviteit van de conventionele antitumor behandeling in patiënten met maligne bijniertumoren benadrukt de noodzaak voor nieuwe en effectieve behandelingsopties.

De "mammalian target of rapamycin (mTOR)" cellulaire route is een van de meest belangrijke mediatoren van de effecten van groeifactoren, waaronder insuline-achtige groei factoren (IGFs), die een mogelijke rol spelen bij de pathogenese van bijnier tumoren. Deregulatie van de mTOR route wordt gevonden in vele soorten menselijke tumoren. De mTOR route is daarom een doelwit voor anti-tumor therapie bij verschillende maligniteiten.

Op het moment dat de studies die gepresenteerd worden in dit proefschrift aanvingen, waren de rol en functies van de cellulaire mTOR route in de normale- en pathologische bijnier, alsmede de effecten van mTOR remmers als nieuwe behandelingsmogelijkheid bij patiënten met maligne bijnier tumoren niet bekend.

Het doel van het in dit proefschrift beschreven onderzoek is de rol van de mTOR route als mogelijk nieuw doelwit voor behandeling van patiënten met bijnier tumoren te onderzoeken.

Hoofdstuk 1 geeft een overzicht van de pathofysiologie en huidige behandelingsmogelijkheden voor bijnier tumoren, alsmede een gedetailleerde beschrijving van de cellulaire mTOR route. Tevens wordt het mechanisme van de mTOR route beschreven als mogelijk doelwit voor behandeling van patiënten met bijnier tumoren.

Hoofdstuk 2 beschrijft de effecten van de mTOR remmers sirolimus en temsirolimus op de groei en cortisol productie in cellijn modellen van menselijke bijnierschorskanker (H295, HAC15 en SW13 cellijnen). Deze studie toont aan dat mTOR remmers de proliferatie op een dosis- en tijdsafhankelijke wijze remmen in alle drie bestudeerde cellijnen.

De anti-proliferatieve effecten bleken voornamelijk gerelateerd aan het remmen van de celcyclus. Bovendien wordt aangetoond dat sirolimus de cortisol productie remt in gekweekte menselijke bijnierschorscarcinoom cellen. De gevoeligheid van de verschillende bijnierschorskanker cellijnen voor het remmende effect van mTOR-remmers bleek echter verschillend, hetgeen suggereert dat mTOR een doelwit kan zijn voor behandeling van het bijnierschorscarcinoom, maar dat variabele responsen verwacht mogen worden. Deze studie suggereert tevens dat de over-expressie van IGF2 een mogelijke oorzaak kan zijn voor een verminderde gevoeligheid van de tumorcellen voor mTOR remmers. In dezelfde studie wordt aangetoond dat het blokkeren van endogeen geproduceerd IGF2 de gevoeligheid voor het antiproliferatieve effect van sirolimus in H295 cellen, een cellijn die een overmatige IGF2 expressie heeft, verhoogd. De in **hoofdstuk 2** beschreven resultaten suggereren dat een combinatie van geneesmiddelen die zowel mTOR als IGF2 als doelwit hebben in bijnierschorscarcinomen een sterker effect heeft dan behandeling met alleen mTOR-remmers.

In **hoofdstuk 3** wordt de mRNA-, eiwit- en phospho-eiwit expressie van de belangrijkste componenten van de cellulaire mTOR route, te weten mTOR, S6K en 4EBP1, beschreven in de normale bijnier, in hyperplastisch bijnier weefsel, en in benigne- en maligne bijnierschors tumoren. Deze studie toont aan dat er een zone specifieke expressie van deze componenten aanwezig is in de normale- en hyperplastische bijnier, hetgeen suggestief is voor een specifieke rol van de mTOR route in bepaalde bijnierfuncties, zoals de regulatie van de androgeen- en/of aldosteron productie. Hoewel bovengenoemde eiwitten van de mTOR route tot expressie komen in de meeste bijnier tumoren, onderschrijven de bevindingen een heterogene expressie van deze eiwitten in bijnierschorskanker, alsmede een down-regulatie van de mTOR route in de meer agressieve tumoren. Deze studie benadrukt daarom het belang van het vinden van biomerkers in bijnierschors tumoren die voorspellend zijn voor een respons op behandeling met mTOR remmers. Dergelijke biomerkers zouden patiënten kunnen identificeren die kandidaat zijn voor dit type behandeling. In een poging om dergelijke biomerkers te vinden hebben we in deze studie tevens de expressie van componenten van de mTOR route onderzocht in relatie tot de *in vitro* effecten van mTOR remmers in primaire kweken van bijnierschors tumoren. Helaas bleek er geen correlatie te bestaan tussen de expressie van de onderzochte eiwitten en de in vitro respons op mTOR remmers. Het aantal onderzochte primaire kweken was echter relatief klein. Op basis van onze pre-klinische resultaten kunnen wij daarom niet een potentiele merker voorstellen die voorspellend is voor het effect van mTOR remmers in bijnierschors tumoren. Voorzichtigheid is daarom geboden voordat het gebruik van mTOR remmers als monotherapie overwogen wordt in niet-geselecteerde patiënten met een bijnierschorscarcinoom.

De huidige gegevens uit pre-klinische studies omtrent het gebruik van mTOR remmers als monobehandeling, in combinatie met de verwachte heterogeniteit van bijnierschorskanker, en het gebrek aan een moleculaire biomerker die een respons op mTOR remmers voorspelt, suggereren dat het combineren van mTOR remmers met andere geneesmiddelen die mogelijk werkzaam kunnen zijn in bijnierschorskanker, een meer voorzichtige klinische benadering is dan het gebruik van mTOR remmers als monotherapie in niet-geselecteerde patiënten met bijnierschorskanker. Aangezien IGF beschouwd wordt als een van de meest aantrekkelijke doelwitten voor een nieuwe behandeling van patiënten met bijnierschorskanker hebben wij in **hoofdstuk 4** de combinatie van geneesmiddelen die gericht zijn tegen zowel de mTOR route als de IGF-route onderzocht. Deze studie toont dat de meeste bijnierschorskankers IGF2, IGF1R en IGF2R eiwitten, maar ook IR-A, tot expressie brengen.

Als zodanig, en voordat definitief besloten wordt tot een "game over" voor de rol van IGF2 in bijnierschors tumorgenese en als potentieel doelwit voor behandeling van patiënten met bijnierschorskanker, is het waarschijnlijk nuttig om terug te gaan naar het laboratorium om de IGF route in bijnierschorskanker in zijn totale complexiteit te onderzoeken. Tevens hebben wij in **hoofdstuk 4** aangetoond dat behandeling met OSI-906, een IGF1R/IR antagonist, de proliferatie van menselijke bijnierschorskanker cellen remt en dat gecombineerde behandeling met OSI-906 en mTOR remmers resulteert in additieve anti-proliferatieve effecten.

Mitotaan is op dit moment het geneesmiddel dat wordt gebruikt bij de behandeling van patiënten met een vergevorderd stadium van bijnierschorskanker (ondanks het feit dat helaas niet alle patiënten reageren op de behandeling en/of intolerant zijn voor het geneesmiddel) en is daarom een andere aantrekkelijke kandidaat voor gecombineerde behandelingsstrategieën in bijnierschorskanker. In **hoofdstuk 5** hebben wij daarom de effecten van behandeling met een combinatie van mTOR remmers en mitotaan onderzocht. Deze studies tonen aan dat sirolimus additieve antiproliferatieve effecten heeft wanneer dit middel gecombineerd wordt een lage dosering mitotaan. Deze onderzochte doseringen mitotaan komen overeen met concentraties die lager zijn dan de therapeutische concentraties van mitotaan, hetgeen suggereert dat het toevoegen van sirolimus aan mitotaan nuttig kan zijn bij patiënten met bijnierschorskanker, wanneer de therapeutische spiegel van mitotaan nog niet bereikt is of niet bereikt wordt.

Naast de eerder beschreven cellulaire mTOR en IGF routes wordt in **hoofdstuk 6** in een grote serie bijnierschors tumoren het voorkomen van moleculaire veranderingen (aangetoond door middel van "hotspot gene sequencing" en "comparative genomic hybridization") die een mogelijk doelwit zijn voor reeds ontwikkelde "targeted drugs" onderzocht en beschreven. Deze studie toont aan dat genetische veranderingen in de belangrijkste componenten van de mTOR route niet frequent voorkomen in bij-

nierschors kanker in een vergevorderd stadium. In de helft van de tumoren werd een verandering in minstens een kopie aantal of een mutatie gevonden. De meest frequente mutaties werden gevonden in *TP53*, *ATM* en *CTNNB1*. De meest frequente kopie aantal veranderingen die gevonden werden zijn amplificatie van het *CDK4* oncogen en deletie van de *CDKN2A* en *CDKN2B* tumor suppressor genen. Op basis van deze bevindingen werd geconcludeerd dat er niet een eenvoudig moleculair doelwit naar voren kwam gebaseerd op genetische veranderingen, en dat de cel cyclus het meest relevante therapeutische doelwit lijkt te zijn voor patiënten met een vergevorderd stadium van bijnierschorskanker.

Hoofdstuk 7 beschrijft de expressie van de belangrijkste componenten van de mTOR route, alsmede de relatie tussen de mTOR- en IGF routes, in feochromocytomen. Tevens worden de effecten van mTOR remmers, alleen en in combinatie met OSI-906, beschreven in een ratten feochromocytoom cellijn (PC12). Deze studie toont aan dat alle onderzochte componenten van de mTOR- en IGF routes tot expressie komen in feochromocytomen. Een hoge expressie van IGF2 mRNA en een verhoogde IR-A/IR-B verhouding werd gevonden. mTOR remmers en OSI-906 remmen de proliferatie van PC12 cellen op een dosis- en tijdsafhankelijke wijze. OSI-906 heeft een sterk apoptose stimulerend effect in PC12 cellen. Gecombineerde behandeling met sirolimus en OSI-906 resulteert in een additief antiproliferatief effect. Deze studie suggereert dat OSI-906, alleen of in combinatie met mTOR remmers, een mogelijk nieuwe behandelingsoptie kan worden bij patiënten met een maligne feochromocytoom.

In **hoofdstuk 8**, de algemene discussie, worden de resultaten van de in dit proefschrift beschreven studies geïntegreerd en nader bediscussieerd. Speciale aandacht wordt besteed aan de rol van de mTOR route als een mogelijk nieuw doelwit voor behandeling van patiënten met bijnierschorskanker of een maligne feochromocytoom. De noodzaak van een meer gepersonaliseerde, op tumor kenmerken gebaseerde aanpak bij de behandeling van patiënten met maligne bijnier tumoren wordt benadrukt.

About the author

List of Publications

PhD Portfolio

Acknowledgements

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ABOUT THE AUTHOR

Maria Cristina De Martino was born on June 4th 1978, in Naples, Italy, where she grew-up with her beloved parents Giuseppe De Martino and Anna Limpido. While studying, she played basketball at agonistic level and she met the love of her life Massimiliano Gambardella. She got more and more fascinated by the natural sciences and became interested in medical research. In 1997 she completed, with the maximum mark, the scientific lyceum at Galileo Galilei and she started her medical study at Federico II University in Naples. In 2003 Maria Cristina graduated in Medicine and Surgery *cum laude* and *honourable mention* to the curriculum studiorum of the candidate and special interest of the thesis. From 2000 to 2007 she performed her pre-graduate and post-graduate clinical and research internship at the Department of Clinical and Molecular Endocrinology and Oncology of Federico II University, under the precious supervision of Prof. G. Lombardi, Prof A. Colao and Dr. R. Pivonello and in 2008 she was appointed of the title of Specialist in Endocrinology. Aiming to became a successful translational researcher in the field of endocrinology, she had several working experiences abroad: the first one was a short pre-graduate fellowship at Institute of Endocrinology of Saint Bartholomew's and the Royal London School of Medicine and Dentistry, London, UK; the second one has been a long working experience, from 2007 to 2011 at the Dept. of Internal Medicine, Section of Endocrinology, Neuroendocrinology Lab.; Erasmus MC Rotterdam, The Netherlands and the last one from 2011 to 2013 at the Departments of Nuclear Medicine and Endocrine Oncology, "Institut Gustave Roussy" of Villejuif; France. During these years Maria Cristina increased her skills in endocrinology, oncology and her ability to develop translational research projects having the occasion to work with experts in the field such as Prof. dr. L.J. Hofland, Dr. R. Feelders, Prof. dr. S.W.J. Lamberts, Dr. E. Baudin and Dr. L. Lacroix. Maria Cristina published several original research studies, and presented her research in national and international congresses. She was awarded several prizes, including the ENETS Translational Research Fellowship, Berlin 2011 and the "Société Française d'Endocrinologie (SFE) bourse de recherché", 2012 Toulouse. After all this travelling, Maria Cristina returned back to her home University where she works as medical researcher, since June 2013. Maria Cristina and Massimiliano got married in 2009 and following their passion for research and the devotion for their job they continue to live their life continuously travelling as enthusiastic "gypsy researchers".

LIST OF PUBLICATIONS

1. Simeoli C, Auriemma RS, Tortora F, De Leo M, Iacuaniello D, Cozzolino A, **De Martino MC**, Pivonello C, Mainolfi CG, Rossi R, Cirillo S, Colao A & Pivonello R. The treatment with pasireotide in Cushing's disease: effects of long-term treatment on tumor mass in the experience of a single center. *Endocrine* 2015.
2. Pivonello C, **De Martino MC**, Negri M, Cuomo G, Cariati F, Izzo F, Colao A, Pivonello R 2014 The GH-IGF-SST system in hepatocellular carcinoma: biological and molecular pathogenetic mechanisms and therapeutic targets. *Infectious agents and cancer* 9:27
3. **De Martino MC**, Feelders RA, de Herder WW, van Koetsveld PM, Dogan F, Janssen JA, Waaijers AM, Pivonello C, Lamberts SW, Colao A, de Krijger RR, Pivonello R, Hofland LJ 2014 Characterization of the mTOR pathway in human normal adrenal and adrenocortical tumors. *Endocrine-related cancer* 21:601-613
4. Pivonello R, **De Martino MC**, Colao A 2014 How should patients with adrenal incidentalomas be followed up? *The lancet. Diabetes & endocrinology* 2:352-354
5. Pivonello R, **De Martino MC**, Auriemma RS, Alviggi C, Grasso LF, Cozzolino A, De Leo M, De Placido G, Colao A, Lombardi G 2014 Pituitary tumors and pregnancy: the interplay between a pathologic condition and a physiologic status. *Journal of endocrinological investigation* 37:99-112
6. van Adrichem RC, Hofland LJ, Feelders RA, **De Martino MC**, van Koetsveld PM, van Eijck CH, de Krijger RR, Sprij-Mooij DM, Janssen JA, de Herder WW 2013 Chromogranin A, Ki-67 index and IGF-related genes in patients with neuroendocrine tumors. *Endocrine connections* 2:172-177
7. **De Martino MC**, Al Ghuzlan A, Aubert S, Assie G, Scoazec JY, Leboulleux S, Do Cao C, Libe R, Nozieres C, Lombes M, Pattou F, Borson-Chazot F, Hescot S, Mazoyer C, Young J, Borget I, Colao A, Pivonello R, Soria JC, Bertherat J, Schlumberger M, Lacroix L, Baudin E 2013 Molecular screening for a personalized treatment approach in advanced adrenocortical cancer. *The Journal of clinical endocrinology and metabolism* 98:4080-4088
8. Auriemma RS, Pivonello R, **De Martino MC**, Cudemo G, Grasso LF, Galdiero M, Perone Y, Colao A 2013 Treatment with GH receptor antagonist in acromegaly: effect on cardiac arrhythmias. *European journal of endocrinology / European Federation of Endocrine Societies* 168:15-22
9. **De Martino MC**, van Koetsveld PM, Feelders RA, Sprij-Mooij D, Waaijers M, Lamberts SW, de Herder WW, Colao A, Pivonello R, Hofland LJ 2012 The role of mTOR inhibitors in the inhibition of growth and cortisol secretion in human adrenocortical carcinoma cells. *Endocrine-related cancer* 19:351-364

10. Pivonello R, De Leo M, Vitale P, Cozzolino A, Simeoli C, **De Martino MC**, Lombardi G, Colao A 2010 Pathophysiology of diabetes mellitus in Cushing's syndrome. Neuroendocrinology 92 Suppl 1:77-81
11. De Leo M, Pivonello R, Auriemma RS, Cozzolino A, Vitale P, Simeoli C, **De Martino MC**, Lombardi G, Colao A 2010 Cardiovascular disease in Cushing's syndrome: heart versus vasculature. *Neuroendocrinology* 92 Suppl 1:50-54
12. **De Martino MC**, van Koetsveld PM, Pivonello R, Hofland LJ 2010 Role of the mTOR pathway in normal and tumoral adrenal cells. *Neuroendocrinology* 92 Suppl 1:28-34
13. **De Martino MC**, Auriemma RS, Brevetti G, Vitale G, Schiano V, Galdiero M, Grasso L, Lombardi G, Colao A, Pivonello R 2010 The treatment with growth hormone receptor antagonist in acromegaly: effect on vascular structure and function in patients resistant to somatostatin analogues. *Journal of endocrinological investigation* 33:663-670
14. **De Martino MC**, Hofland LJ, Lamberts SW 2010 Somatostatin and somatostatin receptors: from basic concepts to clinical applications. *Progress in brain research* 182:255-280
15. Auriemma RS, Galdiero M, **De Martino MC**, De Leo M, Grasso LF, Vitale P, Cozzolino A, Lombardi G, Colao A, Pivonello R 2010 The kidney in acromegaly: renal structure and function in patients with acromegaly during active disease and 1 year after disease remission. *European journal of endocrinology / European Federation of Endocrine Societies* 162:1035-1042
16. Tauchmanova L, Guerra E, Pivonello R, **De Martino MC**, De Leo M, Caggiano F, Lombardi G, Colao A 2009 Weekly clodronate treatment prevents bone loss and vertebral fractures in women with subclinical Cushing's syndrome. *Journal of endocrinological investigation* 32:390-394
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19. Pivonello R, **De Martino MC**, De Leo M, Lombardi G, Colao A 2008 Cushing's Syndrome. *Endocrinology and metabolism clinics of North America* 37:135-149, ix

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22. Pecori Giraldi F, Pivonello R, Ambrogio AG, **De Martino MC**, De Martin M, Scacchi M, Colao A, Toja PM, Lombardi G, Cavagnini F 2007 The dexamethasone-suppressed corticotropin-releasing hormone stimulation test and the desmopressin test to distinguish Cushing's syndrome from pseudo-Cushing's states. *Clinical endocrinology* 66:251-257
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30. Faggiano A, Melis D, Alfieri R, **De Martino M**, Filippella M, Milone F, Lombardi G, Colao A, Pivonello R 2005 Sulfur amino acids in Cushing's disease: insight in homocysteine and taurine levels in patients with active and cured disease. *The Journal of clinical endocrinology and metabolism* 90:6616-6622
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PHD PORTFOLIO

Name PhD student	Maria Cristina De Martino
Erasmus MC department	Internal Medicine, Endocrinology
Research School	Molmed
Promotor	L.J. Hofland
Copromotors	R.A. Feelders and R. Pivonello

1.PhD Training	**Year**
1.1 General academic skills	
Erasmus MC Course on Clinical Neuro-Endocrinology, Rotterdam	2007
Postgraduate course on gastroenteropancreatic neuroendocrine tumors ENETS meeting; Lisbon, Portugal	2011
Postgraduate Diploma in Clinical Oncology (Diplôme Universitaire en Cancérologie Clinique (D.U.C.C)) Université Paris Sud; Paris, France	2011-2012
Postgraduate course on antitumor chemotherapy and cancer treatment (XXVIIe cours de chimiothérapie antitumorale & traitement du cancer) Institut Gustave Roussy, Villejuif, France	2012
1.2 Research skills	
Basic Introduction Course on SPSS, Molmed, Rotterdam	2009
Course Biomedical Research Techniques VIII, Molmed, Rotterdam	2009
Workshop Writing Succesful Grant Proposals, Molmed, Rotterdam	2009
Postgraduate Diploma in Clinical and Translational Research in Oncology (Diplôme Universitaire Européen de Recherche Translationelle et Clinique en Cancérologie (D.U.E.R.T.E.C.C.)) Université Paris Sud; Paris, France	2011-2012
1.3 Oral Presentations (National and International Congresses, Conferences, Meetings, Courses)	
34th National Congress, Italian Society of Endocrinology, Sorrento, Napoli, Italy	2009
Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium	2011
1.4 Poster Presentations (National and International Congresses, Conferences, Meetings, Courses)	
10th Congress of European Society of Endocrinology (ESE), Berlin, Germany	2008
Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium	2009
Molmed Day 2009, Rotterdam, The Netherlands	2009
11th Congress of European Society of Endocrinology (ESE), Istanbul, Turkey	2009
91th Endocrine Society Meeting, Washington, DC, USA	2009
Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium	2010
Molmed Day 2009, Rotterdam, The Netherlands	2010
12th Congress of European Society of Endocrinology (ESE) Prague, Czech Republic	2010
Molmed Day 2010, Rotterdam, The Netherlands	2010
3rd International Adrenal Cancer Symposium, Wurzburg, Germany.	2011
13th Congress of European Society of Endocrinology (ESE), Rotterdam, The Netherlands	2011
Molmed Day 2011, Rotterdam, The Netherlands	2011

	Year
15th International Congress of Endocrinology (ICE)/ 14th Congress of European Society of Endocrinology (ESE), Florence, Italy	2012
1.5 Presentations as Invited Speaker (National and International Congresses, Conferences, Meetings, Courses)	
WORKSHOP: Novel insights in the management of Cushing's syndrome Naples, Italy	2009
8th European Neuroendocrine Tumor Society (ENETS) Congress, Lisboa, Porturgal	2011
6th Italian Meeting of Hypothalamus-Pituitary Diseases, Napoli, Italy	2012
1.6 Attending Other Meetings	
Attending weekly seminars, working discussions and journal club of the Department of Internal Medicine; Erasmus MC; Rotterdam; The Netherlands	2007-2011
Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium	2008
90th Endocrine Society Meeting, San Francisco, USA	2008
7th European Neuroendocrine Tumor Society (ENETS) Congress, Berlin, Germany	2010
29th Congress of French Society, Congrès SFE Toulouse 2012	2012
1.7 Awards	
ESE Young Investigator Awards	2007
ESE Poster prize	2008
Eugenia Rosemberg Abstract Award ENDO, Washington DC	2009
Poster Prize, Presidential Poster Competition, ENDO, Washington DC	2009
Excellence in Translational Medicine in NET Disease (ENETS Translational Research fellowship)	2010
Travel grant, 3rd International Adrenal Cancer Symposium, Wurzburg, Germany.	2011
Travel grant, 14th Congress of European Society of Endocrinology (ESE), Florence, Italy	2012
ISE Travel Grant Award, Florence, Italy	2012
2. Teaching Activities	
2.1. Lectures at (Inter) National Endocrinology Courses	
1st Forum: Aggiornamento in tema di terapia delle malattie endocrine, Naples, Italy	2009
3rd Forum: Aggiornamento in tema di terapia delle malattie endocrine, Naples, Italy	2011

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Many times during the past I dreamt the moment of my PhD defense and even many more times I dreamt to write the following words, feeling the need to express my gratitude, feeling that I could finally write everything I was too timid to express in words.

I would like to thank you Prof. Leo J. Hofland: Dear Leo, I enormously admire you as scientist and as person. I feel lucky that I had the opportunity to develop my project in your lab. Each discussion was always intense and deeply disputed. I wish that you excuse me for having always argued a lot, but I think this has been the perfect exercise for the development of my thinking ability in translational research! Having an Italian or Dutch coffee in your office at Erasmus or *via* Skype from far, during our discussions I learned so much from you! Caro Leo, I also would like to say to you "grazie", for your hospitality and friendship. When I arrived in Rotterdam I felt alone and being far from family I was afraid for all the novelties, but from the very first telephone call with you, I immediately felt relieved due to your friendly welcome. Thanks to you and Nicolette for your kind invitations for dinners at your home, they were all very special.

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When I arrived in Rotterdam my experience in the lab was near to zero, although my enthusiasms and appetite for learning were immense, how could I have managed alone with my project? Everything I learned to do in the lab has been the result of an excellent team, who assisted me day by day in training and helping me. Dear Peter thank you very much to have always been ready to explain to me how to solve any kind of problems with cell cultures, lab methodologies, calculation etc. Thanks to you, Marlijn and Fadime for your help in completing the experiments when I left.

Dear Marlijn and Diana, what a fantastic team you were together! Always kind, always smiling you made the atmosphere in the lab special! Thank you my dear "lab ladies", you both were always very clear in your explanations regarding lab methodologies.

Thank you Marlijn for having invited me for dinner at your place, you were for me a point of reference, teaching me how to deal with any type of "Dutch situation". I cannot forget when Diana and you helped me in moving from my first apartment. I loved our conversations on Friday morning in the cell culture room. You cannot image how many times I felt lonely and yet relieved by our conversations, so many times you helped me to restart my week on Monday morning!

Working aboard offers many opportunities to learn, but it offered me also the opportunity to open my mind and to grow up as strong independent women. I would like to thank all the colleagues for all the fun we had together. Dear Aimee, Christian, Davine, Fadime, Laura, Marije, Michel, Rob, Rogier, Roxanne and Slavka etc., thank you for all the nice times spent together in and out of the lab. I really enjoyed our time together and learned to better know and appreciate the Dutch culture. Due to my period in Rotterdam I made some precious friends. My dear Aimee the affinity I had with you is comparable to the one that I have with my very good friends. Our outings for dinners speaking about female stuff, our dinners at your place or the days you and David visited us in Paris were always great! What a great time we had together! Thank you for all this and to have helped me many times to arrange many practical things during my Dutch experience. Dear Chris, Davine, Laura, Michel you were my first friends at beginning of my time in Rotterdam, thank you for having helped me in adapting. Chris, without your help I would never have found an suitable accommodation. I have a great admiration for you, it has been nice to work together speaking in Italian sometimes, I am grateful for your help. Dear Michel you and your wife helped me in furnishing my apartment, I loved your table! Thank you for your kind help! Dear Marije we had lots of fun together, you have been a nice surprise in my last period in Rotterdam: a new incredible friend that I hope to keep for life. Dear Rogier I really appreciated that you managed to participate to my wedding party, your presence there with Aimee, David and other foreign friends contributed to make that day even more special than expected.

During my first year in Rotterdam, I lost the habit of hearing Italian, so when I met a strange couple, one speaking in Spanish and the other answering in Italian, my attention was polarized. Thanks to a Dutch friend (Davine), I met them again several months later. They were Oscar and Gianluca, two of the "Lega Mediterranea" (a group of friends, predominantly Italians, but also from other countries including some Dutch friends, fond of sea, the sun and Mediterranean food and frequently organizing funny parties) founders together with Francesca, Carmen, Massimiliano and myself. With the addition of Federico you all became my "Italian family", making my stay in Rotterdam much nicer. I always felt there was a person ready to help me in case of need. Cara Francy my time in Rotterdam would not have been the same without you, our conversations, your

wonderful parties and our friendship. Together with other friends such as Francesco, Claudia, Gaia, Antonio, Ermanno, Paolo, Isa, Silvana, Laura, Pierluigi, Valentina, Valerio, Alessandro, Veronica, Alexia, etc. we had a lot of fun! We all had different personalities but shared two common denominators: love for research and the strong determination to success in transforming our passion in a job. I wish all of you the best for life, you deserve it!

A special thanks to my "guide" Dr.Rosario Pivonello. Caro Ross, there are not enough words to thank you. I was only a student when I started to work with you and you trained me as a doctor, as an endocrinologist and as a scientist. My career has been supported by you. Thank you for your teaching and advices and for having invested in me. Now I am very honored to be your eldest co-worker and my enormous admiration and respect for you is in constant growth. I followed your example of dedication and perseverance, you are still my precious guide and I hope we will continue to share the enthusiasm for research also working in a not always easy environment such as Naples. Thank you to have helped me to achieve my dream to become a medical researcher, thank you for your friendly support and comprehension also in my "gipsy" life. I am grateful to Prof. Gaetano Lombardi and Prof. Lucio Zarrilli who met me during my medical studies and being aware of my ambition to become a medical researcher in the field of endocrinology, indicated Prof. Annamaria Colao as the most appropriate person to pursue, becoming the most important person in the design of my career. Dear Prof., if I am "a dreamer who never gives up" it is because you always believed in my capability and supported me, representing a model to emulate as a female scientist.

I would like to express thanks to Antongiulio, Mariagiovanna, Carla, Antonella, Libuse that during my pre-graduate fellowship helped me to integrate in the Dep. of Clinical and Molecular Endocrinology and Oncology of Federico II. Dear Carla thank you for having always been present. I want give an huge hug to Diego and Giovanni, that together with Rosario are the Neapolitans who preceded me in Rotterdam. Dear guys you did a great job there because when I began in the Lab everybody already had been educated to deal with the "Mediterranean behavior". Everyone adapted to me very quickly and it was always very nice to hear stories about you and to see your pictures around the lab.

There is a person that has been always on my side since I was a student, becoming an important friend and witness at my wedding, she is Renata. Cara Reny, thank you for your support and for all the time that we spent and spend together daily. When the time came to return to Naples, I was worried about the possible difficulties in being accepted by the new colleagues, but I felt relieved knowing you would have always been my valuable ally. Thank you! I hope our friendship will last forever and we will continue to sustain one

another, carrying out our aims and dreams. The other person who represented a bridge from Rotterdam to Naples for me, simplifying my return, is my paranymph Claudia. We were in Rotterdam together for some time, so, together with her in Naples, I would have surely continued my so loved lab research. Cara Claudia I hope we will be successful in many projects together. With you I would also like to thanks all the guys and gals in the lab: Cristina, Gaia, Mariarosaria, Maddalena, Federica, Giorgio, Gilda, Donatella, Roberta, Luigi, Imma, Giacomo, etc. You are all a very nice gang! Despite all the daily challenges, you keep the enthusiasm and the passion for research making work with you very pleasant! Going back to my home university I was also positively surprised by the welcome I received from the younger doctors working in our team. For certain reasons I felt I had never left Naples, and immediately felt fully integrated despite there were a lot of new people that I had only occasionally met at congresses. Dear Alessia, Chiara, Davide, Ludovica, Mariano, Monica, Francesco, Rosario etc. despite the unpredictable situations we face daily, working with you is very pleasant. I love the feeling that I have with you and hope to preserve it always. Cara Monica, I returned in a very complicate moment of your life and you proved to be the strong woman you are. I appreciate your speaking directly and benefited from your suggestion on female beauty, thank you, I need that as well! Caro Mary, you have always been a gentleman, a very good person and fortunately this is not changing over time. Please remain like this! Cara Ale I like you very much, you are strong and determinate and I loved our long conversations. We have different personalities, even if we have known each other for short time, but maybe with the complicity of a common Naples-Rome life, I felt very close to you. With mixed feelings I am going to say you goodbye, sad for your departure but very happy for your future life in Rome with your future husband. Cara Chiara, as Rosario anticipated before we met, I have a huge affinity with you, I feel I have a new friend and not only a young co-worker with which I share the enthusiasm and the devotion for our job. Caro Davide you are a very sweet person, in this time we are working together I have seen you grow a lot and I am confident, despite your preferential interest for the clinical part of our job, you will succeed in research as well, you only need to believe in it.

Moving from Rotterdam to Paris, I was happy to finally have more time with Massimiliano, my husband, but the necessity to adapt to a new completely different environment was very tough on me. "*Je voudrais remercier beaucoup Monsieur le Docteur"* Eric Baudin*: "Merci Eric",* I could not expect to be so lucky again in finding a person that would have soon became another "*superbe*" guide for me. It has been a honor and a privilege to work with you, to follow your consultations and to discuss our research projects with you, during consultations, between a patient and the following one, and jumping fast from one subject to another. Our discussions about medicine and science were always rich of new inputs, I learned a lot from you about medicine and science; observing you in great

admiration I learned how to compassionately care for patients affected by cancer. My experience in Paris was of incredible value also thanks to "*Monsieur le Docteur"* Ludovic Lacroix, who gave me the opportunity to work in his Translational Research Lab where I learned new techniques, and to participate to weekly discussions on personalized medicine "from the bench-to-bedside". "*Cher Ludovic merci beaucoup"* for your support during my period at Institut Gustave Roussy and your precious teaching. I would like to express gratitude also to Prof. Schlumberger, for having always been very kind with me and to Dr. Abir Al Ghuzlan, Prof. Jean-Yves Scoazec and other French colleagues who greatly contributed with their work and their knowledge in helping me to carrying out the part of this thesis that has been developed in France.

I would thank my French friends, who contributed to make my time in Paris great. Particularly, I want to thank Rossella, Desirèe, Marta, Marianna and Libuse, friends that I hope to keep for life, and the lovely Tina which is simply special.

Those who have followed me step by step during my life experiences are my parents and my husband, Massimiliano (Massi or Max), all often being far in distance, but always very near to my heart. This thesis is dedicated to them! I feel the need to thank you for all the support you have given me, but I also the need to apologize for the time I did not dedicate to you during the last years.

A special thanks goes to my parents. *Caro papà*, if I love science it is because I am curious like you, because we have always found a great complicity in reading books on biology, watching documentaries or caring for plants and animals. I developed my interest for medicine and research very early, thanks to all the inputs I received from you. Studying and learning have always been a game for me, allowing me to develop my passion and hobby in what now is my job. If I am an hard worker and proud to be, it is because you have been an incredible example of devotion for work and thanks to all your sacrifices we children had the opportunity to study and follow our ambitions. We became adults, but you are always present, ready to offer your help. My aspiration is to be like you. *Caro papà grazie mille* you are very special for me.

To express my gratitude to my mother it is almost impossible….my best friend forever. You dedicated every day of your life to me, even from far, chatting for hours, probably more than we use to do when we were together. You are an amusing person and supporting me always. *Cara Mamma* I love you and *Papà* so much. I cannot forget how often you helped me in preparing my exams and although you knew nothings of the subject, intelligently pointing out useful questions, while as if taking part to our discussions, our little dog Zara stared at us. Many times we painted together and prepared many

cakes and pizzas for parties. You always welcomed our friends, capturing them with loving hospitality. I am proud to be your daughter, I would have never succeed in my life without you and without all the happiness you add to my life.

Caro Massi, since we were sixteen you are adding colors to my life. You encouraged me to follow my ambitions, despite it was difficult and keeping us apart for long time. You supported me in any difficult moment, even from far. When I was a child I dreamt the perfect love, most people would have said that it does not exist, but I was very lucky in finding you very soon in my life. We have been capable to adapt to many different situations, always looking at good side of things. Across the years, growing and developing individually as two very strong people, occasionally disputing before finding a common agreement, but always without prevarication of one on the other. We have always respected each other as individuals independently from being a couple; preserving the juvenile enthusiasm to stay together, we have grown to be two balanced parts of a single dynamic entity. I think this is an incredible formula for a long lasting (more than 20yrs) "perfect love". You are a beautiful person, you developed as an adult with a brilliant carrier but you kept the heart of a child. I suffer a lot when I am far from you and I thank you for all the opportunities you find to make it back home, it is for me of incredible value. When you are with me I am most at peace, I finally relax and my rest becomes serene, feeling loved and protected as when I was a child. You taught me to enjoy life and to find happiness in life's daily occasion. I would have never been capable to succeed in anything without having you by my side.

My sister, Laura, was strongly desired by me, when I was a small child and although she expected for me to be exclusively dedicated to her in some period of her life, she is the greatest present received from my parents. I thank her to have found the way to be close to me even when I was far and I am enjoying a lot the many opportunities to be together now that I am back in Naples. I know I can count on you.

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care of him … but I was not even a medical student at the time. Nonna Maria who has been a second mother for me in the beginning of my life. I'll never forget that white trembling head (due to Parkinson) obstinately waiting outside the office of the faculty to know the results of my admission test to the Faculty of Medicine, I know you would have been proud of me today. Nonno Giovanni from whom I inherited the love of knowledge and painting. Nonna Pasqualina, I am so proud to have a granny like you, always smiling, always open-minded, you are a very strong woman and a lovely person. It is always very pleasant to spend time with you and hear your advices and stories.

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